NOVEL POLYMERS FOR ARTIFICIAL LIVER SUPPORT SYSTEMS

by

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I dedicate this thesis to my
family and the loving memory
of my father and brother.

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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SUMMARY

The work presented in this thesis is concerned with the purpose-design, synthesis and evaluation of hydrogel particulate polymers for use in artificial liver support systems. The introductory chapter concentrates on various aspects of liver functions, diseases of the liver, and the role that hydrogels can play to improve the condition of patients suffering from hepatic failure. The experimental work falls into two main areas. First, the synthesis of hydrogel particulates carried out by employing three suspension polymerisation techniques. Second, the evaluation of hydrogel particulates carried out by determining their particle size, water content, density; examining their internal and external structures with scanning electron microscopy; and studying their adsorption characteristics with respect to certain acidic chemical species used as markers of liver failure.

The three synthetic techniques used were: freeze-thaw polymerisation which involved photopolymerisation of frozen monomer/solvent mixture in a cold n-hexane continuous phase; suspension polymerisation in brine which involved polymerisation of hydrophilic monomers in a high salt content aqueous continuous phase; and inverse-suspension polymerisation of hydrophilic monomers in n-hexane continuous phase. With the use of these combined techniques copolymer and terpolymer particulate hydrogels were synthesised from acidic, neutral and basic monomers. The hydrogel particulates possessed a wide range of surface morphology, porosity, particle size, water content and density. The potential of hydrogel particulates for use as biocompatible adsorbents was studied by static and dynamic adsorption techniques. Hydrogels proved to be good adsorbents for removing bromosulphophthalein, bile acids and amino acids from saline. Hydrogels also showed some adsorption of a lipid soluble hepatic toxin bilirubin from chloroform.

The application of hydrogels in the field of detoxification was further extended by creating a combined hydrogel-enzyme detoxification system by coupling urease to the hydrogel beads. In this way the specificity and efficiency of the adsorbent can be increased. Hydrogel particulates such as those synthesised in this work may provide alternative potential adsorbents for haemoperfusion systems.

KEY PHRASES: Hydrogel synthesis; Haemoperfusion; Adsorption; Hydrogel-enzyme detoxification system.

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LIST OF ABBREVIATIONS

AA - Acrylic Acid
ACR - Acrylamide
APS - Ammonium Persulphate
AZBN - α,α'-Azobisisobutyronitrile
BET - Brunauer, Emmett and Teller
BSP - Bromosulphophthalein
DAA - Diacetone Acrylamide
DMAEMA - Dimethylaminoethyl Methacrylate
DYDA - Diketohydrindylidenediketohydrindamine
EDM - Ethylene Dimethacrylate
EGMA - Ethylene Glycol Methacrylate
EWC - Equilibrium Water Content
GMA - Glycerol Methacrylate
3α HD - 3α Hydroxysteroid Dehydrogenase
HEMA - 2-Hydroxyethyl Methacrylate
ICG - Indocyanine Green
LAD - Artificial Capillary Cultured Cell Liver Assist Device
MAA - Methacrylic Acid
MACR - Methacrylamide
NMACR - N-Methylol Acrylamide
N,N'MBA - N,N'-Methylene bis Acrylamide
PAN - Poly (Acrylonitrile)
PAPS - Adenosine 3'-Phosphate 5'-Phosphosulphate
PETMA - Pentaerithritol Tetramethacrylate
PGA - Propylene Glycol Monoacrylate
Poly(ACR) - Poly (Acrylamide)
Poly(HEMA) - Poly (2-Hydroxyethyl Methacrylate)
PTFE - Poly (Tetrafluoroethylene)
SEM - Scanning Electron Microscopy
SEM'S - Scanning Electron Microphotographs
TEGDMA - Triethylene Glycol Dimethacrylate
TCE - 1,1,2,2-Tetrachloroethane
TPT - Trimethylol Propane Trimethacrylate
UDPGA - Uridine Diphospho Glucuronic Acid
UN - Uranyl Nitrate
UV - Ultra-Violet
Wf - Equilibrium Water Weight Fraction
Wg - The Weight of a Polymer in the Hydrated State
Wp - The Weight of a Polymer in the Dehydrated State

CHAPTER 1

LIVER AND MANAGEMENT OF LIVER FAILURE

The regenerative capacity of the liver cell is almost unlimited. However, for certain forms of liver disease and for critical situations before and after liver surgery, artificial liver support is needed. This thesis is concerned with the technique of haemoperfusion (in which the blood is circulated over an external adsorbent) as a means of artificial liver support and which is in need of adsorbents which are more biocompatible than those currently available. Hydrogels (synthetic water swollen polymer networks) are amongst the most promising polymers for this application. Consequently, the introduction to the thesis deals firstly with aspects of liver failure and secondly the nature of hydrogel polymers. This provides a background for the experimental work subsequently described.

1.1 INTRODUCTION

The liver is an organ of major importance not only because it is the largest organ in the body but also being, from metabolic viewpoint, the most complex internal organ (Harper, 1969). The liver has a heterogeneous distribution of a variety of cell types (hepatocytes being the major cell type in terms of number) that are specialised for performing particular functions and are co-ordinated in a complex three-dimensional arrangement (Elias, 1955). In addition there is a heterogeneous distribution of enzyme activities within the lobular units of the liver.

The liver has been called the body's chemical factory and one could present the functional anatomy of the liver best by reviewing this organ as a purification plant situated between the blood carrying exogenous (organic and inorganic compounds) and endogenous substances (metabolites, hormones and toxins), and the remainder of the body tissues. The substances to be purified flow in through unnumbered rows of various cells. Substances are taken up by the cells whose organelles transform some of them into proper constituents of the body (proteins, carbohydrates and fats), while others are metabolised to non-toxic substances to be excreted. For this activity, the hepatic tissue is arranged in microscopic units and structures around the supplying vessels that deliver the materials to the liver cells. The very numerous and complex functions performed by the liver are summarised below (Keele and Neil, 1973):-

(i) Storage organ - The liver stores glycogen, fat, probably proteins, certain vitamins, other substances concerned in blood formation and regeneration, and blood.

(ii) Synthesis - The liver synthesises the plasma proteins, the blood clotting factors, and (by virtue of its mast cells) heparin.

(iii) Bile secretion - Hepatic cells continuously secrete bile which is a powerful emulsifying agent and aids in the digestion of fatty materials. The constituents of bile have the characteristic property of lowering the surface tension of aqueous solutions, and therefore allowing the formation of stable solutions or emulsions of fatty materials.

(iv) Detoxicating and protective functions - The liver exerts its protective action in a variety of ways. Kupffer cells remove foreign bodies from the blood by phagocytosis. Various chemicals and drugs are detoxified by conjugation, methylation, oxidation and reduction. The removal of ammonia from blood, especially that absorbed from the intestine by way of the portal vein is of particular importance.

(v) Metabolism - The liver is pre-eminently the central organ for metabolism of carbohydrates, proteins, lipids, minerals and vitamins.

(vi) Excretory functions - Linked to detoxification, the liver is an excretory organ and eliminates substances from the body, of which the pigment bilirubin is the most obvious.

There are numerous ways in which the structure and/or function of the liver can be disturbed. Pathological disturbances of the liver, whatever the cause, reflect fundamental disturbances to metabolism and cell structure at a molecular level. The liver injury may result by four different mechanisms; depletion or stimulation of metabolites or co-enzymes, inhibition or stimulation of enzymes, activation of a toxic agent to a more toxic form, and membrane disturbances (Slater, 1978). Failure of liver cell function can occur in all forms of liver disease, but is commonest in portal cirrhosis and acute virus hepatitis (Sherlock, 1975).

As a result of the sheer complexity of the biochemical role played by the liver, severe impairment of hepatic failure is reflected in disturbances in other organs, notably the brain. When a patient's liver fails, it is appropriate to think of replacing various aspects of the liver's function. The synthetic function of the liver can in part, be replaced by intravenous fusion of clotting factors and albumin. The avoidance of certain drugs obviates the need for a part of the liver's detoxification function. The excretory function of the liver is, however, much more difficult to replace. On the other hand, these difficulties are to some extent counterbalanced by the ability of the liver, given the correct conditions, to regenerate rapidly. Thus if functions of the liver can be supplemented during an acute crisis, total recovery is possible in many patients (Dunlop et al., 1975). This has motivated interest in the development of artificial hepatic assist systems.

This chapter is concerned with reviewing: conditions of liver failure that require artificial support; the factors which contribute to the development of such conditions; and various approaches that have been made and are currently being pursued to tide patients over the acute phase of their illness.

1.2 NATURE OF LIVER DISEASES REQUIRING ARTIFICIAL SUPPORT

Patients suffering from a number of conditions could benefit by the development of an artificial liver. It was recognised early that "liver injury" is not a single entity, that the lesion observed in the patient depends not only on the toxic substance involved and the site affected, but also on the period of exposure. Consequently, some forms of liver injury may be acute and can be reversed while others may be chronic and lead to permanent changes. Thus, depending on the severity of the condition, the patients for whom an artificial liver support system is desired can be considered in two main groups:

1.2.1 Fulminant Hepatic Failure

The first group consists of those in acute condition called fulminant hepatic failure. It is that clinical syndrome which develops as a result of massive necrosis of liver cells or following any other cause of sudden and severe impairment of hepatic function in a patient in whom there has been no evidence of liver disease previously (Trey and Davidson, 1970). Acute viral hepatitis, paracetamol overdosage, halothane poisoning, other drug toxicity, amanita phalloides, and fatty liver of pregnancy were found to be the causes of fulminant hepatic failure in a series of 132 patients seen at King's College Hospital over a seven year period.

The first clinical signs of fulminant hepatic failure are a change in mental state, ranging from depression to euphoria, and sometimes accompanied by violent behaviour. Progressive and severe mental changes starting with confusion, and rapidly advancing to stupor or coma are characteristic of this condition. Jaundice appears later and the whole illness from the first symptom to death may be less than a week. Attempts have been made to grade the progressive levels of encephalopathy (Trey et al., 1966). Table 1.1 lists the development of stages leading to coma (Davis and Williams, 1980).

Mortality of fulminant hepatic failure is closely related with the severity of the encephalopathy that develops, average mortality around the world for patients developing the most severe grades (III and IV) is about 85 percent. The detailed management of these patients can be based only on the understanding of the way in which the various signs and symptoms making up the clinical syndrome develop during the course of the illness. This basic supportive therapy is essential; whatever other measures are used (Williams, 1972). The use of artificial liver support in such cases is based on the premise that survival is possible because of the extraordinary capacity of the liver to regenerate.

Table 1.1

Clinical stages in the development of hepatic coma

Grade	Mental State
I	Euphoria, occassionally depression Fluctuant, mild confusion slowness of mentation and affect Untidy, slurred speech Disorder in sleep rhythm
II	Drowsy, but responds to simple commands Inappropriate behaviour
III	Sleeps most of the time, but rousable marked confusion; incoherent speech
IV	Unrousable; may or may not respond to noxious stimuli

1.2.2 Cirrhosis with Liver Failure

There are two categories of people to be considered here. Firstly, there are those in whom signs of liver failure have been precipitated by an event such as bleeding from oesophageal varices, or infection or a metabolic upset. In the case of bleeding from varices, low blood pressure resulting from bleeding leads to inadequate perfusion of the liver, necrosis of regeneration nodules and consequent under-production of blood clotting factors (normally synthesised by the liver) which causes in turn further bleeding. In such cases liver failure is potentially reversible provided the precipitating factor can be controlled. If liver support could be provided for them during the stage of acute liver decompensation, survival figures would almost certainly be improved. There are also patients with acute alcoholic liver damage, or 'hepatitis' which may or may not be superimposed on an underlying cirrhosis. Such patients although deeply jaundiced and usually encephalopathic, can make a remarkable recovery provided they are brought through the acute episode.

The other patient category to be considered is the end-stage cirrhotic liver failure, in whom there is no hope of reversing the course, but in whom liver transplantation is a possibility. This is potentially the largest category of patients, as cirrhosis alone accounts for about 1400 deaths per annum in England and Wales (Dunlop et al., 1975). Kidney transplantation would not be undertaken without dialysis support, yet no equivalent procedure can be carried out before the technically more difficult liver transplant. Likewise, at present, there are no means whereby a patient with liver failure can be kept alive until such time as a donor becomes available.

1.3 ARTIFICIAL LIVER SUPPORT SYSTEMS

The occurrence of encephalopathy is of central importance in the syndrome of hepatic failure. The development of encephalopathy is usually attributed to the effect of toxins which accumulate as a result of impaired hepatic metabolism and detoxification. The exact nature of toxins involved in liver failure is not clearly defined but the current view is that no single substance is responsible but rather the coma is produced due to the cumulative effect of a wide range of toxic chemical species in the middle molecular weight range (1500-5000). These toxins and the possible ways in which they result in the production of coma will be described in section 1.5. Systems of artificial liver support are concerned with the removal of the toxic chemical species from the blood circulation of patients with liver failure and consequently with the reversal of hepatic coma.

To date two different approaches to liver support have been suggested. In the first approach the removal of all known classes of potentially toxic compounds is the basic aim, whereas, the second approach aims at the provision of specific liver functions with the use of perfusion systems containing elements of the liver. These approaches include several techniques that have been applied both clinically and experimentally. These will be described next.

1.3.1 Artificial Liver Support Systems Based on the Removal of All Known Classes of Potentially Toxic Compounds

1.3.1.1 Cross circulation

In this technique blood from the donor is transfused directly into the recipient. The procedure is carried even further by direct cross circulation artery to vein, patient to volunteer (Burnell et al., 1967). The technique is limited to non-infectious cases (Dunlop et al., 1975) but even then it is not without risk to the partner who risks the side effects such as falls in platelet counts and abnormal liver function tests.

Baboons have also been used for cross-circulation with man (Abouna et al., 1972). Some of the patients treated in this way have improved (Hume et al., 1969; Saunders et al., 1968). However, the use of baboons carries potential infectious risks such as virological infections, and therefore necessitates special facilities.

1.3.1.2 Plasma Exchange

This technique involves the removal of patient's blood followed by return of the packed red cells with fresh-frozen, donor plasma (Lepore and Martel, 1970) thus avoiding the need for fresh whole blood. In the largest single study of Lepore et al. (1972), nine patients in fulminant hepatic failure had a mean volume of 36 litres of blood removed for 6 days. All the treated patients died; bronchopneumonia, probably related to volume overload, and thrombocytopenia were seen in all cases. However, greater success (four out of seven survived) was reported by using an initial whole blood exchange followed by plasma exchange (Haapanen and Tuila, 1972). In another study high survival rate (three out of four survived) of patients by exchanging 10 litres of plasma a day using a continuous flow celltrifuge has been reported (Buckner et al., 1973).

The plasma exchange therapy involving the passage of plasma over adsorbents to remove toxic substances in the absence of the cellular elements has also been investigated. This will be discussed in conjunction with haemoperfusion in section 1.3.1.6.

1.3.1.3 Extracorporeal Liver Perfusion

In this technique the patient is connected to an isolated but functional animal liver in an extracorporeal perfusion circuit for the removal of toxic metabolites from circulation of the patient. Ever since the introduction of this technique (Eisman et al., 1965) many investigations have been undertaken using this procedure. Generally livers of porcine or bovine origin were employed by most investigators. The use of bovine liver with no long-term survivors was reported (Condon et al., 1970). Promising results by using baboon and calf livers have been reported (Tung et al., 1981) in the treatment of acute hepatitis and liver cell necrosis. Baboon livers, because of their close genetic disparity to human livers have prolonged extracorporeal function and they exhibit less severe immunological complications. Human cadaver liver was also employed in a limited manner (Abouna et al., 1972). A 50 percent recovery rate has been reported on 14 patients with acute hepatic failure treated by haemoperfusions over baboon and human livers (Lie, 1981).

Major problems encountered in this technique are haematological problems. The use of a liver in the extracorporeal perfusion circuit can produce severe bleeding and can deplete the patient's blood of platelets and clotting factors. The bleeding tendency is aggravated by disseminated intravascular coagulation. The technique is technically difficult and requires well trained team consisting of surgeons, haemodialysis specialists and engineers (Lie, 1981).

1.3.1.4 Dialytic Procedures

The fundamental physiological principle in haemodialysis involves the transfer of a solute or toxin across a semi-permeable membrane in a direction and at a rate consistent with concentration gradients. The use of the artificial kidney (haemodialysis) for the management of severely intoxicated patients who have ingested specific intoxicants (for example alcohols), has been a valuable therapeutic adjunct to the clinician (Sketris and Skoutakis, 1981). However, both haemodialysis and peritoneal dialysis (which differs from haemodialysis mainly in the respect that it makes use of an in-vivo biological membrane) are limited to toxins which are water soluble, exhibit low protein binding and are a size which can pass through the dialysis or peritoneal membrane. Many components of the pattern of plasma abnormalities in fulminant hepatic failure (for example, ammonia, methionine, glutamine) are water soluble and dialysable. The protein bound anions (unconjugated bilirubin, bromosulphophthalein, chenodeoxycholate) are dialysed in negligible amounts (Willson et al., 1972). The fact that excretory functions of the liver deal with a much wider spectrum of compounds (in terms of molecular weights, physico-chemical properties, and protein binding) than do those of the kidney renders the conventional dialysis membranes such as cuprophane unable to remove hepatic toxins efficiently.

This has led to the modification of haemodialysers. Consequently, membranes with high permeability to "middle molecular weight" compounds and with increased membrane area have been developed. The most important new membrane is the polyacrylonitrile (PAN) membrane (Opolon et al., 1976) which is permeable to substances upto a molecular weight 15,000 (but not to plasma proteins). Thus the PAN membrane unlike cuprophane is better in dialysing higher molecular weight substances such as polypeptides which have been hypothesised to play role in the origin of false neurotransmitters or directly on neurotransmission. The false neurotransmitters may contribute to hepatic coma. Cuprophane membrane being permeable to low molecular weight substances only may amplify the imbalance between normal and false neurotransmitters (Opolon et al., 1976).

PAN membrane has been clinically used on patients with viral hepatitis and grades III and IV coma. Out of a total of 24 patients treated, total recovery of consciousness was achieved in 13, 4 patients recovered, death in others being due to a variety of causes (Opolon et al., 1976).

The success obtained both clinically and experimentally by the introduction of new membranes has renewed interest in using haemodialysis as a means of artificial liver support. The power and availability of liver dialysis systems needs to be increased. The natural liver processes the entire blood volume every 4 minutes, but artificial systems rarely achieve 10 percent of this rate (Knell, 1981). For dialysis this implies on the one hand larger and perhaps more permeable membranes, with increasing problems of ultrafiltrate control; and on the other, faster dialysate and blood flows, raising problems of vascular access and doubts about the ability of the patient to tolerate the procedure. Longer haemodialysis times are impracticable.

1.3.1.5 Liquid-Liquid Interfaces

Liquid-liquid interfaces between oils or ion-exchange fluids and plasma provide another potential means of removing metabolites from whole blood. A single liquid-liquid extraction, by choosing the proper oil or modification of an oleaginous liquid, can remove total bilirubin and lipid soluble drugs such as glutethimide selectively (Evenson and De Vos, 1972). Various kinds of oils have been studied. These include: vegetable oils such as cottonseed, linseed, soyabean and corn; mineral oil; ion-exchange oil and silicone and fluorocarbon liquids. Vegetable oils, particularly corn oil are the best studied. It has been shown that liquid ion-exchange materials can cause haemolysis of red blood cells. The toxicity, safety and other potential side effects of silicone and fluorocarbon liquids have not yet been investigated.

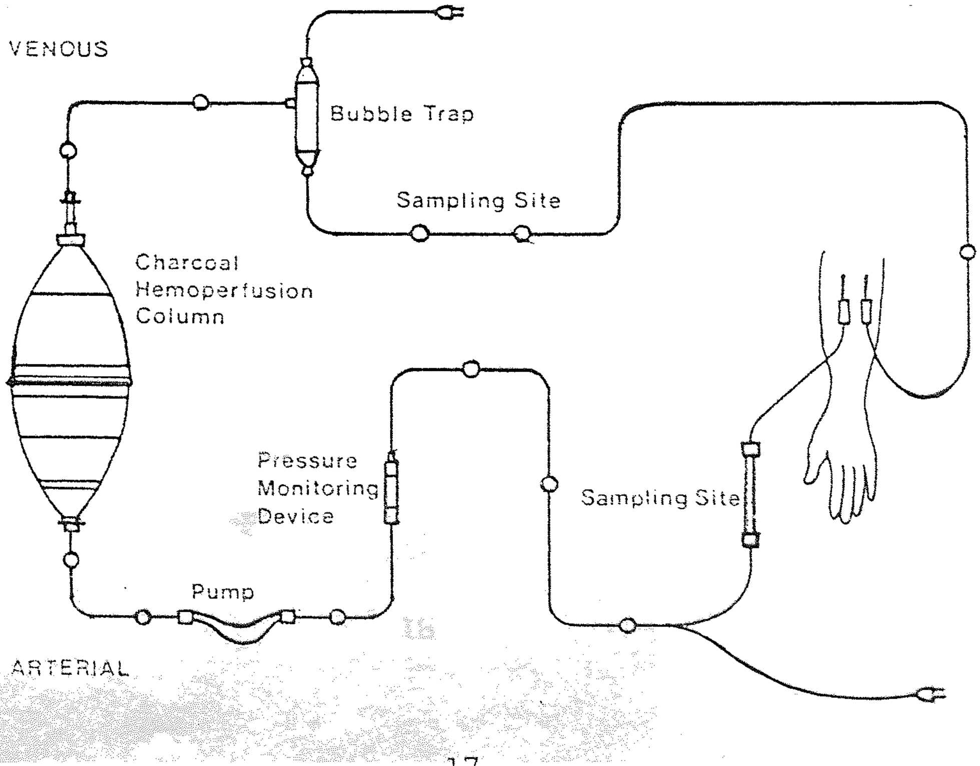
Although experimental use of fluorocarbons in dogs has looked promising (Evenson and De Vos, 1972), this field of research is in its infancy and needs much more development before application to human patients (Dunlop et al., 1975). The technique poses several potential risks to the patients. Firstly, there is danger of forming an emulsion of blood and oil or even small droplets within the liquids. Secondly, pumping oil into the veins could produce life-endangering fat emboli. Third undesirable factor is that some essential substance might also be extracted from blood, such as, lipid-soluble vitamins. However, the last risk may not be so serious as rapid re-equilibration with tissue stores would occur when the oil-haemoperfusion procedure is discontinued.

1.3.1.6 Haemoperfusion

One blood detoxification technique in which there has been still growing interest in the recent years is the haemoperfusion. In this technique the patient's blood is externally circulated over an adsorbent bed. Haemoperfusion in contrast to haemodialysis thus allows the blood to pass directly over an adsorbing surface and is not limited by a dialysis membrane. Basically, the blood is taken from an artery in the patient's arm or leg, pumped through a column of adsorbent material and returned to a vein in a manner similar to dialysis. This is demonstrated in figure 1.1 which shows a circuit for clinical haemoperfusion (Gelfand et al., 1977).

Figure 1.1

Circuit for clinical haemoperfusion



Haemoperfusion has applicability in the treatment of various syndromes associated with the accumulation of toxins in the circulation which include uraemia, hepatic failure and acute poisoning. The effectiveness of its application in toxicology has been described (Okonek, 1981). The simplicity of this technique has much to commend it if a suitable biocompatible adsorbent can be found. The present research is concerned with the design, development and evaluation of biocompatible adsorbents for use in haemoperfusion. This technique will therefore, be considered in some detail. First the development of the idea of haemoperfusion will be reviewed and then various adsorbents used for haemoperfusion will be described.

Although adsorbents, especially charcoal, were known as a medicinal adjunct in ancient Greece and China it is Muirhead and Reid who receive the credit for having introduced the idea of haemoperfusion into clinical medicine (Muirhead and Reid, 1948). They demonstrated in-vitro that certain adsorbents have the capacity to remove toxins directly from blood. Shreiner (1958) provided the classical demonstration of the capacity of an ion-exchange resin column to treat a patient with barbiturate drug poisoning. From these pioneering studies, a number of investigations into the clinical use of adsorbents led to the currently available devices used for the treatment of drug intoxications.

The development of the clinical applicability of haemoperfusion was delayed because of the demonstration that the procedure as performed by Schreiner was associated with significant potential complications including pyrogenic reactions, electrolyte disturbances, and haemolysis of blood passing over the column (Hagstam et al., 1966; Dunea and Kolff, 1965). Clinical trials on patients with drug poisoning (Yatzidis et al., 1965) and subsequent animal studies on charcoal haemoperfusion brought to light more significant complications than originally found. These complications included significant platelet loss and the possibility of embolisation of particles of charcoal from the haemoperfusion device.

Subsequent efforts to develop safe, effective adsorbent haemoperfusion systems, therefore followed four major lines:

(i) The use of ion-exchange resins as adsorbents was continued. Although showing some promise, this did not produce a system safe enough for human clinical use (Rosenbaum et al., 1971).

(ii) The use of non-ionic microporous resins belonging to polystyrene Amberlite series, and macroreticular resin adsorbents was shown to be extremely effective in the removal of a variety of poisons (Rosenbaum et al., 1971).

(iii) The use of charcoal as the basic adsorbent was continued by ingeniously encapsulating the charcoal particles in albumin or albumin-collodion to enhance its biocompatibility and to prevent possibility of embolisation of the charcoal material (Chang et al., 1973). This highly important work demonstrated the applicability of albumin-collodion activated charcoal haemoperfusion system not only in the treatment of toxic ingestions but also in the treatment of hepatic coma and uraemia (Chang, 1976). In subsequent developments charcoal was successfully encapsulated in acrylic hydrogel materials. These encapsulated charcoal systems have developed into the Smith and Nephew Hemacol device (Fenninmore et al., 1974), the Gambro Hemoadsorba device and other commercial systems.

(iv) The use of uncoated activated carbon in which the granular charcoal was fixed to a polyethylene backing and wound into a spiral (Hill et al., 1976). This device, known as the Hemodetoxifier remains the only commercially available uncoated charcoal adsorbent device (Gelfand, 1982).

The adsorbents previously investigated for haemoperfusion which include porous polymer beads of the type commercially used as ion-exchange and non-ionic resins of different types, and activated charcoal are described below.

1.3.1.6.1 Cation-exchange, Anion-exchange and Neutral resins

Cation-exchange resins have been investigated mainly for the removal of ammonia from blood circulation. Ammonia has been shown to be probably the most important coma producing chemical species in liver failure. The main disadvantage of resins of this type is their adverse ion-exchange reactions. Thus Amberlite IR-120, a polystyrene sulphonic acid strong cation-exchange resin is very efficient in the removal of ammonia but has been shown to sequester other cations from the blood. Lowering of the ionic concentration in blood is also known to increase ammonia toxicity (Juggi, 1973). The problems of electrolyte imbalance have been solved by modification of the resins. Thus the use of sodium, potassium, calcium and magnesium forms of the resin have been reported to be a selective and efficient method of removing ammonia from the blood without any significant disturbance in the ionic balance (Juggi, 1973).

Anion-exchange resins, too, have been shown to affect the ionic balance of the blood or plasma which is passed over them. Cholestyramine, a small size anion-exchange resin was the first material of this class which was used to remove cholephilic ions such as bile acids from the stomach. Larger sized anion-exchange resins such as Dowex 1x4 (a polystyrene-divinylbenzene copolymer), and Amberlite IRA 900 (a resin derived from trimethylamine) are being investigated for haemoperfusion. The problem of ionic imbalance is solved again by modifying the resin. Anion-exchange resins, have been demonstrated to remove toxic anions such as barbiturate, salicylate or bile acid ions in exchange for chloride and bicarbonate ions with no appreciable effect on systemic electrolyte balance (Rosenbaum, 1975a).

Uncharged hydrophobic resins have also been used for removing toxins from the blood stream. These resins have included mainly XAD-2, XAD-4 (macroreticular styrene-divinyl benzene copolymers), and XAD-7 (macroreticular acrylic ester resin). These resins have pore sizes which are large in comparison to the blood toxins and drug molecules which they are required to remove and also have very high surface areas. XAD-2 is a good adsorbent for hydrophobic molecules, such as bile acids and certain drugs, but is not a good adsorbent for more water soluble materials such as bromosulphophthalein and salicylates (Willson, 1975). The chemically identical XAD-4 due to its higher surface area has an increased adsorptive capacity for lipid soluble molecules. Haemoperfusion with XAD-2 on patients with life-threatening acute drug intoxication was reported to be associated with high success rate but with adverse affects on blood components (Rosenbaum, 1975b).

XAD-7 has been found to be capable of adsorbing a wide range of compounds. Due to their hydrophobicity they can be used to adsorb through hydrophobic interactions when used in polar solvents (Paleos, 1969). Adsorption of compounds by this resin has been well studied (Dunlop et al., 1975; Simpson, 1972). It has been reported that this resin is more efficient at removing bilirubin than XAD-2 and has a similar capacity with regard to the bile acids (Dunlop et al., 1975).

1.3.1.6.2 Activated Charcoal

Activated charcoal has a highly porous nature and high internal surface area (upto 1500 square metres per gram) and therefore a large adsorption capacity. Two major problems associated with direct haemoperfusion over activated carbon are carbon microparticles and blood incompatibility (Andrade et al., 1975). The microparticles are in part due to the highly porous nature of activated carbon, which makes it inherently fragile. Other additional problems are the ash content and ion-exchange capacity of some carbons, which can result in electrolyte imbalance in perfused blood. One obvious approach to reduce particle generation without coating could be to use activated carbons characterised by a high surface hardness. The criteria for activated carbon selection have been described (Denti and Walker, 1980). Second approach is to coat the adsorbent with polymeric membranes. This, as will be discussed later, also helps to improve biocompatibility. Another approach to reduce particle generation is immobilising the charcoal granules on a special adhesive tape coiled and inserted in a cylindrical container.

Activated charcoal has been shown to be an effective adsorbent to remove a wide range of endogenous and exogenous toxins (Yatzidis, 1964). It has been found that the type of substances adsorbed by charcoal depends on several factors including the size of the internal pores which allow access to the binding sites (Dunlop et al., 1975). Highly fibrous biocompatible carbon fibre adsorbents having good toxic material adsorption properties have been recently reported (Morozova et al., 1984). Adsorption of many different substances such as the amino acids, bilirubin, urea, uric acid, creatinine and drugs such as paracetamol on activated charcoal has been studied. It has been found that water soluble toxins such as amino acids and paracetamol are well adsorbed while the protein-bound toxins such as bilirubin are poorly adsorbed from plasma. The criterion for good adsorption appears to be polarisability of the molecule as distinct from the possession of a permanent dipole (Sing, 1973). Large pore charcoal may adsorb 100 mg of bilirubin per gram from chloroform solution but only 5 mg per gram bilirubin is adsorbed from plasma.

1.3.1.6.3 Biocompatibility of Resins and Activated Charcoal

The problem of resin and charcoal biocompatiblity has been solved by either physically separating the adsorbent with a biocompatible polymer; or by perfusing the adsorbent with plasma only, the formed elements having been removed by continuous centrifugation.

The method of perfusing adsorbents with plasma only is not as impracticable as it seems. The technology has already been developed for the treatment of leukemia, in which white blood cells are selectively removed or irradiated on a continuous basis with continuous flow celltrifuge (Dunlop et al., 1975). The technique of passage of the plasma over adsorbents to remove toxic substances in the absence of cellular elements of blood was first introduced in 1967 (Lepore and Martel, 1967). Since then some investigations have been undertaken in this field (Hughes and Williams, 1980). Unlike exchange transfusion of whole blood, it does not cause haematological problems and for that reason it appears a rational treatment modality for acute liver failure (Inoue et al., 1981).

The potential advantage of plasma exchange is that not only the removal of protein-bound toxins is possible but also with the addition of normal plasma, substances lacking in fulminant hepatic failure such as clotting factors, hormones and co-factors will be replaced (Hughes and Williams, 1980). The use of plasma perfusion over adsorbents also eliminates the possibility of citrate toxicity and the possiblity of hepatitis infection from the large doses of donor plasma required. Initial studies on plasma perfusion of XAD-2 with dogs showed that platelet loss was not significantly different from whole blood perfusion of the same resin (Weston et al., 1975a). By using the same system on two patients with chronic liver failure a clearance of bile acids upto 2.5 litres per hour was obtained with plasma perfusion of XAD-7 (Weston et al., 1975b).

The recent development of hollow fibre membrane plasma separators could be an improvement in this technique (Inoue et al., 1981). Two patients with fulminant hepatic failure have been treated by passing plasma continuously through both charcoal and an anion-exchange resin without a significantly detectable change in blood cellular components (Asanuma et al., 1979). Plasma sorptive detoxification has recently been used clinically on 9 patients with chronic liver disease (Smith et al., 1983). Significant removal of cholylglycine and bilirubin, and reduced pruritis, with long-term effectiveness has been reported (Smith et al., 1983). However, further evaluation of this technique is needed, particularly with regard to detailed studies on biocompatibility. If this proves satisfactory, then the perfusion of plasma through a wide range of adsorbents should produce efficient detoxification systems for the treatment of acute liver failure. The use of membrane plasma separation is a promising patient support technology also for chronic liver failure.

The method of increasing biocompatibility of adsorbents by coating them with biocompatible polymers has been widely investigated. Using the principle of artificial cells (Chang, 1964, 1966, 1972) artificial cell membranes can be formed using a variety of synthetic materials. Thus unlimited extensions in membrane materials and configurations could be used for charcoal haemoperfusion (Chang, 1982). Various types of polymer membranes with different permeability characteristics have been used both for resins and activated charcoal. A complete list of coatings that have been used to coat activated carbons to improve their biocompatibility is given in table 1.2. Characteristics of an ideal coating system have been defined as being:

(i) The coating must be strong enough to eliminate all fragmentation and generation of micro-emboli.

(ii) The coating must be freely permeable to the toxins of interest.

(iii) The coating must be tolerable, particularly with respect to the adhesion of blood cellular elements.

(iv) The coating must permit good flow and low pressure drops in the column.

(v) The coating must be readily sterilisable, non-toxic and non pyrogenic.

Coated activated charcoal adsorbent haemoperfusion has been tried in several patients. One of the biggest clinical trials have been carried out at King's College Hospital on patients suffering from fulminant hepatic failure. Despite high initial success rates this means of treatment had to be discontinued as the initial success rate was not maintained (Trewby, 1975). Poly (hydroxyethyl methacrylate) coated charcoal is used throughout western Europe for cases of drug overdose. An ultrathin cellulose coated system developed in Japan has also been used clinically (Odaka et al., 1980).

Table 1.2

Membrane coatings used for activated charcoal

(a) Albumin (adsorbed) (Herbert et al., 1964)

(b) Albumin (adsorbed on cellulose nitrate) (Chang, 1969)

(c) Albumin (crosslinked) (Andrade, 1971)

(d) Cellulose acetate (Yatzidis, 1966; Rosenbaum et al., 1968; Koning et al., 1982)

(e) Cellulose triacetate (deacetylated) (Denti et al., 1973)

(f) Cellulose nitrate (collodion) (Chang, 1972)

(g) Dextran (adsorbed) (Herbert et al., 1965)

(h) Haemoglobin (adsorbed) (Lau et al., 1965)

(i) Heparin complexed cellulose nitrate (Chang, 1972)

(j) Hydroxyethyl cellulose (Davis et al., 1974)

(k) Methacrylate copolymers (Gilchrist et al., 1975)

(l) Nylon (Chang, 1972)

(m) Poly (hydroxyethyl methacrylate) (Wilson et al., 1973; Langley et al., 1975)

(n) Poly (acrylic acid and styrene copolymer) (Sparks and Robert, 1976)

(o) Poly (acrylonitrile) and Acrylonitrile methyl acrylate copolymer (Morishita and Fukushima, 1976; Morishita et al., 1976)

(p) Poly (acrylic acid butyl methacrylate) (Courtney et al., 1975)

Table 1.2 (continued)

(q) Cross-linked gelatin (Wu et al., 1980)

(r) Silicone and low molecular weight poly (ethylene glycol) (Piskin et al., 1984)

Although coating the activated charcoal does minimise blood damage, it also reduces the rate of uptake of solutes from the blood. Thus in considering the coating by polymers, a compromise has to be made between, on the one hand, the biocompatibility of the system, and on the other, the reduction in the rate of uptake of solutes (Langley et al., 1975).

It has been shown that some polymer coatings lower the overall capacity for adsorption of the activated charcoal, either by blocking active sites on the surface of the charcoal, or by entirely blocking some of the pores which allow access to the internal structure of the charcoal granule. However, equilibrium adsorption isotherms of paracetamol onto uncoated charcoal and to the various coated charcoals are all very similar showing that the polymer does not affect the total capacity of the charcoal for adsorption (Langley et al., 1975). An alternative coating method which does not decrease the adsorption capacity of activated charcoal has recently been reported (Piskin et al., 1984).

1.3.1.7 Affinity Chromatography

This technique was developed for removing protein bound toxins. Agarose-albumin systems have been developed in which the high affinity of albumin for bilirubin is made use of by binding albumin to an inert support such as sepharose and perfusing blood through this combination. This has recently been shown to remove bilirubin in rats (Plotz et al., 1974). The agarose-albumin gels while removing bilirubin efficiently from plasma or whole blood could also be regenerated easily using aqueous ethanol (Plotz et al., 1975). Other compounds removed by this technique include digitoxin, chenodeoxycholate, bromosulphophthalein, and indocyanine green.

Biocompatiblity studies carried out on the system in-vitro showed that blood cells, clotting factors and proteins were virtually unchanged by passage over albumin-agarose gels. The most recent experiments on higher animals (monkeys) have been unsuccessful. However, the weight of albumin-agarose needed to remove significant quantities of toxins in man would probably be too large in terms of expense and volume of blood needed to prime the column of adsorbent. Albumin as an adsorbent for bilirubin would be attractive if a less bulky support system were devised.

1.3.1.8 Hepatic Transplantation

The idea of hepatic transplantation can be thought of as being the most obvious and logical means of treating a patient with liver failure. The definitive therapy of most types of liver disease, particularly if chronic, leaves much to be desired. Transplantation of the liver is a recent form of therapy. The first transplant of a liver in dogs was done in 1955 (Welch, 1955). The first human transplant was performed in 1964 and the recipient lived for 16 days (Absolon et al., 1965). By 1974, 40 transplant teams had performed 227 liver transplants on 219 recipients. After two decades of intensive effort, both in the laboratory and clinically the problems facing hepatic transplantation are unsurmountable. The main two problems being to find a suitable cadaveric donor at the right time, and the bleeding. The immediate post-operative mortality is around 30 percent (leading article, 1974). Deaths are due to such complications as massive haemorrhage, occlusion of the hepatic artery, or portal vein, or cardiac arrest.

At the outset of this therapeutic procedure it was anticipated that rejection of the transplanted liver would be the major complication, but sepsis and biliary tract obstruction and fistulas have assumed equal importance. The increased risk of new hepatic tumours, particularly lymphomas, in transplant recipient is now well recognised and one probable case of reticulum cell sarcoma has already been reported (Williams et al., 1973). At the present time the chances of long-term survival are not sufficiently high for the procedure to be generally recommended, except in a very few special cases and in well-organised centres. At present the recipient of a new liver can be regarded as fortunate if he survives 6 months post-operatively (Sherlock, 1975).

Another procedure which has been investigated and linked to the above mentioned homotransplantation procedure is the auxiliary 'heterotropic' hepatic homografts. Here the donor liver is transplanted but the recipient's diseased liver is left in situ. This technique has been used in experimental animals (Slapak et al., 1970) and also in man (Fortner et al., 1970). The procedure may particularly be of value as a method of temporary hepatic assist in potentially reversible liver failure. It avoids a technically exacting hepatectomy in a gravely ill patient. Moreover, the retention of autogenous liver tissue may be of value if the cadaverically obtained transplanted liver is rejected.

The overall poor results of hepatic transplantation necessitate the continuation of research in discovering alternative means for providing artificial liver support to patients suffering from liver failure. Even in liver transplant opportunity for treatment with artificial liver support arises during the post-transplant period if the graft does not quickly regain its function, or becomes insufficient some time later by rejection episodes or other circulatory disturbances. Certainly, effective support would improve the survival rate after liver transplant and would thus justify enlargement of the spectrum of indication for such treatment (Pichlmayr et al., 1981). The technical achievement of liver transplantation has in part stemmed from the development of the isolated perfused liver (Ritchie and Hardcastle, 1973). Therefore further research in such areas could also contribute more to make hepatic transplantation more successful.

1.3.2 Artificial Liver Support Systems Based on the Use of Elements of the Liver

This approach of liver support aims at the provision of specific liver functions with the use of perfusion systems containing cultured liver cells or slices, and in another known hepatic microsomal enzymes immobilised to inert support materials. This approach may be regarded as something of a return to the earlier approach of using the liver of another organism for support but it has certain advantages over the previous approach. The specificity and greater safety offered by the artificial liver support devices of this category would be expected to result in greater efficiency in the removal of toxins. In this approach the following three techniques have been adopted for the detoxification of blood:

1.3.2.1 Gell-Entraped Liver Cell Microsomes

This involves the entrapping of microsomes from endoplasmic reticulum of liver cells using hydrogels. Liver cell microsomes play an important part in the metabolism of endogenous and exogenous compounds. These microsomes are obtained from liver cell homogenate and are relatively unstable. However, the stability of microsomal enzymes is often improved by their inclusion in cross-linked polymeric matrices. Entrapment of animal liver microsomes in gamma-ray crosslinked polyvinyl pyrrolidone with stabilisation of some enzymatic activities has been demonstrated (Denti and Luboz, 1975).

1.3.2.2 Microsomal Enzymes Bound to Artificial Carriers

The principle of this technique is to eliminate unwanted substances alone from the blood, extracorporeally, by a route which is close to the physiological pathway. This method utilises hydrogels in the form of inert supports to which hepatic microsomal enzymes are immobilised. Hepatic enzymes immobilised on hydrophilic support materials have been experimentally demonstrated to remove toxins (Brunner, 1975). The removal of toxins by using immobilised enzymes will be discussed in detail in chapter 7 which is entirely devoted to the enzymatic detoxification systems.

1.3.2.3 Haemoperfusion Through Liver Cells in Tissue Cultures

The ability of liver cells to grow in culture and propagation of such cell lines is made use of in this method. Liver tissue cultures are incorporated into extracorporeal "artificial livers". Both the synthetic and excretory potential of cultured hepatocytes are utilised in treating the patients. Recent development in this field (Wolf, 1981) has been the development of the artificial capillary cultured cell liver assist device (LAD). This device consists of a tissue-like mass of hepatic cultured cells grown in-vitro on the exterior surfaces of numerous synthetic capillaries made of semipermeable membrane and bunched compactly together within an enclosing shell. Nutrient culture medium is circulated within the capillary lumens during formation of the device by proliferation of the cells. Host animal blood is subsequently circulated through them during its use as an LAD. With experiments on rats, LAD cells have been shown to carry out bilirubin conjugation and excretion of the bilirubin diglucuronide (Wolf, 1981).

1.4 ESSENTIALS OF HAEMOPERFUSION SYSTEMS

The design and development of any haemoperfusion device poses many complex design problems. The work of Dunlop and Langley (1975) indicates the difficulties posed by just one of these problems, that is, the simple mechanical problems in relation to packed bed perfusion columns. The scale of the problems involved can be appreciated by reference to Table 1.3. This shows some of the criteria which must be satisfied in order to design, develop and produce a sterile haemoperfusion column (Fenninmore and Munro, 1975).

Table 1.3

Design criteria for a haemoperfusion column

Performance	Adsorption Kinetics
	Haemodynamics
Safety	Biocompatibility
	Microbiology
	Toxicology
Stability	Chemical and Physicochemical
	Mechanical
Ease of Fabrication	Process reproducibility
	Process and Product quality control
Ease of Use	Minimal in-use preparation

These criteria will be considered individually below, followed by an assessment as to how far the expectations in haemoperfusion for treating hepatic failure have been achieved.

1.4.1 Performance

The column must adsorb from the blood a clinically significant quantity of toxic substance and must do so in an appropriate time. This must be achieved without subjecting blood to physical and chemical conditions which would produce unacceptable blood damage.

1.4.2 Safety

Not only must there be minimal blood damage but the patient being perfused must not be exposed to hazards such as microbial and pyrogenic contaminants. Also toxicological hazards may occur due to elution of other materials (either particulate or chemical) into the blood stream or due to the undesirable uptake of essential blood constituents. From the aspect of the use of haemoperfusion in liver failure, the removal of necessary circulating substances and the occurence of embolism are not desired.

Bruck has listed the effects that materials that are to be useful for blood-contacting biomedical applications should not cause as being: (i) thrombosis, (ii) destruction of the cellular elements of the blood, (iii) alteration of the plasma proteins, (iv) destruction of enzymes, (v) depletion of electrolytes, (vi) adverse immune reactions, (vii) toxic and allergic reactions (Bruck, 1975). These factors must be considered in the design and development of adsorbent materials for use in haemoperfusion. Materials used in the construction of the column must be capable of undergoing normal fabrication procedures including sterilisation, which must not then introduce further hazards due to changes thereby produced in material properties.

1.4.3 Stability

Materials and processes must be chosen in such a way as to combine to produce a product which is chemically and physically stable over an acceptable shelf life period. An important constituent of a stable product, the pack in which it is presented and transported, is often neglected.

1.4.4 Ease of Fabrication

Processes must be designed in such a way that they can be operated in a reproducible manner by normally competent personnel. This implies the imposition of adequate internal standards both for processes and product, quite apart from any relevant external product standards which must also be considered.

1.4.5 Ease of Use

Since a feature of haemoperfusion is its relative simplicity in use compared with other medical techniques, such as haemodialysis (requiring considerable instrumentation and training), it is essential to retain this feature by designing a product which requires minimal in-use preparation.

Currently available haemoperfusion systems are easy to use and of relatively low cost. It is clear that in most instances of serious toxic ingestions, haemoperfusion does in fact provide the best available therapy. Comparing haemoperfusion to other facilitated drug removal treatments, for example peritoneal or haemodialysis reveals that haemoperfusion is two to tenfold superior in the removal of toxins. Animal studies have shown that dogs recovered significantly faster from coma if treated with haemoperfusion compared to other modalities (Medd et al., 1973). Clinical reports over the last 20 years since the first attempt to treat hepatic coma (Chang, 1976) and then the first apparently successful clinical trial (Gazzard et al., 1974) have been quite variable. None of the studies reported on the reversal of hepatic coma are in fact controlled studies thus preventing any solidly based determination of the benefits of haemoperfusion in the reversal of coma (Gelfand, 1982).

The haemoperfusion devices should be compatible within the guidelines set for safety of intravenous fluids and devices coming in contact with circulation. A commercially available acrylic hydrogel-coated column has been demonstrated to have no significant release of particulate material (Fennimore et al., 1974). Other columns have been similarly demonstrated to release the particulate material within the guidelines establised by the British Standards for infusion fluids. The major continuing problem with some haemoperfusion devices is the loss of platelets as a result of this procedure. Although a reasonably biocompatible material, some of the coated or uncoated adsorbents currently in use are nevertheless inherently destructive of platelets which soon thereafter are removed from the circulation. For the most part, in the treatment of toxic ingestions the platelet levels return to haemodialysis reveals that haemoperfusion is two to tenfold superior in the removal of toxins. Animal studies have shown that dogs recovered significantly faster from coma if treated with haemoperfusion compared to other modalities (Medd et al., 1973). Clinical reports over the last 20 years since the first attempt to treat hepatic coma (Chang, 1976) and then the first apparently successful clinical trial (Gazzard et al., 1974) have been quite variable. None of the studies reported on the reversal of hepatic coma are in fact controlled studies thus preventing any solidly based determination of the benefits of haemoperfusion in the reversal of coma (Gelfand, 1982).

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Of special interest in the area of haemoperfusion in the coma of hepatic failure, however, is the additional complication that the patients arrive with already low platelet counts and deficiencies in circulating clotting factors. To overcome these problems the infusion of fresh frozen plasma and platelets at the end of each haemoperfusion procedure has been recommended for patients with hepatic coma (Gelfand et al., 1976).

Although the expectations of the early proponents of haemoperfusion in the treatment of hepatic failure were considerably more restrained than those of early proponents of haemoperfusion in acute ingestions, even these modest expectations have been only partially achieved by clinical results to date. Nevertheless, it is reasonably clear that heamoperfusion is potentially capable of removing a variety of toxins which have been implicated in the pathogenesis of hepatic coma. The chemical agents which are found in abnormal concentrations in patients with hepatic failure will be the subject of discussion of next section.

1.5 POTENTIAL TOXINS AND MARKERS IN LIVER FAILURE

Chemical injury to the liver has many facets. A large number of chemical and biological agents can induce hepatic injury. All agents that can produce hepatic injury might be defined as hepatotoxins. Some hepatotoxins are products of plant or fungal or bacterial metabolism. Some are minerals. Many are products, by-products, or wastes of the chemical or pharmaceutical industry (Zimmerman, 1982). However, with regard to fulminant hepatic failure medicinal agents (drugs) are the most important exogenous toxins. Fulminant hepatic failure is characterised biochemically by a dramatically altered compostion of blood plasma, the most obvious abnormality being the presence in large amounts of several classes of endogenous toxins. The toxicity of these endogenous substances is such that one drop of serum from a patient in fulminant hepatic failure can kill liver cells in culture (Hughes et al., 1976). Although the exact nature of the toxic metabolites that accumulate in the circulation and form the basis of the encephalopathy is uncertain, they are likely to include low to middle molecular weight compounds that are either water soluble or bound to plasma proteins.

Experimental data suggest that known potential toxins listed in table 1.4; possibly in combination with other factors (which have not yet been identified) may be the cause of hepatic encephalopathy.

Table 1.4

Potential Toxins in Liver Failure

1. Amino acids
2. Ammonia
3. Mercaptans
4. Free fatty acids
5. Free phenols
6. Bilirubin
7. Bile acids
8. "False" neurotransmitters octopamine and phenylethanolamine

The mechanism and the extent by which the potential toxins are thought to contribute to the syndrome of hepatic failure will now be dicussed.

1.5.1 Amino Acids

Abnormal concentrations of amino acids in plasma, spinal fluid and the brain are a consistent finding in patients with hepatic failure. Increased concentrations of methionine, phenylalanine, tyrosine and unbound tryptophan are among the most prominent changes in plasma (Iob et al., 1966; Record et al., 1976; Rosen et al., 1977; Morgan et al., 1982). Because the aromatic amino acids compete at the blood-brain barrier for the same carrier-mediated transport system as do the branched chain neutral amino acids, high plasma concentrations of the aromatic amino acids could limit the cerebral uptake of essential branched chain neutral amino acids in patients with hepatic encephalopathy. It was found that the severity of hepatic encephalopathy correlated inversely with the molar concentration ratio [(leucine+isoleucine+valine)/(phenylalanine+tyrosine)] in plasma (Fischer et al., 1976). However, in a large series of patients with liver disease of varying aetiology and severity, a reduction of the above ratio was observed to be secondary to liver disease but was independent of the presence of hepatic encephalopathy (Morgan et al., 1978). Thus it seems unlikely than an imbalance between branched chain and aromatic amino acids in either plasma or brain causes the encephalopathy.

Some amino acids - aspartate, glutamate and arginine, have particular theoretical, if not practical significance, because they have arousal properties in patients with hepatic encephalopathy; they are coma-preventing (Zieve, 1975).

1.5.2 Ammonia, Mercaptans, Fatty Acids

Ammonia is constantly being generated in the brain by the deamination of amino acids, neurotransmitter amines, purines, and other nitrogenous substrates, and by the deamination of glutamine. The concentration of ammonia in the brain is higher than that in blood or spinal fluid and appears to be closely linked to the level of neural activity (Duffy and Plum, 1982). Normally, ammonia is metabolised by hepatic enzymes to urea.

However, in liver failure this process is interrupted and the concentration of ammonia in the body rises (Rafelson and Binkley, 1968). It is well recognised that disturbed ammonia metabolism is associated with hepatic encephalopathy. A great deal of evidence shows that pure ammonia intoxication produces a state which results in convulsions and coma (Zieve, 1975). However, ammonia alone cannot account entirely for the encephalopathy seen in a typical case. In general the correlation between blood ammonia and the degree of hepatic encephalopathy is poor, though positive.

Like ammonia, mercaptans that accumulate during hepatic failure come largely from the gut. From breath analysis it was found that there was four fold increase of methanethiol and a two and a half fold increase of ethanethiol in patients with hepatic coma (Chen et al., 1970). An increase in breath of dimethyl sulphide following the ingestion of methionine was also discovered.

Mercaptans are highly neurotoxic (Ljunggren and Norberg, 1943) and the most toxic of them methanethiol in its lethality is comparable to that of cyanide. That extremely small amounts of the thiol produce toxic manifestations including coma was found from experiments on animals. Blood methanethiol concentrations have been measured in patients with hepatic coma. It was found that as the grade of encephalopathy worsens, the methanethiol concentration generally rises. In 13 consecutive patients who died in hepatic coma, 11 had blood methanethiol changes that correlated closely with the clinical deterioration (Zieve, 1981). Only one half of these patients had changes consistent with clinical deterioration.

Plasma free fatty acid levels are substantially elevated in patients with chronic or fulminant hepatic failure and the increased concentrations of short-chain fatty acids in particular have been linked to the production of encephalopathy of liver failure (Mortiaux and Dawson, 1961; Mays, 1972). Short chain fatty acids are generated in the intestinal tract by bacterial metabolism of the lipid contents of the intestine and by the incomplete oxidation of long-chain fatty acids by the diseased liver. Infusion of short chain fatty acids into animals induces a reversible state of coma accompanied by electroencephalographic changes that resemble those seen in human hepatic coma. Increase in long chain fatty acids was also observed in serum in liver failure, though this was highly variable and could not be correlated with the degree of encephalopathy (Zieve, 1975).

Biochemically, free fatty acids have widespread metabolic effects at very low concentrations such as the inhibition of enzymes and uncoupling oxidative phosphorylation. Normally at low concentrations fatty acids in the blood are not toxic because they are bound to the plasma protein albumin.

The coma producing potential of toxic substances may be multiplied several fold when they are present together, as a result of their interdependent metabolism or interrelated effects. Three of the substances that have shown to act synergistically are ammonia, mercaptans and free fatty acids (Zieve, 1981). Experimental studies have shown that mercaptans enhance the toxicity of ammonia and fatty acids. At pathological concentrations, fatty acids interfere with the disposition of ammonia (Derr and Zieve, 1976) and they augment the coma potential of both ammonia and mercaptans (Zieve, 1981). Increased plasma concentrations of free fatty acids may also contribute to the neurotoxicity of tryptophan by reducing the amount of circulating tryptophan that is bound to albumin (Duffy and Plum, 1982).

1.5.3 Free Phenols

Sixfold increases in free phenols are found in the serum of patients with fulminant hepatic failure (Brunner et al., 1981). From the large number of phenolic substances, phenol itself causes coma when injected. When injected into rabbits phenol has been shown to cause coma at much lower dose compared to fatty acids and ammonia. Injections of coma producing concentration of phenol when added to coma producing concentrations of either ammonium acetate or hexanoate have demonstrated that a significant synergistic effect in coma induction occurs.

Since phenol belongs to the group of lipophilic compounds and is derived from the highly increased aromatic amino acids, tyrosine and phenylalanine, a decrease in the concentration of phenols could be achieved by lowering the aromatic serum amino acid levels (Brunner et al., 1981).

1.5.4 Bilirubin and Bile Acids

Bilirubin and bile acids may be regarded as minor toxins in liver disease. However, their concentrations are raised in the syndrome of hepatic failure.

Bilirubin is a breakdown product of haemoglobin and is responsible for causing the yellow colouration of the skin in jaundice. It is lipophilic and tightly binds to albumin in the blood. Normally it is converted to diglucuronide by the liver by the action of microsomal enzymes. Bilirubin diglucuronide is water soluble and is excreted into the bile. In the event of liver failure due to inefficiency of the liver towards the above process of conjugation, high levels of unconjugated bilirubin occur. Hyperbilirubinaemia, while strikingly obvious, may be of little significance with respect to the ultimate survival of the patient (Berk et al., 1975) with acute hepatic failure. However, in the new born infant in whom the blood brain barrier is immature, unconjugated bilirubin may damage the brain if levels exceed 20mg per 100ml (Dunlop et al., 1975).

Bile acids, the end products of cholesterol metabolism are normally conjugated in the liver with amino acids glycine or taurine and excreted into the bile. Failure of conjugation with taurine and particularly with glycine occurs in the presence of hepatitis or cirrhosis (Ekdahl, 1958). In normal subjects bile acids are not detected in serum. However, in patients with liver failure bile acid levels are raised. It has been shown that the disappearance of intravenously injected bile acids is prolonged in liver failure (Horak et al., 1976). The levels of both dihydroxy (chenodeoxycholic acid, lithocholic acid) and trihydroxy (cholic acid,deoxycholic acid) are raised in chronic and acute hepatitis, obstructive jaundice and cirrhosis of the liver. Accumulation of glycocholate and taurocholate predominates in the serum of patients with fulminant hepatic failure (Horak et al., 1976).

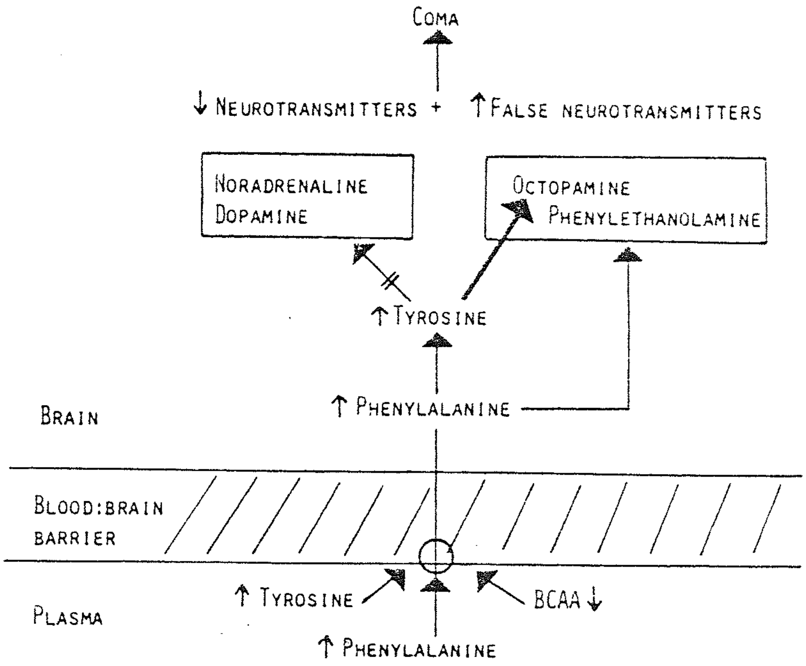
Disorders of bile excretion are sometimes accompanied by severe itching (pruritis) which results from the accumulation of bile acids in the skin (Dunlop et al., 1975).

1.5.5 "False" Neurotransmitter Hypothesis

The finding that the aromatic amino acid levels were increased and the branched chain amino acids were reduced, and that these amino acids both share the same carrier-mediated transport mechanism into the brain, led to the proposal of "false" neurotransmitter hypothesis (Fischer and Baldessarini, 1971). Figure 1.2 shows the proposed relationship between plasma levels of amino acids and hepatic encephalopathy (Hughes et al., 1981). The aromatic amines octopamine, tyramine and β-phenylethanolamine, have been proposed as "false" adrenergic neurotransmitters in hepatic coma, wih the strongest evidence implicating the noradrenaline analogue, octopamine.

Figure 1.2

Proposed relationship between plasma levels of amino acids and hepatic encephalopathy



The neurotransmitters are substances such as noradrenaline, serotonin, histamine and possibly even several highly neuroactive amino acids such as glycine, alanine, taurine, γ-aminobutyrate, glutamate and aspartate, which in addition to acetylcholine may play important roles in normal synaptic transmission in the brain and spinal cord.

Reduced competition at the blood-brain barrier allows increased entry into the brain of tyrosine and phenylalanine, the substrates for catecholamine synthesis. Saturation of the enzyme tyrosine hydroxylase, which normally converts tyrosine to dihydroxyphenylalanine (dopa), leads to preferential decarboxylation of tyrosine to tyramine, followed by β-hydroxylation to the "false" neurotransmitter octopamine. By a similar mechanism phenylalanine can be converted to phenylethanolamine. This process is further enhanced by end-product inhibition of the normal pathway by the "false" neurotransmitters and inhibition by excess phenylalanine and free tryptophan. Now octopamine and β-phenylethanolamine are weak neurotransmitters with approximately 1/50th of the sympathomimetic activity of noradrenaline and they might, in excess, displace noradrenaline at nerve endings and depress cerebral function.

Patients with hepatic coma excrete tyramine and octopamine in urine in increased amounts and there is a good correlation between the serum octopamine concentration and the degree of neurological disability in such patients (Manghani et al., 1975). Concentrations of octopamine and β-phenylethanolamine are seen to increase in brain and spinal fluid in animal models of liver failure (James et al., 1976), and the brain concentrations of these "false" neurotransmitters rise prior to increase in blood levels.

An additional factor, cited in support of the "false" neurotransmitter hypothesis is that the administration of dopa will lighten the coma of some patients with hepatic failure (Fischer and Baldessarini, 1971). From the above observations the changes implicit in the "false" neurotransmitter hypothesis might have some role in the pathogenesis of fulminant hepatic failure. Certainly, recent findings in a surgical model of liver failure in the pig tend to confirm the expected brain amine disturbances (Hughes et al., 1979).

1.5.6 Markers in Liver Failure

When a patient's liver fails several histological, chemical, biochemical and enzymatic changes take place in the liver. Consequently, these changes form the basis of tests which are used for the evaluation of liver injury. Many tests have been proposed in the past years for the quantification of specific hepatic functions (Molino et al., 1983).

The most sensitive index to functional abnormality of liver is a study of the clearance of exogenous or endogenous substances primarily removed from the circulation by the liver. Altered hepatic blood flow, hepatocyte dysfunction, or abnormalities of the biliary passages cause a decrease in clearance capacity. The ability of the liver to excrete foreign dyes has been a measure of hepatic function for a half century (Zimmerman, 1982). The test substance which has been most widely used for this purpose in the past is bromosulphophthalein (BSP). More recently indocyanine green (ICG) has been employed. These dyes are assumed to be treated in the liver in the same manner as naturally occuring organic anions. This is generally true with regard to uptake, intracellular binding, and biliary excretion, but certain differences in the metabolic transformation (for example, conjugation of dyes as compared to endogenous anions) are recognised. However, ICG can be excreted by the liver without conjugation. The sensitivity in detecting abnormalities in liver function for BSP and ICG is the same if a dose is used that approaches the maximium uptake capacity.

Dye clearance is limited by its invasivenss and occasional untoward reactions. In extending the clearance principle, attention has been focussed on non-invasive methodology. This has involved studies on the clearance of endogenous metabolites. Bilirubin clearance studies, in which the ability to clear the blood of an administered bilirubin load is measured, have found special but limited usefulness in the study of the effects of agents on uptake, conjugation and excretion of bilirubin (Vogin et al., 1967) by the hepatocyte.

Bile acid clearance and plasma bile acid levels have recently been used to measure hepatic dysfunction and injury. Thus far however, there appears to have been little application of these tests to experimental hepatotoxicology (Zimmerman, 1978). Release into the blood of substances normally found in the liver such as iron, vitamin B_{12}, and of proteins released by the damaged liver such as enzymes are also useful tools for the study of hepatotoxicity.

However, all the potential toxins described previously form useful markers (at least experimentally) for the evaluation of adsorbents for haemoperfusion if suitable tests for their analysis can be found.

1.6 HYDROGELS, THEIR PREPARATION AND PROPERTIES

A hydrogel can be defined as a polymeric material which exhibits the ability to swell in water and retain a significant fraction (for example greater than 20 percent) of water within its structure, but which will not dissolve in water. Included in this definition are a wide variety of natural and synthetic materials, for example, gelatin, polysaccharides, crosslinked acrylamide polymers, polyelectrolyte complexes etc. Hydrogels have been classified into three main types (Tighe et al., 1971):

(a) Non-ionic hydrogels in which the network is formed by primary chemical bonds between chains of macromolecules. Hydrogels of poly (hydroxyethyl methacrylate) crosslinked with ethylene dimethacrylate are examples of this type.

(b) Second type includes those hydrogels in which the three dimensional structure is held together by weak secondary forces. These are called the thermally reversible hydrogels. For example, hydrogels of polyelectrolyte complexes. Polyelectrolyte complexes were reviewed by Bixler and Michaels (1969).

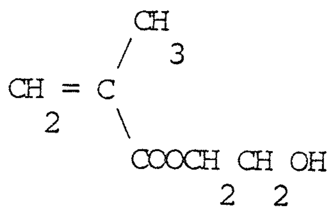
(c) Third type of hydrogels are the novel microcrystal hydrogels. These hydrogels contain discrete, colloidal particles of microcrystals. Examples are the gels of cellulose, nylon and collagen. Microcrystal hydrogels were first proposed by Battista (1950). Certain characteristics of these novel gels were described by Erdi et al. (1968).

All three types of hydrogels have found applications in the biomedical field. Preparation and properties of synthetic hydrogels have been described on the following pages. The potential of these materials as biocompatible adsorbents for Artificial Liver Support Systems will be investigated during the research.

1.6.1 Preparation of Synthetic Hydrogels

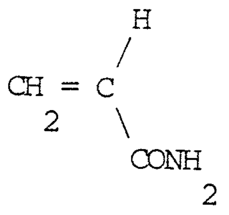
Preparation of insoluble hydrogel polymers is achieved by either copolymerisation of different monomers among the large number available or by chemical modification of preformed hydrophobic polymer networks. Before discussing the two preparatory approaches mentioned above, some important monomers used to prepare synthetic hydrogels for biomedical applications will be described.

Of particular interest in recent years have been those hydrogels derived from polymers and copolymers of methacrylic esters containing at least one hydroxyl group in the side chain. The principal monomer used for this purpose is 2-hydroxyethyl methacrylate (HEMA) whose structure is:



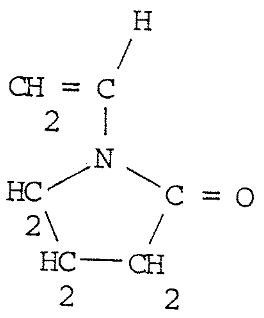
(HEMA)

Another important class of monomers includes substituted and unsubstituted acrylamides and methacrylamides. The parent compound of this class acrylamide (ACR) has the following structure:



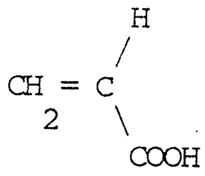
(ACR)

N-vinyl lactams comprise a further class of monomers used for preparing synthetic hydrogels for various biomedical uses.N-vinyl pyrrolidone (NVP) is an example of this class of monomers. The structure of NVP is given below:

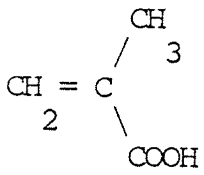


(NVP)

Several other monomers possessing acidic and basic functional groups are available thus extending the range of properties attainable in the hydrogel polymers. Most important acidic monomers are acrylic acid (AA) and methacrylic acid (MAA) which have the following structures:

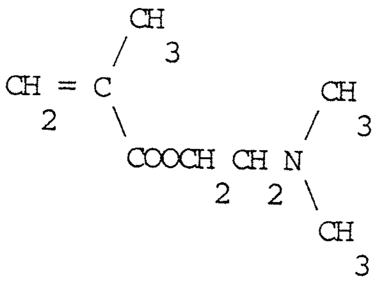

$CH_2=C(H)COOH$

(AA)


$CH_2=C(CH_3)COOH$

(MAA)

Important basic monomers belong to the class of N-substituted amino acrylates and methacrylates. Dimethylaminoethyl methacrylate (DMAEMA) is an important monomer of this class with the following structure:

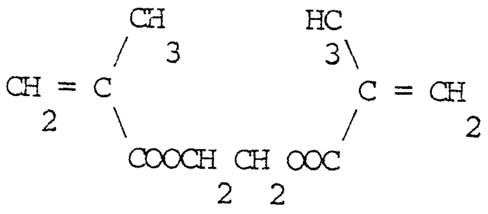

$CH_2=C(CH_3)COOCH_2CH_2N(CH_3)_2$

(DMAEMA)

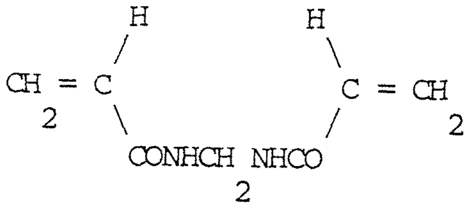
Complete list of monomers to be used in this research will be provided in chapter 2.

1.6.1.1 Preparation of Synthetic Hydrogels by Copolymerisation of Monomers

Preparation is normally carried out in the form of three-dimensional crosslinked gels by copolymerisation of the monomers with crosslinking agents. Free-radical initiators, for example, azo compounds, peroxides, redox catalysts, photochemical systems and high energy radiation are employed for these polymerisations. Several crosslinking agents exhibiting different reactivities and hydrophilicities are available. The most widely used from the viewpoint of application are ethylene dimethacrylate (EDM) and N,N'-methylenebisacrylamide (N,N'MBA) which have the following structures.



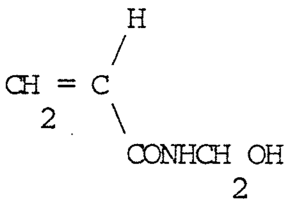
(EDM)



(N,N'MBA)

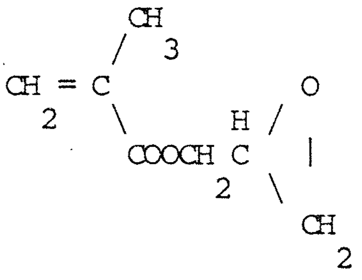
These monomers readily participate in copolymerisation reactions. This facile copolymerisation stems from their highly reactive bonds. In the presence of free-radicals, such monomers polymerise rapidly to high molecular weight polymers (Schildknecht, 1952; Schulz et al., 1954). Both EDM and N,N'MBA possess two vinyl groups each. The vinyl groups on a particular monomer have identical reactivities. These crosslinking agents like any divinyl compound, at first react with one of their double bonds; thus yielding a pendant group. The pendant vinyl group can then form a crosslink by reaction with a radical on another polymer molecule. This results in the production of crosslinked three dimensional networks in the polymer.

It is also possible to prepare crosslinked hydrogels by using a crosslinking agent having two functional groups of different reactivity. Two such monomers are N-methylol acrylamide (NMACR) and glycidyl methacrylate:



$CH_2=C(H)CONHCH_2OH$

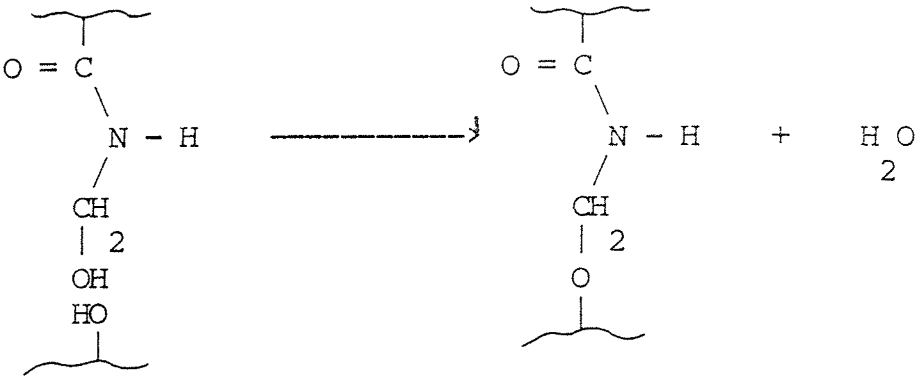
N-methylol acrylamide



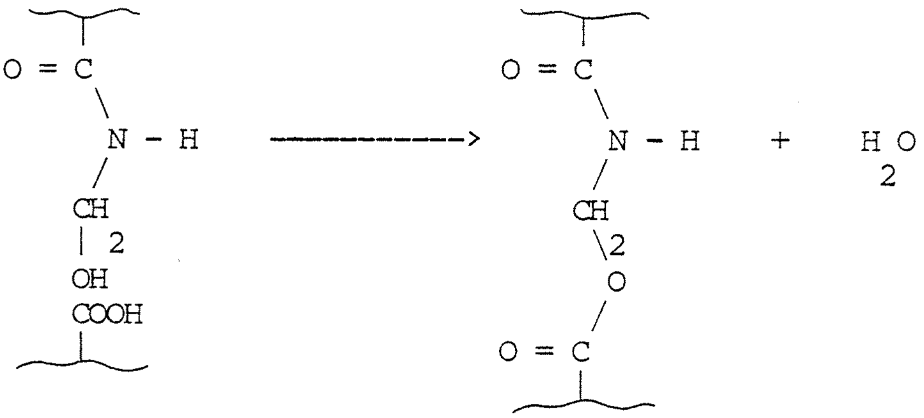
Glycidyl methacrylate

In such monomers the vinyl group can take part in polymerisation with other monomers to produce copolymers. The other functional group with a different reactivity can be activated for example, at higher temperatures to form further crosslinks. Hydrogel polymers containing N-methylol acrylamide can be prepared and crosslinks can be produced as follows:

(i)



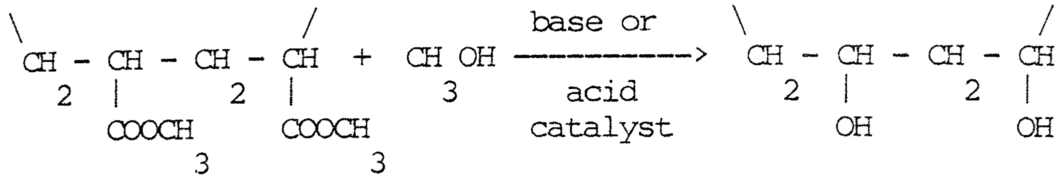
(ii)



The polymerisation processes for preparing hydrogels are performed in homogeneous media, either in bulk or in solution, or in heterogeneous media, either emulsion or suspension. Their properties such as the degree of swelling, modulus of elasticity in the swollen state, permeability and optical properties can be varied over a wide range by changing the polymerisation conditions.

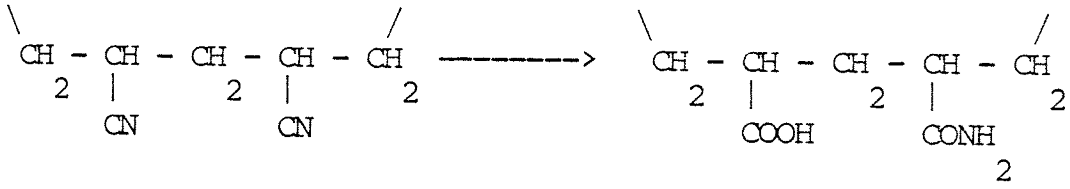
1.6.1.2 Preparation of Hydrogels by Modification of Preformed Polymers

One example of this approach for preparing synthetic hydrogels is the conversion of poly (vinyl acetate) to poly (vinyl alcohol). Poly (vinyl alcohols) are prepared from poly (vinyl acetates) or other poly (vinyl esters) by replacement of acetate groups by hydroxyl groups. The chemical process involved is alcoholysis rather than hydrolysis since the poly (vinyl acetates) do not readily react in the presence of water to give poly (vinyl alcohols). The reaction is given below:



$$\backslash\, CH_2-\underset{|\atop COOCH_3}{CH}-CH_2-\underset{|\atop COOCH_3}{CH}\,/ \;+\; CH_3OH \xrightarrow[\text{acid catalyst}]{\text{base or}} \backslash\, CH_2-\underset{|\atop OH}{CH}-CH_2-\underset{|\atop OH}{CH}\,/$$

Conversion of polymers to prepare synthetic hydrogels can also be achieved by alkaline saponification of poly (acrylonitrile). This reaction produces a copolymer of acrylic acid and acrylamide as shown below:



$$\backslash\, CH_2-\underset{|\atop CN}{CH}-CH_2-\underset{|\atop CN}{CH}-CH_2\,/ \longrightarrow \backslash\, CH_2-\underset{|\atop COOH}{CH}-CH_2-\underset{|\atop CONH_2}{CH}-CH_2\,/$$

In addition, cation-exchanging and anion-exchanging ability can be conferred on hydrogels. Hydrogels with acidic and basic functional groups can be prepared as described below:

1.6.1.3 Anionic and Cationic Hydrogels

Anionic and Cationic hydrogels are usually formed by copolymerising small amounts of anionic or cationic monomers with neutral hydrogel monomers. They can also be prepared by modifying preformed hydrogels such as by the partial hydrolysis of poly (hydroxyalkyl methacrylates).

Hydrogels having acidic, cation-exchanging ability can be prepared by copolymerisation with acrylic or methacrylic acid.

Copolymerisation of acrylic acid with n-butyl methacrylate and further modification of the formed copolymer has been reported (Courtney et al., 1973). In such copolymers the methacrylate constituent determines the mechanical characteristics and acrylic acid gives rise to water sensitive charged species. Reaction of the copolymer with ethylene oxide gas yields a highly hydrophilic hydroxyethylacrylate polymer.

Similarly basic hydrogels having anion-exchanging ability can be prepared by copolymerisation with monomers such as N-substituted amino methacrylates. Thus, copolymers of dimethylaminoethyl methacrylate have been prepared and further modified to alter the surface charge and water sensitivity. Reaction of tertiary amino group in such copolymers with ethylene oxide gas leads to the production of hydroxyethyl primary alcohol groups. The water sensitivity depends on the modification time and temperature (Gilchrist, 1982).

1.6.2 Properties of Hydrogels

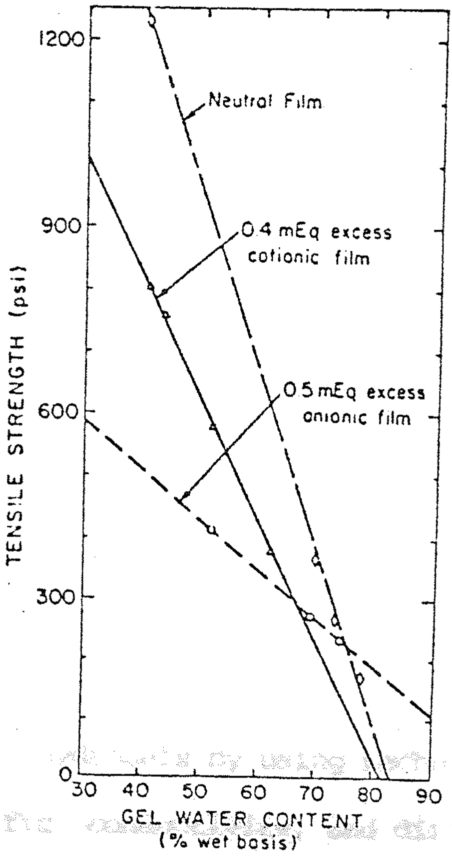
Hydrogel polymers are glossy in the dry state, and become rubberlike after swelling in water or polar solvents. It is in the hydrated state that these materials find applications in the biomedical field. Properties of hydrogels important from the viewpoint of applications in biological systems will be reviewed.

1.6.2.1 Mechanical Properties

Mechanical properties of glycol methacrylate hydrogels have been extensively studied. When dry, these hydrogels are hard and brittle. However, in the hydrated state they exhibit low tear strength and low tensile properties. Most reports in literature relate to the mechanical properties of the dehydrated hydrogel (Xerogel). However, an important relationship between the mechanical strength of the hydrogel in the hydrated state and its water content has been studied. The relationship between tensile strength and water content of several polyelectrolyte complexes is shown in figure 1.3 (Kopecek et al., 1971).

Figure 1.3

Relationship between tensile strength and water content for Ioplex 101 Film



This inverse relationship of decrease in tensile strength with increase in the water content shown for Ioplex 101 is displayed by all hydrogels. Generally, a compromise has to be made between mechanical strength and water content of a hydrogel for achieving acceptance in other properties of the hydrogel, for example, permeability to solutes.

1.6.2.2 Hydrophilicity and Water Content

Hydrophilicity is an important and essential property of hydrogels to be used in biomedical applications. Hydrophilicity of a polymer is the result of the presence of hydrophilic groups on the polymeric chains. This results in swelling of crosslinked polymers on equilibration with water. The equilibrium water content (EWC) is controllable because it depends on the hydrophilicity of the polymer structure and the experimental conditions of the preparation of the hydrogel.

The swelling of non-ionic hydrogels was found to depend on at least three factors.

(i) the interaction parameter between the polymer and solvent

(ii) the extent of crosslinking or the molecular weight of the polymer chain between crosslinks.

(iii) the relative swelling of the polymer at the time of crosslinking.

Studies on synthetic hydrogels by using techniques such as NMR, dilatometry, specific conductivity, and differential scanning calorimetry have resulted in treating water in hydrogels in terms of a three state model. Based upon such data gel water contents would be divided into X water (bulk water), Z water (bound water) and Y water (interfacial water) (Lee et al., 1975). Work conducted in this research group also suggests that there exists a continuum of water states between the two extremes of bound water and free water.

Problems concerning the organisation of water at the molecular level (water structure) are often extremely complex. However, the gross total water contents of swollen hydrogels are most easily measured and most often reported. The water at molecular level may be found to occur in the following forms: (a) polarised around charged ionic groups, (b) orientated around hydrogen bonding groups or other dipoles, (c) structured in "ice-like" configurations around hydrophobic groups, and/or (d) imbibed in large pores as "normal" bulk water.

Studies on states of water in poly (HEMA) gels suggest that the fraction of bulk, bound and interfacial water vary with the total water content of the gel. Increasing fractions of bulk water and decreasing fractions of bound water are found as the water content of the gel increases.

The nature of organisation of water within the hydrated polymeric network may be important in the biomedical applications of hydrogels. This may be one of the factors which will influence the interactions that occur between biological systems and the hydrogels.

1.6.2.3 Permeability

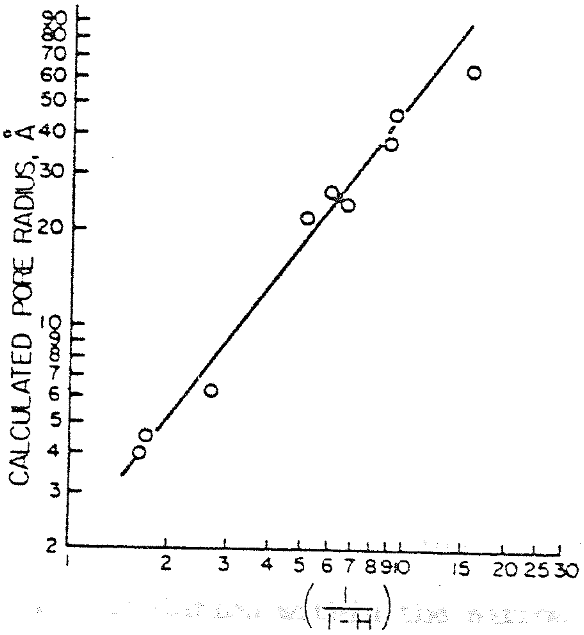
Permeability of hydrogels to water and to metabolites is an important property in their use in biomedical devices. The processes such as adsorption of toxins by an adsorbent, and the transfer of solutes across a semipermeable membrane are made possible by the membrane being permeable to the solutes concerned.

Permeability of solutes depends on several factors which include the type of water present in the gel (whether bound or free), the nature of the gel matrix (whether homogeneous or heterogeneous), and the nature of ionic charges present on the hydrogels (whether cations, anions or both). The term homogeneous gel is used when the water in the gel is homogeneously distributed throughout the structure of the gel and there are no discrete pores present. The gel in homogeneous state is generally transparent. In heterogeneous gels, the water and the gel phases form isolated clusters which make the gels opaque. True porous structure exists in heterogeneous gels. The mechanism of flow of water through the hydrogel may be viscous flow which results from the flow of water molecules in groups; or it may be diffusive flow in which water molecules flow in individual and random manner. It is thought that the transport process of water is a predominantly viscous flow in the polyelectrolyte complexes and in the more dilute glycerylmethacrylate (GMA) hydrogels (74-94 percent water), but is a predominantly diffusive flow in hydroxyethyl methacrylate (HEMA) hydrogels (40-60 percent water).

The relationship between calculated pore radii and corresponding swelling ratios for methacrylate hydrogels was established (Yasuda and Lamaze, 1971). Figure 1.4 shows the dependence of calculated pore radius on the swelling ratio for such gels which could enable to calculate average pore radii for hydrogels of different water contents.

Figure 1.4

Relationship between calculated pore radius and swelling ratio of methacrylate hydrogels.



Therefore, from the knowledge of the average pore radius and the water content, the hydrogel may be prepared which would allow the passage of desired solutes having sizes equal to or less than the average pore size in the hydrogel.

Permeability of solutes can be increased by creating the heterogeneous structure in the hydrogel resulting in the production of discrete pores or by increasing the water content of the hydrogel. However, increasing water content would result in decrease in mechanical strength of the polymer.

The type of ionic charges present in the membrane also effect the permeability. Membranes with permanent positive or negative charges have been shown to exhibit low permeability. Higher permeability is exhibited by membranes having both types of ionic charges (ampholytic membranes) (Kopecek et al., 1971).

1.6.2.4 Biocompatibility

Biocompatibility of hydrogels is the most important property for their application in biomedical devices. In fact it is the most important feature that governs the long term success of any biomaterial application. Biocompatible materials are those which are acceptable to the living physiological environment without adversely affecting the environment. However, in the exact sense, it is very difficult to define this term, since it may also have some correlation within the narrow confines of a specific material produced for a specific purpose. A number of factors which might be deleterious to the performance of materials used for long term internal biomedical applications have been intemized (Bruck, 1973a). Based upon these factors an ideal biomaterial (in terms of biological response) could be defined as one which does not cause thrombosis, destruction of cellular elements, alteration of plasma proteins, destruction of enzymes, depletion of electrolytes, adverse immune reactions, damage to adjacent tissue, cancer and/or toxic or allergic reactions. No synthetic material developed fully satisfies these criteria.

However, hydrogels can be regarded as the most tolerable biomaterials if not the most biocompatible synthetic materials. They resemble in their physical properties living tissues more so than any other class of synthetic biomaterial. Due to their resemblance to living tissues, hydrogels present a less abnormal surface for blood and tissue to interact with than other hydrophobic substances.

Biocompatibility can be regarded as being of two main types: tissue compatibility and blood compatibility. The nature of blood-polymer interaction is exceedingly complex and is far from being completely understood. This interaction involves both the activation of plasma protein clotting factors and platelet function, either of which can result in the formation of thrombus (Bruck, 1972). Mechanisms of interaction of blood with polymer surfaces and methods for studying biocompatibility were extensively reviewed (Skelly, 1979). Three specific areas concerning biocompatibility of polymers: in-vivo degradation of polymers; the effects of polymers on tissues; and the possibility of systemic effects arising from polymer implantation were recently reviewed (Williams, 1982).

The expanded nature of the hydrogel structure and its permeability to small molecules allows polymerisation initiator molecules, initiator decomposition products, polymerisation solvent molecules and other extraneous materials to be efficiently extracted from the gel network before the hydrogel is placed in contact with a living system. The in-vivo leaching of additives used during the fabrication of polymeric materials has been cited as a cause of inflammation and eventual rejection of implanted biomaterials (Homsy, 1970).

Another potential advantage of hydrogels is the low interfacial tension which may be exhibited between a hydrogel surface and an aqueous solution. This low interfacial tension should reduce the tendency of the proteins in the body fluids to adsorb and to unfold upon adsorption (Hoffman, 1974). Minimal protein interaction may be important for the biological acceptance of foreign materials as the denaturation of proteins by surfaces may serve as a trigger mechanism for the initiation of thrombosis or for other biological rejection mechanism.

Although the presence of imbibed water within a polymeric system is not a guarantee of biocompatibility, it is believed that the relatively large fraction of water within certain hydrogel materials is intrinsically related to their high biocompatibility (Bruck, 1973b). Their soft, rubbery consistency gives them strong superficial resemblance to living soft tissue. Such similarity can contribute to their biocompatibility by minimising mechanical (frictional) irritation to surrounding cells and tissue.

Hydrogels with ionic charges have been implicated as good biocompatible materials. The interest in anionic and cationic hydrogel systems stems from observations on the surface charges on blood cells, blood vessel walls, and other tissue types. Under normal conditions the blood vessel walls and blood cells have a negative charge; which is present due to its composition, that of an anionic mucopolysaccharide (Lyman et al., 1965). It is generally thought that negatively charged surfaces should be less thrombogenic than positively charged ones and evidence is available which supports this contention (Leonard, 1969). However, results indicating decreased thrombogenicity for positively charged surfaces have also been presented. It is also believed that it may be that it is the distribution of charge on the surface (surface charge density), which is an important factor in determining whether a surface is thromboresistant or thrombogenic (Ratner and Hoffman, 1976).

At the present time the importance of surface charge in blood-hydrogel interactions is not very clear.

The current three major driving forces behind the development of biomaterials namely (a) low surface free energy (b) hydrophilicity and (c) negative charge density, are not by themselves adequate explanations for biocompatibility. Undoubtedly, each of these parameters is involved, but their role must be considered as only isolated links in a series of events that arise from the interactions between the material and the dynamic and self renewing environment of the living organism (Bruck, 1972).

1.6.2.5 Optical Properties

Hydrogels are known to produce films or membranes having excellent optical clarity. This property of transparency is of great importance in the use of hydrogels in devices such as contact lenses and vitreous implants. Transparency of hydrogels depends very much on the equilibrium water content in the gel and whether the water is homogeneously dispersed throughout the gel. Therefore, any factor which affects the equilibrium water content will also affect the transparency of hydrogels (Wichterle, 1971).

If HEMA monomer containing crosslinking agent is polymerised in the presence of a good solvent (for example, ethylene glycol; ethylene glycol - water) an optically transparent (homogeneous) hydrogel is formed. If the monomer plus crosslinking agent is polymerised in a poor solvent system for the resulting polymer, an opaque, spongy, white (heterogeneous hydrogel) is formed. Ethylene glycol methacrylate (EGMA) gels remain transparent with water content upto 40 percent and propyleneglycolmonoacrylate (PGA) gels upto 50 percent, (Refojo and Yasuda, 1965a). With equilibrium water contents above these limits, hydrogels become translucent and opaque. This change from transparency to opacity has been explained by a microphase separation. Such a separation of phases produces areas within the gel of different refractive indices which result in opacity.

1.7 ROLE OF HYDROGELS IN THE BIOMEDICAL FIELD AND THEIR APPLICATION IN LIVER FAILURE

1.7.1 Role of Hydrogels in the Biomedical Field

Properties of hydrogels (most of which have been discussed in the previous section) make them the materials of choice for use in the biomedical devices. The fact that the starting raw materials for hydrogels are commercially available synthetic monomers of comparatively high purity enables a high reproducibility of properties to be achieved. Due to their very low modulus of elasticity they cause minimal mechanical irritation to the body tissues. In contrast to relatively hard, impermeable plastics, hydrogels are permeable to a wide range of molecules. Therefore, diffusion of low molecular weight metabolites and ions through the hydrogel (when implanted in the body) would occur readily.

Since hydrogels are synthetic copolymers, such materials are not attacked by micro-organisms. The high thermal and hydrolytic stability of hydrogels allows for their sterilisation by heat and chemicals. The ease with which the physical form of a hydrogel can be altered allows the physical properties of the hydrogel to be adjusted specifically for a given application.

Hydrogels of poly (glycol methacrylate) were the first materials of this class of synthetic polymers which were proposed for biomedical applications (Wichterle and Lim, 1960). Replacement of damaged tissue was the main incentive for the implication of these materials in medicine. However, the range of applications of hydrogels has increased considerably during the recent years . Hydrogels, particularly poly (HEMA) gels in the form of implants have been applied in almost all branches of surgery, including as urether prostheses (Kliment et al., 1967), bile duct prostheses (Levowitz et al., 1969), oesophagus prostheses (Levowitz et al., 1968), vascular implants (Singh, 1969), in middle ear surgery and as tympanoplastics, for corneal glaucoma drains and in plastic surgery (Kliment et al., 1968).

A number of biomedical applications of hydrogels reported in literature have been listed in table 1.5. This wide range of applications for hydrogels is made possible both due to their satisfactory performance upon in-vivo implantation in either blood contacting or tissue contacting situations and, as previously mentioned, to their ability to be fabricated into a wide range of morphologies.

Table 1.5

Potential as well as actual biomedical applications of synthetic hydrogels

Coatings:	Sutures, Catheters, Intra uterine devices, Blood Detoxicants, Sensors (electrodes), Vascular grafts, Electrophoresis cells, Cell culture substrates.
"Homogeneous" Materials:	Electrophoresis gels, Contact lenses, Artificial corneas, Vitreous humor replacements, Estrous-inducers, Breast or other Soft tissue substitutes, Burn dressings, Bone ingrowth sponges, Dentures, Ear drum plugs, Synthetic cartilages, Hemodialysis membranes, Particulate carriers of tumour antibodies.
Devices:	Enzyme therapeutic systems, Artificial organs, Drug delivery systems.

In addition to the applications of hydrogels listed above, hydrogels have also been used in the cosmetic field and in other miscellaneous applications in forms such as synthetic physiological mucus, artificial lubricants for joints in arthritis and in artificial joints, and the stabilisation of vaccines. Individual biomedical applications of hydrogels have been discussed in detail (Wichterle, 1971; Skelly, 1979). A detailed coverage of the literature relating to biomedical applications of polymers including hydrogels published during the years 1977 and 1980 has been given by Tighe (1980, 1982). Therefore, a general description of biomedical uses of hydrogels is given in this section with particular emphasis on their use in liver failure.

The use of hydrogels for external purposes requires that they must be kept in contact with body fluids so that they remain hydrated in order to function efficiently. This is the case in their application in soft lining of dentures and in contact lens applications. Hydrogels of poly (glycerol methacrylate) were suggested for possible use as corneal implants (Refojo, 1969). Advantages of using poly (HEMA) containing soft contact lens materials over hard lens materials have been reviewed (Tighe, 1981). One of the greatest advantages of poly (HEMA) not only over other synthetic biomaterials but also over other hydrogels is its stability to varying conditions of pH, temperature and tonicity.

Adsorption and desorption properties of hydrogels can be made use of in applications such as vehicles for drugs and medication. For example, a hydrogel device can be used to create a high drug concentration in the region of a tumour growth (Drobnik et al., 1974) or in a contact lens form to provide controlled release of therapeutic drugs to the eye (Refojo, 1972). Hydrogels have also been considered as vehicles for the release of antibiotics, antinarcotic agents and prostaglandins. The use of hydrogels as rectal and vaginal pessaries, as oral dosage forms, in wound healing and targetted drug delivery has recently been reviewed (Graham, 1983).

Semi-permeable property of hydrogel membranes for use in separation processes and their wide range of transport properties have resulted in the use of hydrogels as membranes in haemodialysis (Muir et al., 1971). High biocompatibility of poly (HEMA) has been used for surface treating other materials to improve their physiological tolerance, such as in the coating of surgical sutures (Singh and Melrose, 1971) and latex catheters (Miller, 1975).

1.7.2 Applications of Hydrogels in Liver Failure

One important application of hydrogels in which currently there is growing interest is the technique of haemoperfusion. Substantial progress has been made in the detoxification of blood and in the treatment of uraemia by using haemoperfusion. However, the wider application of this technique as the basis of artificial liver support systems is still faced with several problems. Attempts are constantly being made to overcome problems such as micro-emboli formation, low blood compatibility and non-specific adsorption, encountered with the use of charcoal and ion-exchange resins as adsorbents.

Hydrogels with their outstanding properties provide means for improving the existing adsorbents by producing permeable, biocompatible coatings. They are also being investigated for producing biocompatible adsorbents by polymerising the components of the membranes in particulate form. This may result in the development of more efficient haemoperfusion systems.

Several synthetic hydrogels have been investigated for achieving physical separation of the adsorbent from the formed elements of the blood. Studies have been carried out on the properties of poly (HEMA) coating of various sorts in improving the biocompatibility of activated charcoal (Willson et al., 1973; Fennimore and Munro, 1975). Another synthetic hydrogel coating comprised of a copolymer of acrylonitrile and dimethylaminoethyl methacrylate is being investigated (Lindsay et al., 1973). A further type of hydrogel membrane system under investigation for coating purposes is based on a copolymer of acrylic acid and n-butyl methacrylate (Lindsay et al., 1973).

In several cases the coating is thought to be incomplete. In order to achieve completeness of the coating its thickness has to be increased which improves the biocompatibility of the adsorbent. However, the thicker the coating the lower the rate of permeation will be, causing each haemoperfusion to be less effective. Therefore, the use of an adsorbent which is itself biocompatible remains the ideal solution. The possibility of producing a macroporous hydrogel bead having strength, blood compatibility and adsorption properties appropriate to use in haemoperfusion has been investigated (Tighe and Skelly, 1978).

In addition to the problems, mentioned previously, associated with the use of conventional adsorbents for haemoperfusion, there are other factors to be considered. For example, the haemoperfusion columns using uncoated charcoal are also susceptible to sludging and channeling which leads to pressure gradients and often reduced clearances. High heparin doses are generally required for haemoperfusion of uncoated activated carbon. The use of hydrogels in the form of biocompatible, porous particles or uniform spherical beads provide potential alternative as adsorbents for haemoperfusion systems. The porous nature of the particles would increase the internal surface area greatly, thus providing efficiency in the adsorption of toxins. The spherical particles used in the column would allow work to be conducted at high flow rates with minimum pressure losses. In addition, uniform hydrogel beads would be expected to possess similar diffusion properties.

The presence of a large number of polar reactable sites on hydrogel beads make them suitable for use in artificial liver support systems for another reason. The reactive sites on hydrogels can be used to immobilise certain enzymes by a variety of techniques. In addition, enzymes and other important biological molecules such as heparin can be entrapped within the network structure of crosslinked hydrogel beads. The enzymes bound to the insoluble hydrogel support can then be used to carry out the detoxification reactions for the removal of toxins from the circulation of a patient with liver failure. The entrapment of liver cell microsomes in crosslinked poly (vinyl pyrrolidone) has been reported (Denti and Luboz, 1975). Successful covalent binding of a microsomal enzyme to an artificial carrier on an acrylamide base has been described (Brunner, 1975), and this enzymatic system has been demonstrated to eliminate (in-vivo) phenols in rabbits.

Thus, by the use of hydrogels, the problems of poor biocompatibility, non-specific adsorption and inefficiency in the removal of toxins posed by the existing adsorbents could be overcome.

1.8 SCOPE AND OBJECTIVES OF PRESENT RESEARCH

The technique of adsorbent haemoperfusion, as a means of artificial liver support is still faced with several problems. The main problems being non-specific adsorption, poor biocompatibility, and low efficiency of currently available adsorbents. Therefore, the development of porous, biocompatible adsorbents showing some degree of specificity and good efficiency for the removal of a range of hepatic toxins is desired.

This research is concerned with the purpose-design, synthesis and evaluation of hydrogel particulate materials for use in artificial liver support systems. The techniques available for synthesising hydrogel particulates centre on conventional suspension polymerisation. However, due to the hydrophilic nature of the monomers involved, alternatives to water for the continuous phase will be investigated. By studying various synthetic techniques, it is intended to produce a range of hydrogel particulates with varying physical and chemical properties. The efficiency of adsorbents synthesised in this way for the removal of a range of chemical compounds, particularly acid species will be investigated in-vitro. Adsorption studies will be carried out using conventional static adsorption techniques. In addition, a dynamic continuous flow technique will be studied for adsorption of certain compounds.

Immobilisation of certain detoxifying enzymes on the surface of hydrogel beads will be attempted to create an enzymatic detoxification system for use in haemoperfusion. Covalent binding of enzymes in this way will be carried out to increase specificity and efficiency of the adsorbent towards certain model compounds.

CHAPTER 2

MATERIALS AND METHODS

This chapter concentrates on various techniques employed in this work to synthesise and evaluate hydrogel particulate materials. Additionally, the sources of monomers, catalysts, and other chemicals used in the course of the project are described.

2.1 MATERIALS

All the chemicals and reagents used during the research work together with their suppliers are described in this section. The solvents and reagents were used as supplied by the manufacturers unless stated otherwise. Distilled water was used except where stated.

2.1.1 Monomers and their Purification

The monomers and crosslinking agents used are listed in table 2.1. Monomers were purified by conventional methods and stored in the refrigerator.

Table 2.1

Monomers and Suppliers

Monomer	Supplier
Acrylamide	Fisons Ltd.
Acrylic acid	Koch-Light Ltd.
Diacetone acrylamide	Ubichem Ltd.
Dimethylaminoethyl methacrylate	ICI Ltd.
Ethylene dimethacrylate	BDH Ltd.

Table 2.1 continued

Hydroxyethyl methacrylate	BDH Ltd.
N-Hydroxy methacrylamide	BDH Ltd.
Methacrylic acid	Aldrich Chemical Co. Ltd.
N,N'-Methylene bis acrylamide	Cambrian Chemicals

2.1.2 Initiators, Catalysts and Enzymes

Initiators and enzymes used to catalyse the reactions are listed in table 2.2. Suppliers of these materials are also indicated.

Table 2.2

Suppliers of Initiators and Enzymes

Initiator	Supplier
Ammonium persulphate (APS)	Hopkin and Williams Ltd.
α,α'-Azobisisobutyronitrile (AZBN)	BDH Ltd.
Dimethyl amino propionitrile (DMAPN)	BDH Ltd.
Uranyl Nitrate (UN)	Sigma Ltd.

Chemical formulae of initiators and catalysts are shown in figure 2.1.

Enzyme	Supplier
3α-Hydroxysteriod Dehydrogenase (Dried cells of Pseudomonas Testosteroni)	Sigma Ltd
Urease (From Jack Beans)	Sigma Ltd

Figure 2.1

Initiatiors and Catalysts used in the project

$(NH_4)_2S_2O_8$	(APS)
$(CH_3)_2C\ CN\ N=NC\ CN\ (CH_3)_2$	(AZBN)
$N\equiv C-(CH_2)_2-N(CH_3)_2$	(DMAPN)
$UO_2(NO_3)_2$	(UN)

2.1.3 Miscellaneous Chemicals

A list of miscellaneous chemicals used in the project is given in table 2.3.

Table 2.3

Miscellaneous chemicals used in the project

Chemical	Supplier
Bilirubin (From Bovine gall stones)	Sigma Ltd.
Bromosulphophthalein (Sodium salt)	Koch-Light Ltd.
Cholic acid (Sodium salt)	Sigma Ltd.
Cyanogen bromide	Aldrich Chemical Co. Inc.
Deoxycholic acid (Sodium salt)	Sigma Ltd.
Ethylene glycol	BDH Ltd.
Glutaraldehyde	BDH Ltd.

Table 2.3 continued

Methionine	BDH Ltd.
2-Mercaptoethanol	Aldrich Chemical Co. Inc.
β-Nicotinamide adenine dinucleotide	Sigma Ltd
Ninhydrin	BDH Ltd.
Phenol	BDH Ltd.
Phenylalanine	BDH Ltd.
Pyridine	BDH Ltd.
Pyrophosphate buffer	Sigma Ltd.
Sodium chloride	Fisons Ltd.
Sodium Hydrogen Carbonate	Fisons Ltd.
Sodium Hydroxide	Cambrian Chemicals
Urea	Hopkin and Williams Ltd.
Suspension Stabilisers:	
Arlecel C (Sorbitan sesquioleate)	Honeywell-Atlas Ltd.
Hydroxypropylmethyl cellulose	British Celanese Ltd.
Span 20 (Sorbitan monolaurate)	Honeywell-Atlas Ltd.
Xanthan Gum	Sigma Ltd.

All other reagents and solvents used were of standard laboratory reagent grade.

2.2 METHODS

This section of the chapter includes a general description of the methods used to prepare hydrogel particulate materials, and the techniques employed for their evaluation. Methods used for the immobilisation of enzymes on hydrogel particulates, and characterisation of the immobilised enzyme urease have also been outlined.

2.2.1 Synthesis of Hydrogel Particulates

Synthesis of hydrogel polymer particulates is difficult because conventional methods of polymerisation, for example suspension polymerisation can not be applied directly. This is because of the hydrophilic nature of the monomers involved. The successful polymerisation of such monomers requires the use of a continuous phase in which the monomers are not miscible.

Therefore, the fundamental requirement in order to produce spherical hydrogel polymer particles is maintenance of suspension of the monomer phase in the continuous phase until such time that the polymerisation has proceeded to a stage at which insoluble polymer is produced. In this research three different techniques of polymerisation were used; Freeze-thaw polymerisation, Suspension polymerisation in brine, and Inverse-suspension polymerisation in hexane. Polymerisation processes employed were of free-radical addition type. Free-radical polymerisation was effected by either heating (thermal polymerisation) using mainly AZBN as the initiator, or by ultra-violet (UV) radiation (photopolymerisation) using UN as the photo-sensitizer. Methods used to synthesise hydrogel particulates are described below.

2.2.1.1 Freeze - Thaw Polymerisation

Hydrogel beads were prepared by making a homogeneous solution of monomers in the solvent (ethylene glycol and water mixture) containing UN initiator. This was followed by the addition of monomer solution in the form of droplets to stirred cold non-solvent (n-hexane) in a large Dewar flask. The non-solvent was kept at -70°C by the addition of powdered solid carbon dioxide. The formation of monomer droplets was caused by using either a burette or a rapid injection from an automatic syringe injector. The frozen monomer-solvent droplets were subjected to UV radiation to initiate polymerisation while stirring was maintained at a constant rate of 300 rpm After polymerisation had been completed, the beads were filtered and added to a large volume of distilled water.

2.2.1.2 Suspension Polymerisation in Brine

The technique of polymerising water soluble monomers in brine was described by Hamann and Stellwagen (1975). During this research the technique has been modified to produce hydrogel particulates. Basically, the method consisted of suspending a homogeneous solution of monomers, prepared in an organic solvent, in an aqueous continuous phase of a high salt content.

The monomer phase and the continuous phase had previously been deoxygenated by purging nitrogen through them. Stable suspension of the monomer phase in the continuous phase was formed and maintained by stirring and adding suspension stabilisers. Polymerisation was performed in a split resin flask at 70°C using AZBN as the initiator. Upon completion of polymerisation the solvent was removed form the hydrogel particulates which were then stored in a large volume of distilled water.

2.2.1.3 Inverse - Suspension Polymerisation in Hexane

This technique of synthesis resembles closely to the suspension polymerisation technique. The only differences were that the continuous phase used in this case was n-hexane, and polymerisation reaction was carried out in presence of ammonium persulphate initiator at 45°C. A saturated aqueous solution of monomers was dispersed in the continuous phase by stirring and dispersion was stabilised by adding suspension stabilisers. After polymerisation the hydrogel beads were thoroughly washed with and stored in distilled water.

2.2.2 Characterisation of Hydrogel Particulates

Various methods used to characterise hydrogel particulates are described below.

2.2.2.1 Density Measurements

The method for measuring density of hydrogel polymers has previously been described by Ng (1974). This method was adapted to determine the densities of both hydrated and dehydrated hydrogel particulates. A density gradient column was set up by using a mixture of two miscible liquids carbon tetrachloride (Density = 1.59) and xylene (Density = 0.86). Using 50 ml measuring cylinders, solutions of carbon tetrachloride and xylene in various proportions were added to a 250 ml stoppered measuring cylinder. Using a glass rod, the mixture was stirred gently without shaking (to avoid excessive mixing) and the column was allowed to equilibriate for 15 minutes. Table 2.4 lists the solutions in the order in which they were added.

Table 2.4

Volumes of carbon tetrachloride and xylene used to prepare the density gradient column

Solution Number	1	2	3	4	5	6	7	8	9	10
Volume of carbon tetrachloride (ml)	25	22	20	17	14	11	8	5	3	-
Volume of xylene (ml)	-	3	5	8	11	14	17	20	22	25

Calibration of the column was carried out by placing coloured marker floats of known densities in the column and measuring their positions at equilibrium with a cathetometer. Densities of hydrated samples were measured by wiping off surface water of beads with a soft tissue paper. The test samples were then moistened with carbon tetrachloride and gently dropped into the gradient column at 22°C. The positions of test samples were followed with the cathetometer at various time intervals, until an equilibrium was reached. Values for densities of samples were read from the calibration graph.

For measurement of densities of the dehydrated samples, they were dried in a vacuum oven at 60°C to constant weight. The dried samples were moistened with carbon tetrachloride and placed in the column at 22°C. Cathetometer readings were taken and densities of the dehydrated samples were obtained as previously described.

Calibration graphs for density gradient columns obtained for dehydrated and hydrated samples are shown in figures 2.2 and 2.3 respectively. These graphs show variation in density with height of the column. It was found in calibration of the column that density may not be linear with the height of the liquid column over the entire range. An example of this deviation from linearity is shown in figure 2.4. This could be due to some errors resulting from difficulties faced in preparing the column. These possible errors will be discussed in chapter 3 along with further description of density measurement of hydrogel polymers. For a particular polymer, densities of four different samples were measured. When a marked change in the density gradient was observed in the column, a fresh column was made.

Figure 2.2

Calibration graph for measurement of densities of dehydrated hydrogel particulates

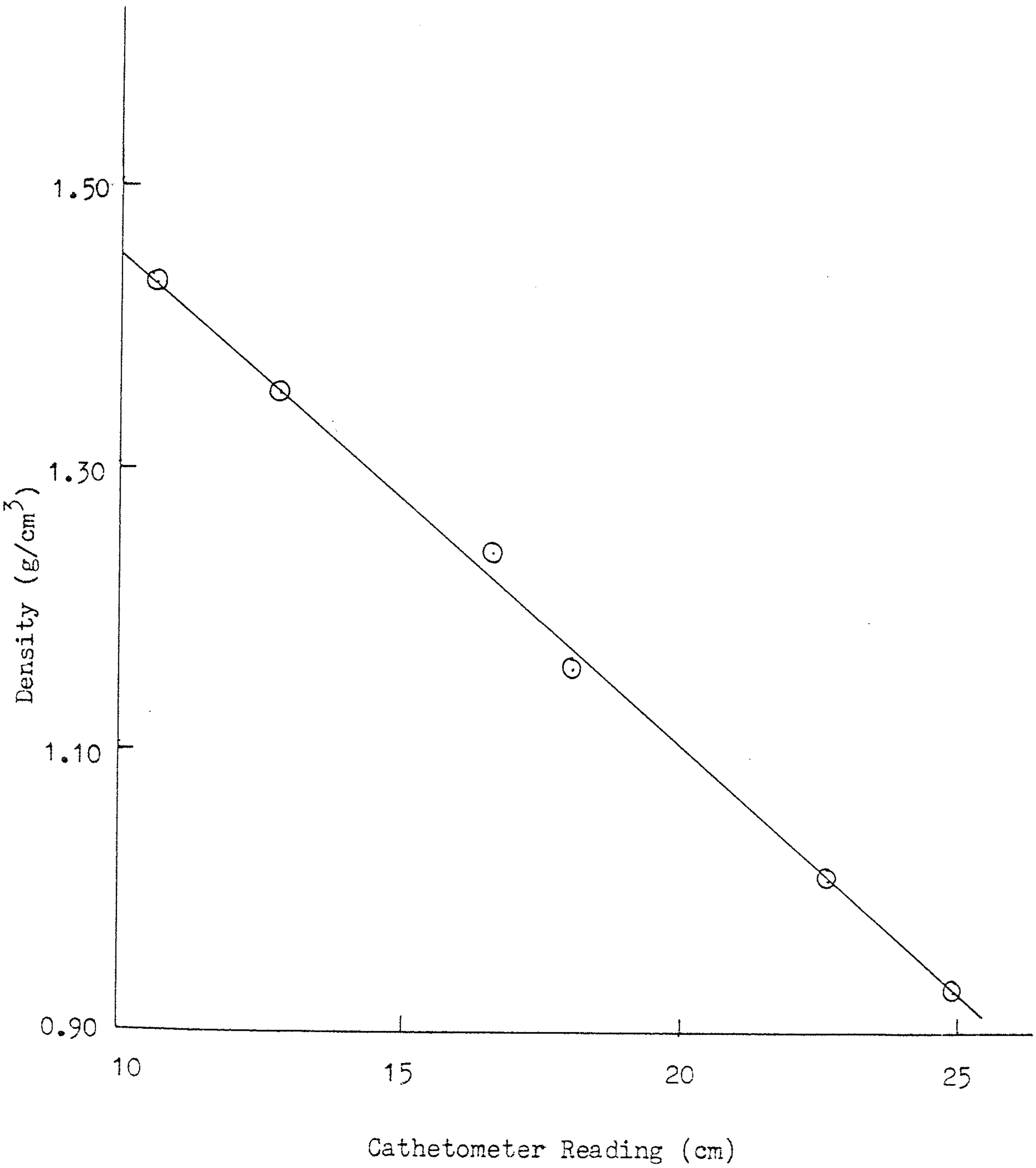


Figure 2.3

Calibration graph for measurement of densities of hydrated hydrogel particulates

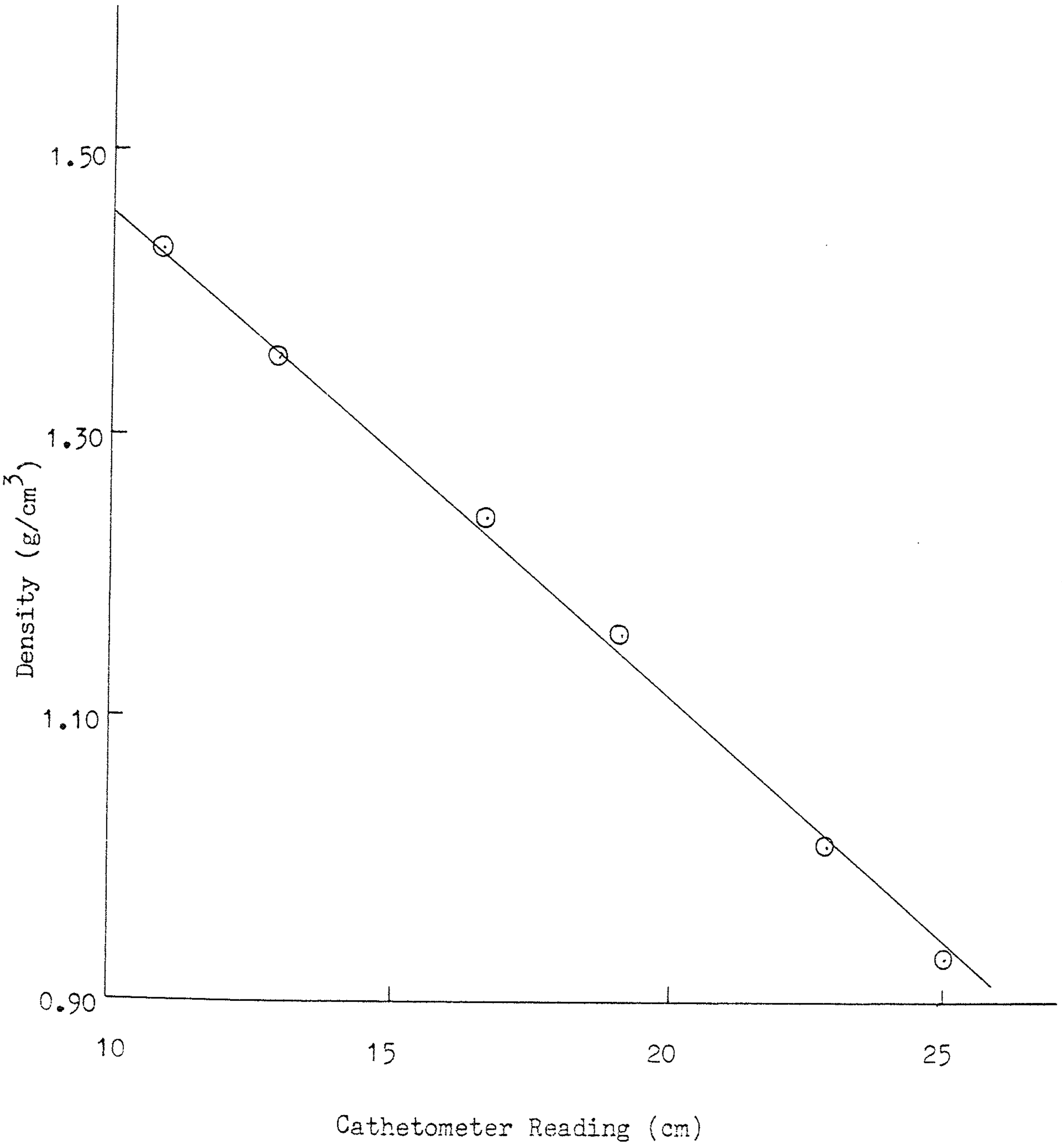
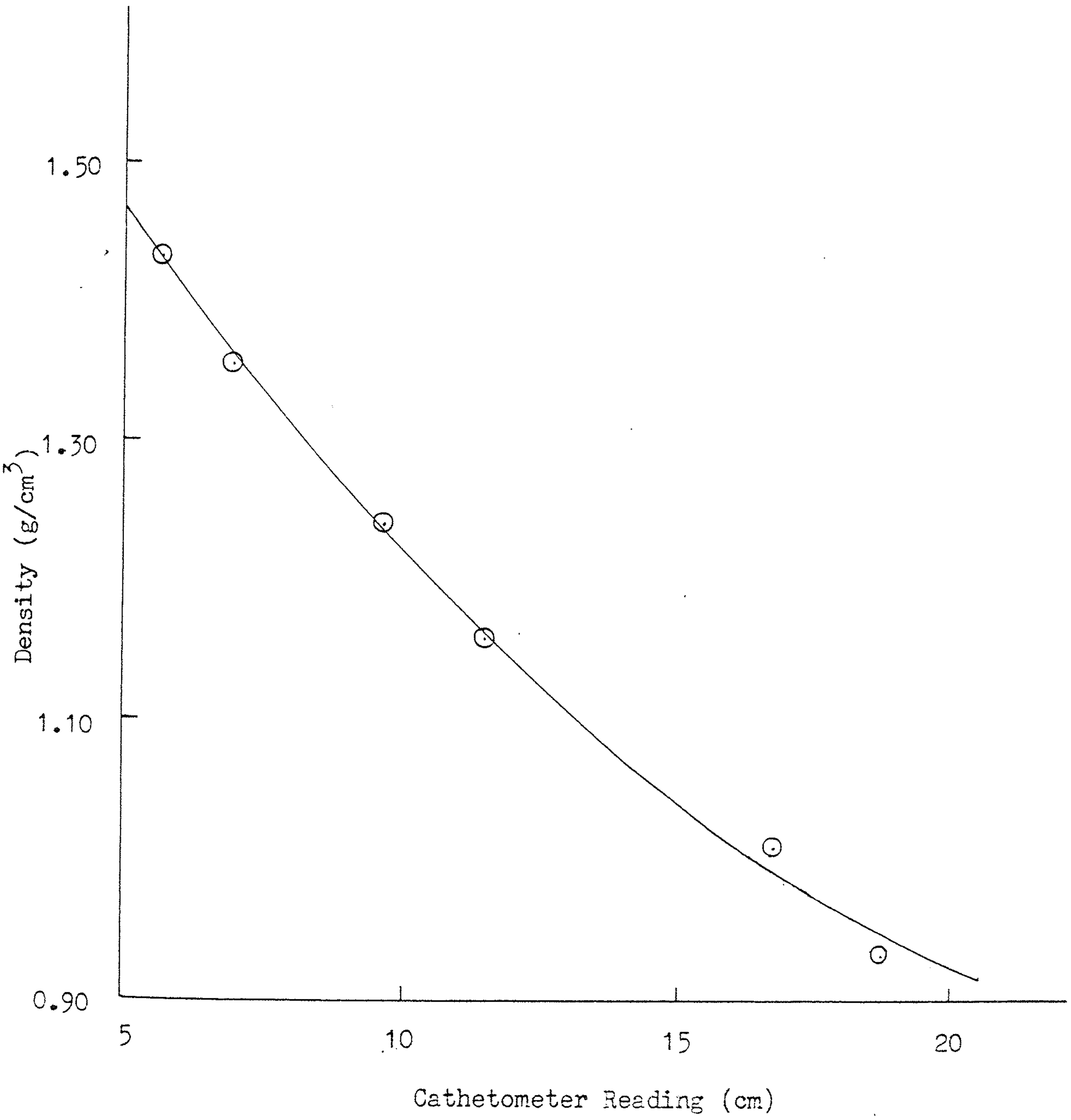


Figure 2.4

Calibration graph for measurement of densities showing non-linearity in certain experiments



2.2.2.2 Equilibrium Water Content (EWC) Measurements

Measurement of equilibrium water content of a hydrogel is a useful technique of characterising the hydrogel. This is because water plays an important role in the behaviour of hydrogel in a tissue environment. Equilibrium water content of a hydrogel is the water uptake by hydrogel at the point at which an equilibrium exists between the osmotic pressure of the polymer segments causing swelling and the opposing forces arising as the chains between cross links retract and elongate. Such an equilibrium exists when hydrogel is placed in water.

The technique of measuring EWC of a hydrogel has been previously studied (Ng, 1974; Barnes, 1976; Pedley, 1976; Skelly 1979). The method for determining EWC of hydrogel particulates involved hydration of the particulates in distilled water for at least three weeks for equilibrium to reach. Hydrated beads were taken out of the sample bottle and their surface water was removed by pressing gently between two filter papers. The sample was weighed in a weighing bottle and the weight of the hydrated hydrogel was noted. The sample was then dried in a vacuum oven at 60°C to constant weight and the weight of dehydrated hydrogel was noted. EWC of the hydrogel was calculated using the following equation and expressed as a percentage

$$EWC = \frac{Wg - Wp}{Wg} \times 100\,\%$$

where, Wg = The weight of hydrogel in the hydrated state

Wp = The weight of hydrogel in the dehydrated state

$$\text{Thus, EWC} = \frac{\text{weight of water in hydrogel}}{\text{weight of hydrogel in hydrated state}} \times 100\ \%$$

A mean value of four separate determinations was taken for each sample of hydrogel to obtain EWC of the sample.

The possibility of calculating the EWC of hydrated hydrogel from the knowledge of the densities in both dehydrated and hydrated states was examined using the following equation:

$$\frac{W_w}{W_g} = \frac{1 - \frac{d_g}{d_p}}{d_g\left(\frac{1}{d_w} - \frac{1}{d_p}\right)} \times 100\ \%$$

Where Wg = the weight of a polymer in the hydrated state

dg = the density of a polymer in the hydrated state

dp = the density of a polymer in the dehydrated state

Ww, and dw = the weight and density of water in hydrogel

Derivation of the above equation is given in Appendix (A). Two assumptions have been made in deriving the above equation. These assumptions are as follows:

(i) the volume of the hydrogel is the sum of the volumes of the polymer and water in the network.

(ii) the volume of the polymer is the ratio of the weight of the polymer to the density of the dehydrated polymer.

2.2.2.3 Scanning Electron Microscopy (SEM)

External and internal surfaces of the hydrogel particulates were examined by scanning electron microscopy carried out on dehydrated samples.

Sample preparation involved dehydrating the hydrogel particulates in a vacuum oven at 60°C for several hours until samples were completely dry. The dried samples were mounted on a stub and treated with the electron conducting spray. Scanning electron microscope was operated by following the instruction manual supplied with the instrument.

Surfaces and interiors of the samples were examined at various magnifications and photographs were taken. Scanning electron micrographs (SEM's) of the interior of samples were taken by cutting the particulate with a sharp knife. Care was taken to avoid the contamination of the sample from moisture and dust particles. It was found that sometimes particles of dust from air clung to the surface of the sample thus modifying the true appearance of the surface. To avoid contamination, samples were stored in a clean desiccator over anhydrous calcium chloride and removed just prior to examination.

2.2.2.4 Particle Size Determination

Hydrogel particulates were graded into different size fractions by manual sieving. A set of British Standard test sieves obtained from Endecotts (Filters) Limited was used. Majority of beads were sieved in the dry state. Fractions or combination of fractions in the size range 125-250 μm; 250-500 μm; 500-1000 μm and greater than 1000 μm were collected. Sieving operation was carried out by using the following method:

Metallic sieves of appropriate mesh sizes were assembled in a row with mesh size increasing from bottom to top. Sample was added to the top most sieve and the whole assembly was shaken for 15 minutes. Fractions retained on each of the sieves used in the test were weighed and listed as percentages of the original test sample weight by using the following equation:

$$\text{weight percentage retained} = \frac{\text{weight of beads retained}}{\text{total sample weight}} \times 100\,\%$$

The results were expressed as histograms by plotting fractional weight percent retained by a particluar sieve against the width of that sieve aperture on ordinary graph paper.

2.2.2.5 Adsorption Studies

Characteristics of hydrogel particulates to adsorb certain chemical species (markers of liver failure) were examined. Three different types of chemical species with acidic functional groups were used for these studies. These compounds are categorised below.

(i) Bromosulphophthalein (BSP)

(ii) Bile acids (Cholic acid and Deoxycholic acid)

(iii) Amino acids (Methionine and Phenylalanine)

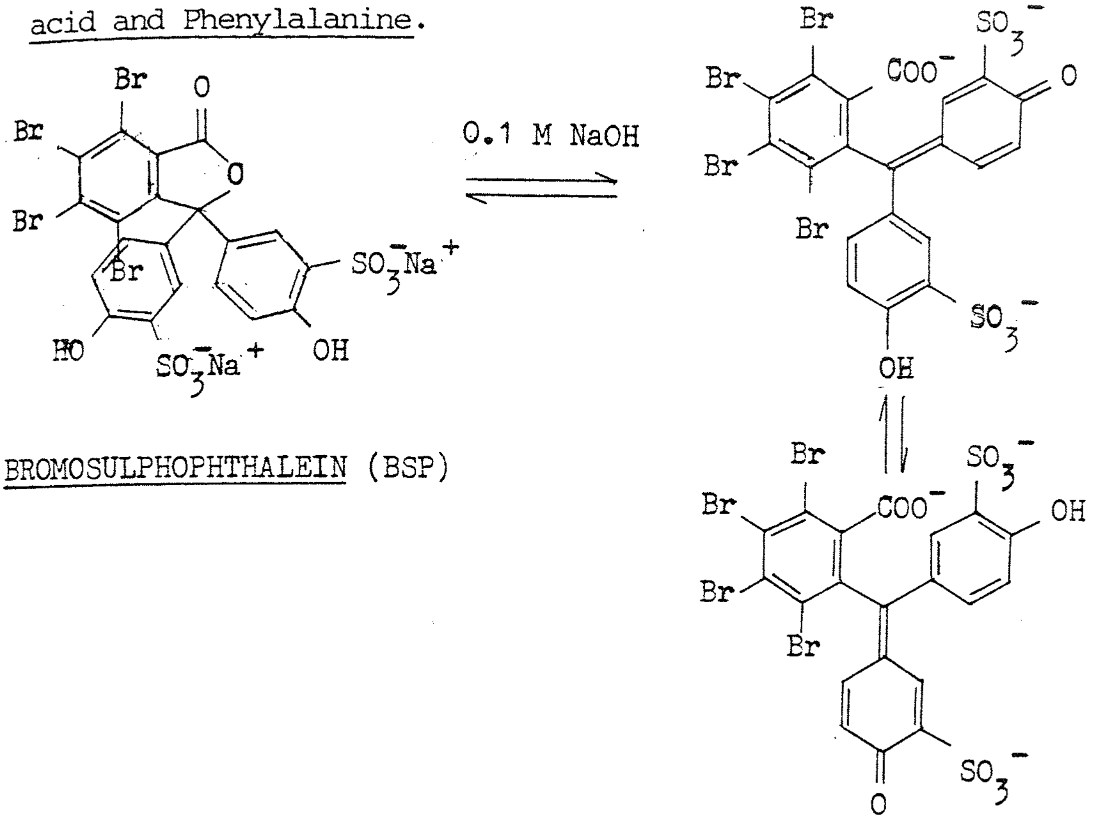
BSP and bile acids were used in the form of their sodium salts. In addition to the chemical species listed above, some experimental work was carried out to study the adsorption of another hepatic toxin, bilirubin, by selected samples of hydrogel particulates. Adsorption studies with BSP, bile acids and amino acids were carried out from saline. Adsorption of bilirubin was studied from chloroform.

Adsorption studies were carried out by the construction of adsorption isotherms using static technique, and by using a dynamic technique which involved the continuous flow of solution of the toxin over a bed of hydrogel adsorbent. The details of methods used in carrying out adsorption studies will be described in chapter 6.

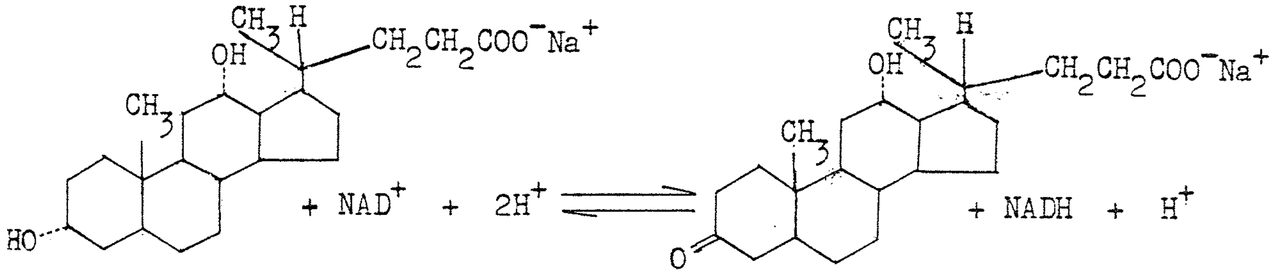
The extent of adsorption of BSP and amino acids was determined spectrophotometrically by colour reactions. Bile acid determination was carried out enzymatically. The analytical techniques used to assay BSP, bile acids, amino acids and bilirubin will now be described.

Figure 2.5

Reactions involved in the determination of BSP, Deoxycholic acid and Phenylalanine.



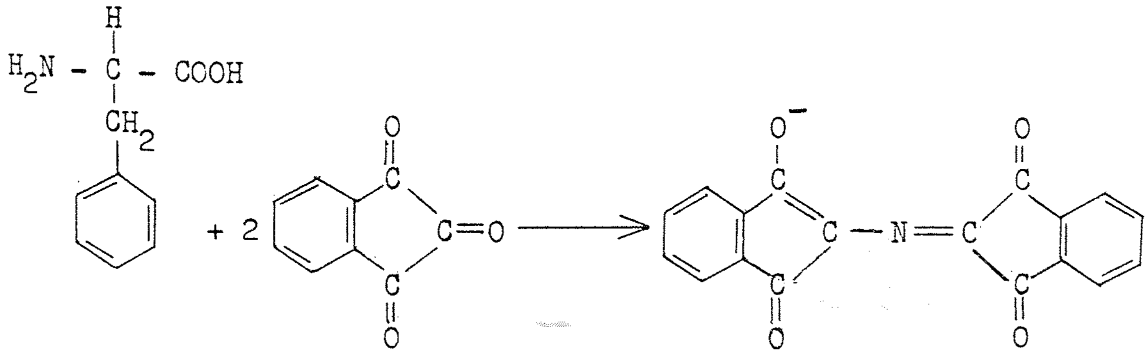
BROMOSULPHOPHTHALEIN (BSP)



3 - hydroxysteroid

3 - ketosteroid

SODIUM DEOXYCHOLATE



PHENYLALANINE

Ninhydrin

DYDA

2.2.2.5.1 Analysis of BSP

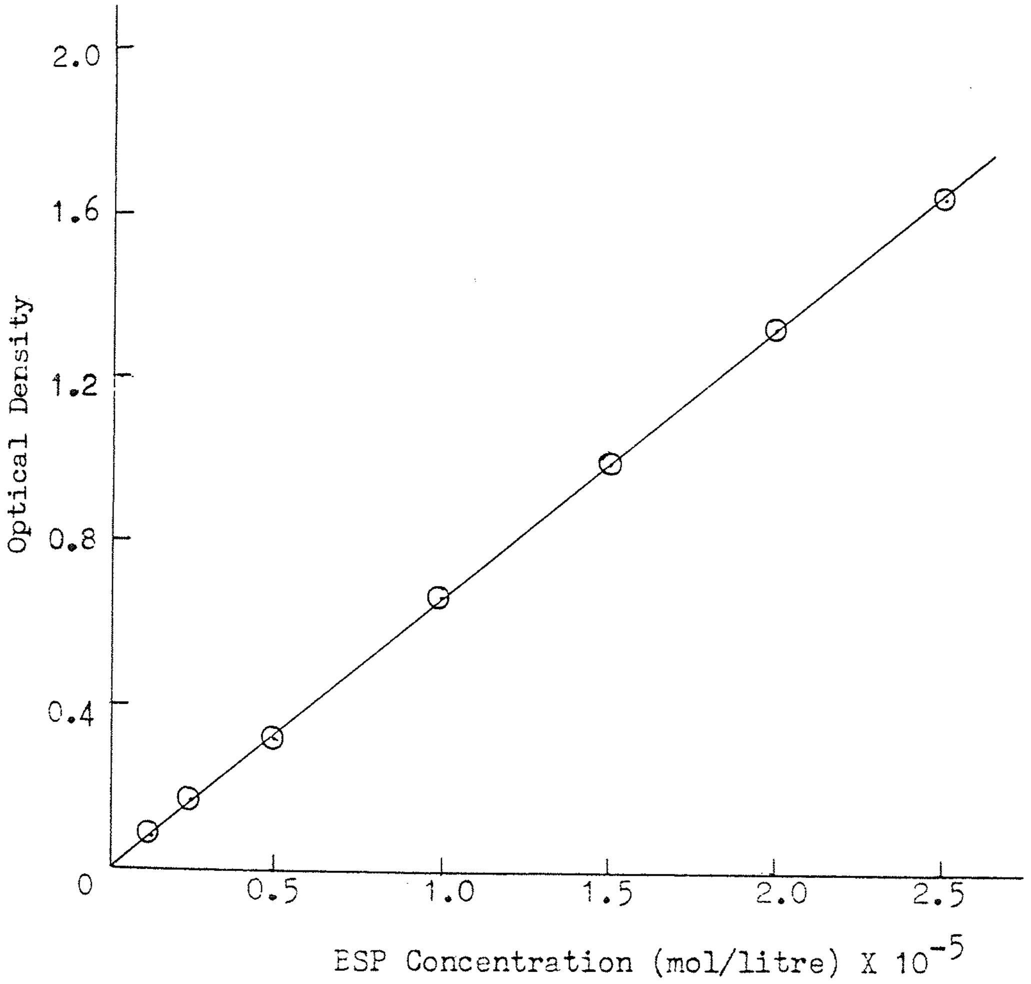
BSP was analysed by its reaction with 0.1M sodium hydroxide. When BSP reacts with sodium hydroxide solution, the chemical reaction results in the creation of a highly conjugated system which produces purple colour in the solution. The reaction is shown in figure 2.5. This system has a maximum absorbance at 580 nm which allows easy colorimetric determination to be carried out. A calibration graph was constructed by preparing standard solutions of BSP of varying concentration in 0.1M sodium hydroxide. The optical density of samples was read in a SP 800 UV/Visible spectrophotometr, and readings of optical density, thus obtained, were plotted against the corresponding concentration of the solution. The calibration graph (figure 2.6) was then used to obtain concentrations of test solutions from observed optical densities in subsequent experiments.

2.2.2.5.2 Analysis of Bile Acids

Sodium salts of cholic acid and deoxycholic acid were analysed by the method of Turnberg and Anthony-Mote (1969). This method is based on the enzymatic oxidation of the 3-hydroxysteroid to 3-ketosteroid by nicotinamide adenine dinucleotide (NAD) by the reaction shown in figure 2.5 for deoxycholic acid. The bile acid was reacted with NAD quantitatively in the presence of 3α-hydroxysteroid dehydrogenase (3αHD), and the NADH formed was determined spectrophotometrically at 340 nm (wavelength of maximum absorption). The preparation of bile acid solutions

Figure 2.6

Calibration graph for BSP



for analysis was carried out as follows. A 100 mg of crude freeze-dried preparation of the organism Pseudomonas Testosteroni containing hydroxysteroid dehydrogenase were ground with 100 mg alumina in 10 ml water at 4^{o}C. The enzyme was extracted by separating the residue by centrifugation. The clear supernatant contained the enzyme used for assays.

To 0.2 ml of test solution of the bile salt were added 1 ml of 0.005 M NAD, 0.6 ml of enzyme solution, and 1.2 ml of 0.1 M sodium pyrophosphate buffer solution (pH 10.8). The final pH of 3 ml solution was 10.4. Blanks were prepared containing an extra 0.2 ml buffer instead of bile salt solution. The samples were incubated at 26^{o}C for 60 minutes. Readings of optical density were then made at 340 nm. One mole of bile acid formed one mole of NADH in presence of 3αHD. Thus, the amount of bile acid present in the mixture, expressed as micro moles was calculated from the formula:

$$\text{Amount of bile acid} = \frac{A \times 3.0}{6.22} \text{ micro moles}$$

where, A = the increase in extinction, and 6.22×10^{6} is the molar extinction of NADH at 340 nm.

2.2.2.5.3 Analysis of Amino Acids

Analysis of amino acids was carried out by using a modified ninhydrin method (Troll and Cannon, 1953). The method involves the quantitative deamination of amino acids by ninhydrin. The first step in the reaction is the oxidation of the amino acid with the formation of ammonia, carbon dioxide and an aldehyde, and the reduction of ninhydrin to hydrindantin. The ammonia thus formed condenses with the hydrindantin to form diketohydrindylidenediketohydrindamine (DYDA) which has a blue colouration. The reaction is shown in figure 2.5. The anionic DYDA has an absorbance maximum at 570 nm and, therefore, can be readily analysed colorimetrically. The reagents used and the method involved were as follows.

Reagents:

(i) Ninhydrin solution - 500 mg of ninhydrin dissolved in 10 ml of absolute alcohol.

(ii) 80 percent phenol solution - 80 g of reagent grade phenol dissolved in 20 ml of absolute alcohol. The solution was shaken with 1 g of permutit for about 20 minutes to remove traces of ammonia, and was decanted.

(iii) Potassium cyanide-pyridine reagent - 2 ml of 0.01 M solution of KCN diluted to 100 ml with ammonia free pyridine.

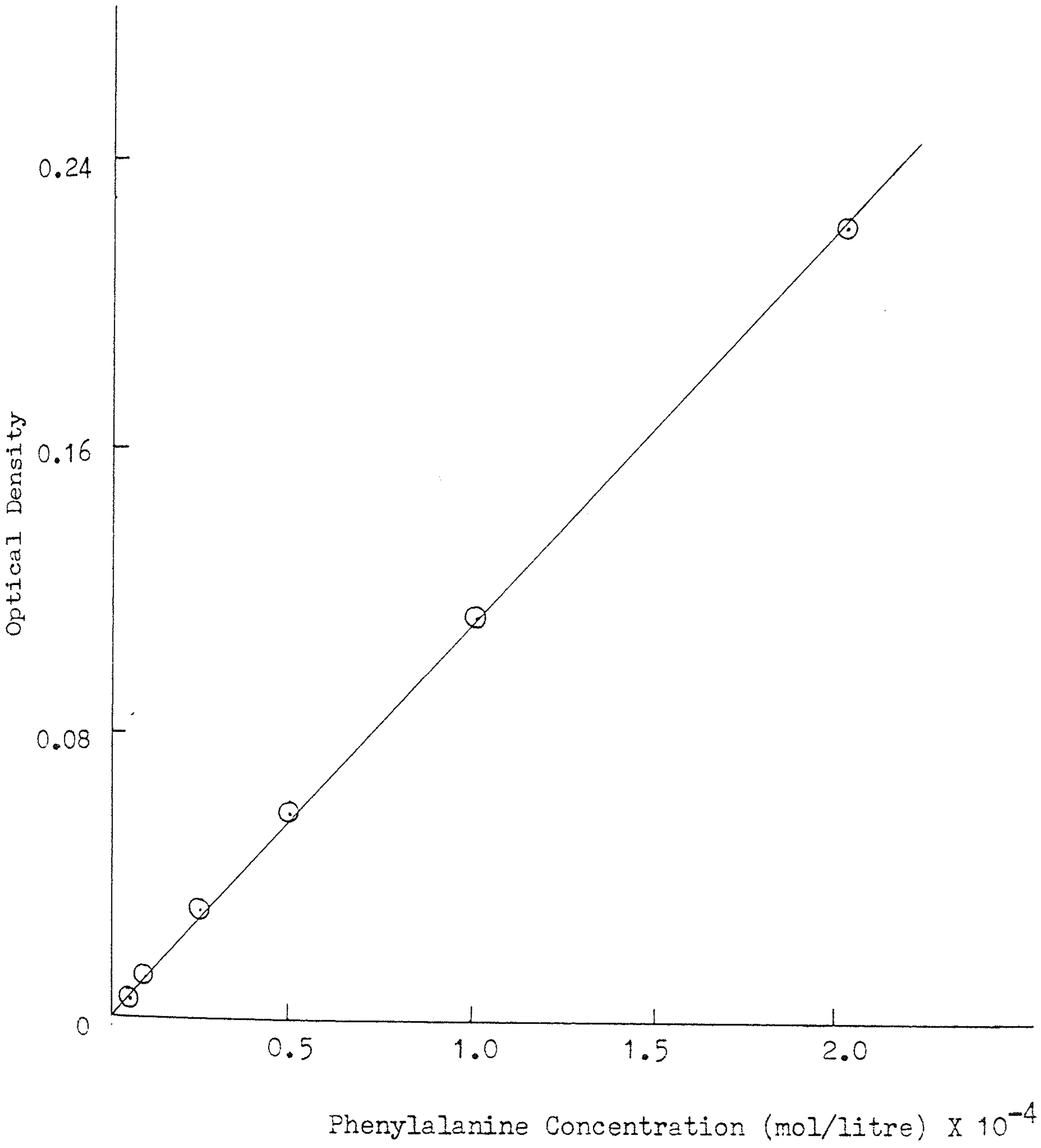
(iv) 60 percent solution of ethanol (by volume).

Method:

0.5 ml of the amino acid solution was heated with 1 ml of reagent (iii) and 1 ml of reagent (ii) in a boiling water bath. When the mixture had reached the temperature of the bath, 0.2 ml of reagent (i) was added, the reaction vessel was stoppered and reaction was allowed to proceed for 3-5 minutes. The solution was cooled and made upto 10 ml with reagent (iv). 0.5 ml of ammonia free water subjected to the same procedure was

Figure 2.7

Calibration graph for Phenylalanine



used as the reagent blank. Optical density readings were taken at 570 nm against the reagent blank. Standard calibration graphs were prepared for amino acids which were used to determine concentration of test samples in the latter experiments. Figure 2.7 shows the calibration graph for phenylalanine.

2.2.2.5.4 Analysis of Bilirubin

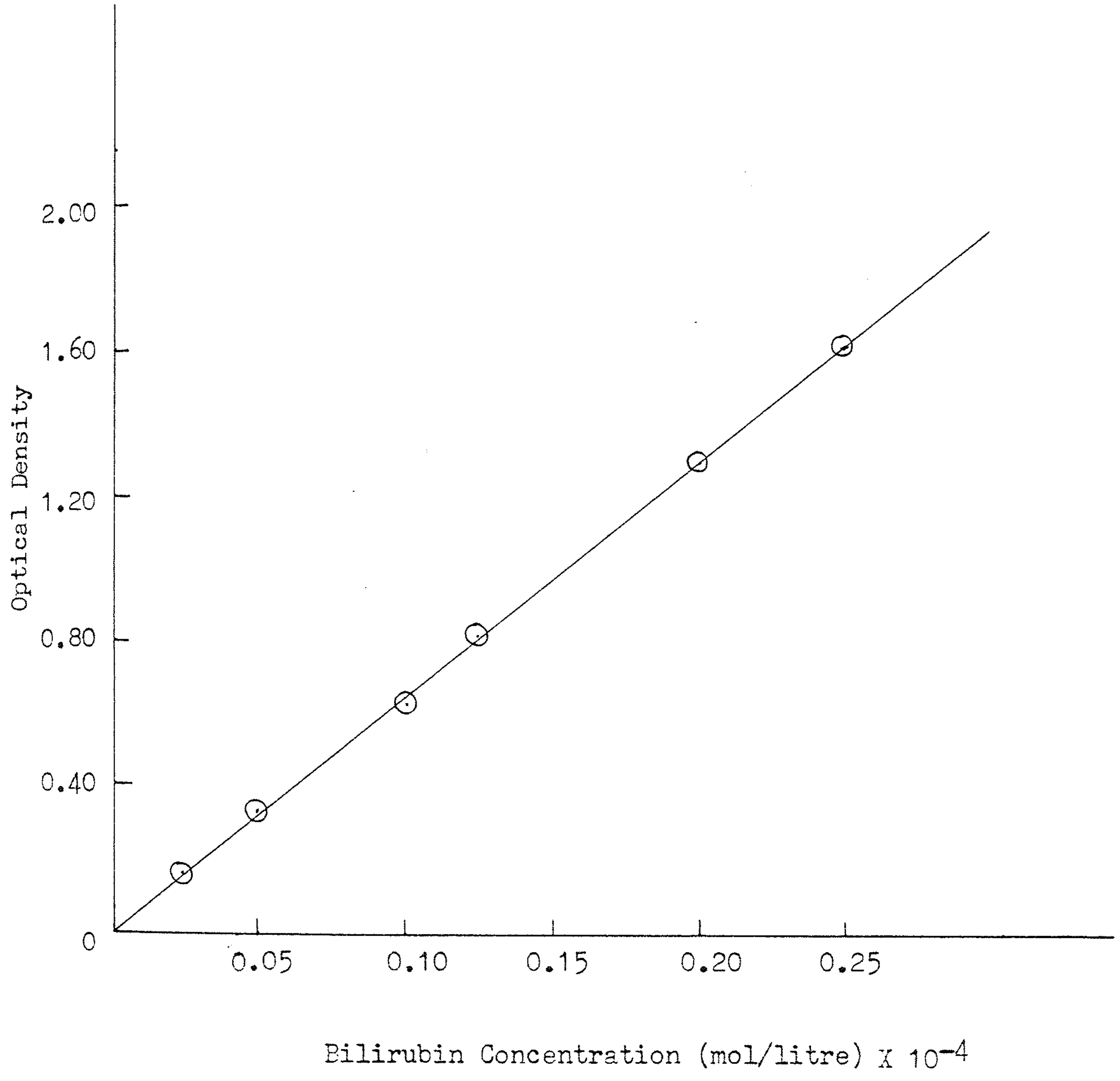
Adsorption studies with bilirubin were carried out by dissolving this compound in chloroform since it is not very soluble in saline. The method involved the preparation of a calibration graph (figure 2.8) by plotting the readings of optical density against concentration of various standard solutions of bilirubin in chloroform. Optical densities were read at 453 nm. Concentration of bilirubin in test samples was then obtained from the calibration graph.

2.2.3 Immobilisation of Detoxifying Enzymes on Hydrogel Beads

The model enzyme urease was immobilised on various hydrogel bead samples synthesised in this work by suspension polymerisation techniques. Immobilisation was carried out by the covalent binding method. The immobilised enzyme was characterised by measuring its activity using a pH meter. The detailed experimental methods for covalent binding, and characterisation of the bound enzyme will be described in chapter 7.

Figure 2.8

Calibration graph for Bilirubin



CHAPTER 3

SYNTHESIS OF HYDROGEL PARTICULATES; FREEZE - THAW POLYMERISATION

3.1 INTRODUCTION

In this chapter the polymerisation of hydrophilic monomers to produce beaded hydrogels by using the frozen solvent/monomer technique has been described. The freeze-thaw technique, as previously outlined in chapter 2, involves the polymerisation of monomers while maintained 'frozen' in a cold n-hexane continuous phase. The technique has previously been demonstrated to produce macroporous hydrogels possessing good mechanical and water flux properties. The macroporous hydrogels produced in this way were reported to have higher permeability to substances such as bromosulphophthalein than their homogeneous counterparts (Skelly and Tighe, 1979). Since substances in comparable size range to bromosuphophthalein have been implicated as potential hepatic toxins, this technique can be used to synthesise adsorbents for the removal of such toxins from blood.

The principle of polymerisation on a crystalline matrix of a solidified solvent to form fibrous structures was reported by Krauch and Sanner (1968). This method was adopted and extended by Haldon and Lee (1972) to produce Poly (hydroxyethyl methacrylate) films with a wide range of structures and related properties. In this technique a mixture of monomers 2-hydroxyethyl methacrylate and ethylene dimethacrylate was added to a solvent, aqueous ethylene glycol containing a photosensitizer (uranyl nitrate). This mixture was then injected between glass plates held together by spring clips.

The solution was frozen by pouring powdered solid carbon dioxide on top. The frozen monomer/solvent mixture was photopolymerised by irradiating with light from an ultra-violet (UV) lamp.

A porous film is produced by this method because when the solution is frozen the monomer occupies the interstices between the solvent crystals. Therefore, after polymerisation and removal of the solvent this configuration is retained and a porous structure results. The technique can be applied to any monomer/solvent system in which the solvent can be solidified by cooling. The size of the pores will be a direct function of the size of the crystalline solvent particles. Haldon and Lee measured approximate pore sizes from photomicrographs of films taken at right angles to the pore axes and found the pore size to be 1 to 2 μm for the very porous samples and 0.5 to 1.0 μm for the slowly cooled.

The above method was modified by Skelly (1979) for producing macroporous hydrogel beads by droplet techniques. The process involved dropwise addition of monomer/solvent (water and ethylene glycol) mixture containing uranyl nitrate to n-heptane at -70°C. The frozen monomer/solvent spheres thus formed were irradiated with UV light. Upon completion of polymerisation and subsequent thawing, porous hydrogel beads were obtained.

In this method of polymerisation, the most porous structures are produced by rapid cooling which gives unidirectional pores at right angles to the surface of the polymer. By comparison, the slow cooling results in small random pores. Therefore, two important parameters in altering the permeability of such polymers are the direction of the thermal gradient and the rate of cooling (Haldon and Lee, 1972).

Changes in structure can also be brought about by varying the monomer concentration. An increase in monomer concentration yields stronger, more compact polymer. A further gradation in structure and related permeability can be made by varying the water/ethylene glycol ratio of the solvent because the temperature at which ice separates from a solution depends on this ratio.

In this research project preparation of a series of hydrogel beads having a range of composition by modification of the above mentioned techniques was undertaken. The principal monomer used for preparing hydrogel beads was 2-hydroxyethyl methacrylate (HEMA).

Acidic and basic functional groups were incorporated by using appropriate monomers to produce copolymers and terpolymers with HEMA. The hydrogel adsorbent beads with a variety of physical structures and chemical properties, thus prepared, were evaluated by determining their equilibrium water contents, densities, size distributions, appearances of their external and internal surfaces, and their potential to adsorb certain marker substances of liver failure.

The dropwise addition of the monomer solution to the non-solvent continuous phase was achieved in two different ways. Initial experiments were conducted by using a burette which enabled the slow addition of monomers to the n-hexane, thus producing beads having diameter greater than 0.5 mm. In order to reduce the particle size an alternative for the addition of monomers to the n-hexane was then investigated. This second approach involved the use of an automatic syringe injector.

Details of the synthesis and evaluation of hydrogel beads will be described on the following pages. The adsorption characteristics of the beads with respect to markers of liver failure will be described in chapter 6.

3.2 PRODUCTION OF LARGE BEADS (DIAMETER GREATER THAN 0.5 mm)

Hydrogel beads comprising various compositions were synthesised by the use of a burette for producing monomer droplets. Synthesis of hydroxyethyl methacrylate and ethylene dimethacrylate copolymer beads serving as a typical example of this method has been detailed below:

Polymerisation of Hydroxyethyl Methacrylate Cross-linked with Ethylene Dimethacrylate in the Bead Form

2-hydroxyethyl methacrylate (10.0347 g, 0.0771 mol) and ethylene dimethacrylate (1.5263 g, 0.0077 mol) were dissolved in a mixture of ethylene glycol:water (11.5610 g, 1:4 ratio by weight) containing uranyl nitrate (0.2508 g). The solution was filtered and transferred to a burette. The n-hexane was cooled to -70°C in a Dewar flask by addition of powdered solid carbon dioxide. The burette was clamped above the non-solvent reservoir and its tip was kept just above the surface of cold n-hexane. The n-hexane was stirred at 300 rpm by an anchor type glass stirrer. The homogenous monomer solution was then added dropwise to the non-solvent.

After the addition was complete, the frozen droplets were allowed to settle and then transferred to a beaker containing n-hexane and a small amount of powdered dry ice. A magnetic follower was added and the beaker was irradiated from above with a UV lamp for one and a half hours. After polymerisation was completed the beads were added to a large volume of distilled water for hydration and removal of the solvent. 10.9814g (94.99% yield) spherical beads having a particle size in the range (3000 - 500 μm) were obtained. Equilibrium water content of beads was 51%. By using this method spherical and uniform beads were produced. However, it was found to be time consuming due to the slow addition of individual droplets to the non-solvent. Another problem encountered with this method was that the tip of the burette was frequently found to be blocked due to freezing of the monomer solution at the outlet. This problem was overcome by occasionally dipping the tip of the burette in a beaker of water. One further point to note concerning this method was the size range of the particles as it produced beads larger in size than 0.5 mm diameter. Reduction in particle size was desired from the viewpoint of adsorption properties of the polymer. In general, the adsorption of a solute from a solution would be expected to proceed at a faster rate with smaller particles than with larger particles of the same adsorbent.

In view of the above problems, a different approach for the addition of monomer phase to the cold n-hexane was then studied. A dispensing system was used which could dispense reagents at a high, constant flow rate in a contaminant free manner. This system will now be described.

3.3 RAPID INJECTION TECHNIQUE

The technique involved rapid injection of monomer solution to the cold n-hexane using a motorised syringe. The syringe was used in conjunction with type LFA/10 dispenser (Marketed by Fisons of Loughborough) described below.

Operation of Automatic Syringe Injector

The dispenser consists of a specially designed PTFE twist valve into which the syringe is fitted, operated by a synchronous motor. This motor also drives a rack on which a bracket to hold the end of the syringe plunger is mounted. The syringe barrel is held firmly against the twist valve by an L-shaped clamp. As the rack moves backwards and forwards, so the plunger moves up and down the barrel sucking up and dispensing liquid. The inlet of the dispenser is connected to the monomer/solvent reservoir and the outlet to the mixing head. The rack operates at a constant speed, therefore, the rate of flow could only be varied by changing the size of the syringe. The larger the syringe used, the faster the injection speed. Table 3.1 lists the flow rates obtainable using three standard syringe sizes (Sutton, 1974).

Table 3.1

Syringe volumes and the obtainable flow rates

Syringe Volume ml	Time Taken For Full Stroke (i.e. half cycle) sec	Flow Rate ml/sec
5	5	1
10	6	1.7
20	6	3.3

The instrument is shown diagrammatically in figure 3.1 and also in plate 3.1. The commercial dispensers are supplied with 'Viton' or 'Neoprene' 'O' rings to stop leakages of reagents. However, the leakages were not prevented with these rings. This problem was overcome by cutting 'O' rings from 1/8 inch diameter PTFE tubing which were found to be adequate for 1/16 inch diameter fluid lines.

A series of monomer composition was polymerised by using the fast injection technique. The method will be exemplified by describing the preparation of acrylic acid and ethylene dimethacrylate copolymer beads.

Polymerisation of Acrylic Acid Cross-linked with Ethylene Dimethacrylate in the Bead Form

The following formulation was used:

Acrylic acid = 10.2040 g (0.1416 mol); Ethylene dimethacrylate = 2.3882 g (0.0121 mol); uranyl nitrate = 0.2584 g;

Ethylene glycol and water = 12.5922 g (1:4 ratio by weight). Monomers were mixed with aqueous ethylene glycol containing uranyl nitrate. The mixture was filtered and added to the reservoir. A syringe needle (G 18, 3 inch length) was connected to the outlet PTFE tubing from the dispenser. The needle was clamped about 6 inches above the surface of cold n-hexane (-70 °C) contained in a large double walled glass vessel equipped with a stirrer and a thermometer. Stirring was initiated and dispenser was switched on. The monomer/solvent

Figure 3.1

Apparatus for freeze-thaw polymerisation

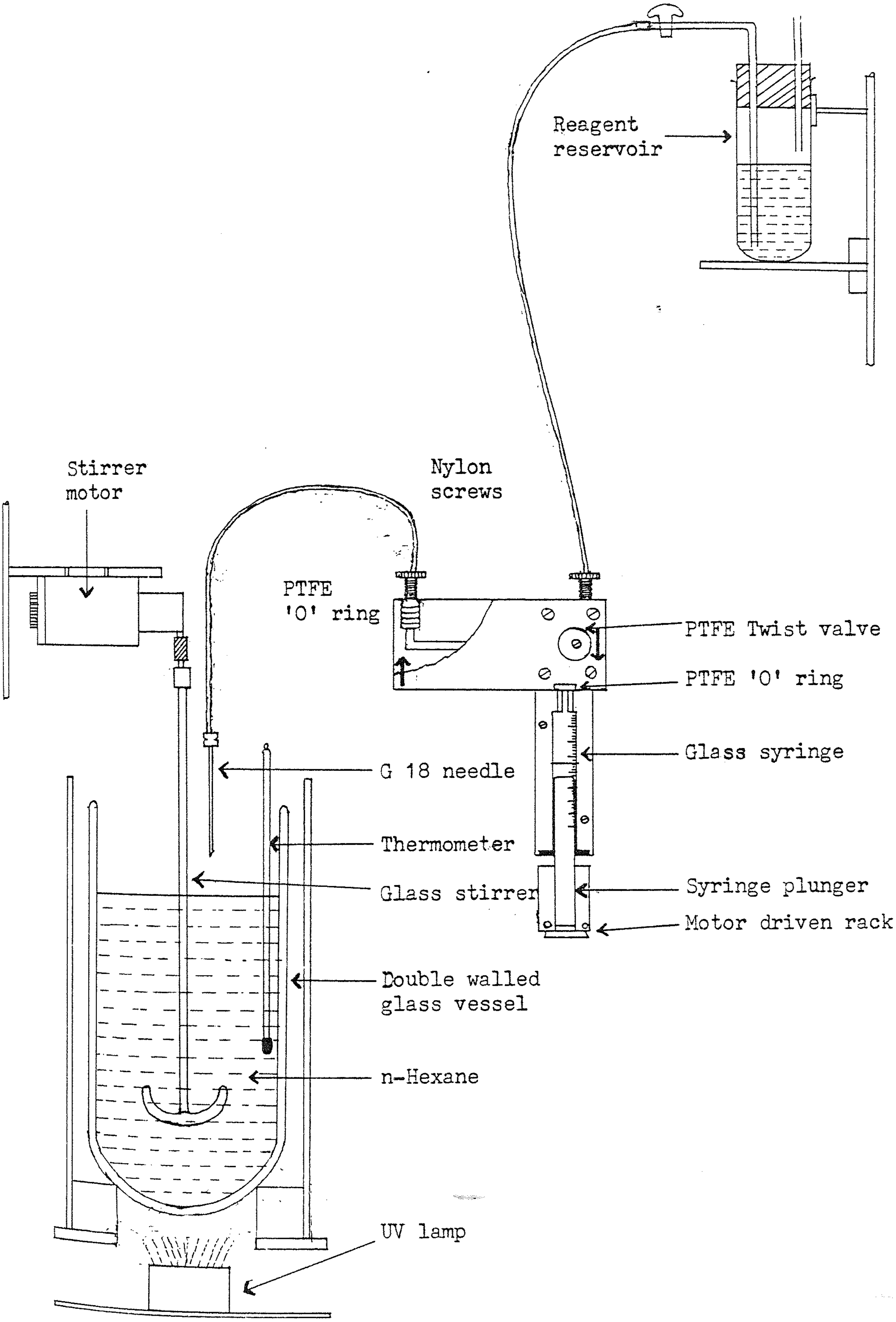


Plate 3.1

Apparatus for freeze-thaw polymerisation

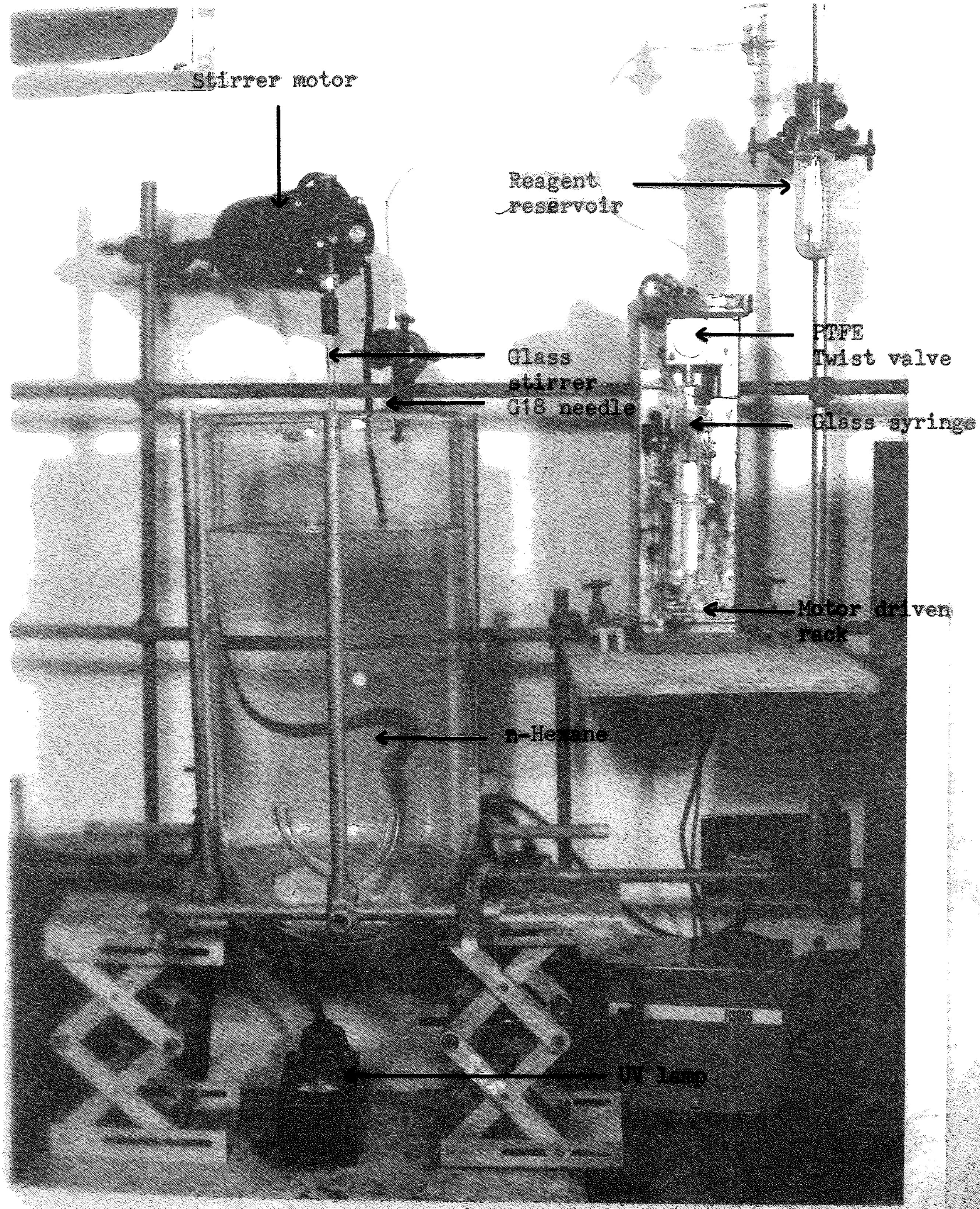
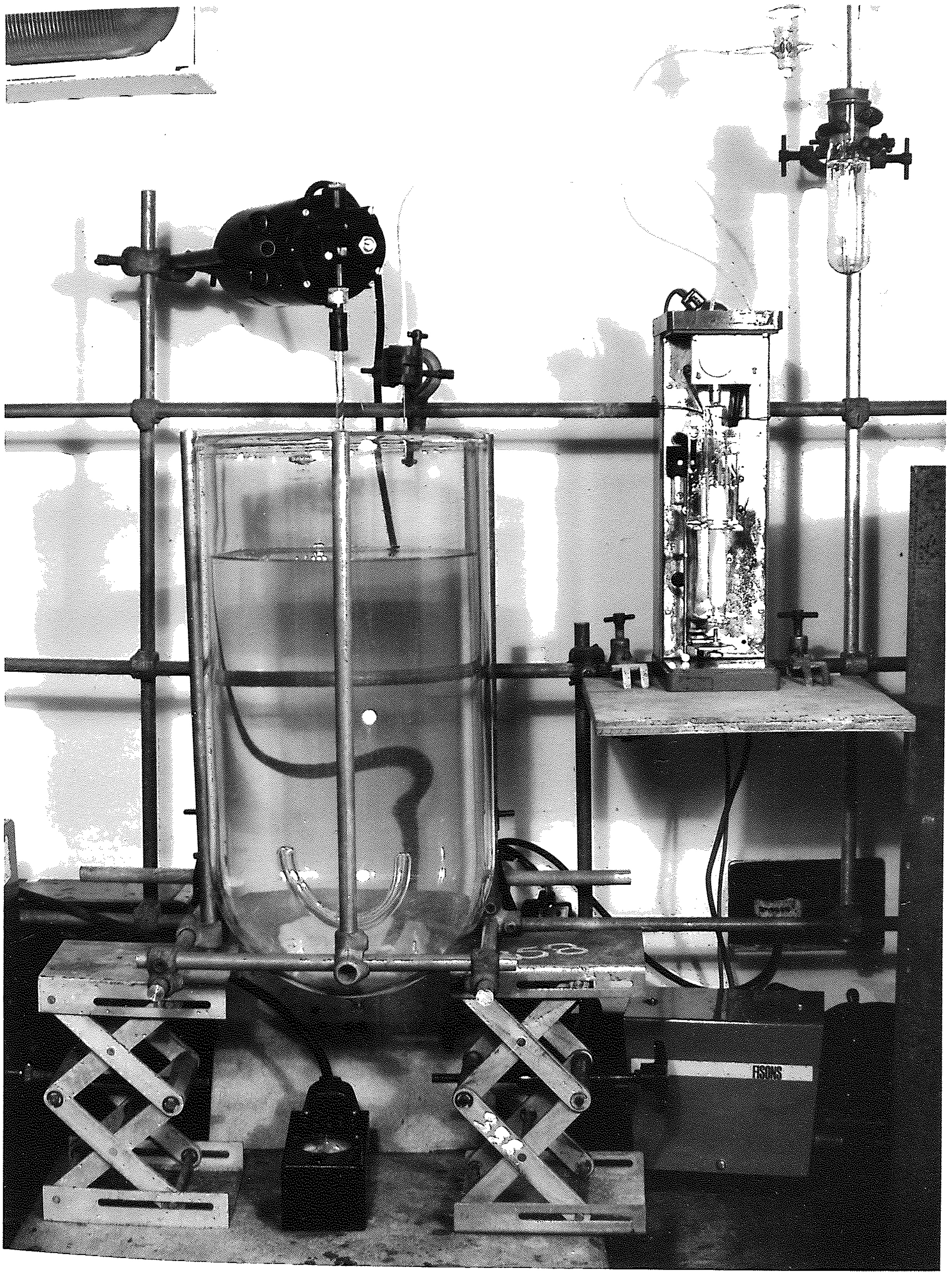


Plate 3.1

Apparatus for freeze-thaw polymerisation



mixture was injected at the rate of 1 ml per second using a 5 ml syringe. Frozen spheres of monomer/solvent were clearly visible in the stirred non-solvent. Polymerisation was initiated by irradiating the glass vessel with a high intensity UV lamp placed under the vessel. The stirring was maintained at 300 rpm until polymerisation was complete. Beads were then transferred to a large volume of water for hydration and removal of ethylene glycol. 12.2020 g (96.90% yield) of spherical beads having a size range 2000 μm - 250 μm were obtained. Equilibrium water content was found to be 76%.

The rapid injection technique, therefore, enabled polymerisation to be carried out 'in situ'. This avoided the step, encountered in the burette process, of transferring frozen monomer/solvent spheres to a beaker of n-hexane before initiation of polymerisation. Certain variations in the original set up were made in order to create best conditons for producing beads in sufficient quantity and acceptable particle size range. For example by increasing the size of the reservoir and the vessel containing n-hexane, large quantity of beads can be synthesised. Injection rates, 1 ml per second and 1.7 ml per second enabled to produce beads in the size range of 2000 μm - 125 μm suitable for carrying out further studies.

The following important points should be considered concerning the operation of the dispenser and the technique in general:

These considerations enabled to increase the efficiency of the technique:

(i) It was found necessary to add powdered dry ice to the cold bath of n-hexane frequently as the heat from the UV lamp tended to raise the temperature of the bath.

(ii) Anchor type glass stirrer was found to be most suitable to effect stirring. The stirrer type is illustrated in appendix (B). The rate of stirring (300 rpm) was such that beads could be moved around gently inside the bath. Slower rates of stirring caused coalescence of beads and faster rates tended to distort the shape of otherwise spherical beads.

(iii) The inlet and outlet tubings of the dispenser were cleaned 'in situ' by flushing with water quickly after each run. The cleaning was found to be necessary due to two reasons: (a) to avoid the blockage of fluid lines and dispenser head due to the possible polymerisation of monomer droplets, and (b) to avoid contamination of solutions in the subsequent run from solutions of the preceding run. The whole dispensing system was however, dismantled and cleaned afterwards.

Initially copolymers of HEMA/EDM, AA/EDM and ACR/N,N'MBA were synthesised by this technique. The process was then extended to polymerise compositions to produce terpolymers. These compositions were composed of HEMA, EDM and either an acidic or a basic monomer. In this way a range of hydrogel beads with varying composition were synthesised. Table 3.2 shows a complete list of hydrogel beads prepared by the freezing droplet technique.

Table 3.2

Samples of hydrogel beads synthesised by freeze-thaw polymerisation

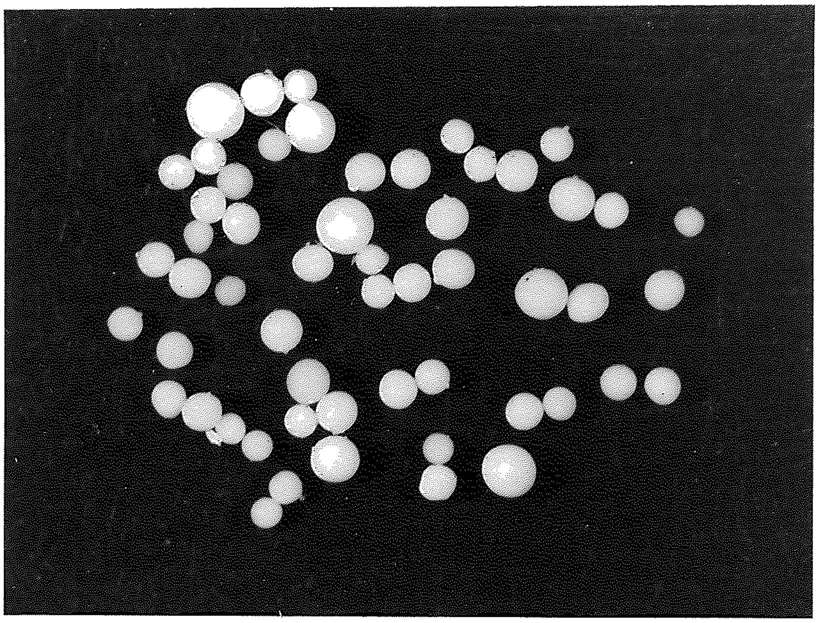
Sample	Monomer Composition	Initial Molar Ratio	Monomer:Solvent* Ratio
Acidic:			
Sample I	AA:EDM	100:8.5	50:50
Sample II	AA:HEMA:EDM	75:25:8.5	50:50
Sample III	AA:HEMA:EDM	60:40:8.5	50:50
Sample IV	MAA:HEMA:EDM	50:50:4.6	50:50
Neutral:			
Sample V	HEMA:EDM	100:10	50:50
Basic:			
Sample VI	ACR:N,N'MBA	100:10	50:50
Sample VII	HEMA:DAA:EDM	100:25:9	50:50
Sample VIII	HEMA:NMACR:EDM	90:10:8.5	50:50
Sample IX	HEMA:DMAEMA:EDM	90:10:8.5	50:50

* Solvent: Mixture of Ethylene Glycol and Water in the ratio 1:4 (by weight) respectively.

The samples have been split into acidic, neutral and basic groups depending upon the type of monomers used in their preparation. Although generalisation can be made about monomers, it is apparent from the table that some monomers may fall into slightly acidic or slightly basic categories. For example, although acrylamide is generally considered to be neutral, the nitrogen atom has tendency to accept proton and therefore has basic tendencies. The hydrogens on the nitrogen are sufficiently acidic to allow the formation of hydrogen bonds between amide groups in the polymer.

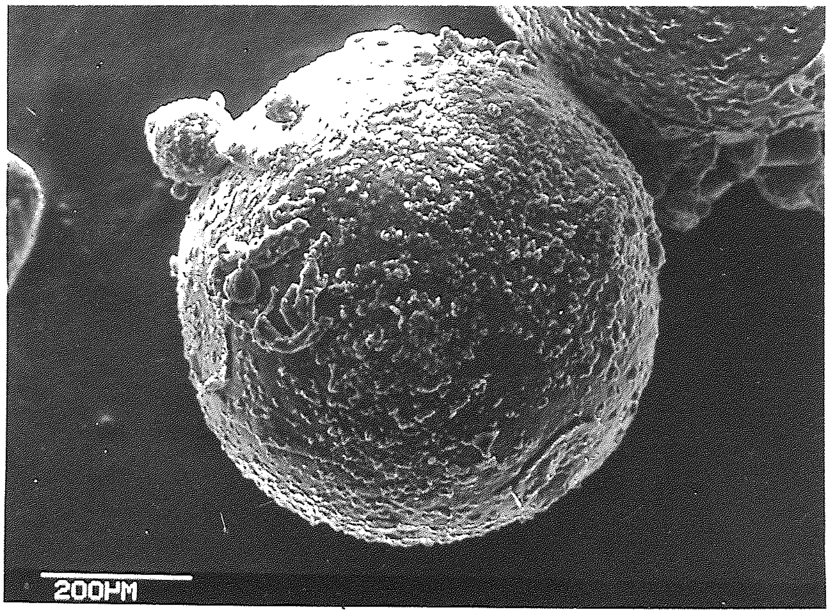
Plates 3.2 and 3.3 show photographs of hydrogel beads of a sample, terpolymer of HEMA/DMAEMA/EDM prepared by the freezing droplet technique. Plate 3.2 shows the regularity in spherical nature of the beads. Plate 3.3 is the low magnification scanning electron microphotograph of a single hydrogel bead demonstrating the general appearance of the hydrogel.

Plate 3.2



Hydrogel beads synthesised by freeze-thaw polymerisation

Plate 3.3



Low magnification scanning electron microphotograph of a single bead

3.4 INCORPORATION OF POWDERED CHARCOAL IN HYDROGELS

Some experimental work was performed concerning the possible incorporation of powdered activated carbon in the macroporous matrix of hydrogel beads. Attempts were made to freeze the monomer/solvent and charcoal mixture in cold n-hexane and subsequently polymerising the frozen droplets. It was thought that by including charcoal the adsorption characteristics of the hydrogel bead could be modified.

However, it was found difficult to disperse charcoal uniformly in the monomer solution. Due to its higher density, charcoal separated out from the solution and settled at the bottom of the reservoir. Some success was achieved by shaking the monomer/solvent and charcoal mixture rapidly but even in this case some separation occured.

The method used was basically the same as previously described in this chapter. Charcoal had tendency to block the syringe needle during injection. Therefore, addition of charcoal/monomer mixture was achieved by using either a burette or a syringe without the needle attached.

A typical formulation used for these experiments was as follows:

Acrylic acid = 10.2040 g

Ethylene dimethacrylate = 2.3882 g

Activated Carbon = 0.6296 g (5% by weight of total monomer content)

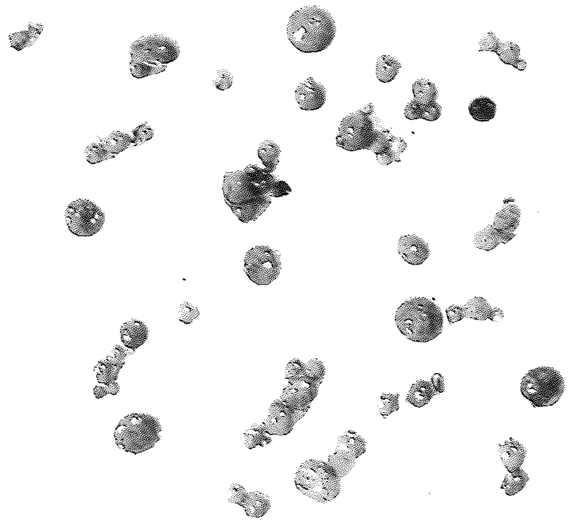
Ethylene glycol = 2.5177 g

Water = 10.0708 g

Uranyl nitrate = 0.2584 g (2% by weight of total monomers)

A small amount of charcoal was successfully incorporated into the hydrogel beads. However, upon hydration the beads were found to release powdered charcoal slowly. This process rendered the beads soft and fragile. Plate 3.4 shows the successful incorporation of carbon in some beads.

Plate 3.4



Activated charcoal dispersed in AA/EDM copolymer beads

However, due to lack in mechanical strength and due to consideration that the released powdered charcoal could cause the formation of micro-emboli if used in haemoperfusion, the beads were not considered to be useful for adsorption studies. It has previously been reported that incorporation of powdered charcoal in the macroporous hydrogel membranes could destroy the activity of the charcoal due to adsorption of the monomer (Skelly, 1974) by charcoal.

The evaluation of hydrogel beads by determination of equilibrium water content, density and particle size distribution, and by using scanning electron microscopy will now be described.

3.5 SCANNING ELECTRON MICROSCOPY (SEM) OF HYDROGEL BEADS

Surfaces and interiors of hydrogel beads were examined by performing SEM on the dehydrated samples. This study enabled to distinguish between macroporous and non-macroporous samples and to examine the surface smoothness of beads. SEM on the interior of the beads was done in order to get some idea about the extent of macroporous character (whether the macropores extended throughout the bead structure) and its directionality. The differences created in the bead structure by varying the composition were also elucidated by SEM.

Scanning electronmicrophotographs of selected samples are presented on the following pages. They have been divided into two categories: those displaying macroporous and those exhibiting non-macroporous nature of the bead. SEM on the surface of a commercial polymeric adsorbent (Amberlite XAD-7) was also performed for comparison purposes. These microphotographs are also included.

3.5.1 Scanning Electron Microphotographs (SEM's) of Macroporous Beads

In this category are included SEM's of beads with following compositions: AA/EDM, AA/HEMA/EDM, HEMA/EDM, and HEMA/DMAEMA/EDM. Table 3.3 lists various magnifications of photographs obtained for the above compositions.

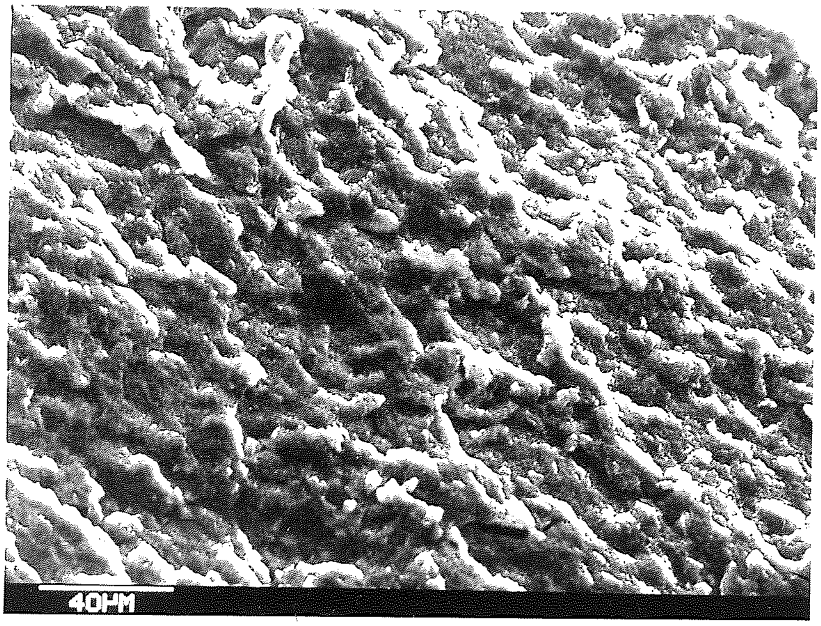
Table 3.3

SEM's of macroporous beads synthesised by freeze-thaw polymerisation

Composition (Initial Molar Ratio)	Plate No	Surface/ Interior	Magnification
AA : EDM	3.5	Surface	X 500
(100):(8.5)	3.6	Interior	X 2000
AA :HEMA: EDM	3.7	Surface	X 560
(60):(40):(8.5)	3.8	Surface	X 1600
	3.9	Interior	X 160
	3.10	Interior	X 560
HEMA:EDM	3.11	Surface	X 570
(100):(10)	3.12	Surface	X 1700
	3.13	Surface	X 2750
	3.14	A pore on the surface	X 6000
	3.15	Interior	X 2000
	3.16	Interior	X 5000
HEMA:DMAEMA:EDM	3.17	Surface	X 500
(90): (10) :(8.5)	3.18	Surface	X 1000
	3.19	Surface	X 2000
	3.20	Surface	X 5000

Scanning Electron Microphotographs of Macroporous Beads

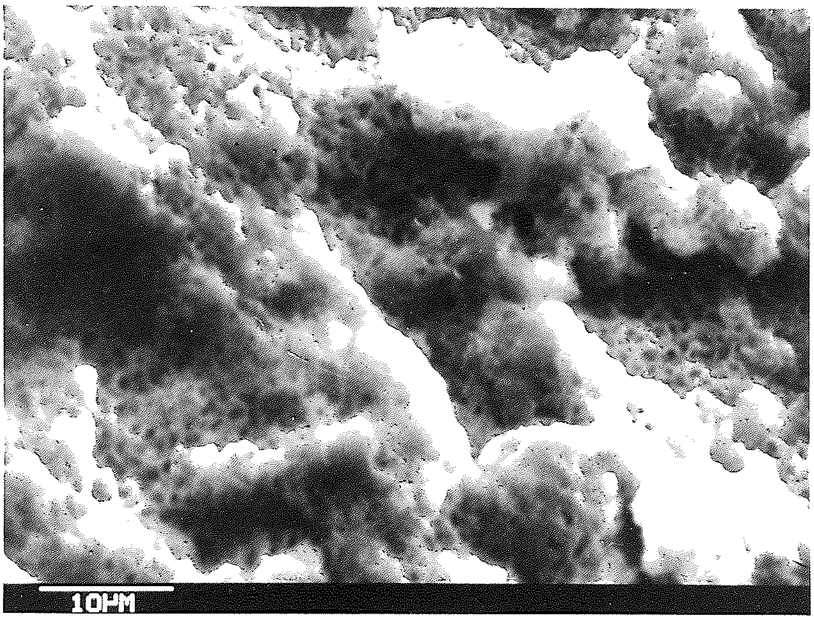
Plate 3.5



Surface of AA/EDM copolymer

Magnification X 500

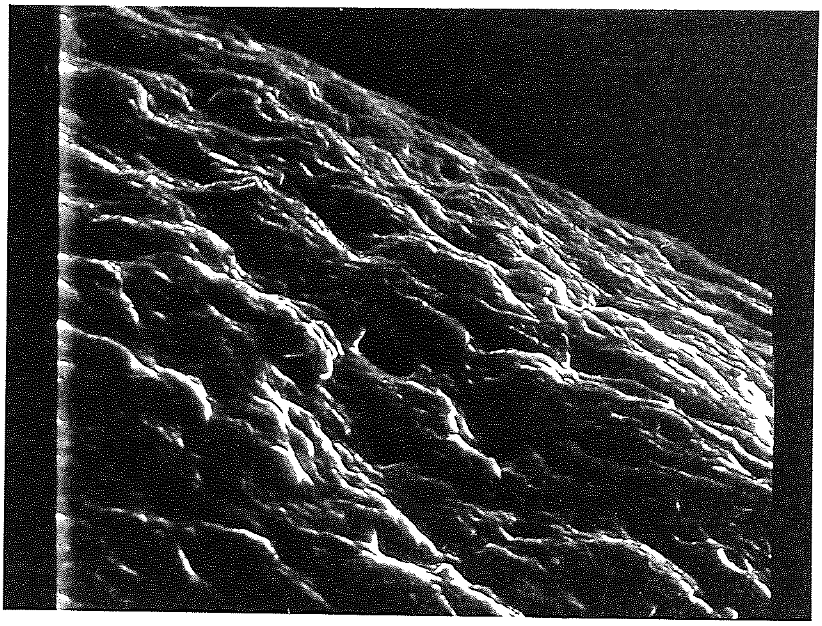
Plate 3.6



Interior of AA/EDM copolymer

Magnification X 2000

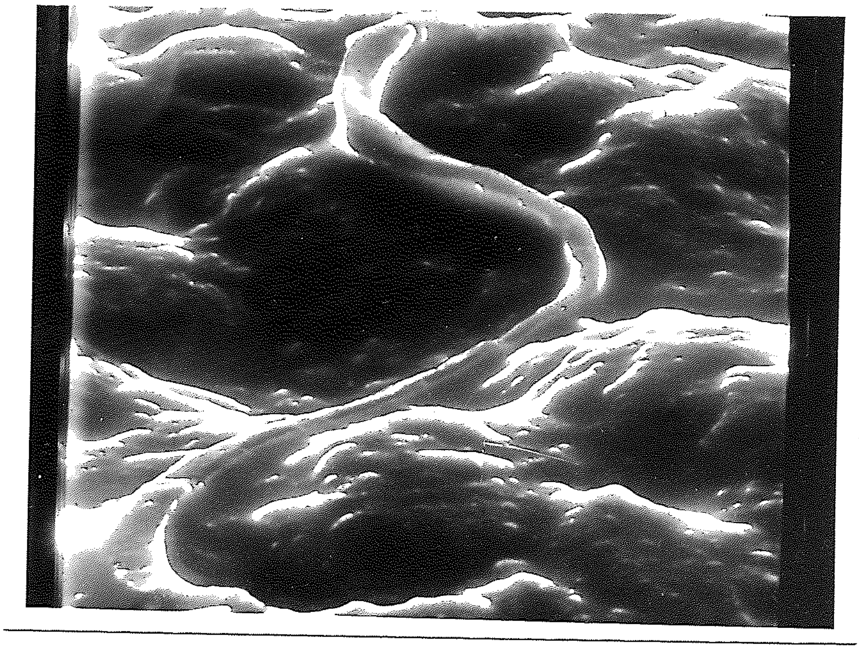
Plate 3.7



Surface of AA/HEMA/EDM terpolymer

Magnification X 560

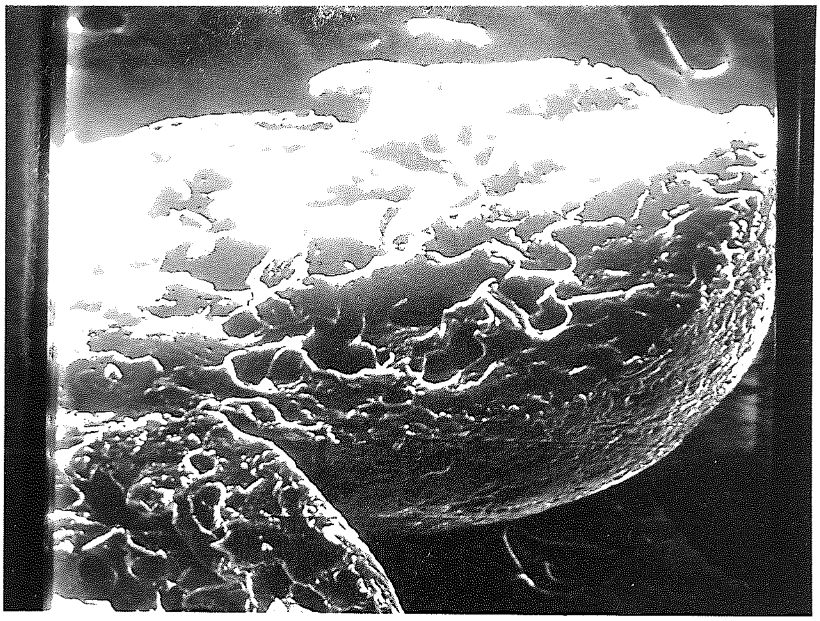
Plate 3.8



Surface of AA/HEMA/EDM terpolymer

Magnification X 1600

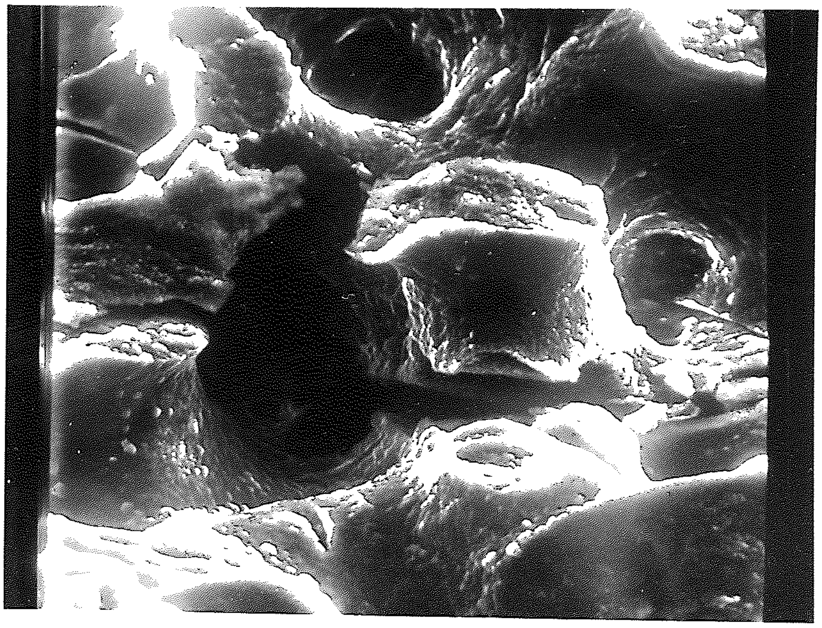
Plate 3.9



Interior of AA/HEMA/EDM terpolymer

Magnification X 160

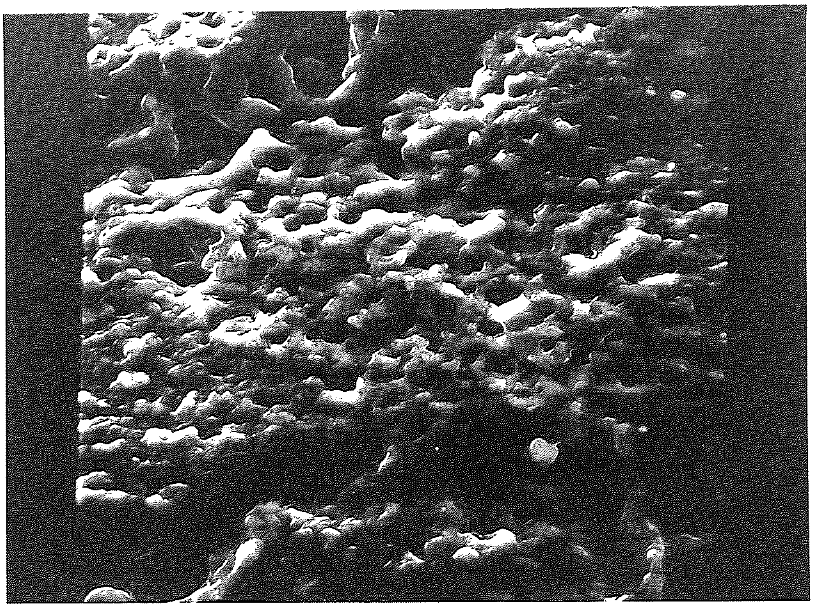
Plate 3.10



Interior of AA/HEMA/EDM terpolymer

Magnification X 560

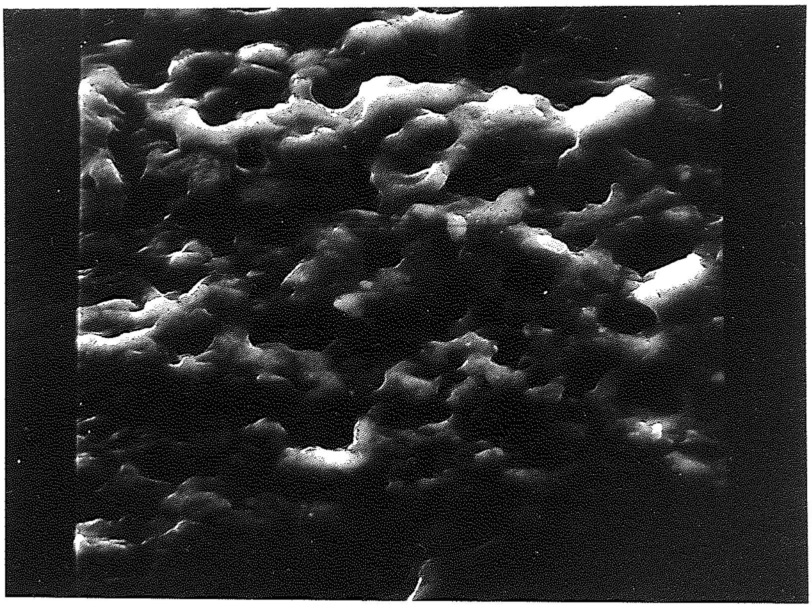
Plate 3.11



Surface of HEMA/EDM copolymer

Magnification X 570

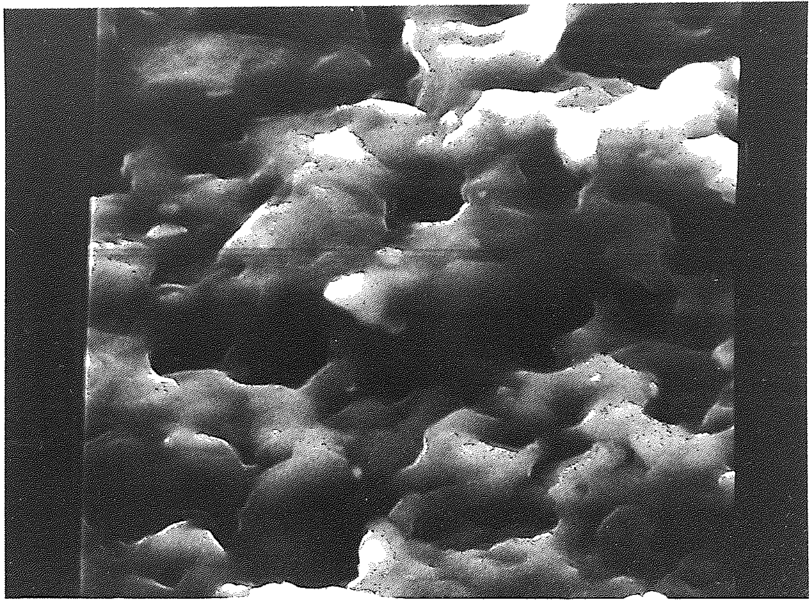
Plate 3.12



Surface of HEMA/EDM copolymer

Magnification X 1700

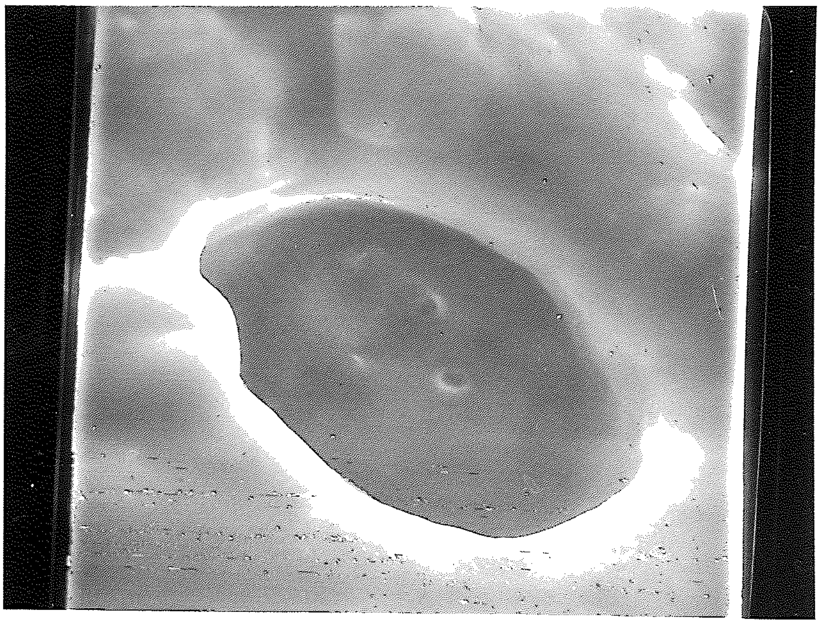
Plate 3.13



Surface of HEMA/EDM copolymer

Magnification X 2750

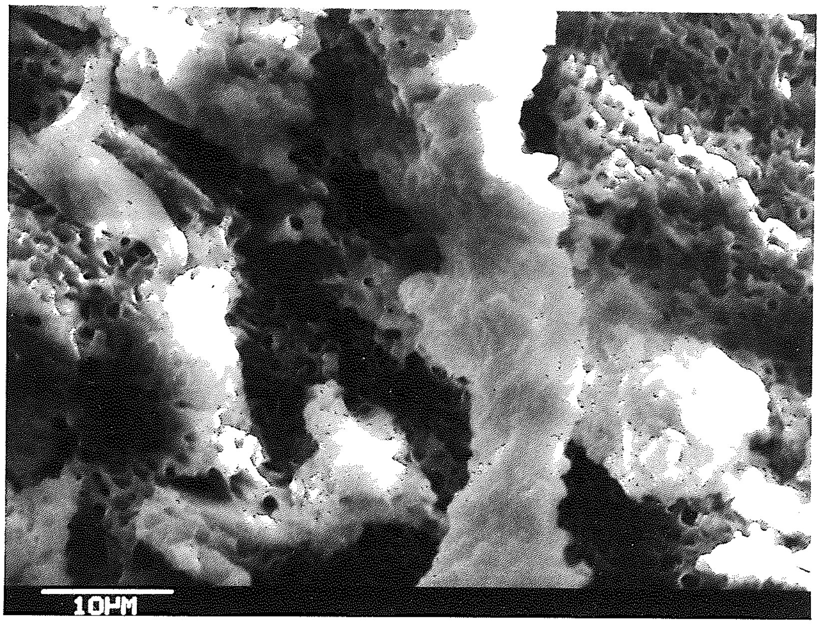
Plate 3.14



A single macropore on the surface of HEMA/EDM copolymer

Magnification X 6000

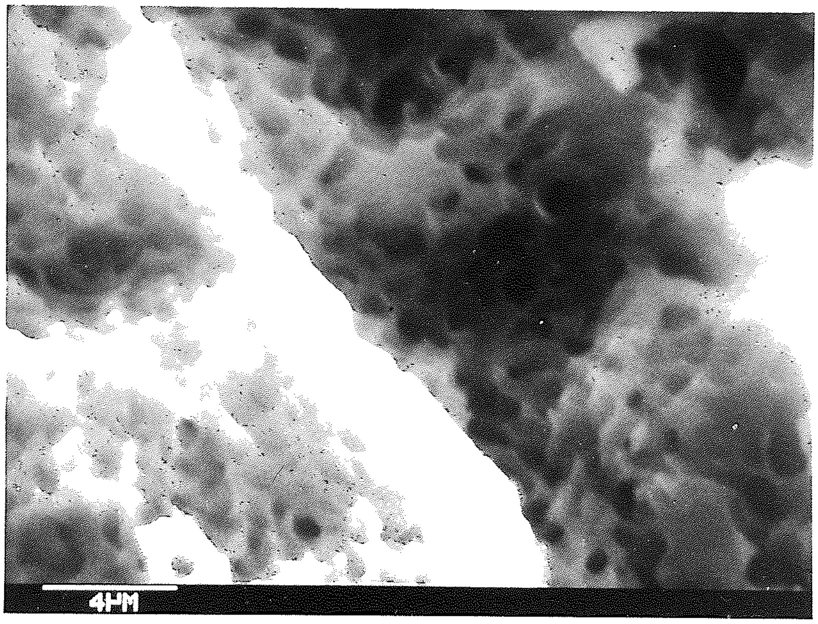
Plate 3.15



Interior of HEMA/EDM copolymer

Magnification X 2000

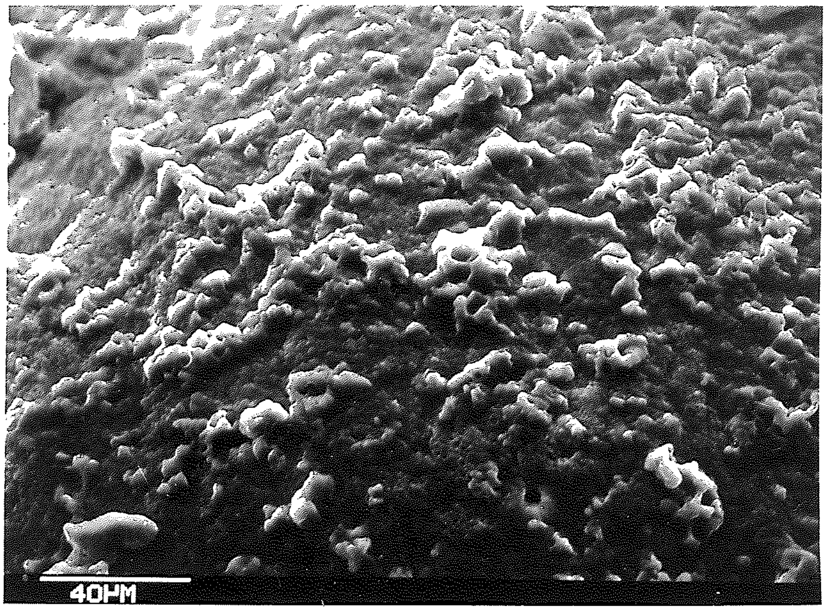
Plate 3.16



Interior of HEMA/EDM copolymer

Magnification X 5000

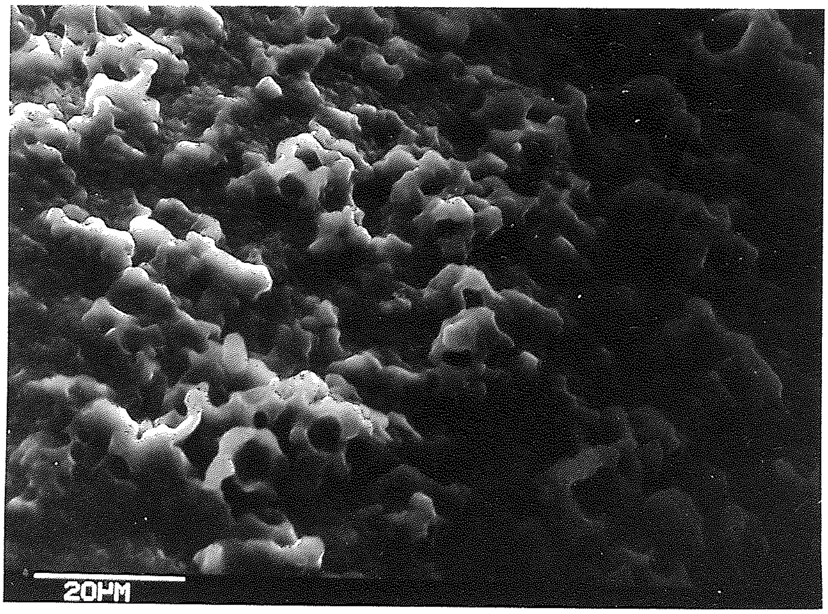
Plate 3.17



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 500

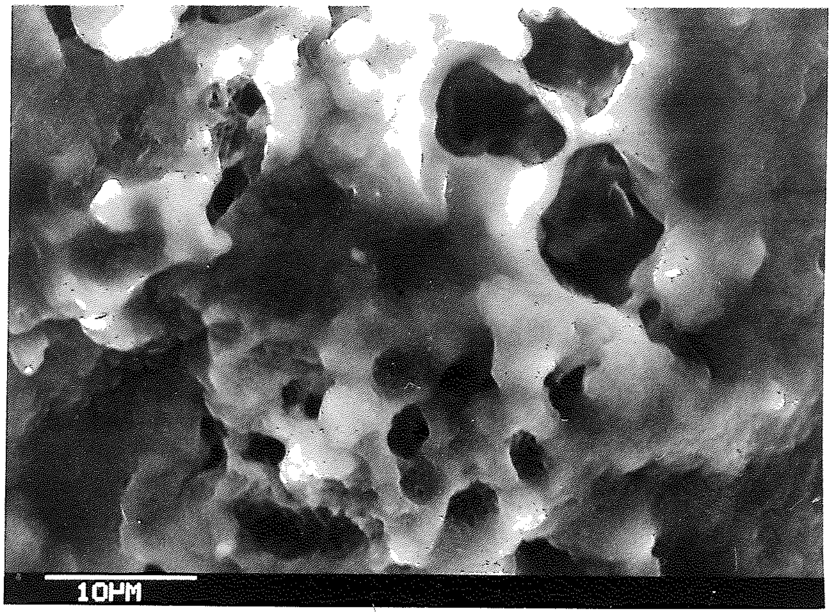
Plate 3.18



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 1000

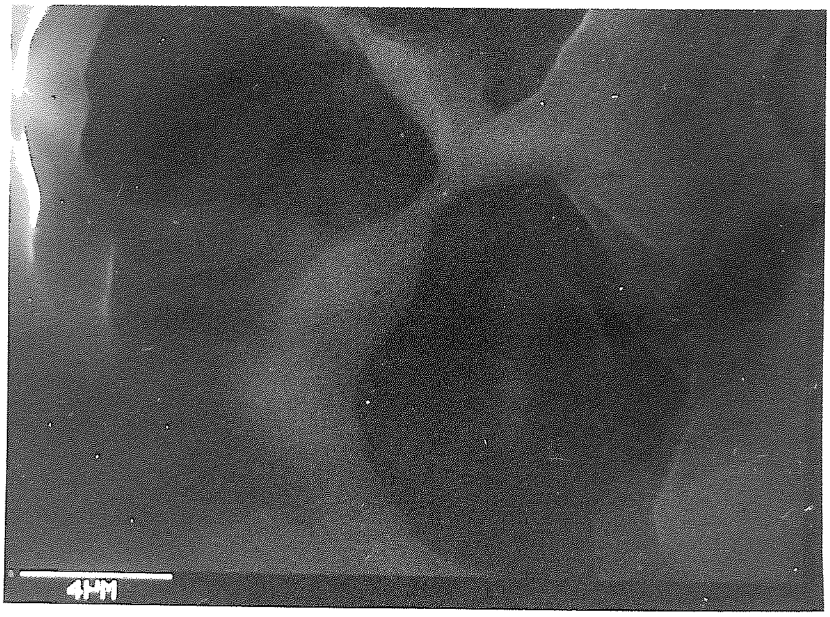
Plate 3.19



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 2000

Plate 3.20



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 5000

3.5.2 Scanning Electron Microphotographs of Non-Macroporous Beads

This category includes SEM's of the beads synthesised from compositions: ACR/NN'MBA, HEMA/DAA/EDM, and HEMA/NNACR/EDM. SEM's obtained at various magnifications for the above compositions are listed in table 3.4.

Table 3.4

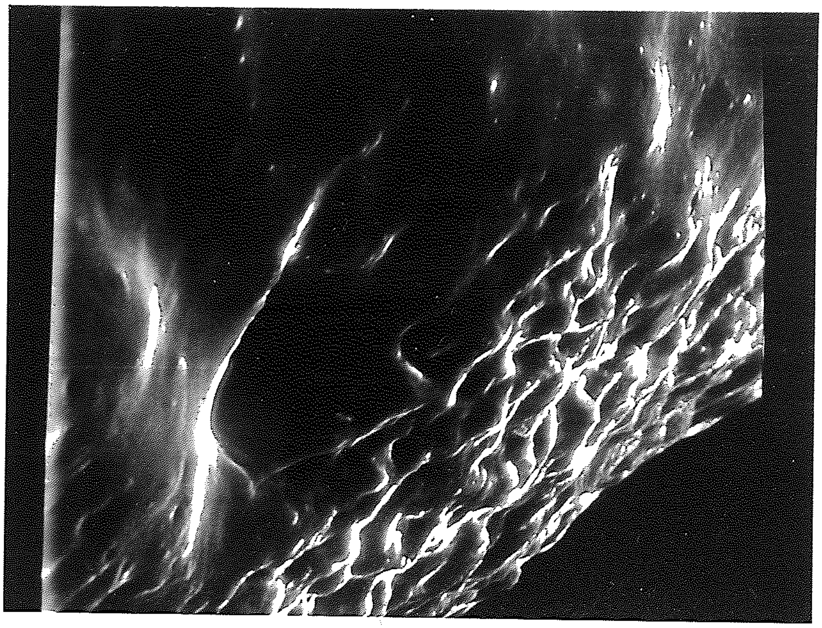
SEM's of non-macroporous beads synthesised by freeze-thaw polymerisation.

Composition (Initial Molar Ratio)	Plate No.	Surface/ Interior	Magnification
ACR:N,N'MBA	3.21	Surface	X 550
(100):(10)	3.22	Surface	X 1550
	3.23	Interior	X 250
	3.24	Interior	X 550
	3.25	Interior	X 1500
	3.26	Interior	X 2500
HEMA : DAA: EDM	3.27	Surface	X 550
(100):(25):(9)	3.28	Surface	X 1500
HEMA:NMACR:EDM	3.29	Surface	X 1500
(90): (10): (8.5)	3.30	Surface	X 2500

Scanning electron microphotographs of the Amberlite XAD-7 resin are shown in plates 3.31 and 3.32.

Scanning Electron Microphotographs of Non-Macroporous Beads

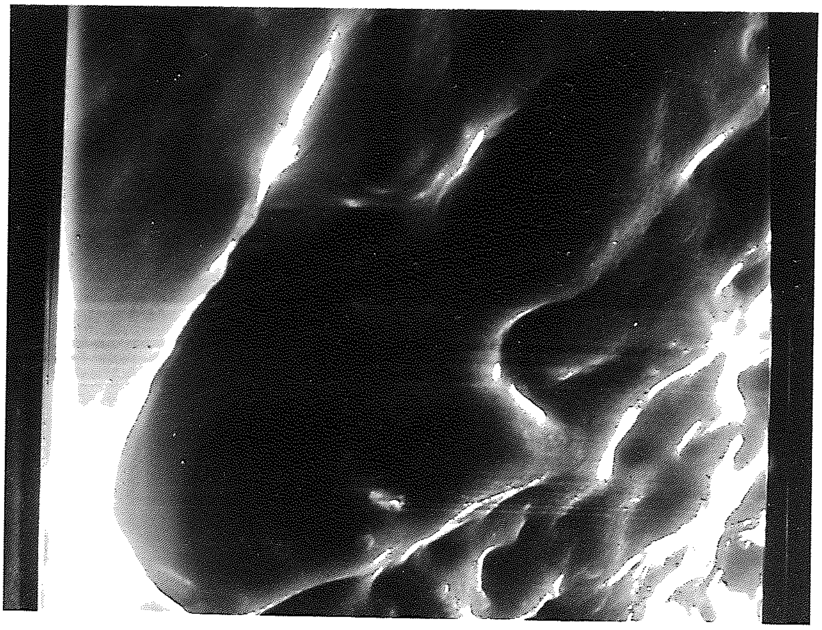
Plate 3.21



Surface of ACR/N,N'MBA copolymer

Magnification X 550

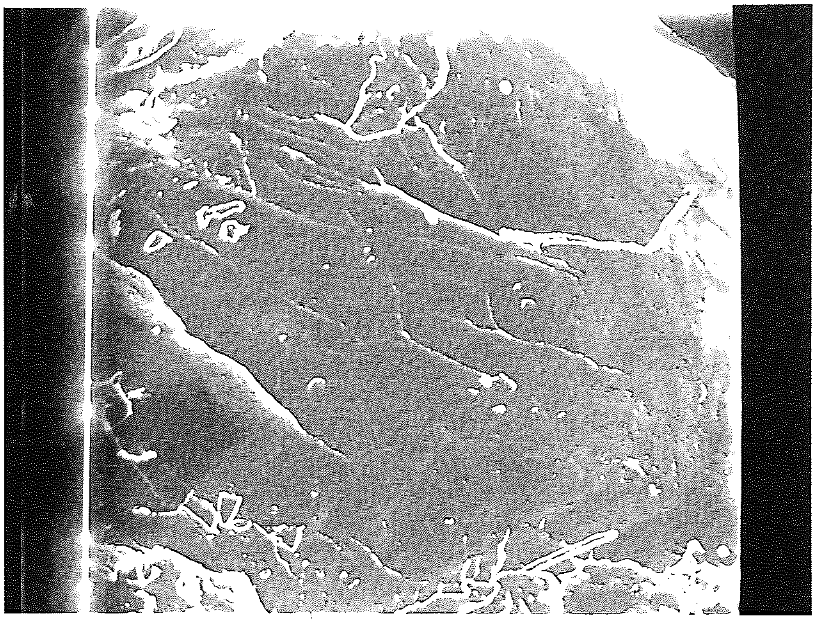
Plate 3.22



Surface of ACR/N,N'MBA copolymer

Magnification X 1550

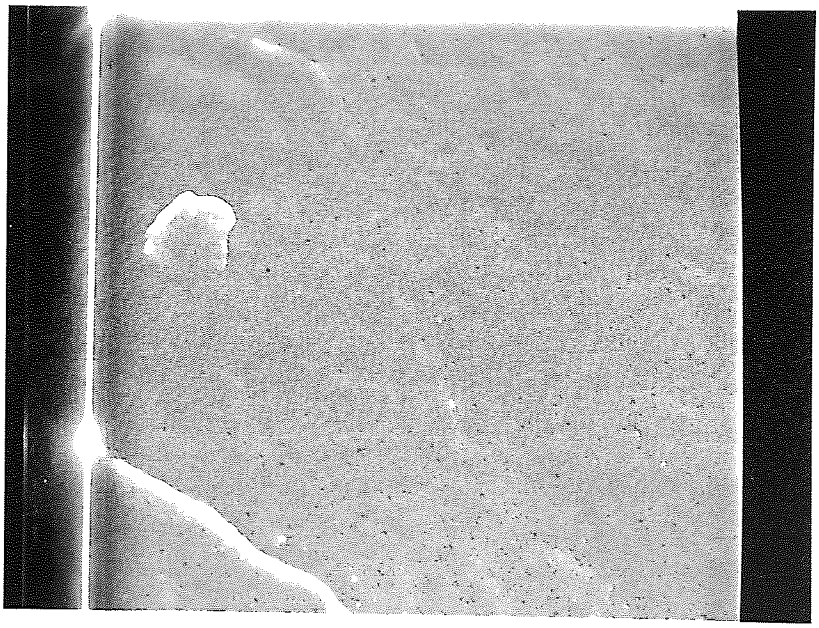
Plate 3.23



Interior of ACR/N,N'MBA copolymer

Magnification X 250

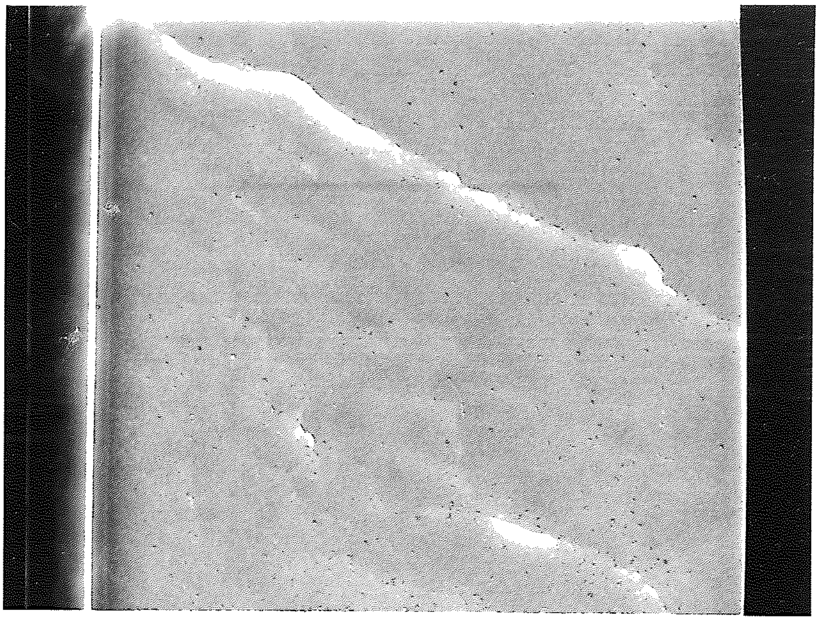
Plate 3.24



Interior of ACR/N,N'MBA copolymer

Magnification X 550

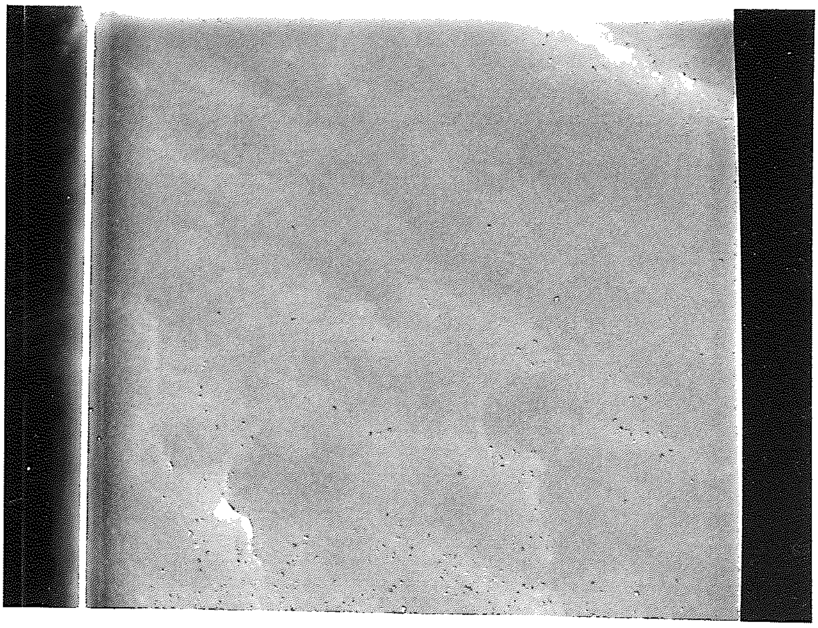
Plate 3.25



Interior of ACR/N,N'MBA copolymer

Magnification X 1500

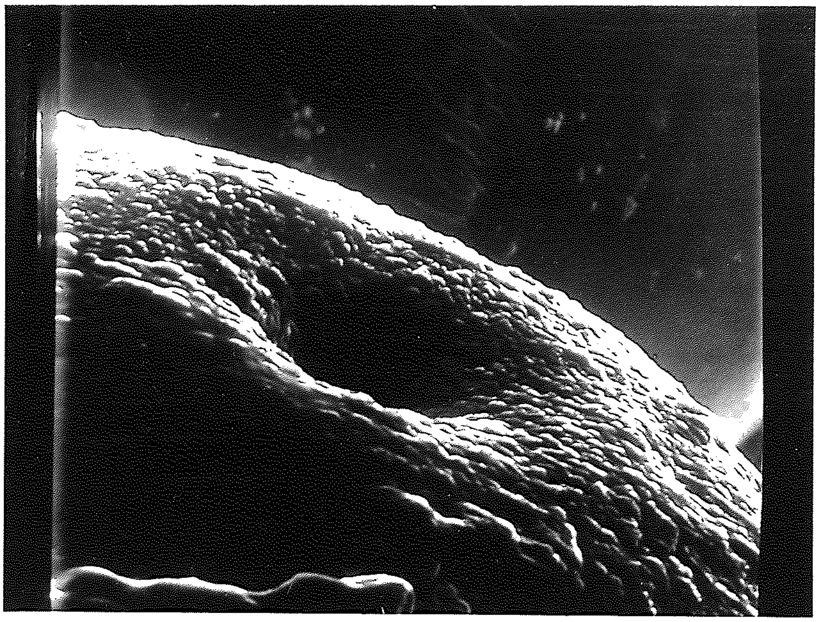
Plate 3.26



Interior of ACR/N,N'MBA copolymer

Magnification X 2500

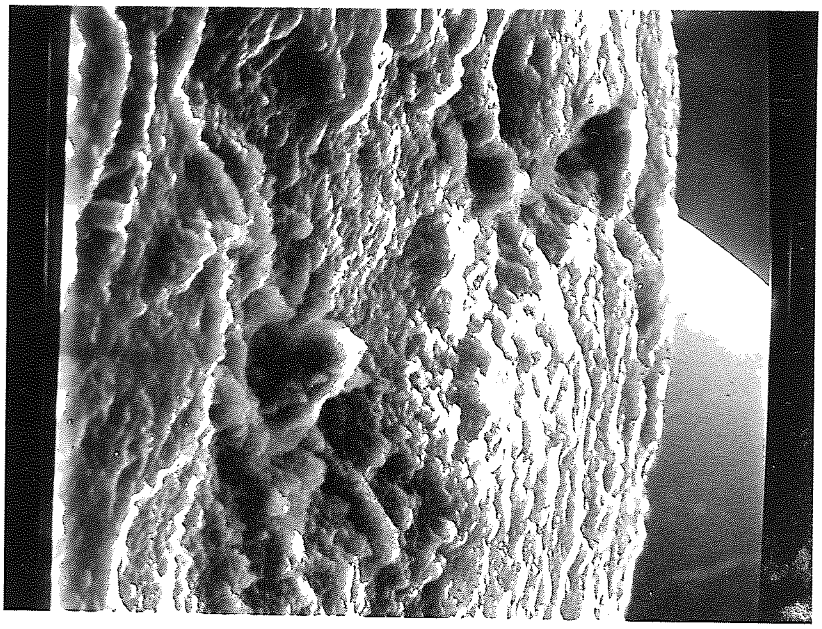
Plate 3.27



Surface of HEMA/DAA/EDM terpolymer

Magnification X 550

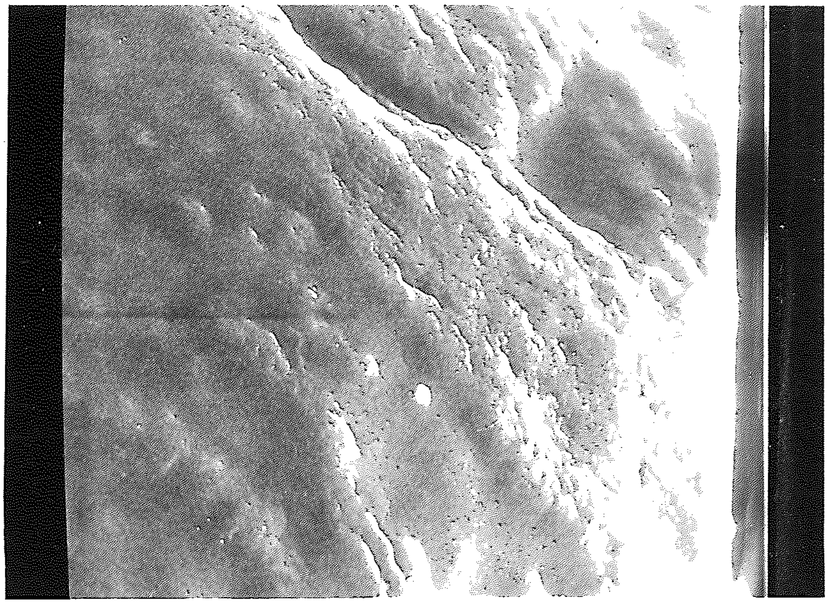
Plate 3.28



Surface of HEMA/DAA/EDM terpolymer

Magnification X 1500

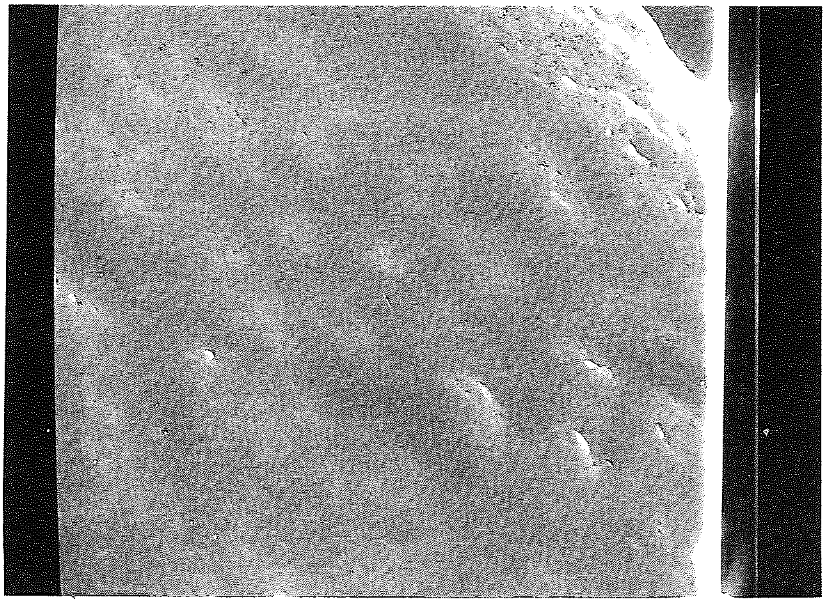
Plate 3.29



Surface of HEMA/NMACR/EDM terpolymer

Magnification X 1500

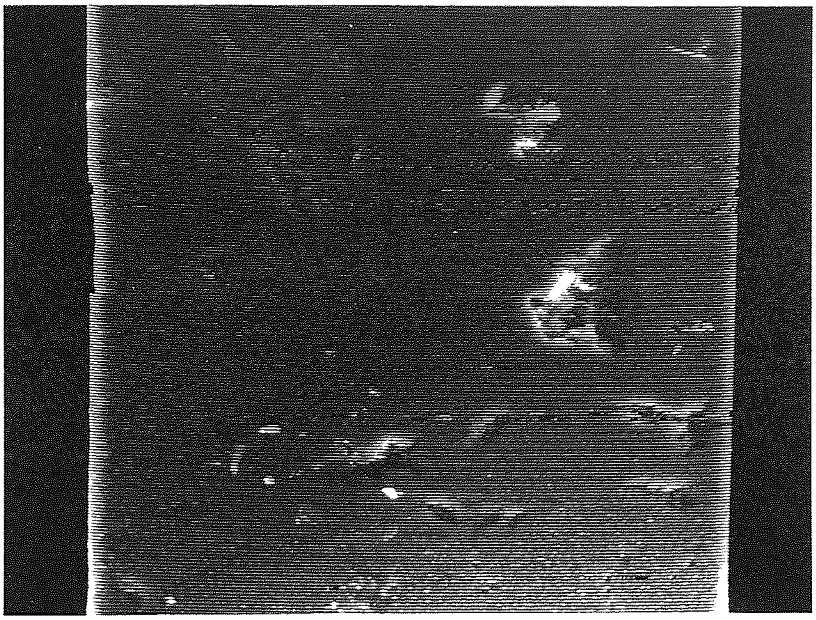
Plate 3.30



Surface of HEMA/NMACR/EDM terpolymer

Magnification X 2500

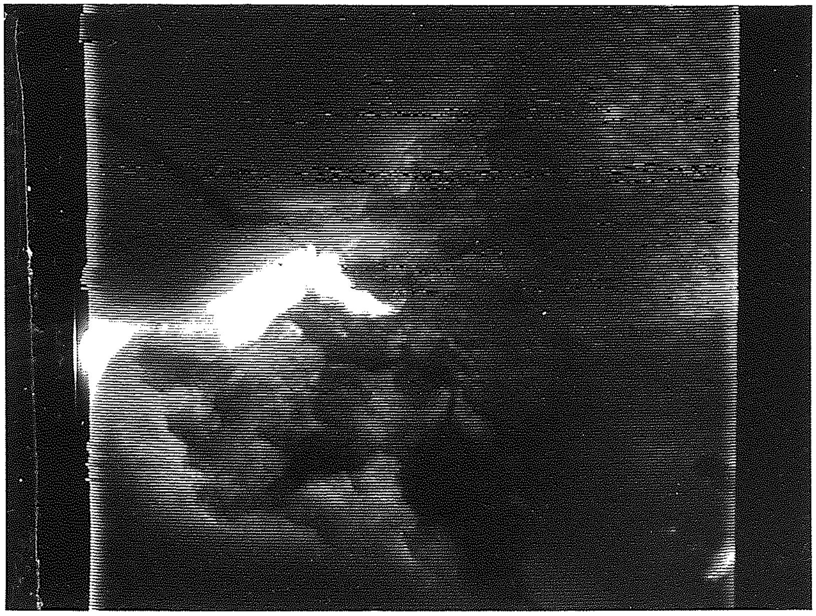
Plate 3.31



Surface of Amberlite XAD-7 Resin

Magnification X 560

Plate 3.32



Surface of Amberlite XAD-7 Resin

Magnification X 2500

3.6 EQUILIBRIUM WATER CONTENT AND DENSITY OF HYDROGEL BEADS

Table 3.5 lists the equilibrium water contents of hydrogel beads as determined experimentally and as calculated from the knowledge of densities of dehydrated and hydrated samples. Each value of EWC determined experimentally represents the mean of four separate determinations.

Table 3.5

Equilibrium water content of hydrogel beads

Monomer Composition	Initial Molar Ratio	EWC %	
		Experimental	Calculated
AA:EDM	100:8.5	76	80
AA:HEMA:EDM	75:25:8.5	60	40
AA:HEMA:EDM	60:40:8.5	58	30
MAA:HEMA:EDM	50:50:4.6	56	42
HEMA:EDM	100:10	51	45
ACR:N,N'MBA	100:10	80	39
HEMA:DAA:EDM	100:25:9	53	23
HEMA:NMACR:EDM	90:10:8.5	59	61
HEMA:DMAEMA:EDM	90:10:8.5	62	50

Densities of dehydrated and hydrated hydrogel beads as measured by density gradient column are summarised in table 3.6.

Table 3.6

Density of dehydrated and hydrated hydrogel beads

Monomer Composition	Density (g/cm^3)	
(Initial Molar Ratio)	Dehydrated	Hydrated
AA : EDM	1.232 1.240	1.040 1.042
(100:8.5)	1.242 1.259	1.069 1.080
AA:HEMA:EDM	1.240 1.252	1.145 1.150
(75: 25 :8.5)	1.252 1.260	1.150 1.160
AA:HEMA:EDM	1.200 1.210	1.150 1.157
(60: 40 :8.5)	1.220 1.231	1.160 1.160
MAA:HEMA:EDM	1.260 1.272	1.150 1.152
(50: 50 :4.6)	1.281 1.290	1.155 1.160
HEMA:EDM	1.275 1.279	1.129 1.138
(100:10)	1.280 1.292	1.140 1.140
ACR :N,N'MBA	1.280 1.285	1.160 1.160
(100:10)	1.285 1.290	1.160 1.170
HEMA:DAA:EDM	1.160 1.162	1.140 1.150
(100:25 :9)	1.182 1.186	1.151 1.165
HEMA:NMACR:EDM	1.260 1.266	1.090 1.140
(90 : 10 :8.5)	1.270 1.272	1.142 1.142
HEMA:DMAEMA:EDM	1.260 1.266	1.120 1.120
(90 : 10 :8.5)	1.270 1.270	1.130 1.132

It will be noticed that in table 3.6 the mean density has not been quoted, instead four separate determinations of density of samples of a particular composition are stated. These values are expected to indicate the nature of the polymer network and the porosity of the samples. Porosity may in turn yield information regarding the surface area of the sample which is important from the aspect of adsorption of solutes by the adsorbent.

3.7 PARTICLE SIZE DISTRIBUTION OF HYDROGEL BEADS

Samples of hydrogel beads were graded into different size ranges by manual sieving. The overall particle size range obtained for samples is indicated in table 3.7. Figures 3.2 and 3.3 show plots of weight percentage of different size ranges against the particle diameter for a representative sample. These figures show variation in particle size range caused by varying the rate of injection of monomer solution into the non-solvent.

Table 3.7

Particle size range of hydrogel beads

Monomer Composition	Initial Molar Ratio	Particle Size Range (μm)
AA:EDM	100:8.5	2000 - 250
AA:HEMA:EDM	75:25:8.5	3000 - 500
AA:HEMA:EDM	60:40:8.5	3000 - 500
MAA:HEMA:EDM	50:50:4.6	2000 - 125
HEMA:EDM	100:10	2000 - 250
ACR:N,N'MBA	100:10	3000 - 500
HEMA:DAA:EDM	100:25:9	2000 - 125
HEMA:NNACR:EDM	90:10:8.5	2000 - 125
HEMA:DMAEMA:EDM	90:10:8.5	2000 - 125

Figure 3.2

Histogram of the fractional data for AA:EDM, (100:8.5) copolymer

Injection rate: 1 ml/sec

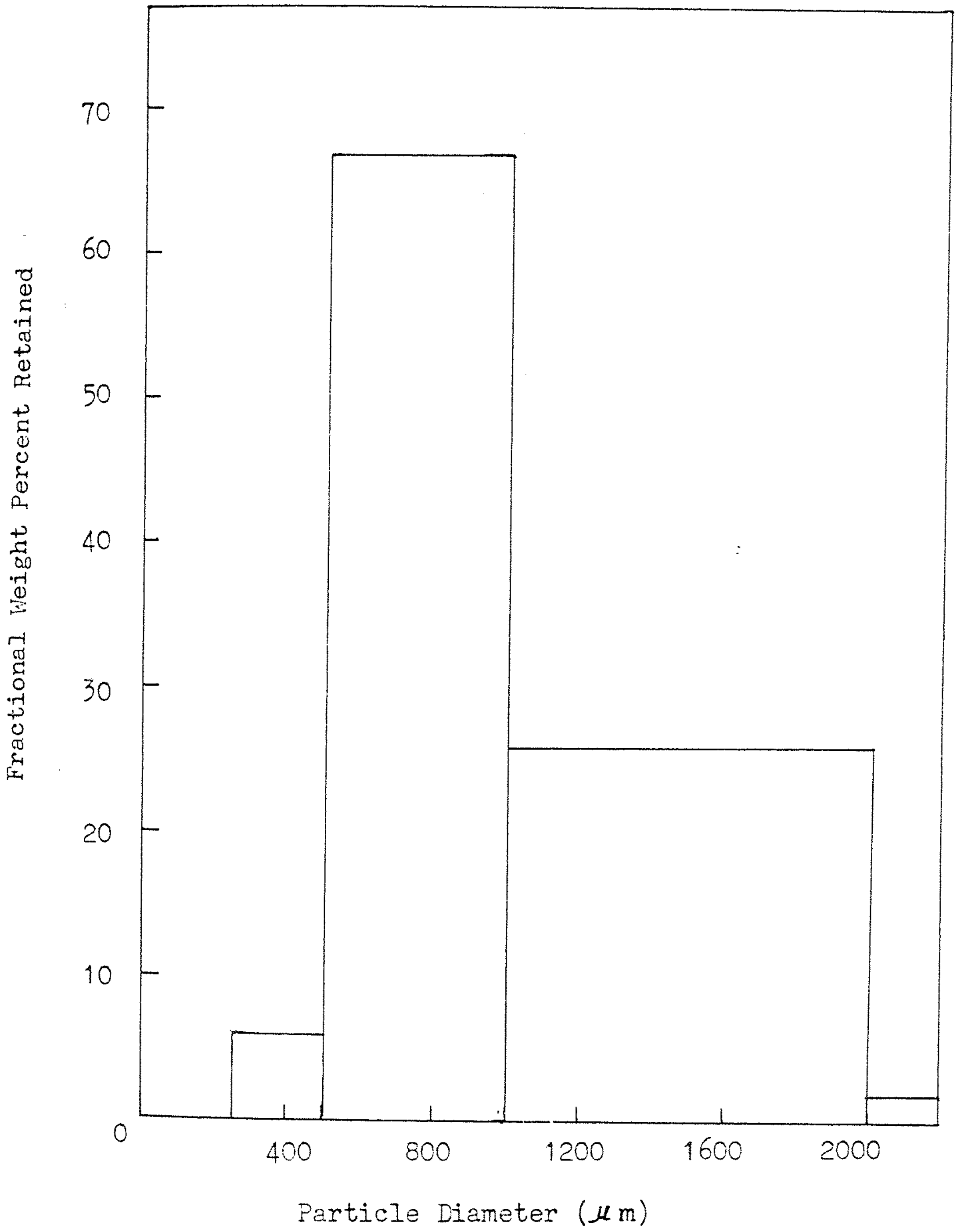
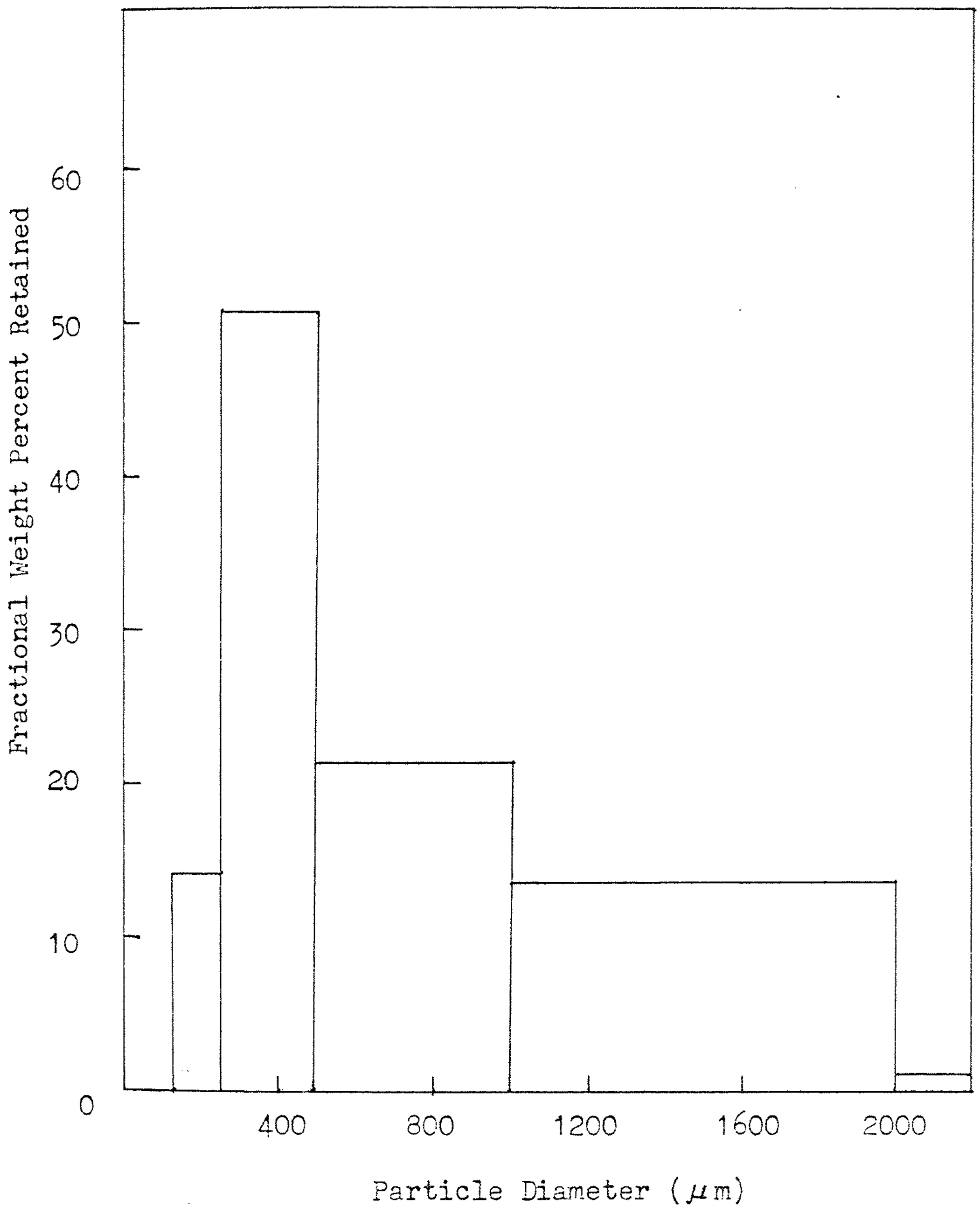


Figure 3.3

Histogram of the fractional data for AA:EDM, (100:8.5) copolymer

Injection rate: 1.7 ml/sec



3.8 DISCUSSION

The method of polymerisation by trapping monomers in frozen solvent crystals was successfully modified to produce a variety of hydrogel beads. The modifications included polymerisation 'in situ', and using n-hexane instead of heptane as the non-solvent for freezing the monomer/solvent mixture. Compositions (ACR/N,N'MBA), (HEMA/DAA/EDM), (HEMA/NMACR/EDM) and (HEMA/DMAEMA/EDM) were new additions to the previously reported range polymerised in the bead form by using this method.

Results indicate that hydrogel beads with a range of chemical composition, particle size, porosity, density, water content, and surface morphology can be prepared. The resultant beads prepared by this method were white and opaque. This is thought to be due to the formation of a dispersion of separated water clusters and hydrogel phases. This two phase-heterogeneous system with different refractive indices in the two phases results in turbidity in the polymer matrix. This is in sharp contrast to homogeneous gels which are single phase systems in which the imbibed water is held immobile, more or less uniformly throughout the gel structure.

The effects of various parameters upon the structure of hydrogel beads prepared with the freeze-thaw technique will be discussed below.

3.8.1 Monomer Composition

Although all the compositions considered were polymerised in the bead form, compositions including monomers ACR, DAA and NMACR did not produce macroporous beads. Hydrogel matrices of beads prepared from compositions containing HEMA, AA, MAA and DMAEMA showed the existence of pores in the macroporous size range. Differences in macroporosity can be attributed to the differences in the freezing behaviour of monomers. Ice separates from solutions of different monomer compositions at different temperatures. This affects the rate of cooling and the subsequent nature of polymer matrix during polymerisation. Acrylamide has a high solvation energy. Therefore, compositions containing acrylamides freeze slowly when added to cold hexane but the separation of phases does not occur which explains the absence of macroporous structure in hydrogels containing such monomers. In sample VI, ACR/N,N'MBA copolymer, the presence of non-macroporosity was also favoured by the factor that it contained a hydrophilic crosslinking agent N,N'MBA. The observation of Haldon and Lee (1972) that a hydrophobic crosslinking agent such as EDM is required for phase separation to take place upon freezing, is held. This phase separation is the determining factor in the creation of macroporous structure.

Compositions of samples containing AA, MAA, HEMA and DMAEMA produced macroporosity because of their almost instantaneous freezing behaviour after dropping into the cold hexane followed by phase separation.

In the terpolymer beads containing (HEMA/DAA/EDM) and (HEMA/NMACR/EDM) there was some tendency for porous structure to be produced due to the presence of HEMA and EDM in their composition.

Increasing the monomer concentration would result in fewer and smaller pores. Increasing the amount of EDM in the monomer composition would mean to increase the number of crosslinks. This would in turn lead to tightening up of the polymer network. The amount of EDM in the monomer composition would also affect the nature of freezing as ice separates more readily from solutions of high EDM content.

Although increasing the amounts of monomers would provide more strength to the bead it may adversely affect other important properties such as permeability to water. Permeability to water and solutes is important from the aspect of adsorption properties of the beads. Haldon and Lee (1972) found from studies on HEMA/EDM system that at a monomer concentration of 62 percent the polymer prepared in water rich solvents showed a minimum permeability at appromixately 6 percent EDM. Above this EDM level the permeability co-efficient increased quite dramatically. Beyond 10 percent EDM the permeability co-efficient of rapidly frozen system was constant. Solutions containing low amounts of EDM showed no unidirectional character of porous structure on freezing. The effect of crosslink density upon EWC is considered later in this chapter.

Ethylene glycol and water content of the solvent also affects the polymerisation conditions. The higher the ethylene glycol content the slower the rate of cooling. This affects by providing lower freezable content of the solvent and by lowering the rate of freezing. Ethylene glycol is also a better solvent for EDM than water and its presence allows more EDM to be dissolved.

3.8.2 Particle Size and Pore Size

Table 3.7 shows that particle size range 3000μm - 125μm was obtained under the experimental conditions used. Variation in injection rate provided versatility in particle size. Figures 3.2 and 3.3 demonstrate the size of beads for a particular sample, AA/EDM copolymer obtained by two different injection rates. As the injection rate was varied from 1.0 ml/sec to 1.7 ml/sec, while keeping the stirring rate constant, the particle size range shifted to lower values. Beads produced by these two injection rates were in the adequate size range to be used for adsorption studies.

Pores having diameter upto 5 μm were found to exist in the macroporous hydrogel beads. Pore sizes were estimated from

SEM's. Sizes of pores on the surface and the interior of the bead differed. This could be explained by the manner in which the monomer droplet freezes. The solution near the surface freezes quickly, therefore, probably not allowing sufficient phase separation to occur. The solution inside the droplet has longer time to freeze, thus resulting in the creation of different porous sturcture inside the bead. This effect is evidently demonstrated on microphotographs in plates 3.5 and 3.6, 3.8 and 3.9, and 3.13 and 3.15. These photographs show external and internal surfaces of beads of three different samples. However, effective porosity of macroporous beads differs from the porosity of non-macroporous beads.

In the broadest sense, the porosity of a solid is defined as the ratio of the volume of interstices of the material to the volume of its mass. Whereas homogeneous gels are uniform for all practical purposes, the heterogeneity of the two-phase macroporous gels must be taken into account in any consideration of their porosity. Their effective porosity is related directly to the volume fraction of pores in the gel and inversely to the degree of crosslinking in the solid gel phase. Porous structure of macroporous hydrogel beads would differ in hydrated and dehydrated states. By removing the imbibed water on dehydration, the microporosity of the gel beads would collapse. Therefore, in the dehydrated state only the macroporosity would be evident.

3.8.3 Density

Table 3.6 summarises densities of dehydrated and hydrated hydrogel beads. It was hoped that density measurements would shed some light upon the nature of the polymer matrix. As expected, hydrated beads had lower densities than the dehydrated beads. This was found to be the case with all samples. Density can in general be regarded as the measure of looseness of the bead structure. Thus density values may be expected to affect or determine other properties such as permeability and surface area of the beads. Beads having lower density might be expected to have more porous structure than the beads of the same material having higher density, although, this is not always the case as observed from density figures. In general, the more porous the structure the higher the surface area and permeability will be.

In table 3.6 four density values obtained for each sample are quoted. In some cases there is variation in density values. These discrepancies can be attributed to several possible errors encountered during the density measurements. The errors involved in experiments using the density gradient column will be discussed below.

In the density gradient column the positioning of partially mixed liquids results in the column having a range of densities, and the density of the liquid mixture increases from the top to the bottom. In certain experiments the graph of density against cathetometer reading deviated from linearity as shown in figure 2.4 (chapter 2). This reflects the difficulties faced in keeping control upon the proper rate of mixing of the two miscible liquids used to prepare the column, and also upon the proper flow rate of the liquid mixture into the column. The addition and removal of samples by using the sweep basket also subjected the column liquid to disturbances. When the column liquid was allowed to stand over a period of several days and was used again, the results obtained varied slightly from those obtained when column was freshly prepared. This was thought to be due to the diffusion of one part of the liquid into another and, probably, also due to the impurities leached out from test samples into the liquid of the column.

The above problems were avoided upto some extent by preparing fresh column whenever a slight change in the density gradient was discovered. A new calibration graph was then constructed. Other difficulties faced were in taking the cathetometer readings at equilibrium position of the sample. In some cases it was found difficult to obtain the samples in stationary state before taking the reading. In such cases the sample was allowed sufficient time to become stationary after introducing into the column.

Errors may also have been introduced by the possible attachment of air bubbles to the beads even after moistening with carbon tetrachloride. The difficulties in obtaining completely dehydrated samples may have contributed further to the discrepancies in density values. These difficulties in connection with density measurements have been reported by other workers also (Shen et al., 1967; Llavsky et al., 1968; Ng, 1974). Incomplete drying of samples would result in a lower apparent density of the dehydrated samples.

It is difficult to find references in literature to densities of such copolymer and terpolymer systems as prepared in this research. However, the densities of poly (HEMA) in the dehydrated state (1.275-1.292) and the hydrated state (1.129-1.140) obtained in this work are quite close to the values reported in literatures: 1.279 the dehydrated state and 1.169 the hydrated state (Ng, 1974), 1.284 the dehydrated state and 1.189 the hydrated state (Atherton, 1982). Results differ because different polymerisation conditions were used for synthesising poly (HEMA) hydrogels.

3.8.4 Equilibrium Water Content (EWC)

Equilibrium water content values summarised in table 3.5 show variation with different copolymer and terpolymer systems. Out of the hydrogel beads synthesised in this work, ACR/N,N'MBA copolymer displayed the highest equilibrium water content (80 percent) and the HEMA/EDM copolymer the lowest (51 percent). Other compositions had water contents which fall between the ACR/N,N'MBA and HEMA/EDM systems. At first glance it is indicated that the higher the proportion of hydrophilic groups in the hydrogel structure the higher the water content. However, this may only be partly applicable as several other factors determine the swelling of hydrogels. Swelling of non-ionic hydrogels was reported to depend on at least three factors:

(i) the interaction parameter between the polymer and solvent

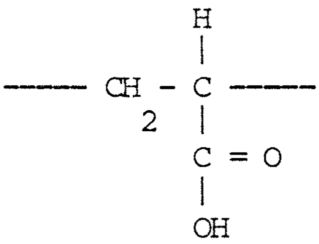
(ii) the extent of crosslinking or the molecular weight of the polymer chain between crosslinks

(iii) the relative swelling of the polymer at the time of crosslinking

The interaction parameter between polymer and water is a most important factor in determining EWC of the polymer. If it is low then water is a bad solvent for the polymer for example, poly (HEMA) and if it is high the polymer will dissolve unless factors such as crosslinks prevent it. Therefore, an increase in the polarity of the monomer units wll lead to increased hydrophilicity and increased solvation. The presence of highly polar amide group in ACR/N,N'MBA copolymer made it very hydrophilic, whereas HEMA/EDM with its less polar hydroxyl group attracts lesser amount of water. This situation is further favoured by the presence of more hydrophilic (N,N'MBA) and less hydrophilic (EDM) respectively crosslinking agents.

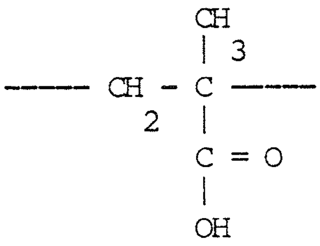
In the acrylic acid series (samples I to IV) a gradual decrease in EWC was observed with increasing amount of HEMA added to the composition. AA/EDM copolymer with its polar carboxyl group equilibrated with 76 percent water. Addition of 25 mole percent HEMA in sample II reduced the water content to 60 percent. With a further 15 mole percent of HEMA added in sample III the EWC was further reduced to 58 percent. This reduction in EWC can be attributed to the more hydrophobic nature of the hydroxyl group of HEMA than the carboxyl group of Acrylic acid.

MAA/HEMA/EDM terpolymer had 56 percent EWC. In this case it was not only the fact that another hydrophobic group had been added that caused lowering of the EWC; it was also due to decreased chain rotation about the polymer backbone caused by the presence of methyl group as is demonstrated by structures of AA and MAA:



$$-CH_2-\overset{H}{\underset{COOH}{C}}-$$

Acrylic Acid



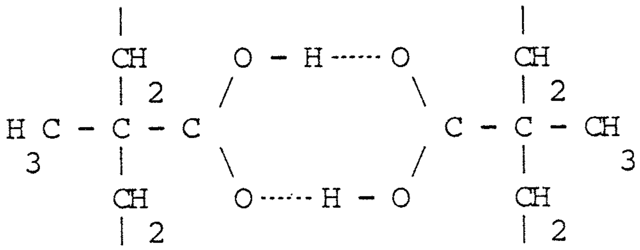
$$-CH_2-\overset{CH_3}{\underset{COOH}{C}}-$$

Methacrylic Acid

Another factor which could affect EWC in systems containing AA and HEMA or MAA and HEMA was thought to be the occurrence of interchain hydrogen bonding between carboxylic acid groups of polymer backbones. This kind of interaction would create secondary crosslinks and restrict the ability of the polar hydrophilic groups to cluster more water molecules. This type of interaction was also reported by Middleton (1981), with the HEMA/MAA system where a minimum in EWC at 50:50 mole percent composition was observed. This would be expected under these circumstances because hydrogen bonding will reach a maximum as an equimolar composition is attained. The inter-chain hydrogen bonding between two MAA units is illustrated in figure 3.4.

Figure 3.4

The interchain hydrogen bonding between two MAA units



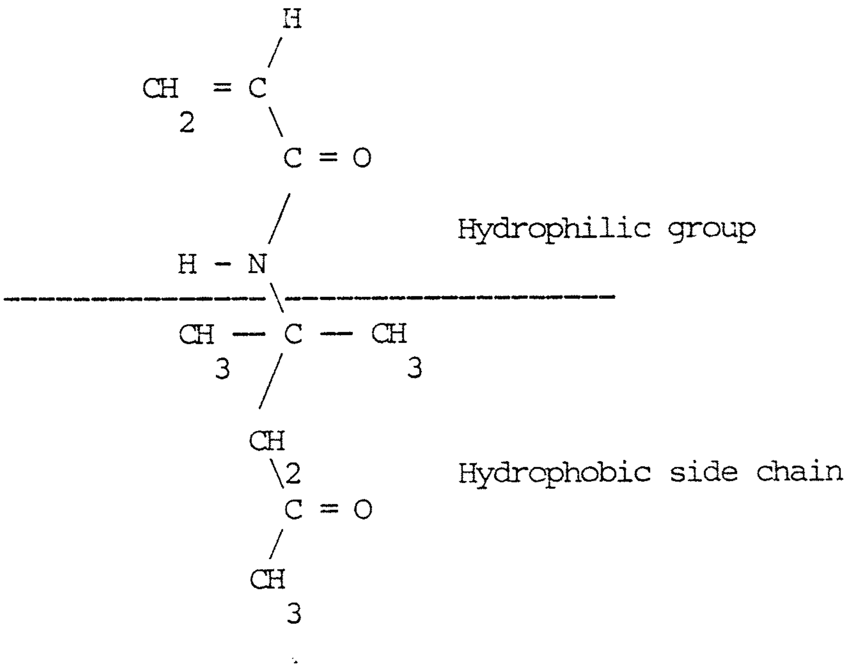
In the MAA/HEMA/EDM terpolymer the reduction in EWC would probably be greater than in AA/HEMA/EDM terpolymer. The replacement of AA with MAA would probably affect EWC in addition to providing hydrophobicity of methyl groups, by the possibility of hydrophobic bonding. This would lead to a stabilisation of the hydrogen bond and hence a lower water content, as the hydrogel would be effectively more crosslinked.

In ACR series (samples VI to VIII), there was observed a lowering of EWC content of hydrogel beads as more hydrophobic monomers were incorporated. EWC of 80 percent in ACR/N,N'MBA copolymer decreased to 53 percent in HEMA/DAA/EDM terpolymer and to 59 percent in HEMA/NMACR/EDM terpolymer system. In this case two hydrophobic components had been added which made differences in water contents. One was introduced by EDM and the other by DAA and NMACR monomers. DAA being more hydrophobic than NMACR had a greater influence in reducing the EWC.

HEMA/DAA/EDM terpolymer may also be expected to possess high bulk hydrophilicity and low surface hydrophilicity. Considering the structure of DAA (figure 3.5) it can be seen that this monomer has a large hydrophobic side chain and a highly hydrophilic amide group. Thus the polymer containing

Figure 3.5

Structure of Diacetone Acrylamide (DAA)



DAA will possess a polymer backbone with large hydrophobic side groups. Between hydrophobic backbone and the hydrophobic side group there is highly polar amide group, which is shielded by the hydrophobic group and backbone. This would, therefore, give HEMA/DAA/EDM terpolymer high bulk hydrophilicity.

Interchain hydrogen bonding which was thought to occur in MAA/HEMA system would also be expected to exist in ACR/HEMA systems. Acrylamide, which is itself known to form plurimolecular aggregates in various solvents does in fact strongly hydrogen bond which results in high boiling point of many amides. However, in copolymers the carbonyl group of acrylamide is shielded so that hydrogen bonding between chains cannot readily take place.

HEMA/DMAEMA/EDM terpolymer beads displayed water content of 62 percent. This value compared with water content of 51 percent of HEMA/EDM beads was higher. The increase in EWC was due to the incorporation of DMAEMA component. DMAEMA contains a water sensitive tertiary amine group. Upon self polymerisation DMAEMA gives a water soluble material but when blended into other monomers and copolymerised such as HEMA/DMAEMA/EDM system, the tendency towards solubility decreases due to the formation of crosslinks in the terpolymer. However, the cationic property of the tertiary amine group is retained in the copolymers.

The effects of introducing ionic groups in the hydrogel on the water swelling were studied by Gregonis et al. (1976). MAA was used as a carrier of anionic charges and DMAEMA as a carrier of cationic charges. As shown in figures 3.6 and 3.7 poly (HEMA) gels polymerised with upto 5 mole percent MAA or DMAEMA show little change in EWC from that of pure poly (HEMA) polymerised at identical conditions. This is because carboxylic acids and tertiary amines being weak acids and bases do not ionise to an appreciable extent at neutral pH.

Figure 3.6

Equilibrium water weight fraction, wf, of HEMA/MAA copolymers

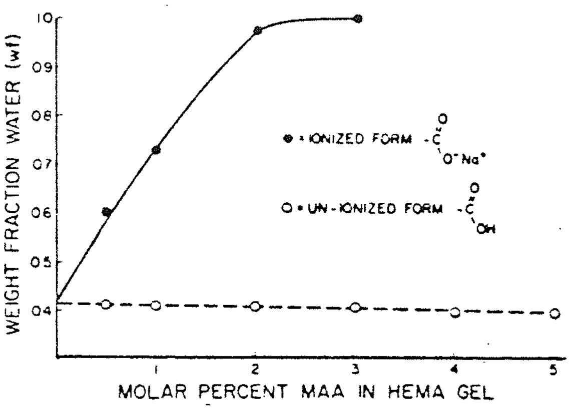
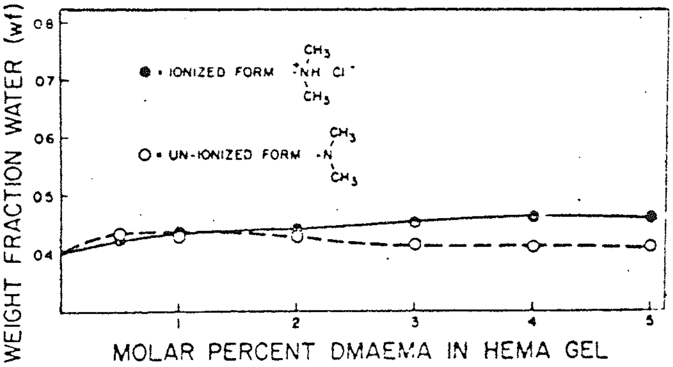


Figure 3.7

Equilibrium water weight fraction, wf, of HEMA/DMAEMA copolymers

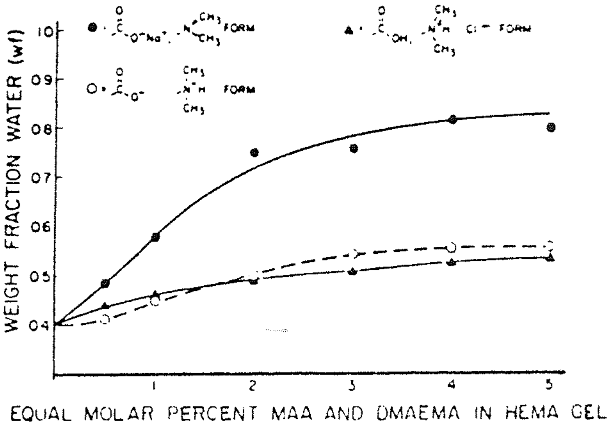


The conversion of MAA to its carboxylate salt in the gels dramatically increases the EWC as shown in figure 3.8. Converting HEMA/DMAEMA gel to its hydrochloride salt did not show the same dramatic swelling effect, even though the water fraction increased with increasing concentration of DMAEMA. One possible reason for greater swelling of carboxylate salt gels than the amine hydrochloride gels was thought to be the increase in crosslink density due to chain transfer mechanisms found in tertiary amine groups. It is known that tertiary amines act as chain transfer agents with methacrylate system (Gregonis et al., 1976). Figure 3.8 shows EWC of HEMA gels containing equal molar quantities of MAA and DMAEMA. Again the conversion of gels into carboxylate salts showed greater water contents than the conversion of gels into hydrochloride salts.

Results obtained in this research indicate that complexing between functional groups, and the presence of ionic charges play an important role in determining the EWC of copolymer and terpolymer systems studied. There are other parameters which affect water swelling of hydrogels. These parameters include crosslink density, polymerisation conditions, equilibrating solution and pre-swelling. These will now be discussed.

Figure 3.8

Equilibrium water weight fraction, wf, of HEMA/MAA/DMAEMA terpolymers. MAA and DMAEMA are polymerised at equal molar concentrations.



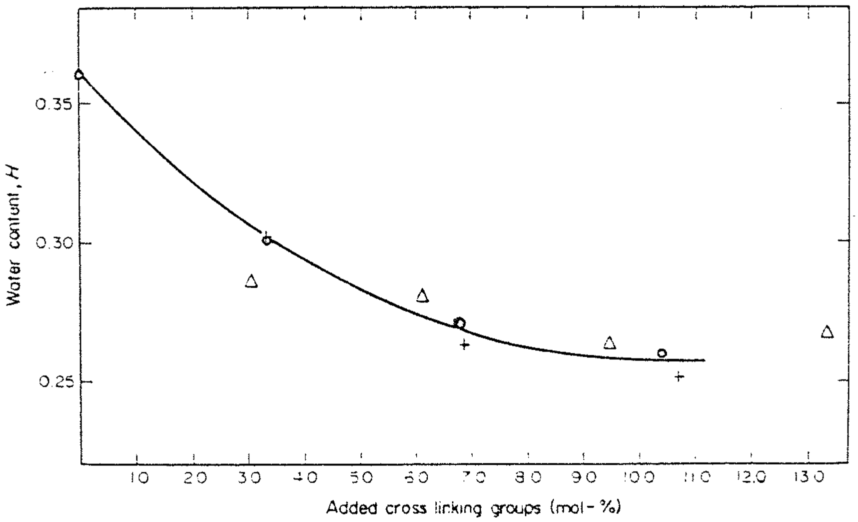
(a) Crosslink Density.

Crosslink density can be an important determining factor especially for high water content hydrogels such as poly(ACR). However, for gels having low interaction parameter with water such as poly(HEMA), the extent of crosslinking does not have a marked influence on the EWC until very high levels of crosslinking agent are used. This is demonstrated in figure 3.9 (Haldon and Lee, 1972).

With HEMA/TEGDMA system it was reported by Refojo and Yasuda (1965a) that increasing the concentration of TEGDMA from 0.3 percent to 3 percent had little effect on the EWC of poly(HEMA) hydrogel. Jolly (1981) reported the effect on the EWC of

Figure 3.9

The EWC of poly(HEMA) crosslinked with EDM, TPT, PETMA in ethylene glycol:water 1:4



HEMA cross-linked with (○), EDM , (△), TPT, and (+), PETMA homogeneous poly(HEMA) preparations of increasing amount of crosslinking agent EDM. The effects on the water structure caused by increasing amounts of EDM resulted overall in lowering the EWC of poly(HEMA). The increasing amounts of crosslinking agent affected the water swelling of hydrogel in two ways. In the 0 - 10 mole percent EDM range the amount of freezing water was markedly affected because the increasing amount of EDM tightens up the matrix thus reducing the space available for freezing. In the 10 - 20 mole percent EDM range the non-freezing water was thought to be affected as the tightening up effect becomes less important. The crosslinking agent now acts as a hydrophobic monomer and reduces the hydrophilicity of the system. In this way layer of water molecules most closely associated with hydrophilic groups in the matrix could be influenced. Whereas in the homogeneous hydrogels the non-freezing water content decreases with increasing crosslinking, in the macroporous systems the effect is different. In macroporous poly(HEMA) it was found that as EWC and freezing water content decreased, the non-freezing water remained fairly constant. This indicates that in macroporous hydrogels of poly(HEMA) the non-freezing water is present mostly in the gel network.

(b) Polymerisation Conditions

EWC in a hydrogel was found to be affected by the initiator concentration (Yasuda et al., 1966) and equilibrating temperature of the hydrogel (Refojo and Yasuda, 1965b). For

HEMA hydrogel, the EWC increased with increasing initiator (ammonium persulphate) concentration. This is related to the number of growing radicals. A minimum in the water content (around 37 percent) in the equilibrated poly (ethylene glycol methacrylate) gel at about 60°C, and an increased water content upto the freezing and boiling points was observed. It was suggested that swelling of glycerolmethacrylate (GMA) hydrogels was an endothermic process whereas swelling of propyleneglycolmonoacrylate (PGA) hydrogels was an exothermic process.

(c) Equilibrating Solution

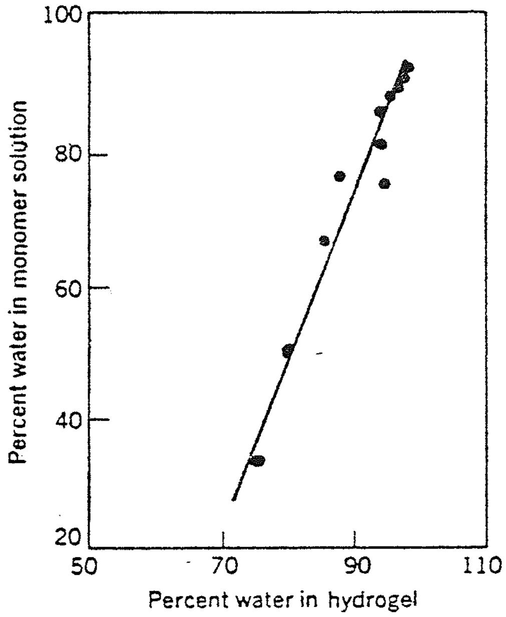
EWC was also found to be dependent on the equilibrating solution being used. A decrease of EWC from 40 percent to 38 percent was observed if the HEMA hydrogel was equilibrated in 0.9 percent sodium chloride solution, but an increase to 46 percent if 1.4 percent sodium chloride was used (Refojo and Yasuda, 1965a). That poly (HEMA) hydrogel deswells in sodium chloride solutions was reported by Jadivin et al. (1970). The presence of sodium chloride in water enhances the water structure resulting in an increase in the hydrophobic bondings in the hydrogel and hence a decrease in EWC.

(d) Pre - Swelling

Additionally, other factors such as relative swelling of the polymer at the time of crosslinking can be important in cases where solution rather than bulk polymerisation is used. This observation is exemplified in figure 3.10 which shows the effects of the amounts of water in the monomer solution upon the amount of water in GMA hydrogels.

Figure 3.10

The effect of the amount of water in the monomer solution upon final water content



3.8.5 Scanning Electron Microscopy (SEM)

SEM provided a useful means of visualising surface morphology and, because of its great depth of field, it allowed appreciation of three dimensional relationship of the structure of hydrogel beads. Microphotographs of porous samples (plate 3.9, 3.14) exhibit regularity and unidirectionality in porous structure. Compositions AA/HEMA, HEMA/EDM and HEMA/DMAEMA/EDM were found to be most porous. In general (comparing microphotographs in plates 3.11, 3.12 and 3.15, 3.16 for example) it is observed that the outer regions of beads of porous samples have higher degree of order. This is thought to be due to rapid freezing on the surface and slower rate of freezing in the interior of the bead. This results in disordered structure in the interior of the bead.

ACR/N,N'MBA copolymer beads (plates 3.21 and 3.22) show the presence of depressions on the surface and in general an uneven surface. This could be due to the slower freezing of this composition and the disturbance caused during polymerisation by the impacts between the bead surface and the stirrer. Such impacts would result in deformed surfaces. This is also observed with HEMA/DAA/EDM (plate 3.27) and HEMA/NMACR/EDM (plate 3.29) beads. Comparing with XAD-7 (plates 3.31 and 3.32) the surfaces of more porous hydrogel beads are rougher than the surfaces of the macroreticular acrylic acid resin. However, the surface of the resin also exhibits little roughness.

In general, the hydrogel beads synthesised by the freeze-thaw technique have clean, debris free surfaces. This is important from the aspect of application of the beads as adsorbents in haemoperfusion. If the adsorbent has detachable particles on its surface, there may be the possibility of induction of micro-emboli formation which is not desired in haemoperfusion.

CHAPTER 4

SYNTHESIS OF HYDROGEL PARTICULATES; SUSPENSION POLYMERISATION IN BRINE

4.1 INTRODUCTION

With the use of the freezing technique described in chapter 3, a variety of monomer compositions were shown to be polymerisable to produce hydrogel beads having a range of properties. However, the inability of certain compositions to produce macroporous structures in the beads was observed. In addition, the technique of freeze-thaw polymerisation posed limitations on the control of physical properties such as pore size and particle size distribution. In order to synthesise hydrogel particulates with an even wider range of properties, the technique of suspension polymerisation, previously outlined in chapter 2 was investigated.

The principle of suspension polymerisation is to disperse a monomer or a mixture of several monomers in the presence of a monomer-soluble initiator by strong mechanical agitation in a non-solvent continuous phase in the form of small globules. The suspension may be heated to increase the rate of polymerisation. The resultant polymer is obtained as a dispersed solid phase and may be in the same spherical form in which the monomer was dispersed. Each isolated monomer droplet undergoes in effect a self-contained bulk polymerisation, and thus the kinetics of suspension polymerisation are similar to those for bulk polymerisation. However, the correspondence between bulk and suspension polymerisation is not complete. There can be differences, for example, when relatively water soluble monomers or auxiliary agents are present (Munzer et al., 1977).

In suspension polymerisation the continuous non-solvent phase, provides a medium for dissipation of the heat evolved during the reaction. Hence problems of poor heat transfer resulting in polymers of low molecular weights and broad molecular weight distributions, common in bulk polymerisation, are mostly avoided. Suspension polymerisations are mostly free radical polymerisation processes, as they have found little application with ionic and with condensation polymerisation (Lesek and Khromecek, 1961). The literature on suspension polymerisation was reviewed (Hohenstein and Mark, 1946; Munzer et al., 1977). The important features of suspension polymerisation have been discussed by Farber (1970), and Wolf and Eckert (1971).

In the initial stages of suspension polymerisation when the monomer is liquid it can be kept in droplet form by use of stirring. Towards the end of the reaction, when the polymer beads form tiny, hard spheres slightly denser than water, again, agitation presents no problems. In the intermediate stage, however, when the droplets are partially polymerised and are sticky, they may tend to adhere to one another and to the stirrer. Therefore, the prime consideration is to prevent coalescence of the beads, particularly, at the point of gelation by adding suitable suspension stabilisers to the continuous phase. The patent literature provides very many examples of additives and procedures developed for its mitigation (Munzer et al., 1977).

Early solution to the problem of coalescence of droplets was the addition of finely divided powders, such as talc. The latest techniques employ water-soluble high polymeric suspending agents. These are of two types - those which mostly alter the viscosity of the continuous phase (viscosity modifiers), and those which affect the interface between the continuous and dispersed phases (interfacial surface tension modifiers). Examples of water soluble suspending agents are poly(vinyl alcohol), poly(vinyl pyrrolidone) and gelatin.

The exact mechanisms by which agents stabilise monomer phase suspensions are still not fully understood. It has been postulated that stabilisation is achieved due to a steric or solvation barrier caused by adsorption of the stabilising agent upon the monomer-polymer droplet or granule (Black, 1968). The conditions for a stable dispersion of liquid globules in a continuous medium have been listed (Church and Shinnar, 1961).

The use of suspension stabilisers also affects particle-size, and shape. It has been reported (Winslow and Matreyek, 1951) that increasing the molecular weight of a suspension stabiliser produces smaller particles from a suspension polymerisation, as well as increases in the concentration of the suspension stabiliser.

Increased suspension stability in suspension polymerisation can be achieved by still other means. Interfacial tension between the water and monomer phases can be increased by dissolving electrolytes in the aqueous phase. This also serves to decrease the solubility of the monomer in water and tends to result in smaller particles (Farber, 1970). By reducing the relative concentration of the monomer phase to the continuous phase the effective collisions amongst monomer droplets can be reduced. The density of the aqueous phase can be adjusted to that of the monomer-polymer phase so that the polymerising mass neither sinks nor floats in the aqueous phase. Also, the viscosity of the continuous phase can be increased so that the dispersed globules do not collide vigorously enough to merge.

Another vital feature in producing discrete particles is the rate of stirring which should be such as to maintain the dispersion in suspension whilst not causing forced agglomeration by violent interdroplet collisions. Uniform stirring renders scale-up a less risky procedure in processes involving dispersions stabilised by additives. Continuous agitation thus maintains dispersion and the size of the resulting particles is a function of energy input or rate of agitation.

Substantial progress made over the years in the art and application of suspension polymerisation has resulted in the development of several processes which give reproducible results. These processes today form the basis for commercial preparation of polymers from many monomers, among the most important being acrylic esters, methacrylic esters, styrene, vinyl acetate, vinyl chloride and these with monofunctional and polyfunctional comonomers. However, literature relating to the production of hydrogel beads, for specific biomedical applications, by suspension polymerisation is scarce. The preparation of hydrogel suspension beads is complicated due to the hydrophilic nature of the monomers involved. Consequently, it has not been easy in the past to apply ordinary suspension polymerisation for the synthesis of hydrogel beads.

With the use of suspension polymerisation, macroreticular (Kunin et al., 1962) and macroporous (Millar et al., 1963) polymers can be produced. This is, in fact, made use of in the production of commercial ion-exchange resins. They have large internal surface resulting from added solvents or swelling agents that are removed after suspension copolymerisation. In such polymers the water is inhomogeneously distributed throughout the gel, between a polymer network of low water content and a porous network in which water molecules predominate. In haemoperfusion, it is desired to produce biocompatible adsorbents possessing pores in the macroporous range for the efficient removal of 'middle molecular' weight toxins from the circulation. Therefore, the technique of suspension polymerisation may be regarded as an obvious choice for producing porous hydrogels in the bead form. This can be achieved if techniques can be developed to avoid the dissolution of monomers in the continuous phase, and stable dispersion of monomers in the continuous phase can be formed and maintained.

This chapter is concerned with the synthesis of hydrogel particulates by a suspension polymerisation technique carried out in a high salt content aqueous medium, and the evaluation of such particulates using various techniques. Suspension polymerisation in brine has been used for the first time for the synthesis of biocompatible hydrogel particulates with a range of physical and chemical properties.

4.2 SUSPENSION POLYMERISATION IN BRINE

This technique relates to a free-radical suspension polymerisation process of water soluble monomers in brine carried out in the presence of a dispersion system comprising a combination of a high molecular weight linear polysaccharide and a hydroxyalkylmethyl cellulose. In this method the high salt content of the medium limits the solubility of the monomer or monomers in the aqueous phase. It has been shown that a continuous phase containing xanthan gum as a dispersant (viscosity modifier), together with hydroxyalkylmethyl cellulose as a droplet stabiliser (interfacial tension modifier) leads to successful suspension polymerisations in which the monomers are dispersed in various discrete organic phases (Hamann and Stellwagen, 1975). The technique was originally developed for polymerising hydrophilic monomers that are useful in the manufacture of ion-exchange resins. Subsequently, the method was used for preparing crosslinked polymer beads of acryloyl morpholine with acrylamides, suitable for applications in solid (gel) phase peptide synthesis (Epton et al., 1979; Goddard, 1980).

4.2.1 Suspending Agents for Suspension Polymerisation in Brine

Xanthan gum, an extracellular polysaccharide from 'Xanthomonas Campesteris' forms a highly viscous homogeneous solution in water. The solution is stable to acids and at low concentrations relatively insensitive to salts. In fact low concentrations of salt have a beneficial effect insofar as it makes the viscosity less liable to dimunition on temperature rise (Sloneker and Jeanes, 1962). In contrast, the use of a salt medium more often adversely affects the performance of many commonly used dispersants in aqueous suspension polymerisations. The structure of xanthan gum investigated by Jansson et al. (1975) is illustrated in Appendix (C). Hydroxyalkylmethyl cellulose gums are derived from and have a polymeric backbone of cellulose, a natural carbohydrate that contains a basic repeating structure of anhydroglucose units. The general formula of cellulose gum is illustrated in Appendix (C).

4.2.2 Development of The Technique

In the present research, the technique of suspension polymerisation in brine was modified to produce copolymer and terpolymer hydrogel beads from starting materials which are known to form biocompatible products. The monomers which included AA, MAA, HEMA, ACR, NMACR, DAA and DMAEMA, and the related crosslinking agents EDM and N,N'MBA, were copolymerised while maintained 'salted in' an organic phase dispersed in saturated brine, containing xanthan gum and hydroxypropylmethyl cellulose. Development of the technique starting with initial attempts leading to the successful synthesis of hydrogel particulates will now be described.

4.2.2.1 Initial Experiments

Initial experiments were carried out on a small scale with simple monomers such as AA, HEMA and the crosslinker EDM. The monomers dissolved in 1,1,2,2-tetrachloroethane (TCE) solvent in the presence of AZBN/DMAPN initiator system were polymerised in xanthan gum continuous phase. The following experiment used for the synthesis of HEMA/EDM copolymer will serve as an example to describe the initial attempts.

Organic Phase Copolymerisation of 2-Hydroxyethyl Methacrylate and Ethylene Dimethacrylate in a Continuous Xanthan Gum Aqueous Phase

Organic phase: HEMA (7.8084 g, 60 mmol), EDM (1.1893 g, 6 mmol), AZBN and DMAPN (0.1799 g each, 2% w/w of the monomer content), and 1,1,2,2-tetrachloroethane (40 ml)

Aqueous phase: 0.20% (w/v) solution of xanthan gum in water (42.4 ml), 0.16% (w/v) aqueous solution of hydroxypropylmethyl cellulose (8.4 ml), sodium chloride (18 g) and distilled water (128 ml).

The organic monomer phase of approximately 20% (v/v) of the total polymerisation system was deoxygenated by passing a nitrogen purge for 30 minutes. The aqueous phase with sodium chloride dissolved was transferred to a 250 ml round-bottom polymerisation flask fitted with a stirrer (type a, Appendix B), a nitrogen inlet and a water condenser. The aqueous phase was deoxygenated using a nitrogen purge for 30 minutes. The organic phase was added to the continuous phase and the mixture was stirred at 400 rpm for 15 minutes to form a dispersion of monomer droplets in the aqueous phase. The temperature of the water bath was raised to 65°C and stirring was reduced to 300 rpm. Nitrogen was continually passed over the suspension until the end of the experiment. The stirring was stopped after 3 hours and the polymer was allowed to cure at a lower temperature for a further 6 hours. Subsequent washing of the polymer was carefully controlled. The copolymer was thoroughly washed with water in order to remove sodium chloride completely. The absence of the salt in washings was confirmed by a negative silver nitrate test. Equilibration with ethanol was carried out to remove the solvent TCE. The copolymer was then shrunk with ether. After drying in vacuum oven at 60°C for 12 hours soft, agglomerated mass of beads (8.6909 g, 96.59% yield) was obtained. Agglomerated particulates were separated by using a macerator.

Some compositions, however, produced beads with the above method but suffered from the disadvantage of tailing of the beads. In general, the main problems associated with the procedure as carried out above were agglomeration, poor regularity and general lack in mechanical strength of the particulates. Agglomeration was thought to be due to insufficient mechanical agitation and/or due to lack of suspension stabilisers. Poor regularity was thought to have resulted from inappropriate stirrer action. The softness of the particulates was attributed to the presence of the solvent and ethanol in the resultant polymer. Various modifications in the original set up were, therefore, felt necessary in order to overcome these problems.

Further developments in the technique were made empirically. For example, experiments were repeated by varying the concentration of the initiator, the concentration of suspension stabilisers, and the sodium chloride content of the reaction mixture. The amount of the solvent TCE was varied, and the effects on the polymer structure caused by the complete lack of the solvent in the case of liquid monomers were examined. To obtain an efficient 'salting out' effect the salt content of the mixture was increased so as to produce saturation of the continuous phase. To obtain an acceptable bead size it was found appropriate to stir the suspension at high speed prior to the initiation of the reaction. In order to improve stirrer action, stirrers having a range of size and shape were tried. Special polypropylene paddles were designed and the size and shape to effect most suitable dispersion were determined. Type (c) polypropylene stirrer paddle (Appendix B) in conjunction with stainless steel rod was best suited for producing uniform spherical particles.

Type of the polymerisation flask also affected the bead dimensions. Flat bottomed 2 litre split resin flask enabled to obtain uniform dispersion of monomer phase, and to scale up the procedure to produce spherical beads in sufficient quantity for use in further studies.

4.2.2.2 Subsequent Experiments

A large number of experiments performed resulted in varying degree of success. After various modifications of the initial procedure, a formulation was used which enabled to synthesise porous, uniform and spherical hydrogel particulates from a range of monomer composition. HEMA/DMAEMA/EDM terpolymer bead synthesis described below exemplifies the typical procedure adopted for the subsequent experiments. Figure 4.1 and plate 4.1 illustrate the apparatus used for these experiments.

Organic Phase Polymerisation of 2-Hydroxyethyl Methacrylate, Dimethylaminoethyl Methacrylate and Ethylene Dimethacrylate Terpolymer in the Bead Form in Xanthan Gum Continuous Phase

The following formulation was used:-

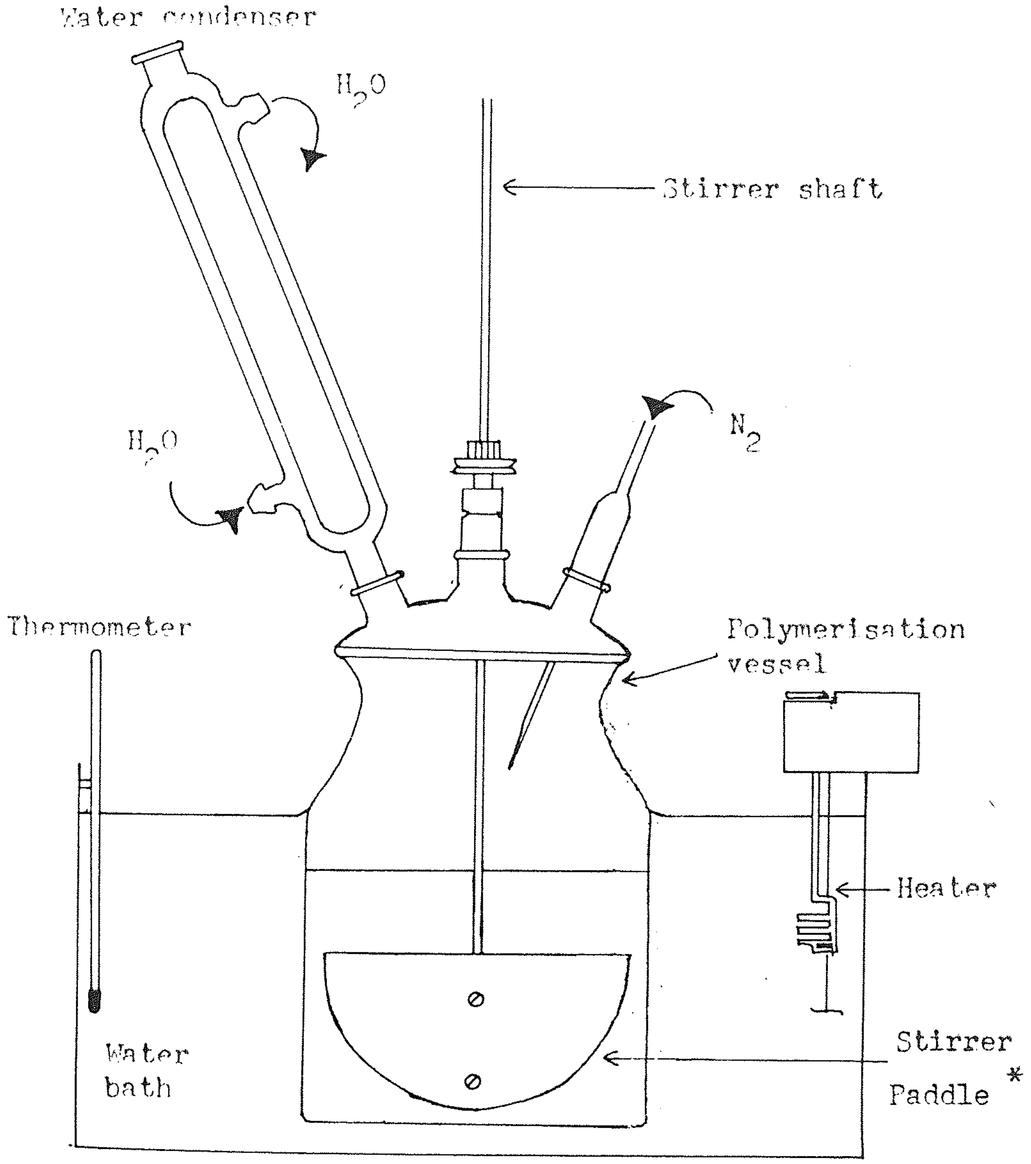
Organic phase: HEMA (93.7008 g), DMAEMA (12.5600 g), EDM (3.4292 g), tetrachloroethane (199 g), AZBN (2.3938 g, 2% w/w of the monomer content), DMAPN (2.3938 g).

Aqueous phase: Distilled water (480 g), Sodium chloride (290g), Xanthan gum, a 0.2% (w/v) aqueous solution (160 g), and Hydroxypropylmethyl cellulose, a 0.16% (w/v) aqueous solution (35 g).

A 2 litre flat-bottom polymerisation flask was equipped with a stirrer, type (c), a nitrogen inlet and a condenser. The pre-mixed aqueous phase was added to the flask and deoxygenated using a nitrogen purge sufficient to produce an efficient aerosol effect over the solution for 1 hour.

Figure 4.1

Apparatus for suspension polymerisation techniques



* Stirrer Paddles are illustrated in Appendix (B)

Plate 4.1

Apparatus for suspension polymerisation techniques

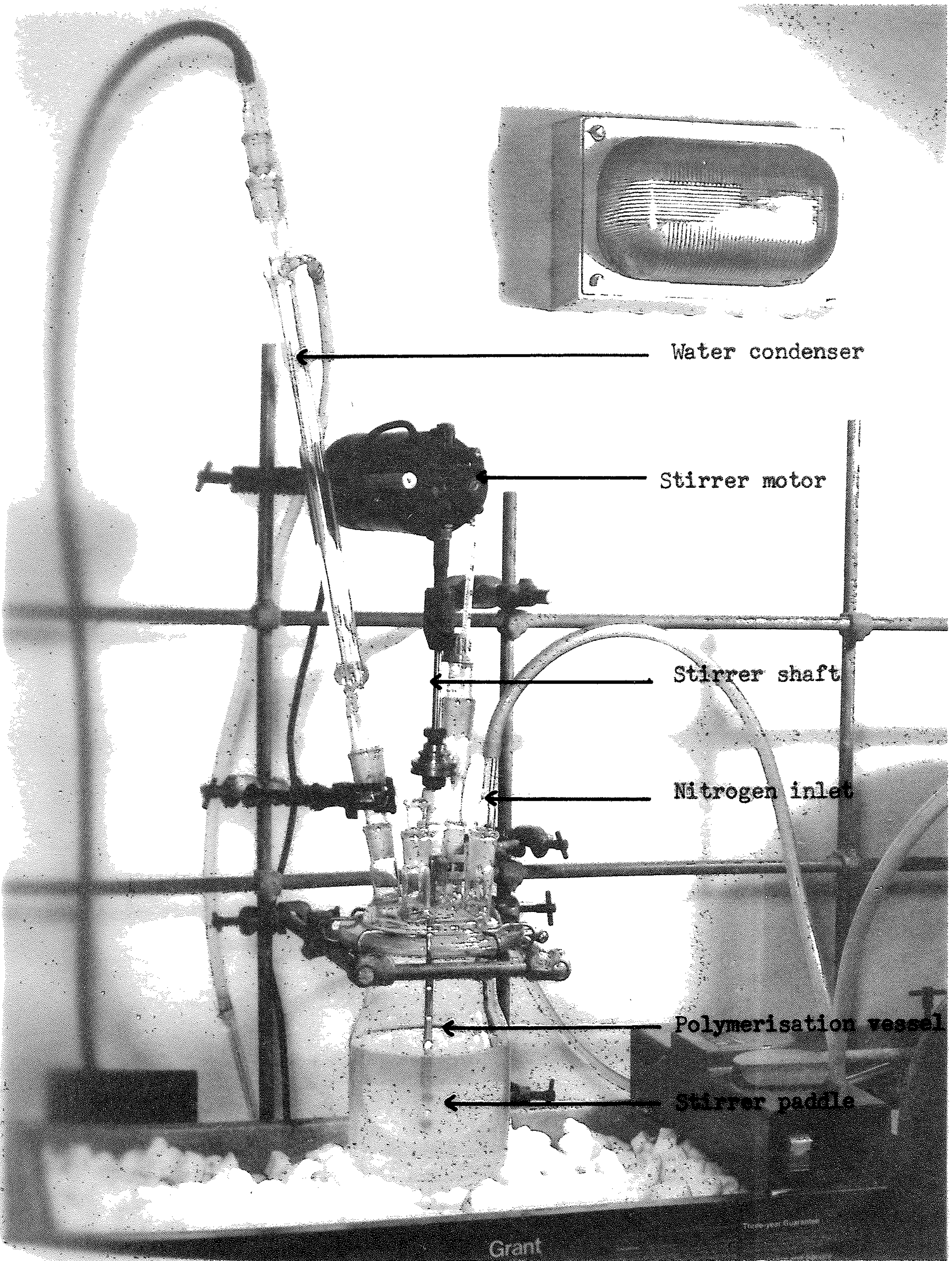
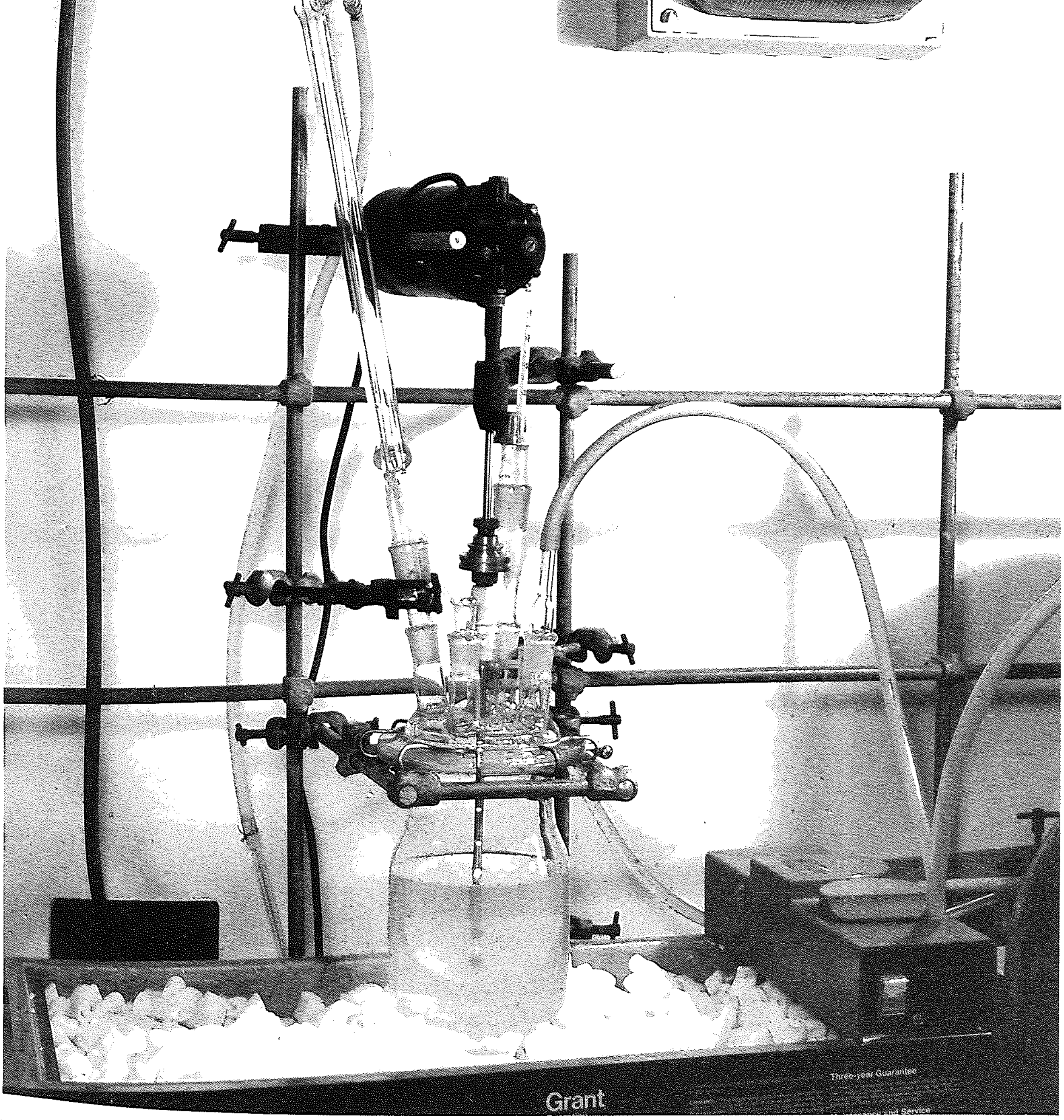


Plate 4.1

Apparatus for suspension polymerisation techniques

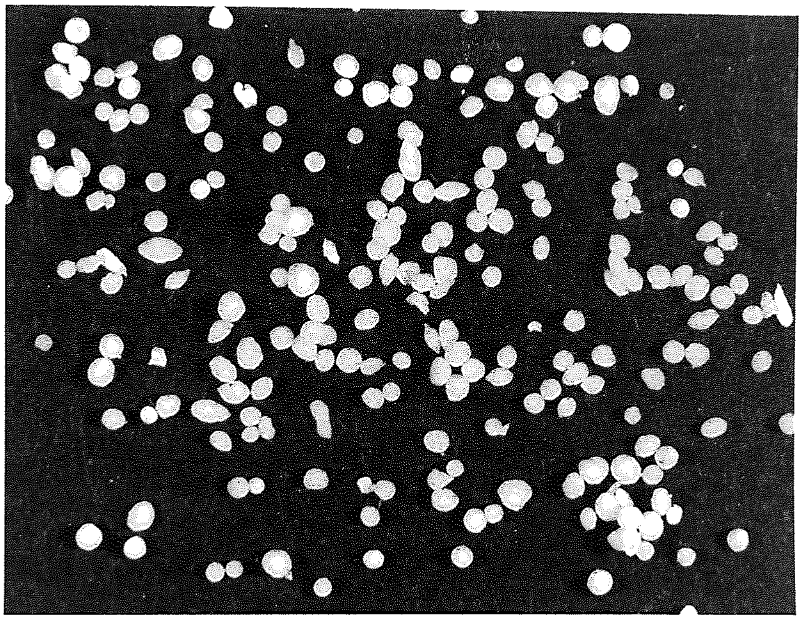


Monomers were dissolved in one half of the solvent, and the initiator in the other, similarly deoxygenated, mixed together and immediately added to the slowly stirred aqueous phase. The mixture was whisked at approximately 900 rpm for 15 minutes. The temperature of water bath was raised to 70°C. After gelation had begun the stirring rate was reduced to approximately 400 rpm and maintained for a further 3 hours. The beginning of gelation was determined by withdrawing samples from the flask with a PTFE tubing connected to a syringe barrel. A nitrogen atmosphere was maintained above the suspension whilst the polymerisation was in progress. After 3 hours the temperature of the water bath surrounding the reaction vessel was allowed to fall and stirring stopped. The polymer was allowed to cure for a further 12 hours after which it was washed rigorously with water and ethanol, and finally shrunk with ether. After drying in vacuum oven at 60°C, white, opaque and spherical beads (95.90 g, 87.43% yield) were obtained. Hydrogel beads had a size range of $1000\mu m$ - $250\mu m$ and after hydration in distilled water, an equilibrium water content of 77% was determined.

The above formulation of aqueous phase was used and the previously attempted synthesis of HEMA/EDM copolymer beads was repeated. The problems of agglomeration, tailing and poor regularity were largly overcome and uniform, spherical beads were produced by the modified technique. The procedure was then extended to copolymerise other monomer combinations. The possibility of synthesising hydrogel particulates by photopolymerisation in addition to thermal polymerisation was also investigated. The initiator used in these experiments was either AZBN or uranyl nitrate. Essentially the same experimental procedure as described for thermal polymerisation was used. However, in uranyl nitrate initiated polymerisations the reaction vessel was irradiated with high intensity UV lamp placed alongside the vessel, at room temperature. Photopolymerisation also produced spherical hydrogel beads. However, the yield of hydrogel beads from such experiments was low as some of the polymer appeared as amorphous material. Copolymer beads having a range of composition, particle size, water content and density were thus synthesised. Photographs of the HEMA/EDM copolymer and HEMA/DAA/EDM terpolymer beads prepared in this way are shown in plates 4.2 and 4.3 respectively. Plates 4.4 and 4.5 compare low magnification SEM's of a typical hydrogel bead synthesised by suspension polymerisation in brine, and a bead of a commercial macroreticular acrylic ester resin XAD-7 respectively.

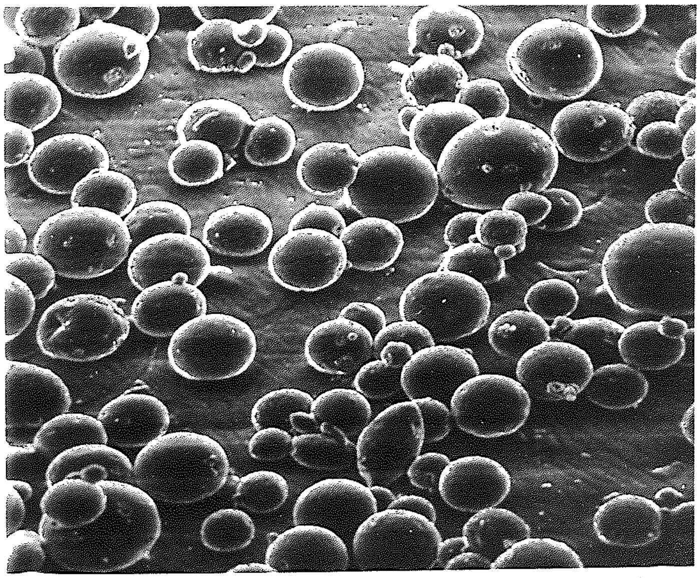
Samples of hydrogel particluates having varying composition synthesised by using the xanthan gum suspension polymerisation procedure are tabulated in table 4.1. Sample 5 and sample 7 were photopolymerised using UN as the initiator. All other samples were synthesised by thermal polymerisation initiated by AZBN.

Plate 4.2



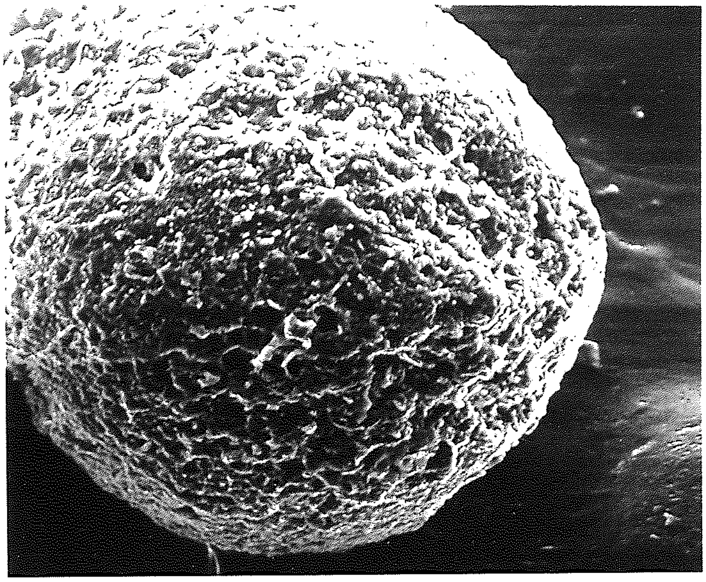
Hydrogel beads of hydroxyethyl methacrylate and ethylene dimethacrylate copolymer

Plate 4.3



Hydrogel beads of hydroxyethyl methacrylate, diacetoneacrylamide and ethylene dimethacrylate terpolymer

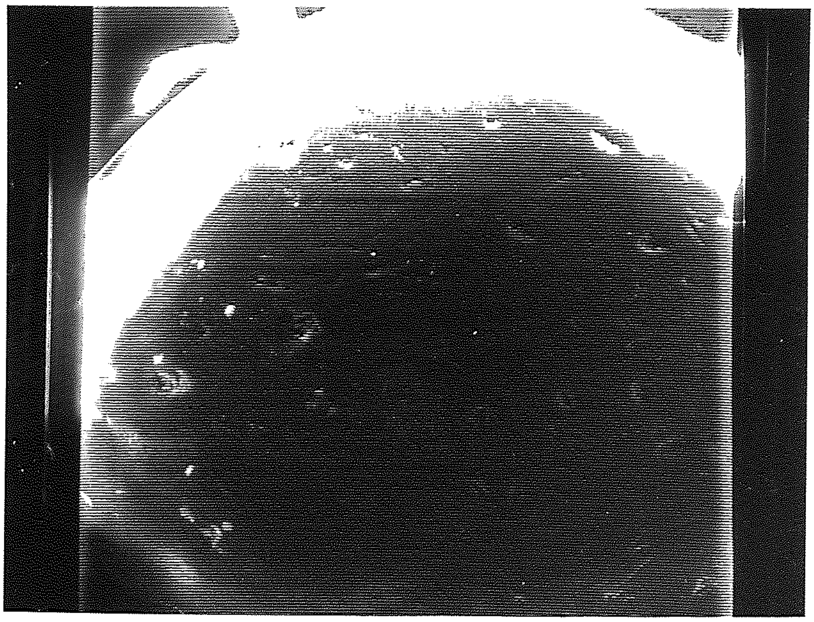
Plate 4.4



HEMA/EDM copolymer bead

Magnification X 250

Plate 4.5



Acrylic Ester (XAD-7) Resin Bead

Magnification X 250

Table 4.1

Samples of hydrogel particulates synthesised by suspension polymerisation in brine

Sample	Monomer Composition	Initial Molar Ratio	Solvent
Acidic:			
Sample 1	AA:EDM	100:8.5	TCE
Sample 2	AA:HEMA:EDM	60:40:8.5	TCE
Sample 3	MAA:HEMA:EDM	50:50:4.6	----
Neutral:			
Sample 4	HEMA:EDM	100:10	TCE
Sample 5	HEMA:EDM	100:8.5	----
Basic:			
Sample 6	ACR:N,N'MBA	100:10	TCE
Sample 7	HEMA:ACR:EDM	100:20:5.2	TCE
Sample 8	HEMA:DAA:EDM	100:25:9	----
Sample 9	HEMA:NMACR:EDM	90:10:8.5	TCE
Sample 10	HEMA:DMAEMA:EDM	90:10:8.5	TCE

In table 4.1 samples have been grouped into acidic, neutral and basic groups depending upon the type of monomers present in the composition. However, this is a broad classification since some monomers may be grouped into more than one class. For example, ACR may be regarded as a neutral monomer, and HEMA was used as the principal monomer in most of the samples. Table 4.2

shows the amounts of monomer and continuous phases used as percentage of the total polymerisation system.

Table 4.2

Percentages of the monomer and continuous phases in the polymerisation system

Sample	Monomer Content of Organic Phase (%)	Organic Phase Content of the Total System (%)
Sample 1	33	13.8
Sample 2	41	11
Sample 3	100	20
Sample 4	50	4.1
Sample 5	100	2.2
Sample 6	13.5	25
Sample 7	50	2.2
Sample 8	100	2.3
Sample 9	61	20
Sample 10	35	25

4.2.3 Characterisation of Hydrogel Particulates

After successful preparation of hydrogel copolymer and terpolymer particulates of varying composition their characterisation was carried out. This was achieved by determination of their size distribution, equilibrium water content, density, adsorption characteristics with respect to certain markers of liver failure, and by examination of the surfaces and interiors using scanning electron microscopy. The results of characterisation studies except those of adsorption characteristics will be given below. The adsorption characteristics of the particulates will be described in chapter 6.

4.2.3.1 Size Distribution

Determination of particle size range of the hydrogel particulates was mainly done by manual sieving. The size range of samples having particulates with diameter less than about 100 μm was determined by scanning electron microscopy. The method of gradation of the particulates into different size fractions has already been described in chapter 2. The size ranges obtained for all samples are tabulated in table 4.3. Fractional data for two samples: HEMA/DAA/EDM terpolymer and HEMA/EDM copolymer beads have been shown in figures 4.2 and 4.3 respectively in the form of histograms. The rates of stirring in these figures refer to the initial rates at which the dispersion of the monomer phase in the continuous phase was formed.

Table 4.3

Size distribution of hydrogel particulates synthesised by suspension polymerisation in brine

Monomer Composition	Initial Molar Ratio	Size Range (μm)
AA:EDM	100:8.5	100 - 25
AA:HEMA:EDM	60:40:8.5	150 - 50
MAA:HEMA:EDM	50:50:4.6	150 - 25
HEMA:EDM	100:10	500 - 125
HEMA:EDM	100:8.5	1000 - 250
ACR:N,N'MBA	100:10	80 - 20
HEMA:ACR:EDM	100:20:5.2	1000 - 250
HEMA:DAA:EDM	100:25:9	150 - 25
HEMA:NMACR:EDM	90:10:8.5	1000 - 250
HEMA:DMAEMA:EDM	90:10:8.5	1000 - 250

4.2.3.2 Equilibrium Water Content and Density

Table 4.4 lists the equilibrium water contents of hydrogel particulates. Experimental values refer to the water content obtained after drying the hydrogels in a vacuum oven at 60°C, whereas, calculated values refer to the water content worked out from the knowledge of the densities of the dehydrated and hydrated samples.

Figure 4.2

Histogram of fractional data for HEMA/DAA/EDM terpolymer beads

Initial rate of stirring: 600 rpm

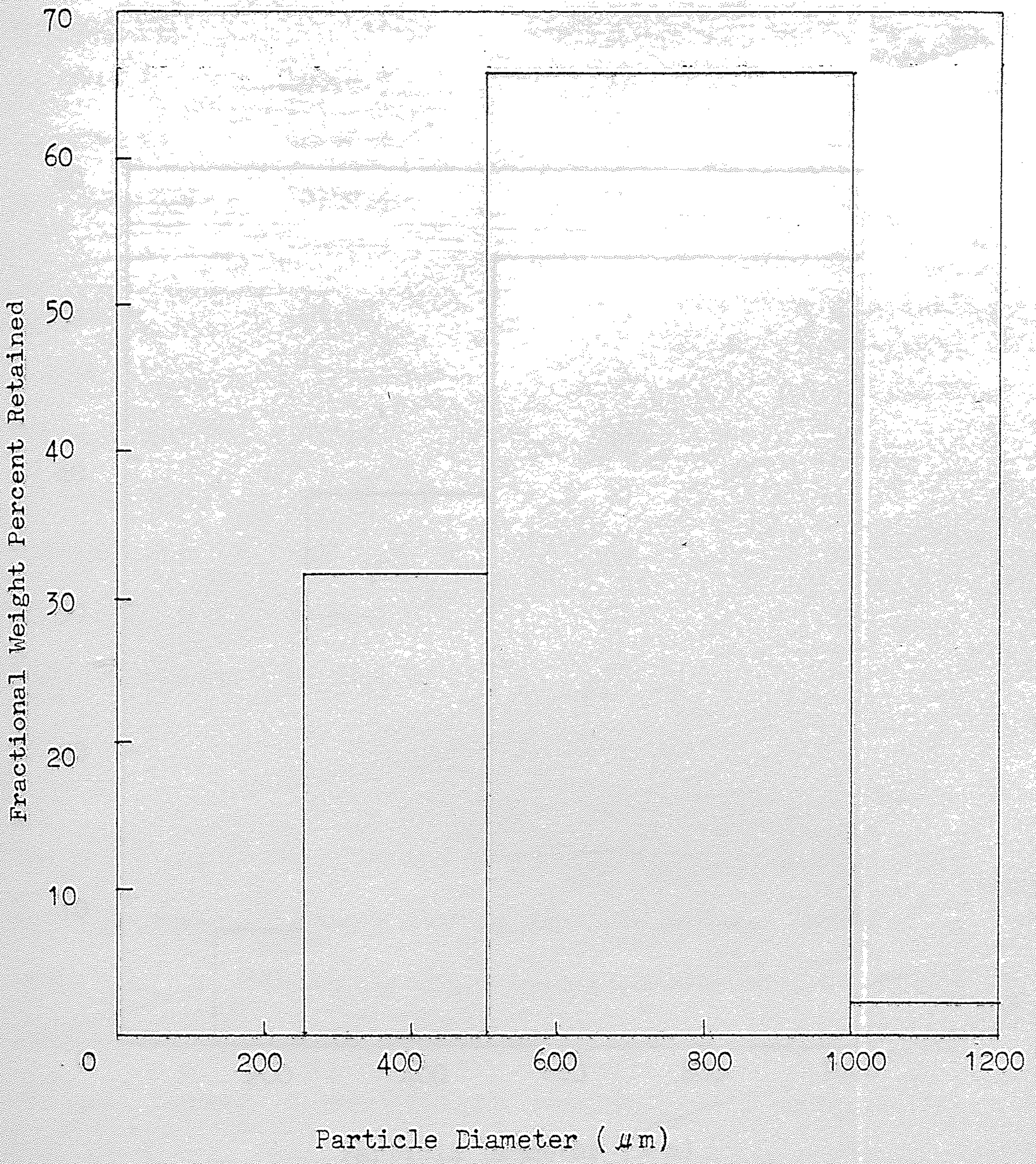


Figure 4.3

Histogram of fractional data for HEMA/EDM copolymer beads

Initial rate of stirring: 800 rpm

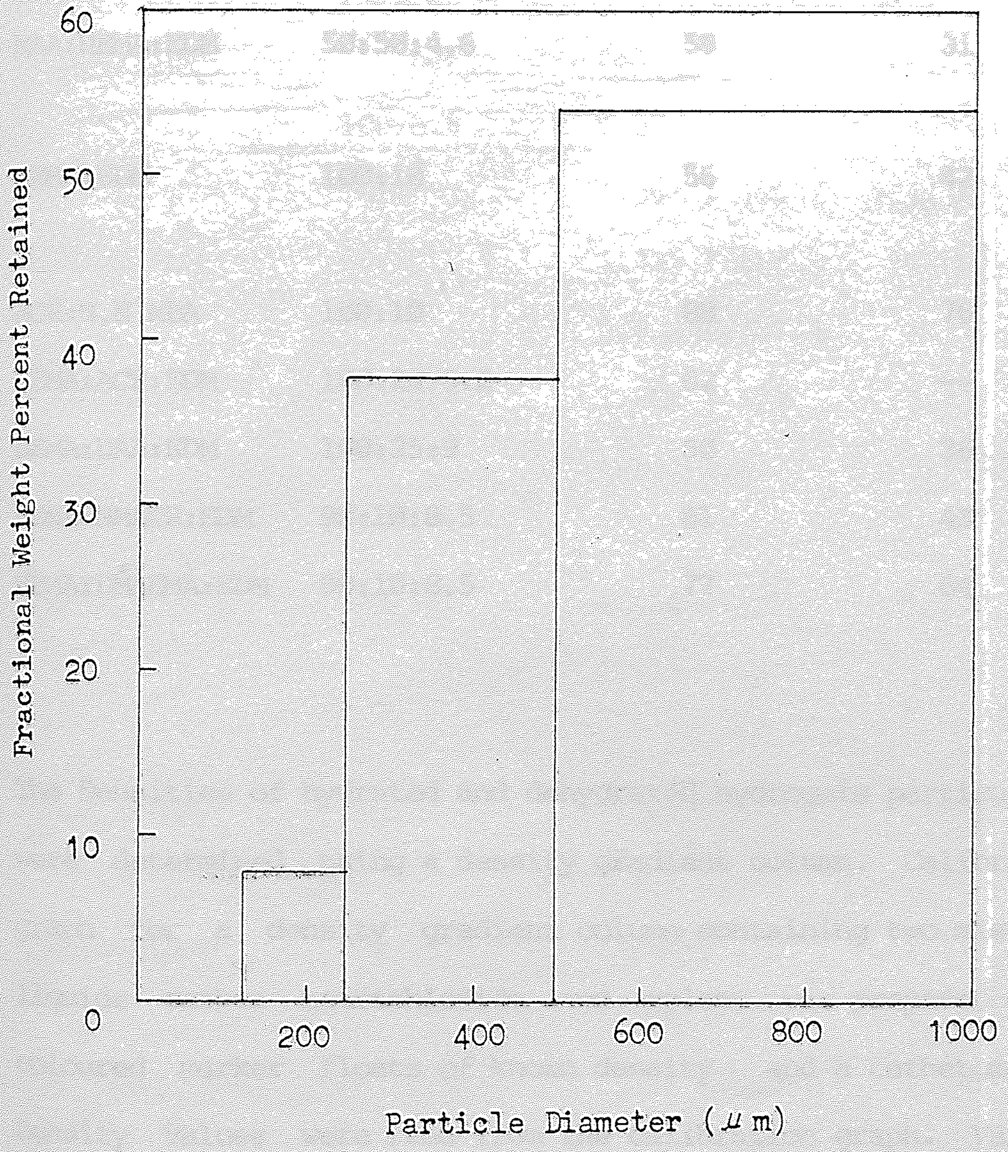


Table 4.4

Equilibrium water content of hydrogel particulates synthesised by suspension polymerisation in brine

Monomer Composition	Initial Molar Ratio	EWC % Experimental	EWC % Calculated
AA:EDM	100:8.5	77	70
AA:HEMA:EDM	60:40:8.5	64	67
MAA:HEMA:EDM	50:50:4.6	58	31
HEMA:EDM	100:10	56	42
ACR:N,N'MBA	100:10	88	76
HEMA:ACR:EDM	100:20:5.2	62	---
HEMA:DAA:EDM	100:25:9	50	36
HEMA:NMACR:EDM	90:10:8.5	61	43
HEMA:DMAEMA:EDM	90:10:8.5	77	64

The Densities of hydrated and dehydrated hydrogels particulates were determined using a density gradient column. Calibration graph for a density gradient column containing two miscible liquids carbon tetrachloride and xylene was prepared using coloured marker floats of known density and a cathetometer. Density values were read from the calibration graph. Various possible errors associated with the preparation of the column and in taking cathetometer readings were discussed in chapter 3.

The preparation of the column was described in chapter 2. Table 4.5 lists the density of hydrated and dehyrated samples.

Table 4.5

Density of hydrated particulates synthesised by suspension polymerisation in brine

Monomer Composition	Initial Molar Ratio	Density (g/cm^3) Dehydrated		Hydrated	
AA:EDM	100:8.5	1.361	1.365	1.095	1.089
		1.369	1.366	1.100	1.137
AA:HEMA:EDM	60:40:8.5	1.325	1.117	1.087	1.088
		1.327	1.325	1.112	1.131
MAA:HEMA:EDM	50:50:4.6	1.281	1.279	1.192	1.205
		1.282	1.282	1.179	1.180
HEMA:EDM	100:10	1.130	1.127	1.107	1.077
		1.122	1.130	1.071	1.089
ACR:N,N'MBA	100:10	1.303	1.317	1.070	1.069
		1.311	1.317	1.071	1.061
HEMA:DAA:EDM	100:25:9	1.234	1.229	1.139	1.142
		1.224	1.215	1.156	1.140
HEMA:NMACR:EDM	90:10:8.5	1.165	1.151	1.087	1.110
		1.159	1.148	1.095	1.122
HEMA:DMAEMA:EDM	90:10:8.5	1.260	1.249	1.080	1.073
		1.254	1.260	1.082	1.082

4.2.3.3 Scanning Electron Microscopy

The physical appearances of the surfaces and interiors of the particulates were examined by scanning electron microscopy performed on the dehydrated samples. For examining the interior of the particualte, it was gently cut with a sharp knife into two parts. Photographs of the samples were taken at various magnifications. Scanning electron microphotographs of selected samples are shown on the following pages. SEM's of particulates representing samples from acidic, neutral and basic classes are listed in tables 4.6, 4.7 and 4.8 respectively.

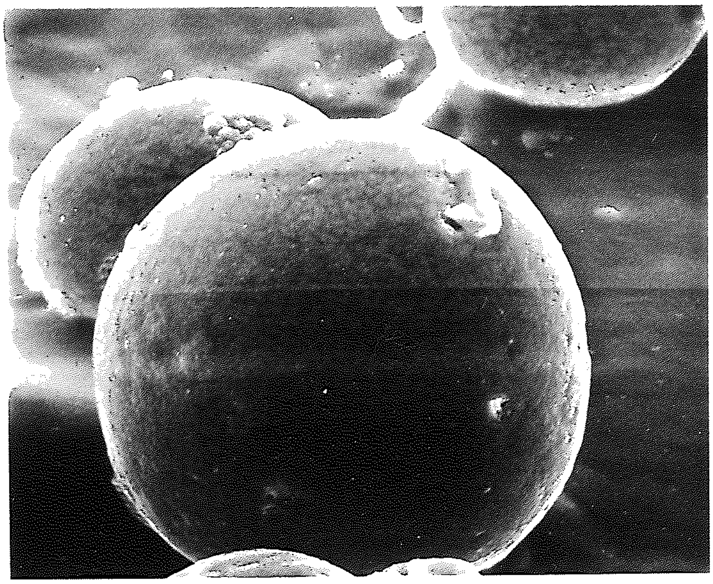
Table 4.6

Key to the SEM's of hydrogel particulates containing acidic monomers

Monomer Composition (Initial Molar Ratio)	Plate No	Surface/ Interior	Magnification
AA : EDM	4.6	Surface	X 550
(100:8.5)	4.7	Surface	X 1600
MAA:HEMA:EDM	4.8	Surface	X 1500
(50 :50 :4.6)	4.9	Surface	X 2500

Scanning Electron Microphotographs of Hydrogel Particulates Containing Acidic Monomers

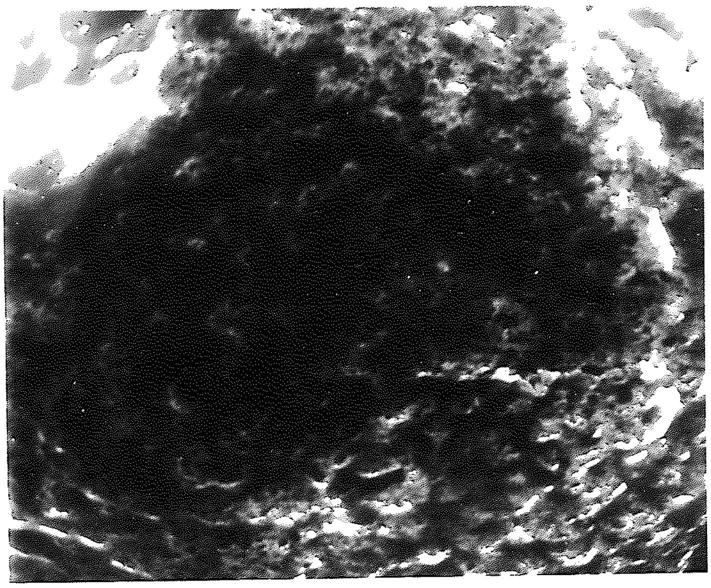
Plate 4.6



Surface of AA/EDM copolymer

Magnification X 550

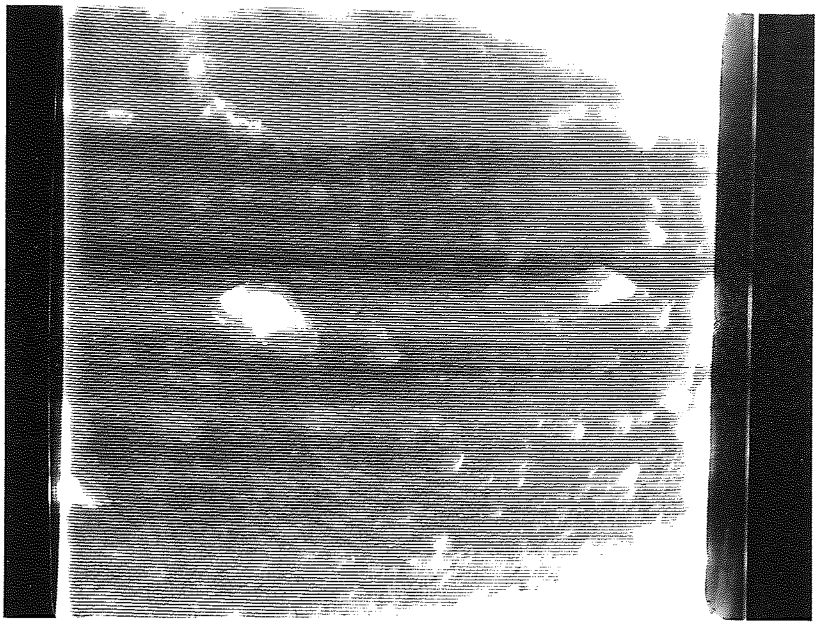
Plate 4.7



Surface of AA/EDM copolymer

Magnification X 1600

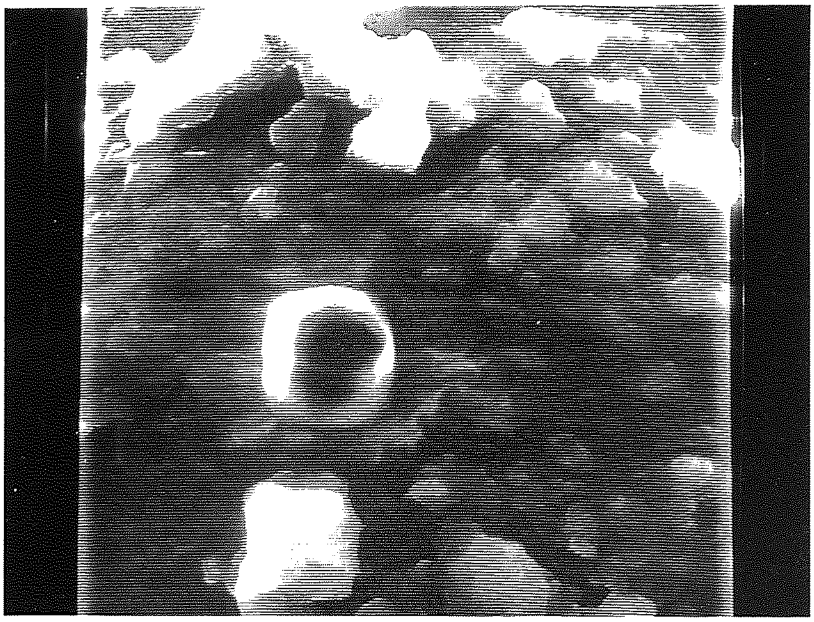
Plate 4.8



Surface of HEMA/MAA/EDM terpolymer

Magnification X 1500

Plate 4.9



Surface of HEMA/MAA/EDM terpolymer

Magnification X 2500

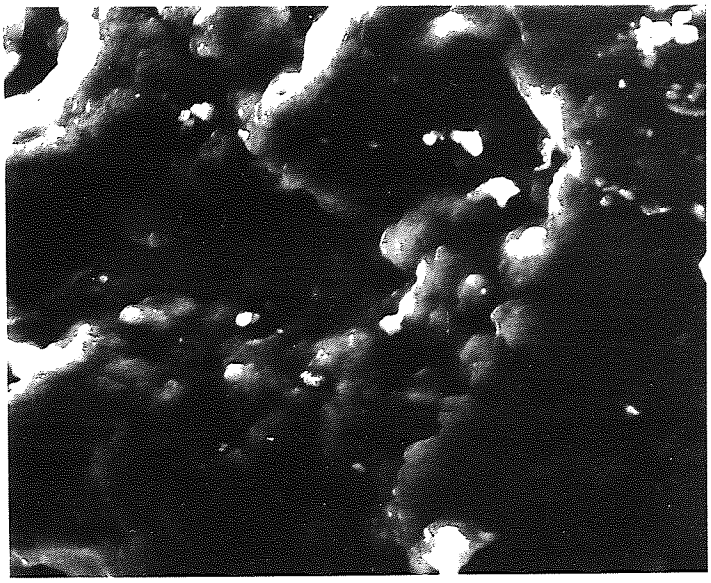
Table 4.7

Key to the SEM's of hydrogel particulates containing neutral monomers

Monomer Composition (Initial Molar Ratio)	Plate No	Surface/ Interior	Magnification
HEMA:EDM	4.10	Surface	X 1600
(100:10)	4.11	Surface	X 2600
	4.12	Interior	X 160
	4.13	Interior	X 260
	4.14	Interior	X 560
	4.15	Interior	X 1600
HEMA:EDM	4.16	Surface	X 560
(100:8.5)	4.17	Surface	X 2560

Scanning Electron Microphotographs of Hydrogel Particulates Containing Neutral Monomers

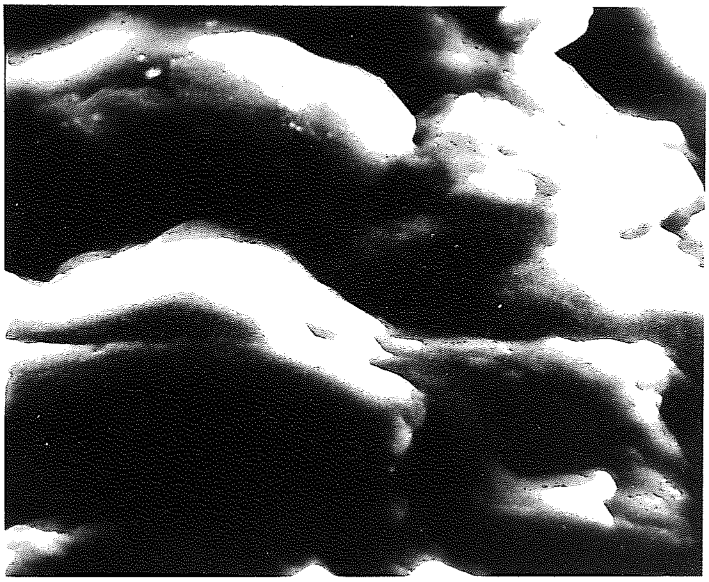
Plate 4.10



Surface of HEMA/EDM copolymer

Magnification X 1600

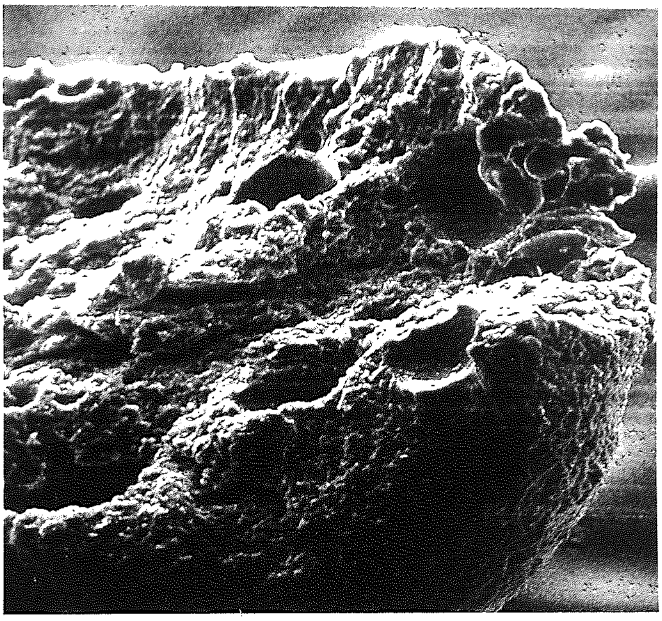
Plate 4.11



Surface of HEMA/EDM copolymer

Magnification X 2600

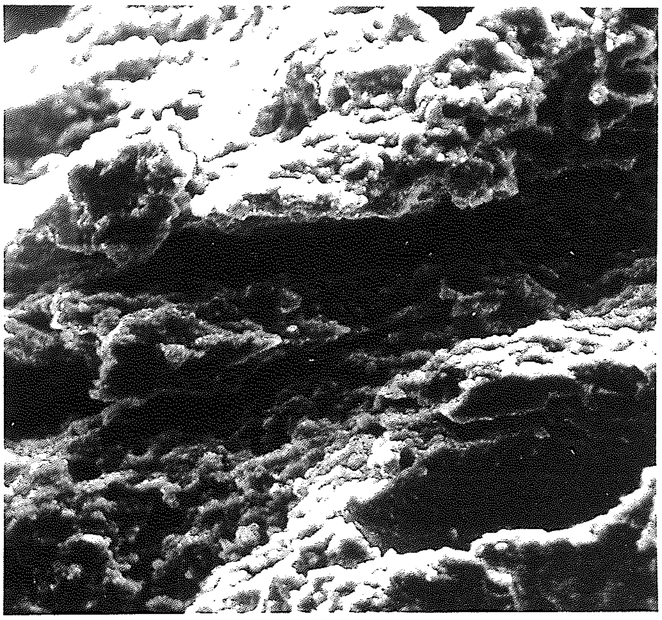
Plate 4.12



Interior of HEMA/EDM copolymer

Magnification X 160

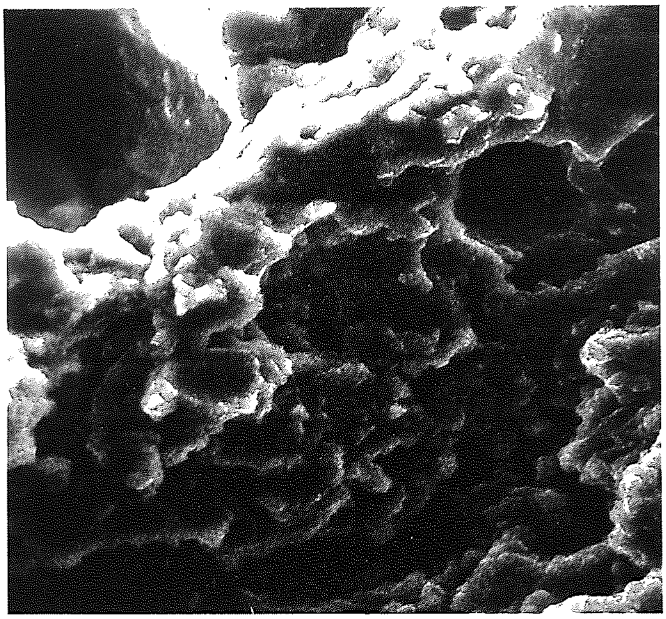
Plate 4.13



Interior of HEMA/EDM copolymer

Magnification X 260

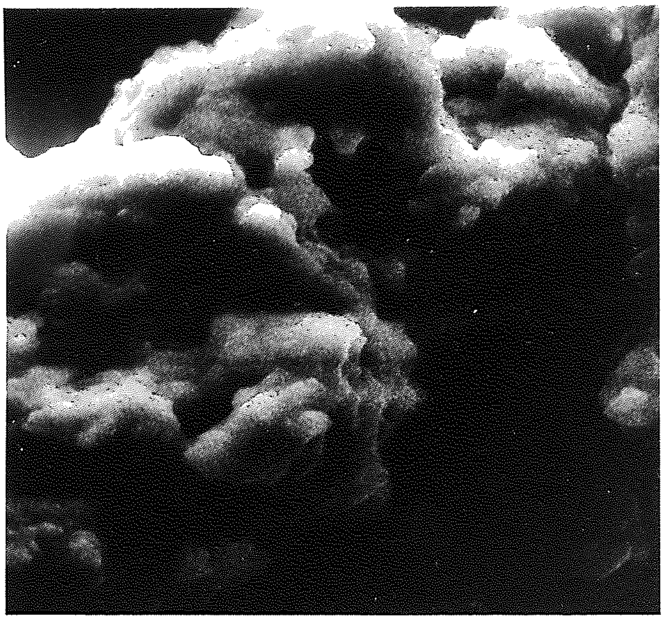
Plate 4.14



Interior of HEMA/EDM copolymer

Magnification X 560

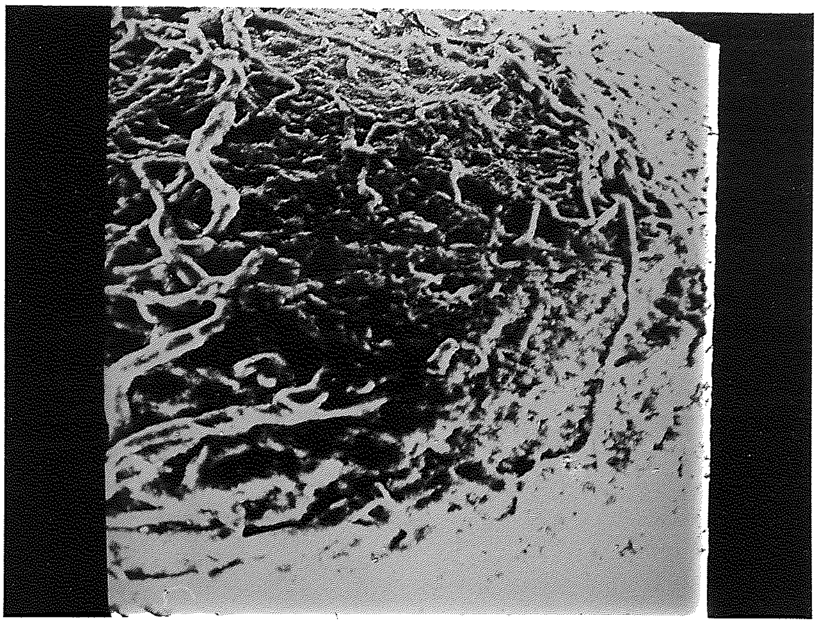
Plate 4.15



Interior of HEMA/EDM copolymer

Magnification X 1600

Plate 4.16



Surface of HEMA/EDM copolymer

Magnification X 560

Plate 4.17

Surface of HEMA/EDM copolymer

Magnification X 2560

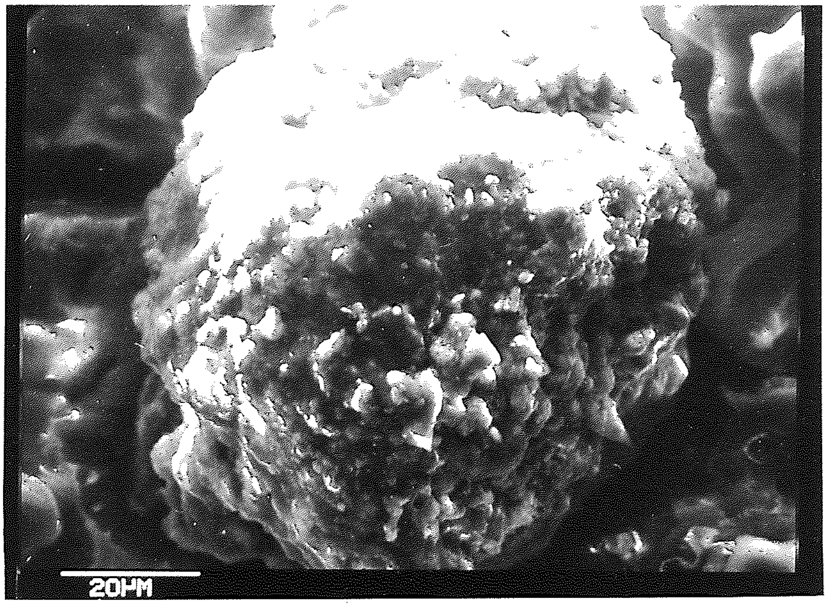
Table 4.8

Key to the SEM's of hydrogel particulates containing basic monomers

Monomer Composition (Initial Molar Ratio)	Plate No	Surface/ Interior	Magnification
ACR:N,N'MBA	4.18	Surface	X 1000
(100:10)	4.19	Surface	X 5000
HEMA:ACR:EDM	4.20	Surface	X 1600
(100:20 :5.2)	4.21	Surface	X 2600
HEMA:NMACR:EDM	4.22	Surface	X 500
(90 : 10 :8.5)	4.23	Surface	X 1000
	4.24	Surface	X 2000
	4.25	Surface	X 5000
	4.26	Interior	X 260
	4.27	Interior	X 560
	4.28	Interior	X 1600
	4.29	Interior	X 2600
HEMA:DMAEMA:EDM	4.30	Surface	X 100
(90 : 10 : 8.5)	4.31	Surface	X 200
	4.32	Surface	X 500
	4.33	Surface	X 1000
	4.34	Surface	X 2000
	4.35	Surface	X 5000
	4.36	Interior	X 570
	4.37	Interior	X 1700
	4.38	Interior	X 2700
	4.39	Interior	X 5700

Scanning Electron Microphotographs of Hydrogel Particulates Containing Basic Monomers

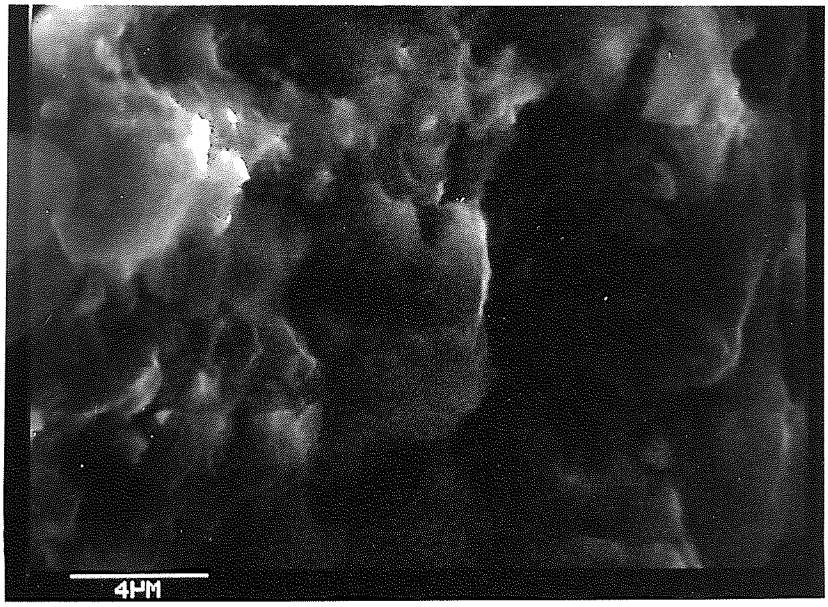
Plate 4.18



Surface of ACR/N,N'MBA copolymer

Magnification X 1000

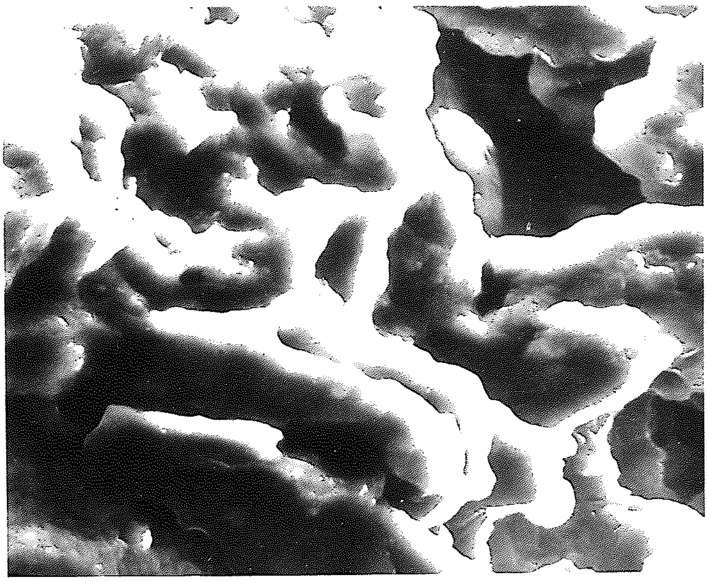
Plate 4.19



Surface of ACR/N,NMBA copolymer

Magnification X 5000

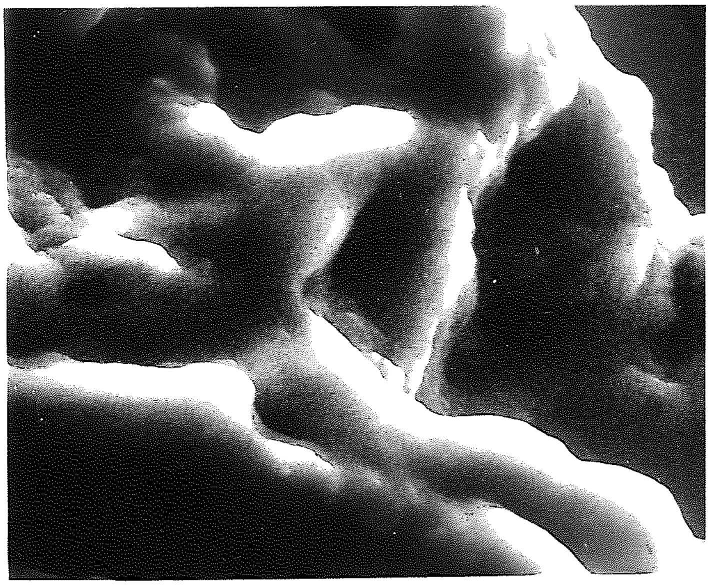
Plate 4.20



Surface of HEMA/ACR/EDM terpolymer

Magnification X 1600

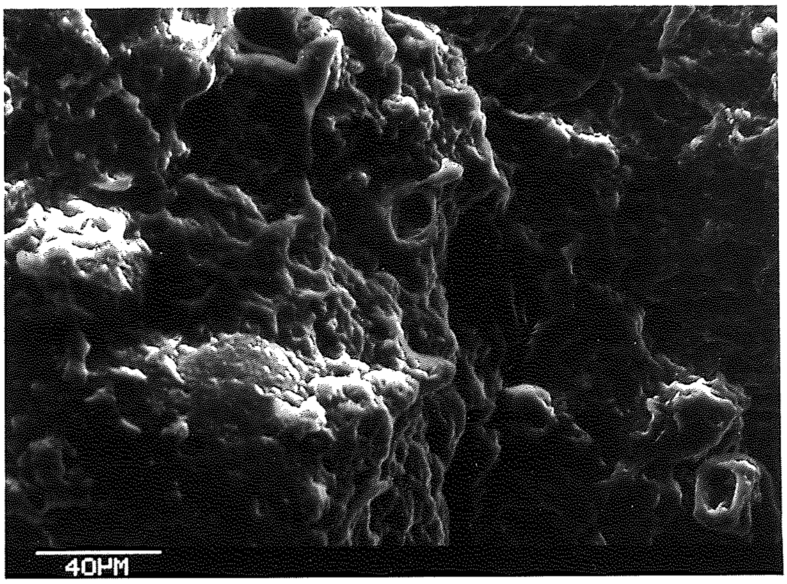
Plate 4.21



Surface of HEMA/ACR/EDM copolymer

Magnification X 2600

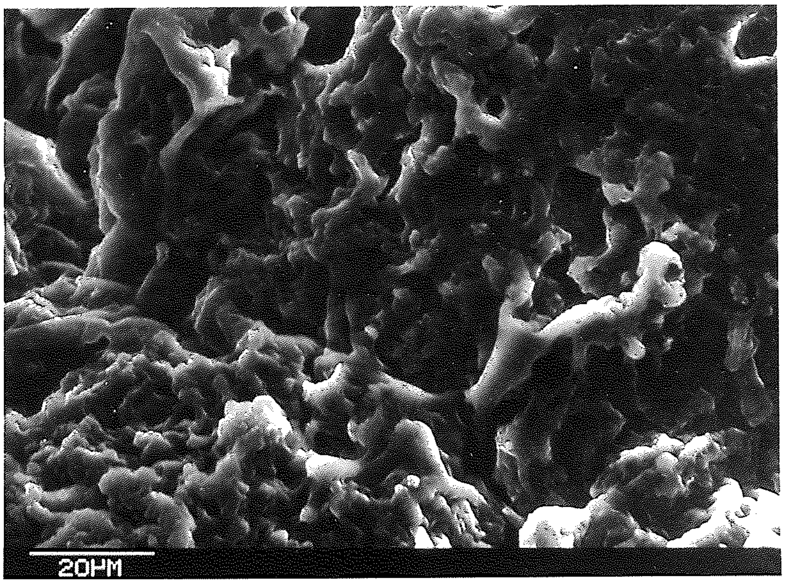
Plate 4.22



Surface of HEMA/NMACR/EDM terpolymer

Magnification X 500

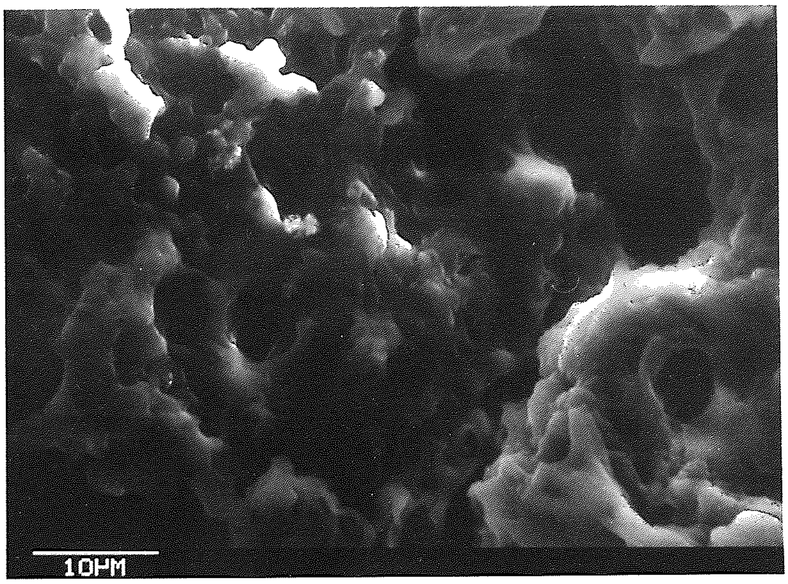
Plate 4.23



Surface of HEMA/NMACR/EDM terpolymer

Magnification X 1000

Plate 4.24



Surface of HEMA/NMACR/EDM terpolymer

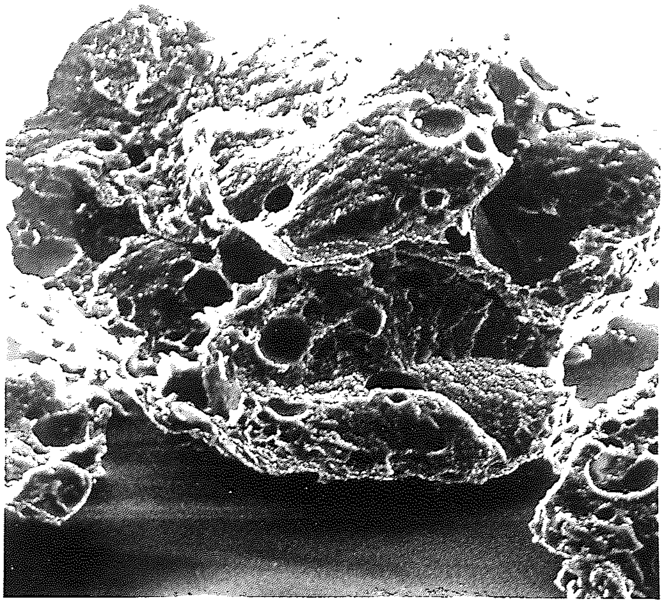
Magnification X 2000

Plate 4.25

Surface of HEMA/NMACR/EDM terpolymer

Magnification X 5000

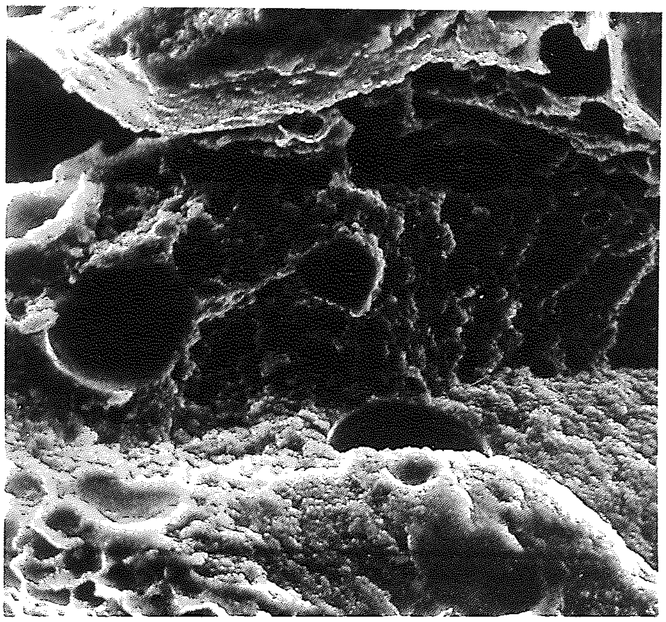
Plate 4.26



Interior of HEMA/NMACR/EDM terpolymer

Magnification X 260

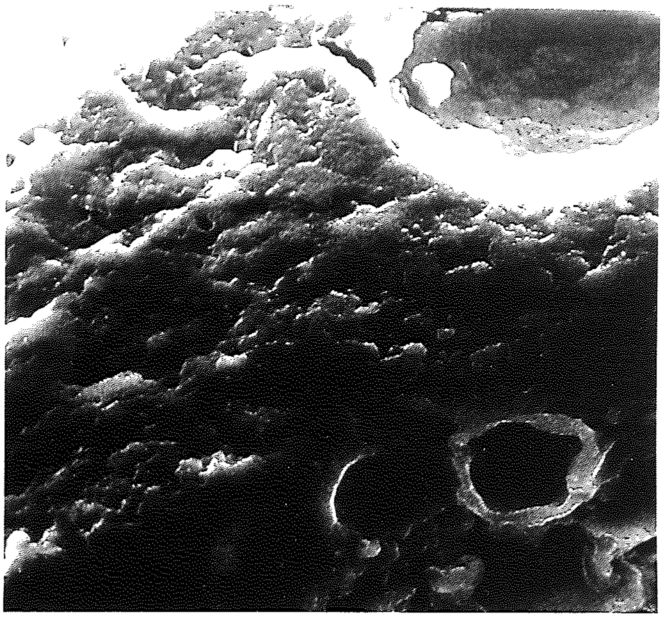
Plate 4.27



Interior of HEMA/NMACR/EDM terpolymer

Magnification X 560

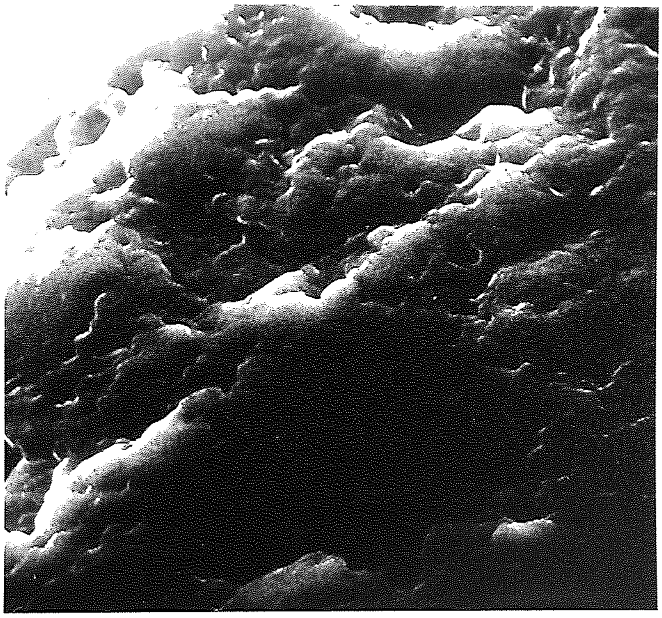
Plate 4.28



Interior of HEMA/NMACR/EDM terpolymer

Magnification X 1600

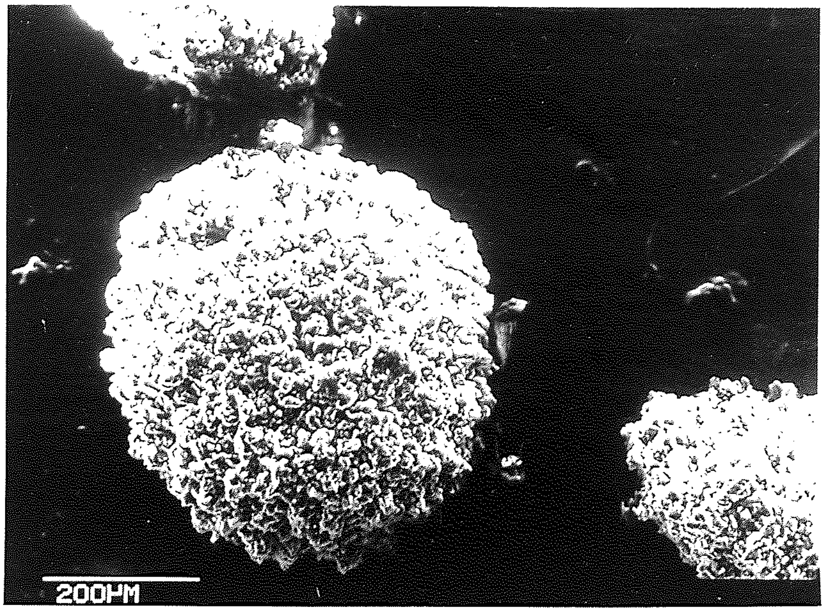
Plate 4.29



Interior of HEMA/NMACR/EDM terpolymer

Magnification X 2600

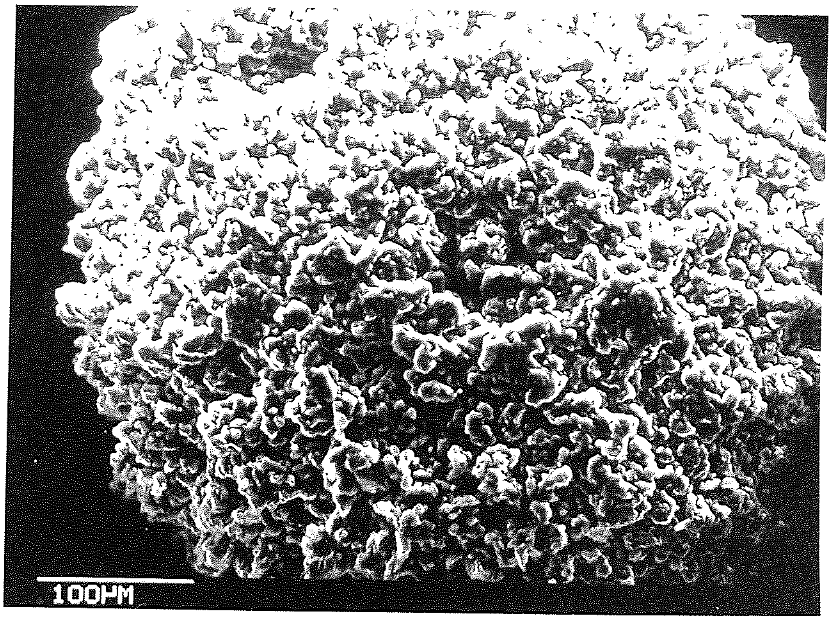
Plate 4.30



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 100

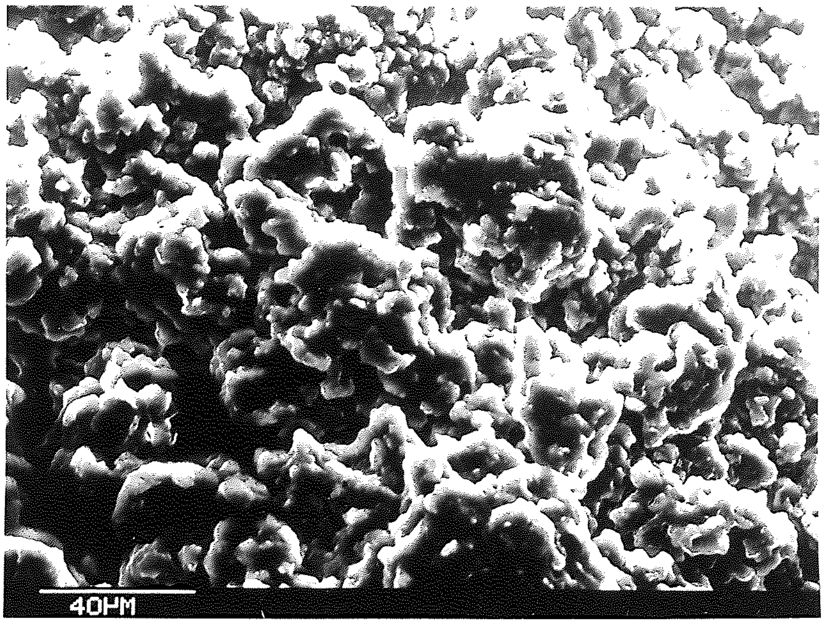
Plate 4.31



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 200

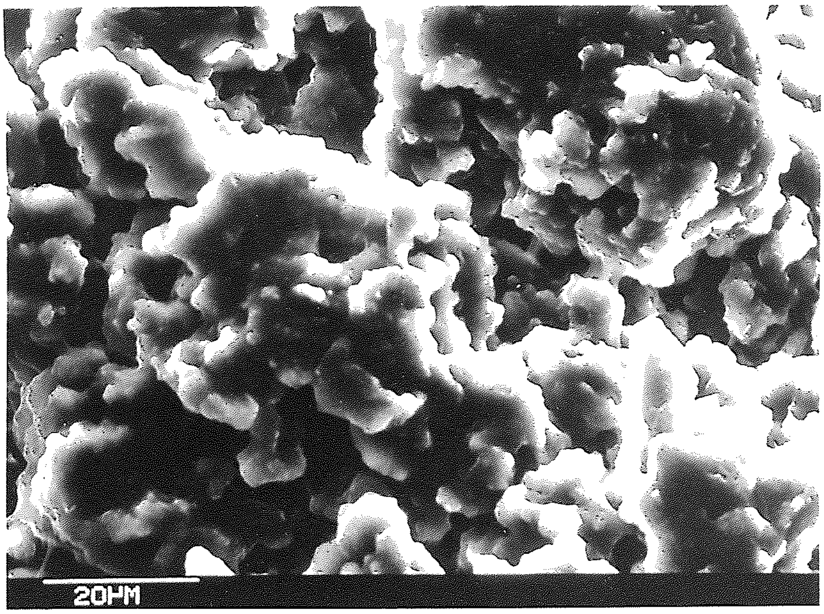
Plate 4.32



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 500

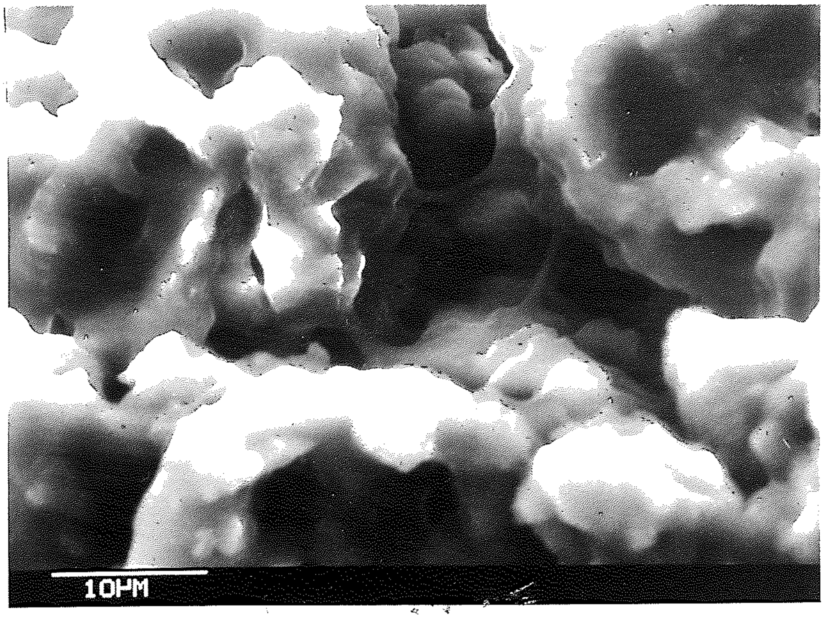
Plate 4.33



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 1000

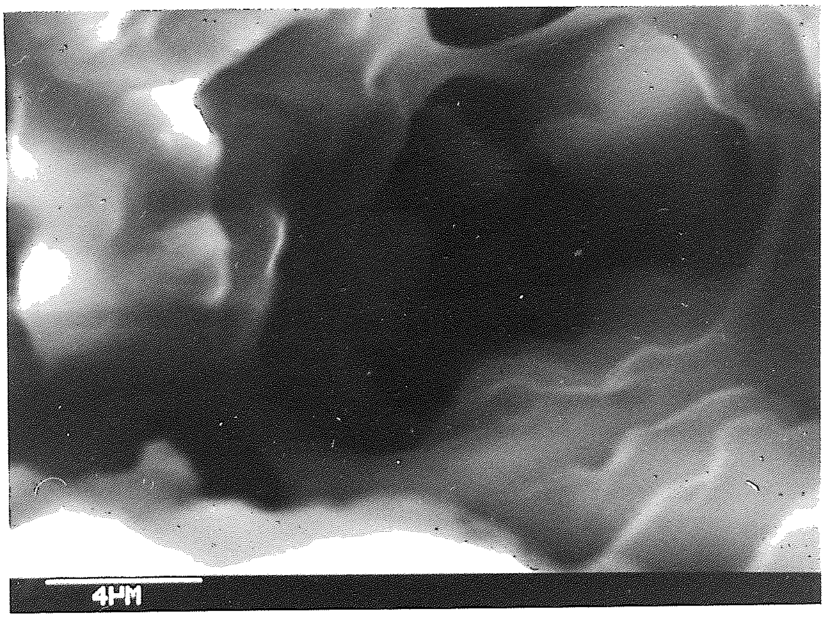
Plate 4.34



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 2000

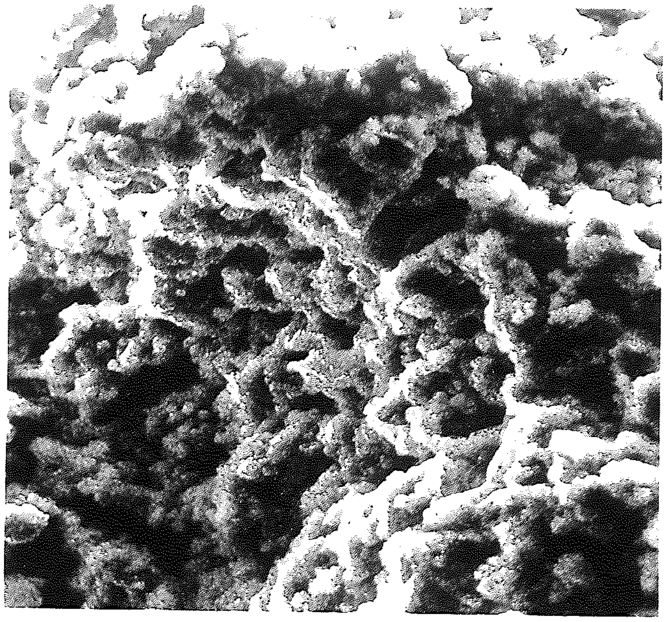
Plate 4.35



Surface of HEMA/DMAEMA/EDM terpolymer

Magnification X 5000

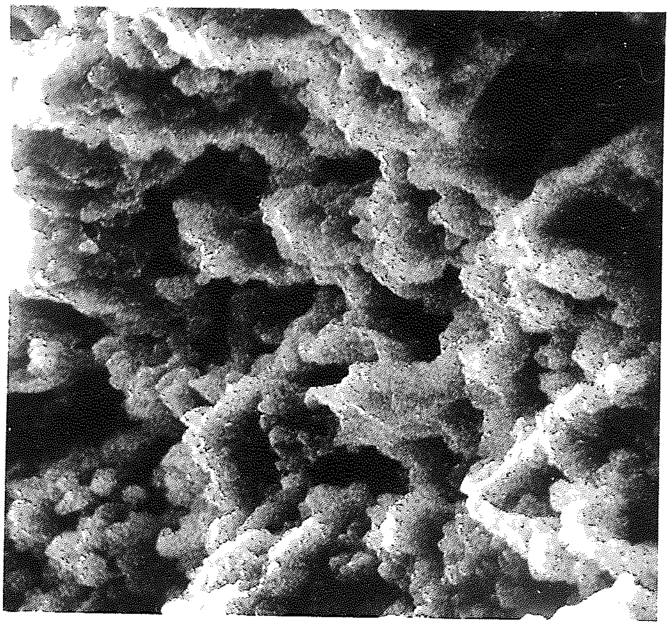
Plate 4.36



Interior of HEMA/DMAEMA/EDM terpolymer

Magnification X 570

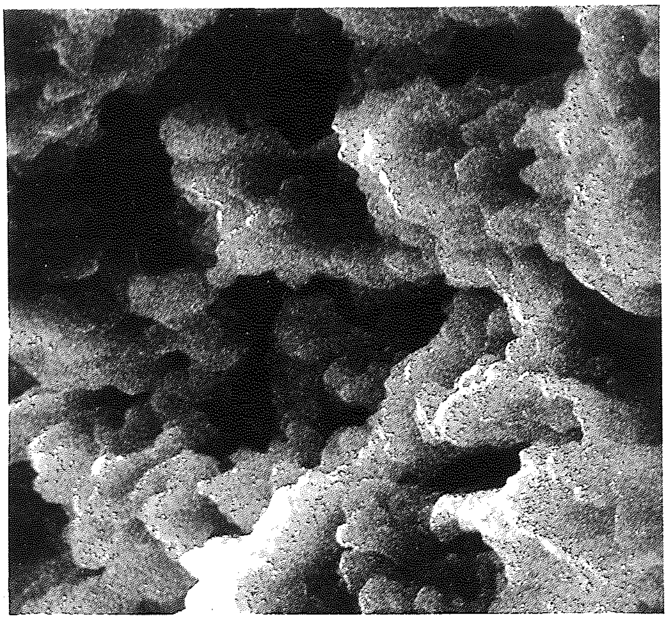
Plate 4.37



Interior of HEMA/DMAEMA/EDM terpolymer

Magnification X 1700

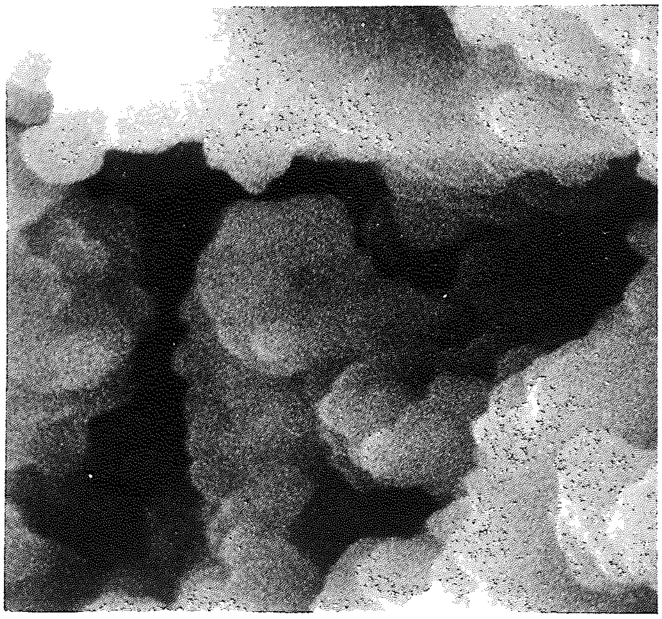
Plate 4.38



Interior of HEMA/DMAEMA/EDM terpolymer

Magnification X 2700

Plate 4.39



Interior of HEMA/DMAEMA/EDM terpolymer

Magnification X 5700

4.3 DISCUSSION

It had long been found desirable to apply the suspension polymerisation technique for the synthesis of hydrogel particulates for their application as adsorbents in haemoperfusion. The technique of suspension polymerisation in a high salt content xanthan gum continuous phase was successfully adapted and modified for producing hydrogel particulates with varying composition, particle size, water content, density and surface morphology. Depending upon the proportion of the solvent and of the crosslinking agent a gradation in properties from those of an expanded network to those of a macroreticular material was obtained. The parameters that can be varied to produce a variety of structures with suspension polymerisation technique will be discussed below.

4.3.1 Monomer Composition, Crosslink Density and Solvent

It was found possible to produce hydrogel particulates from all the monomer compositions considered, with suspension polymerisation in brine. It has been reported in the past that monomers with considerable solubility in water such as acrylamide and acrylic acid did not undergo successful aqueous suspension polymerisation. These monomers were, however, reported to polymerise with reverse suspension polymerisation methods. In the present work since there was a high proportion of water soluble reactants, the salting-out effect caused by the presence of a large amount of sodium chloride limited the solubility of such reactants in water. The suspension stabilising effect of xanthan gum and hydroxypropylmethyl cellulose combination worked remarkably well.

Most monomers were suspension polymerised in the solvent TCE, although the absence of the solvent also produced beaded hydrogels. Hydrogel particulates prepared in the presence of increasing amount of solvent exhibited an increasing turbidity, and possessed macroreticular character, being highly porous even in the dry state. This phenomenon is typical of the polymerisation of monomer mixture and crosslinking agent in the presence of an inert and soluble compound acting as a solvent for monomers but which only swells the polymer. When a monovinyl compound and a divinyl compound are polymerised in such a solvent, the growing polymer has only a limited swelling capacity and phase separation occurs. The phase separation is more pronounced the higher the amount of the crosslinking agent and the lower the solvating power of the solvent. In the extreme case a process like a precipitation polymerisation in each polymerising droplet occurs, and microgel particles which are formed within these droplets at an early stage of the polymerisation gradually grow together. After removal of the solvent a crosslinked polymer with permanent porosity is obtained. Such heterogeneously crosslinked copolymers also have a swelling porosity besides the permanent porosity. The main factors governing heterogeneity are the amount of crosslinking agent as well as the amount of the inert solvent as has been indicated previously. Hydrogel beads having this type of structure were obtained from all the monomer compositions when the solvent was used. This is clearly demonstrated by the SEM's of the particulates prepared from compositions HEMA/EDM, HEMA/NMACR/EDM, HEMA/DMAEMA/EDM etc.

Hydrogel particulates synthesised in the absence of the solvent tetrachloroethane (samples 3, 5 and 8) show different structures on the electron microphotographs. As is shown by SEM's in plates 4.8, 4.9, 4.20 and 4.21 of these samples, the macroreticular structure is absent. In such particulates only the amount of the crosslinking agent determines the porosity. On swelling these crosslinked hydrogel particulates, the single polymer chains become solvated and form a loose statistical network, the porosity is only present in the swollen state and is called the swelling porosity. These particulates may in statistical sense, be regarded as homogeneously, crosslinked. It has been reported that copolymerisation of hydroxyl containing monoesters of methacrylic acid with the corresponding diesters is typical of a polymerisation accompanied by pronounced gel effect (Kopecek et al., 1968). Even in heterogeneous polymerisation the reaction in the initial stages proceeds via a homogeneous mechanism, with growing conversion separation of phases takes place resulting in heterogeneous polymers. It should be noted that chemically different polymers, though polymerised with similar amounts of crosslinking agent will differ in pore structure according to the efficiency of the divinyl compound in crosslinking, and changes in solvation properties upon swelling (Heitz, 1970).

Table 4.3 illustrates that monomer compositions containing less hydrophilic monomers such as HEMA produced hydrogel particulates in the size range 1000 μm - 250 μm. Compositions containing more hydrophilic monomers such as ACR and AA produced particulates in a lower size range 150 μm - 20 μm. Due to the high polarity of the bonds in more hydrophilic monomers, the dipole forces between the monomer molecules and dipole attractions between the water molecules are easily replaced by similar dipole attractions between the water and monomer molecules. Therefore, more hydrophilic monomers may have a tendency to dissolve in water even in high salt content medium. Solvent tetrachloroethane was more efficient in preventing the extraction of less hydrophilic monomers by the aqueous continuous phase, than the extraction of more hydrophilic monomers. It is also indicated from table 4.3 that it was possible to prepare beads in higher size range from more hydrophilic monomers in the presence of less hydrophilic monomer and crosslinking agent. This is explained by the diffusion of more water soluble monomer into the organic phase for copolymerisation thus providing favourable distribution in the two phases. The effect of producing smaller particulates from more water soluble monomers was more pronounced in case of ACR/N,N'MBA copolymer, because in this case both the principal monomer and the crosslinking agent were more hydrophilic than for example, in AA/EDM copolymer.

These differences in bead size exhibited by different monomer compositions cannot be attributed to any single factor since bead formation is a complex process involving several factors. The various parameters which determine bead size will be discussed in the next section on particle size distribution. However, interfacial phenomona play an important role in determining the stability of suspensions of monomer phase in the continuous phase. It is known that as the interfacial tension between monomer and continuous phase increases, the particle size decreases. Therefore, solubility considerations together with surface tension effects result, in the case of more water soluble monomers, in the stabilisation of smaller sized dispersions which in turn form smaller beads.

4.3.2 Alternative Solvents for the Polymerisation of More Hydrophilic Monomers

After successful polymerisation of more water soluble monomers in the bead form, attempts were made to increase the particle size by using alternative solvents for such monomers as acrylamide. Several experiments were conducted in order to find a better solvent than TCE so that a more suitable dispersion of monomers in the continuous phase could be formed. It was found that acrylamide was not very soluble in most organic solvents and the solvents in which it was soluble were either partially or completely miscible with the xanthan gum continuous phase. Some success was achieved using chloroform as the solvent and tertiary butyl cyclo per dicarbonate as the initiator. However, crosslinked poly (ACR) beads produced in this way were mechanically very weak. Another problem with chloroform (boiling point 61-62°C) was its evaporation during polymerisation. Other possible solvents considered for acrylamide included tetrahydrofuran, diethylketone and di-isobutylketone. However, the use of these solvents did not lead to successful bead polymerisation.

In order to synthesise beads in the higher size range from ACR/N,N'MBA combination, the technique of inverse suspension polymerisation was investigated. This technique will be described in chapter 5.

4.3.3 Particle Size Distribution

Table 4.3 is indicative of the finding that hydrogel particulates with varying size range can be produced by suspension polymerisation in brine. Gradation of particulates by manual sieving indicated that the particle size followed a normal Gaussian distribution. This is in agreement with the results of bead sizes obtained with suspension polymerisation reported in literature. It has been reported with control tests on bead polymers that the average bead sizes from a large number of production lots followed a normal Gaussian distribution (Munzer et al., 1977).

During the development of suspension polymerisation in brine technique a large number of experiments were carried out to produce suitable and stable suspension of monomer phase in the continuous phase. Stirrers of varying shape and size, both commercially available and purpose-designed were used. Various stirrers used are illustrated in Appendix (B). Type (c) stirrer made from a stainless steel rod, and a polypropylene paddle (9 cm wide and 4.7 cm deep) with circular holes drilled at 5 mm intervals worked most satisfactorily in conjunction with a flat-bottom 2 litre polymerisation flask. Metal stirrers, type (a) and (e), used had a tendency to produce a large amount of amorphous material and to accumulate polymer on their surfaces. Anchor type glass stirrer, type (b) produced granular particles. Other stirrer types tended to produce tailing and irregular beads. In general, impeller stirrers produce finer beads than leaf or anchor type stirrers. The principal problem in suspension polymerisation was the formation of as uniform a suspension as possible of monomer droplets in the aqueous continuous phase and the prevention of coalescence of these droplets during the polymerisation process. Particle size and size distribution are important properties for specific applications. Therefore, various factors which are thought to affect size and stability of monomer dispersion and consequently bead size, and the mechanism of bead formation will be discussed next.

4.3.4 Factors Affecting Bead Size, and Mechanism of Bead Formation

Under favourable conditions, through the shearing action from the stirring and local turbulence, together with surface tension forces, the monomer assumes more or less stable spherical form. The equilibrium distribution of droplet size was reported to obey a Gaussian distribution (Munzer et al., 1977). Since the distribution of shearing action in the polymerisation flask can be very non-uniform, an equilibrium condition is only possible if all the particles pass through a zone of maximum shear. The time necessary for equilibrium increases with viscosity of the continuous phase, with decreasing droplet size, and with decreasing rate of stirring. The overturning action resulting from stirring must be strong enough that loss of the dispersion through settling and difference in density is hindered. Generally, particle size decreases with increasing rate of agitation. This is indicated from figures 4.2 and 4.3 which show histograms of beads synthesised at two different rates of stirring. However, the intensity of stirring normally is limited by the desired size of dispersed monomer droplets. In a system of two immiscible liquids at a fixed rate of stirring and stirrer design, there exists a minimum droplet size above which stabilisation by stirring becomes possible. If the average size is much smaller than this, the droplets will coalesce as in an unstabilised dispersion, until they reach a minimum value. The mechanism of droplet stabilisation in an agitated dispersion was described by Church and Shinnar (1961).

The determination of bead size upon rates of stirring depends upon the presence of sufficient suspension stabliser. On the other hand the dynamic equilibrium between coalescence and dispersion becomes more difficult as the viscosity of the dispersed monomer phase increases with polymerisation. Theoretical studies of liquid-liquid dispersion support the idea that bead size can be controlled by rates of stirring only within a limited range (Church and Shinnar, 1961). Studies on the mechanism of action of water soluble suspension stabilisers have been reported (Wolf et al., 1972). According to these studies the viscosity in the phase boundary layers is much higher in the presence of added suspension stabliser. It has also been suggested that the suspending agent may form a gel-like protective layer that keeps monomer droplets separated and hinders coalescence.

A few of the variables one would have to consider in correlating the suspension stabliser to particle size are listed below:

(i) type and concentration of suspension stabliser
(ii) rate of stirring and design of stirrer
(iii) design of polymerisation vessel
(iv) ratio of monomer phase - aqueous phase

(v) reaction temperature and time
(vi) concentration of free-radical initiator
(vii) type and concentration of monomers
(viii) densities and viscosities of monomer phase and aqueous phase
(ix) surface tension.

The effect of each variable itself and/or synergistic effects of two or more of these variables give an indication of the degree of complexity involved in determining the bead size.

4.3.5 Equilibrium Water Content (EWC)

EWC values from table 4.4 demonstrate the relationship between hydrophilicity of the polymer particulates and the nature of groups on the polymer chains. A gradation in EWC values with different samples is observed. Table 3.5 (chapter 3) listed the EWC of samples having same composition as in table 4.4 but synthesised by the freezing technique. Comparing the two tables, it is observed that the water contents follow the same pattern in both cases.

The underlying trend of increase in water content with increase in hydrophilicity of monomers is seen. ACR/N,N'MBA copolymer due to the presence of highly polar amide groups and hydrophilic crosslinking agent absorbs large amount of water (88 percent), whereas, HEMA/EDM copolymer with its less hydrophilic hydroxyl groups and more hydrophobic crosslinking agent absorbs lesser amount of water (56 percent). AA/EDM copolymer has more polar carboxyl groups than the hydroxyls of HEMA, and therefore this copolymer has a higher water content (77 percent) than the HEMA/EDM copolymer. In acrylic acid series, the incorporation of more hydrophobic monomer HEMA in HEMA/AA/EDM terpolymer reduces the EWC to 64 percent. By the replacement of acrylic acid by methacrylic acid as in HEMA/MAA/EDM copolymer the water content is reduced even further. In these terpolymer systems other factors such as decreased chain rotation due to the presence of bulky methyl group in HEMA/MAA/EDM, and interchain hydrogen bonding in both terpolymers also play role in determining EWC.

Acrylamide series also show similar decrease in EWC with increase in the number of hydrophobic constituents in the terpolymers. HEMA/DMAEMA/EDM terpolymer equilibrates with large amount of water (77 percent). This is due to the presence of water sensitive tertiary amine group of DMAEMA. Thus complexing between functional groups and the presence of ionic charges on polymeric chains play important role in determining the amount of water absorbed by a particular system.

Several other factors determine the swelling of hydrogels. These include crosslink density, polymerisation conditions, equilibriating solution, and pre-swelling of the polymer at the time of crosslinking. These parameters have previously been discussed in some detail in chapter 3 on freeze-thaw polymerisation.

4.3.6 Scanning Electron Microscopy, Porosity and Density

Morphology of the surfaces and interiors of hydrogel particulates of various samples (visualised by scanning electron microscopy) indicates that a variety of structures can be created during the polymerisation of hydrogel particulates by the suspension polymerisation in brine technique. The crosslinked particulates synthesised in the presence of large amounts of solvent tetrachloroethane exhibit macroreticular character. The macroreticular nature of the hydrogels is observed both on the external surfaces and interiors of such samples. The macroreticular nature of the matrix is best demonstrated by SEM's of samples: HEMA/EDM (plates 4.10 - 4.15). HEMA/NMACR/EDM (plates 4.22 - 4.29), and HEMA/DMAEMA/EDM (plates 4.30 - 4.39). The polymer in these systems can be seen to consist of agglomerates of microspheres which are crudely linked together (plates 4.30 - 4.39) to form crevices and pores within a semirigid matrix. Macroreticular hydrogel beads contain macropores (several hundred Angstroms wide) with a large inner surface area. The structural part in these beads is composed of a highly crosslinked gel with narrow micropores. Hydrogel beads synthesised in the present research in comparison with the commercially available macroreticular resin XAD-7 (SEM's of which are illustrated in plate 4.5, and also in plates 3.31 and 3.32) show superior macroporous character, and would therefore be expected to possess much higher available surface area for the adsorption of solutes.

SEM's of hydrogels synthesised in the absence of solvent, for example, HEMA/MAA/EDM (plates 4.8 and 4.9), and HEMA/ACR/EDM (plates 4.20 and 4.21) lack macroreticular character. These microreticular hydrogel particulates are composed of gel containing micropores.

Apart from demonstrating the three-dimensional arrangement of macropores, other features which scanning electron micrographs delineate are the shape, surface regularity, and the presence of any debris on the surface of the beads. Most of the samples prepared were in uniform and spherical shape. The surfaces of beads are shown to be, in most cases debris free. However, some samples, for example HEMA/MAA/EDM (plates 4.8 and 4.9) show the presence of slight protrusions from the surface. These may possibly be the polymer microspheres extending from the surface. The microphotograph of the copolymer bead of AA/EDM (plate 4.6) indicates the presence of depressions on the surface. These are thought to have been caused by either direct impacts of the bead with stirrer paddle or collisions with other beads. Such surface depressions can also be observed on the microphotograph of the commercial resin bead XAD-7 (plate 4.5).

Due to highly porous nature of macroreticular hydrogels their densities are lower than micorporous hydrogels. Comparing the density of samples of hydrogel beads prepared by suspension polymerisation with their counterparts from freeze-thaw technique, the former have lower density. For example, density of dehydrated terpolymer bead of HEMA/NMACR/EDM from freeze-thaw technique was in the range 1.266 - 1.272. Corresponding value from suspension polymerisation is in the range 1.148 - 1.165. This is so because the terpolymer hydrogel synthesised by suspension polymerisation technique is macroreticular whereas macroporous character was not produced by the freeze-thaw technique. Similar results are observed for several other samples. However, generalisation in this respect can not be made as different polymerisation conditions were used in the two techniques.

CHAPTER 5

SYNTHESIS OF HYDROGEL PARTICULATES; INVERSE-SUSPENSION POLYMERISATION IN HEXANE

5.1 INTRODUCTION

One further technique which was investigated for the creation of hydrogel beads was the inverse-suspension polymerisation. In this method a concentrated aqueous solution of monomers is dispersed in the immiscible hydrophobic continuous phase (Barrett and Thomas, 1969). In the recent years processes have been developed in which organic liquids such as aliphatic hydrocarbons or less frequently toluene, xylene, and chlorinated hydrocarbons form the outer, dispersing phase. Liquid paraffin and silicone fluid have also been used as non-solvent continuous phases. The dispersion of the monomer phase in the continuous phase is achieved by mechanical agitation.

Inverse-suspension polymerisation reactions are initiated using water soluble initiators such as persulphates, hydrogen peroxide, and redox activators of these. Effective suspension stabilisers used in the reverse-phase polymerisation include fine powders such as hydrophobic kaolin and bentonite, or mineral fillers together with emulsifiers that are dispersible in organic medium.

The inverse-suspension polymerisation technique is particularly useful with hydrophilic monomers such as acrylamide, methacrylamide, salts of corresponding acids, and quaternary ammonium monomers such as quaternised diethylaminoethyl methacrylate and their mixtures. Nilsson et al. (1972)

reported the preparation of spherical acrylic copolymers by bead polymerisation in hydrophobic continuous phase comprised of a mixture of chloroform and toluene. Preparation of similarly synthesised acrylic copolymer beads for use as matrices for enzyme immobilisation was reported by Johansson and Mosbach (1974). Crosslinked poly(acrylamide) beads have been prepared within an organic continuous phase prepared from a mixture of tetrachloromethane and hexane (Stahl et al., 1979).

Previous attempts made in the department (Pasha, 1976; Skelly, 1979) to synthesise crosslinked poly(acrylamide) beads by inverse-suspension polymerisation have not been very successful. These attempts, to produce biocompatible adsorbents for haemoperfusion, used either paraffin oil or silicone oil as the dispersing medium. The main problem reported with the previous attempts was the agglomeration of hydrogel particulates caused by the production of unstable dispersion of monomers in the non-solvent. It was, therefore, felt necessary to explore and adopt an alternative hydrophobic non-solvent for the continuous phase. In this work the hydrocarbon n-hexane (density 0.66 g/cm , boiling point 69°C) was used as the dispersing medium. Due to the low boiling point of hexane, ammonium persulphate-dimethylamino propionitrile (APS-DMAPN) catalyst system was used to initiate polymerisation at 50° C. Span 20 (sorbitan monolaurate) and

Arlecel C (sorbitan sesquioleate) were used as suspension stabilisers.

5.2 DEVELOPMENT OF THE TECHNIQUE

The investigation with inverse-suspension polymerisation was carried out mainly for the preparation of crosslinked poly (acrylamide) beads. Experiments were centered upon the acrylamide /N,N'methylene bis acrylamide (ACR/N,N'MBA) system for the following reasons.

(i) The freeze-thaw technique although enabled to polymerise ACR/N,N'MBA in the bead form, did not result in the creation of macroporous structure in the beads. Macroporosity was thought to be essential from the viewpoint of permeation of molecules with size in the 'middle molecular' range.

(ii) Suspension polymerisation in brine, too, provided ACR/N,N'MBA in the bead form. However, difficulties were encountered in producing the ACR/N,N'MBA beads in size greaterthan 80 μm in diameter with the use of that technique.

(iii) It has previously been reported that poly(acrylamide) is one of the most promising biocompatible synthetic materials (Bruck et al., 1973).

(iv) Preformed poly(acrylamide) can be chemically modified for the purpose of introducing any of a wide variety of functional groups to a predetermined level. This can be made use of for removing toxins in various ways. For example, the conversion of poly(acrylamide) beads into a variety of ion-exchange media might be useful for toxin removal by ion-exchange (Inman and Dintiz, 1969). Poly (acrylamide) can also be used as a template for protein molecules and hence for the removal of protein bound toxins.

(v) The high solubility of ACR and N,N'MBA monomers in water enables water to be used as a convenient solvent thus avoiding contamination of the polymer which might occur if other solvents are used. Also N,N'MBA is quite soluble in an acrylamide/water solution. Therefore, highly porous structures can be produced by incorporating high amounts of the crosslinker by the same method used for conventional ion-exchange resins. Also the solubility behaviour of ACR/N,N'MBA system in relation to temperature, monomer-solvent ratio, and ratio of the monomers has been studied (Skelly, 1979).

(vi) Some previous work has been carried out for synthesising ACR/N,N'MBA suspension beads (Halpern and Greenberg, 1969; Johansson and Mosbach, 1974).

(vii) Finally, crosslinked poly(acrylamide) beads can be used as supports to immobilise hepatic enzymes for the removal of specific hepatic toxins.

Initial experiments were performed with the ACR/N,N'MBA system to investigate the possibility of producing beads from these comonomers. Further attempts were then made to standardise the technique, and to incorporate other monomers to produce copolymer and terpolymer beads with varying composition, water content and particle size. Hydrogel beads synthesised by the inverse-suspension technique were then examined by scanning electron microscopy, and adsorption characteristics of selected samples were studied in relation to certain chemical species. On the following pages, the description of the synthetic approach starting with initial attempts followed by subsequent experiments to produce hydrogel beads will be given.

5.2.1 Initial Experiments

For these experiments the following basic formulation was used: Monomer phase: Acrylamide (20 g, 0.2814 mol), N,N'Methylene bis acrylamide (5 g, 0.0324 mol), Water (40 g), APS (0.25 g, 1% w/w total monomer content), and DMAPN (0.25 g, 1% w/w total monomer content).

Organic phase: n-Hexane (659 g), Span 20 (0.75 g, 3% w/w total monomer content).

Solution of ACR and N,N'MBA in water was prepared by heating at 50°C for 30 minutes. The solution was filtered and initiators were dissolved in this solution. The suspending agent Span 20 was dissolved in n-hexane which was then transferred to a 1 litre round bottom split resin flask fitted with a stirrer (type e, Appendix B), a water condenser, a thermometer, and a nitrogen inlet. The continuous phase was deoxygenated by purging nitrogen for 30 minutes. Similarly pre-purged monomer solution was then dispersed in the continuous phase by stirring at 900 rpm for 10 minutes. The temperature of water bath was increased to 50°C and the stirring was reduced to 400 rpm and maintained at this rate for a further 3 hours. The stirring was then stopped and the polymer was allowed to cure in slowly falling temperature for a further 12 hours. The hydrogel was washed thoroughly with water and dried under reduced pressure at 60°C. Granular hydrogel particulates were obtained (23 g, 92% yield). After hydration the particulates were found to have an equilibrium water content of 56%.

After successful polymerisation of ACR/N,N'MBA system in granular form, several modifications were made to obtain the copolymer in spherical bead form. During deoxygenation of monomer solution it was observed that the aqueous monomer solution polymerised even without the addition of further initiators. This effect was attributed to air oxidation of monomers to form peroxides and hydroperoxides which may initiate polymerisation. Since light can also accelerate the initiation process, the deoxygenation was carried out in the dark by wrapping an aluminium foil around the flask containing monomer solution, in subsequent experiments. The use of ethanol as solvent was also investigated since it has been reported that, although the rate of polymerisation of acrylamide in water is fast, in certain anhydrous polar solvents like alcohols and halides the rate is slower.

5.2.2 Subsequent Experiments

Further experiments were conducted by making modifications in the above described procedure. These included variations in the: stirrer type, solvent, monomer-solvent ratio, monomer phase-continuous phase ratio, and the type and concentration of suspension stabiliser. Experiments carried out in this way enabled to produce a formulation which lead to the synthesis of ACR/N,N'MBA copolymer in uniform spherical beaded form. A typical experiment carried out with this formulation is described next.

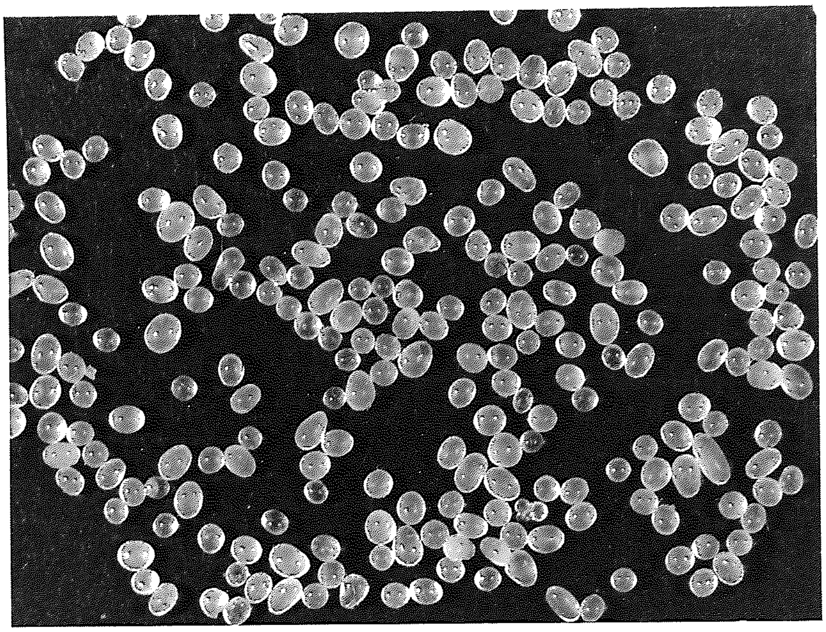
Copolymerisation of Acrylamide and N,N'Methylene Bis Acrylamide in Bead form in a n-Hexane Continuous Phase

Monomer phase: Acrylamide (17.2900 g, 0.2432 mol), N,N'Methylene bis acrylamide (2.4080 g, 0.0156 mol), Solvent: Mixture of water (18.5 g) and ethanol (20 g), Initiators: APS and DMAPN (0.3939 g each, 2% w/w total monomer content).
Continuous phase: n-Hexane (1123 g), Span 20 (0.5908 g, 3% w/w total monomer content).
The following procedure was used:

Dissolved oxygen was displaced from the continuous phase contained in a 2 litre flat bottom polymerisation vessel fitted with an anchor type glass stirrer (type b, Appendix B), a water condenser, nitrogen inlet, and a thermometer.The apparatus used is shown in figure 4.1 and plate 4.1 (chapter 4). Initiators dissolved in one third of the solvent, and monomers in the remainder of the solvent were deoxygenated separately. Initiators were mixed with the monomer solution and added immediately to the slowly stirred continuous phase which was then stirred rapidly (1000 rpm) for 15 minutes. Polymerisation was initiated by increasing the temperature of water bath to 50°C, and stirring rate was reduced to 400 rpm and continued for 3 hours. The polymer was allowed to cure for a further 12 hours with the heating turned off. The hydrogel was washed thoroughly with water and dried under reduced pressure at 60 ° C. 18.80g (95.44% yield) of spherical, beads were obtained. The particle size range was found to be 1000 μm - 250 μm. After equilibration with water, the water content was determined to be 69 percent. Plates 5.1 and 5.2 illustrate samples of dehydrated and hydrated crosslinked poly(acrylamide) beads synthesised by this method.

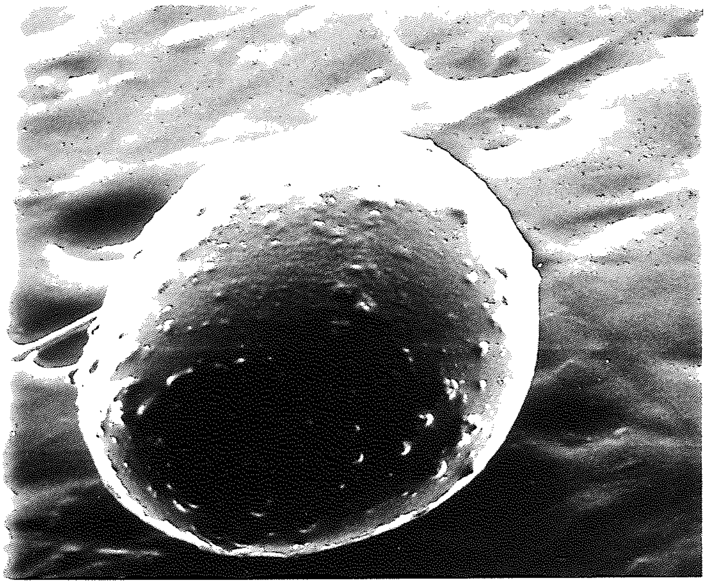
Due to the successful synthesis of ACR/N,N'MBA copolymer beads in the spherical shape and acceptable size range for their possible application in artificial liver support systems, the technique was extended to prepare hydrogel beads with varying composition. Incorporation of HEMA and MACR was successfully achieved. However, attempts to obtain beaded hydrogels with compositions containing EDM for example, HEMA/EDM and AA/EDM were not met with much success. A range of compositions polymerised by the inverse-suspension method is listed in table 5.1. Monomer phase and aqueous phase contents of the polymerisation system are tabulated in table 5.2.

Plate 5.1



Hydrated beads of ACR/N,N'MBA copolymer synthesised by inverse-suspension polymerisation in n-hexane.

Plate 5.2



Dehydrated bead of ACR/N,N'MBA copolymer synthesised by inverse-suspension polymerisation in n-hexane

Table 5.1

Samples of hydrogel particulates synthesised by inverse-suspension polymerisation in n-hexane

Sample	Monomer Composition	Initial Molar Ratio	Solvent
S 1	ACR:N,N'MBA	100:6.4	Water and Ethanol
S 2	ACR:N,N'MBA	100:9.2	Water and Ethanol
S 3	ACR:N,N'MBA	100:10	Water and Ethanol
S 4	ACR:N,N'MBA	100:11.5	Water
S 5	ACR:N,N'MBA	100:11.5	Ethanol
S 6	MACR:N,N'MBA	100:7	Water and Ethanol
S 7	HEMA:N,N'MBA	100:10	Water
S 8	ACR:HEMA:N,N'MBA	80:44:8	Water

Table 5.2

Percentage of the monomer and the continuous phase in the polymerisation system

Sample	Monomer Composition	Monomer Content of Aqueous Phase (%)	Aqueous Phase Content of Total System (%)
S 1	ACR:N,N'MBA	33	5
S 2	ACR:N,N'MBA	35	5
S 3	ACR:N,N'MBA	22	7
S 4	ACR:N,N'MBA	38.5	9
S 5	ACR:N,N'MBA	38.5	9
S 6	MACR:N,N'MBA	33	5
S 7	HEMA:N,N'MBA	32.7	5
S 8	ACR:HEMA:N,N'MBA	50	6

5.3 CHARACTERISATION OF HYDROGEL PARTICULATES

Characterisation of hydrogel beads of the acrylamide series was carried out by determination of their equilibrium water content and size distribution, and examination of their surfaces and interiors by scanning electron microscopy. Methods for carrying out these determinations were described in chapter 2. Equilibrium water contents of the samples are tabulated in table 5.3. Particle size range obtained from sieve analysis is shown in table 5.4. Table 5.4 also lists the type of stirrer used for individual samples. Size distribution for a typical sample is illustrated in figure 5.1. Results of adsorption characteristics of ACR/N,N'MBA copolymer will be included in chapter 6.

Table 5.3

Equilibrium water content of hydrogel beads

Monomer Composition	Initial Molar Ratio	Equilibrium Water Content (%)
ACR:N,N'MBA	100:6.4	69
ACR:N,N'MBA	100:9.2	61
ACR:N,N'MBA	100:10	80
ACR:N,N'MBA (S 5)	100:11.5	57
MACR:N,N'MBA	100:7	52

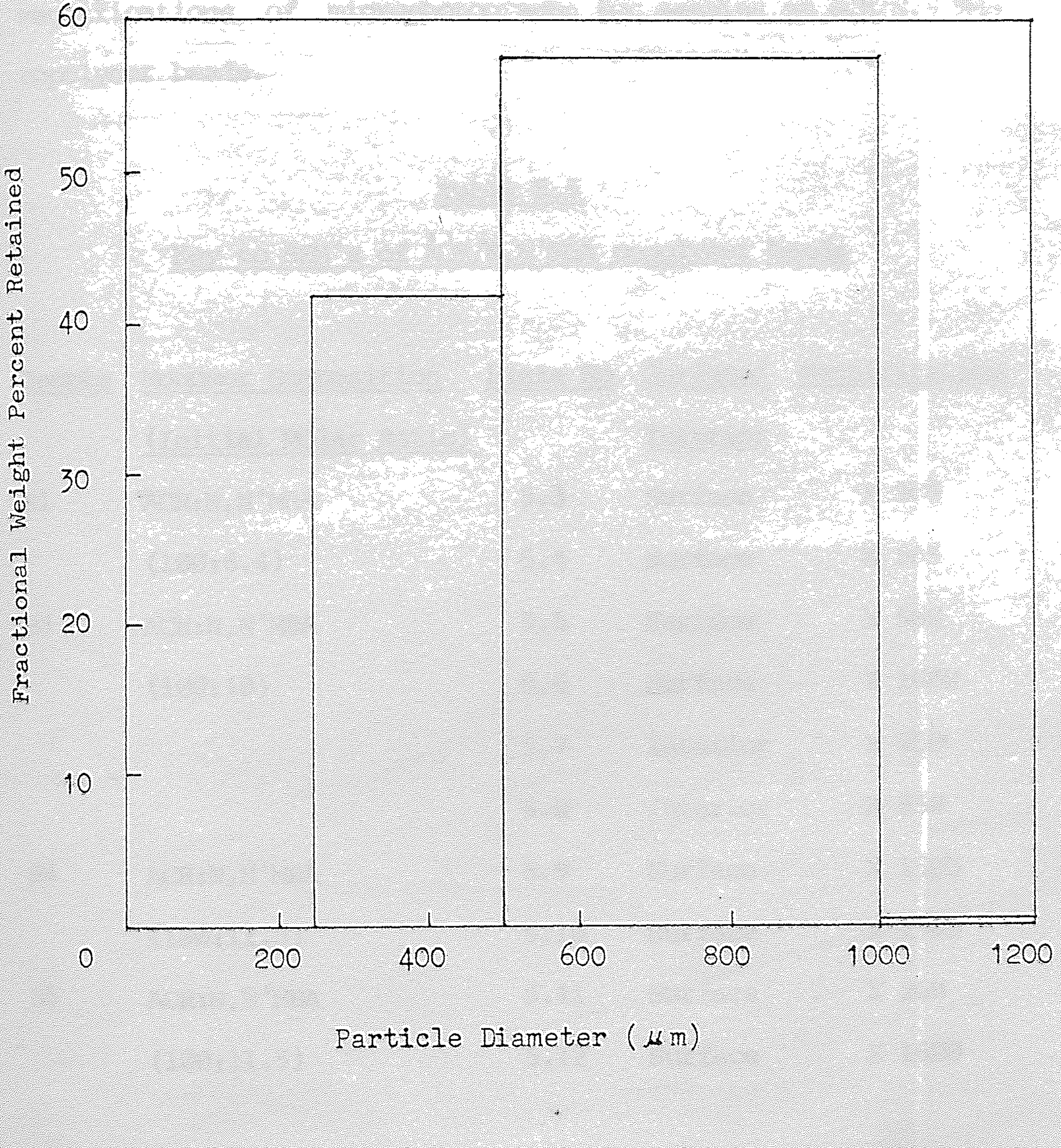
Table 5.4

Particle size range of hydrogel beads

Monomer Composition	Initial Molar Ratio	Stirrer Type	Size Range (μm)
ACR:N,N'MBA	100:6.4	b	1000 - 250
ACR:N,N'MBA	100:9.2	d	2000 - 250
ACR:N,N'MBA	100:10	b	1000 - 250
ACR:N,N'MBA	100:11.5	c	125 - 50
MACR:N,N'MBA	100:7	b	4000 - 500

Figure 5.1

Histogram of fractional data for ACR/N,N'MBA (100/10) copolymer beads



Scanning electron microscopy was performed on dehydrated samples of beads to view the external and internal surface of selected samples. Scanning electron microphotographs (SEM's) delineating the surfaces and interiors of beads are illustrated on the following pages. Table 5.5 lists various magnifications of microphotographs for samples of ACR/N,N'MBA copolymer beads.

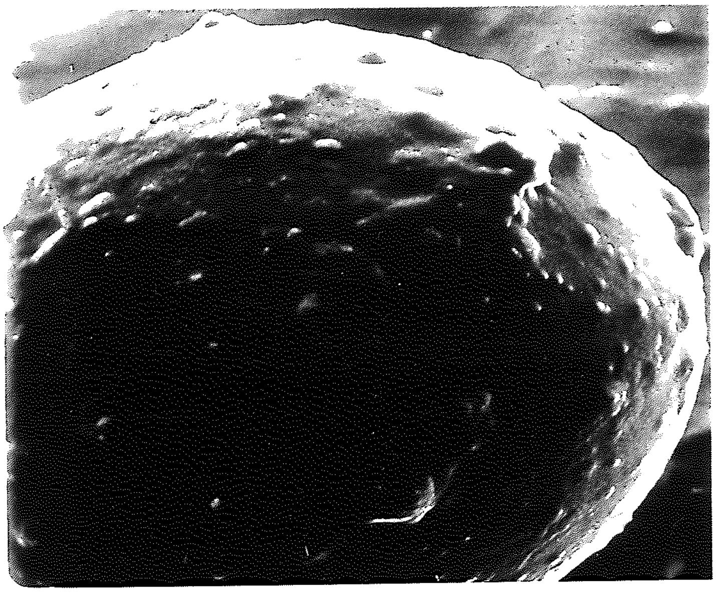
Table 5.5

Key to SEM's of ACR/N,N'MBA copolymer beads

Sample	Monomer Composition (Initial Molar Ratio)	Plate No	Surface/ Interior	Magnification
S1	ACR:N,N'MBA	5.3	Surface	X 265
	(100:6.4)	5.4	Surface	X 565
S3	ACR:N,N'MBA	5.5	Surface	X 560
	(100:10)	5.6	Surface	X 1600
		5.7	Interior	X 250
		5.8	Interior	X 550
S4	ACR:N,N'MBA	5.9	Surface	X 1500
	(100:11.5)	5.10	Surface	X 2500
S5	ACR:N,N'MBA	5.11	Surface	X 260
	(100:11.5)	5.12	Surface	X 1600

Scanning Electron Microphotographs of ACR/N,N'MBA Copolymer Beads

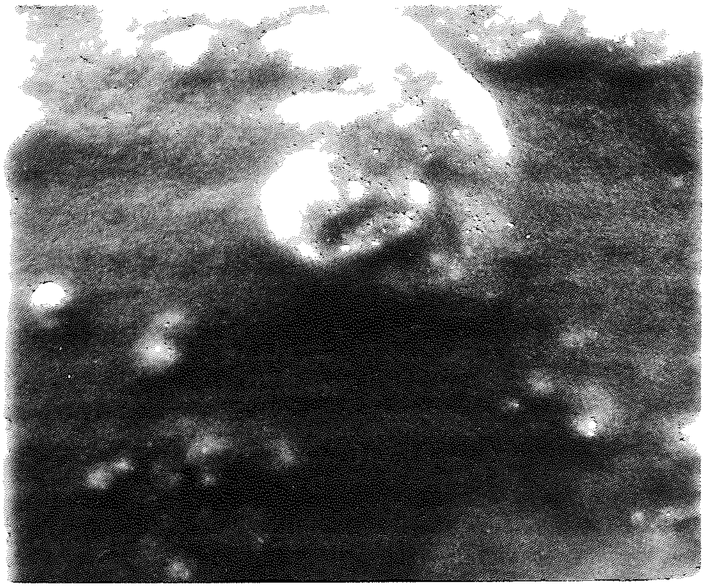
Plate 5.3



Surface of ACR/N,N'MBA (100/6.4) copolymer

Magnification X 265

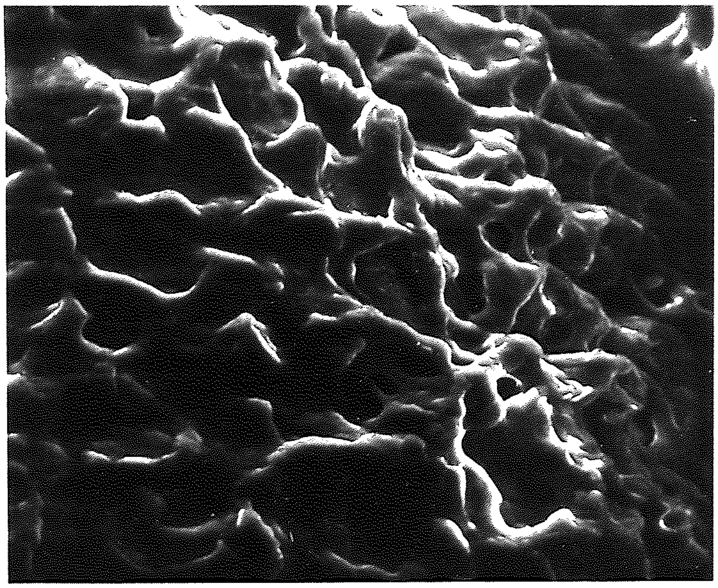
Plate 5.4



Surface of ACR/N,N'MBA (100/6.4) copolymer

Magnification X 565

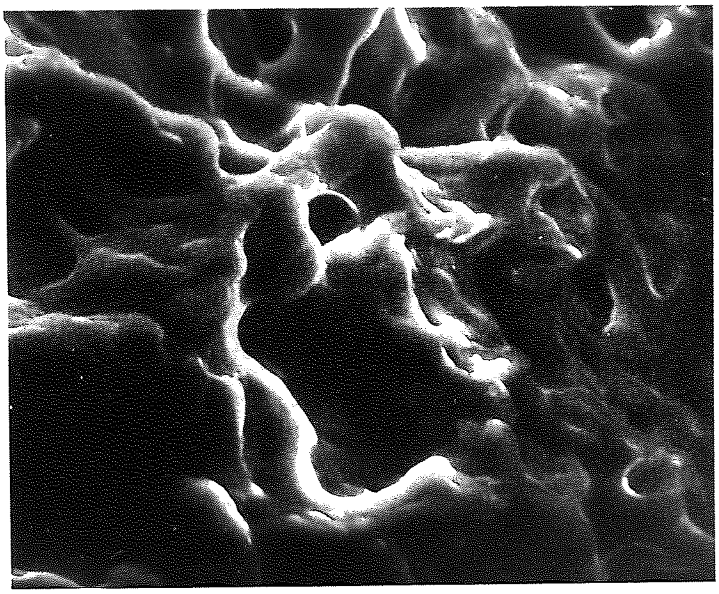
Plate 5.5



Surface of ACR/N,N'MBA (100/10) copolymer

Magnification X 560

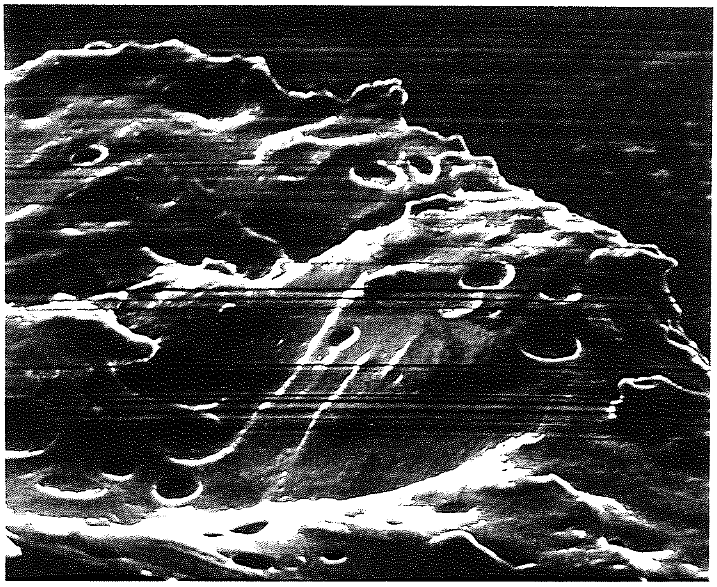
Plate 5.6



Surface of ACR/N,N'MBA (100/10) copolymer

Magnification X 1600

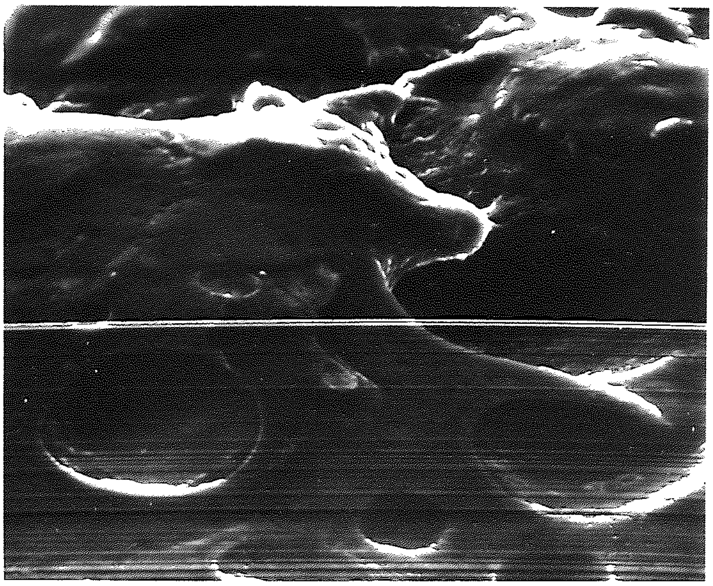
Plate 5.7



Interior of ACR/N,N'MBA (100/10) copolymer

Magnification X 250

Plate 5.8



Interior of ACR/N,N'MBA (100/10) copolymer

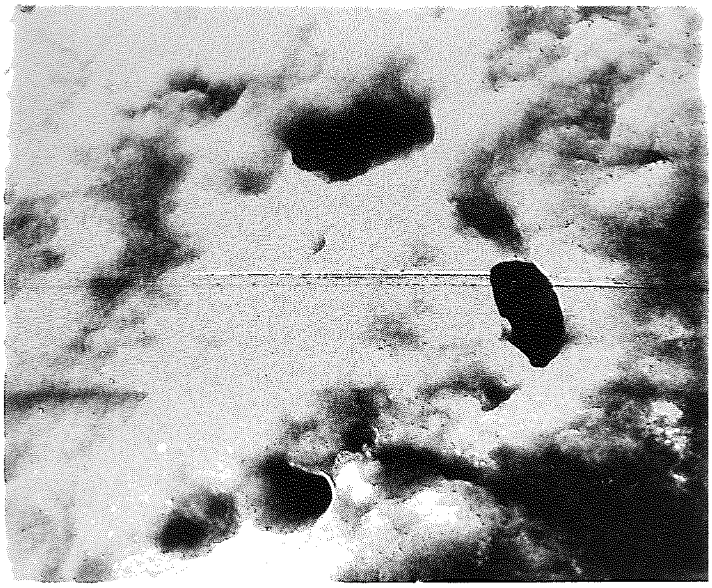
Magnification X 550

Plate 5.9

Surface of ACR/N,N'MBA (100/11.5) copolymer (Sample 4)

Magnification X 1500

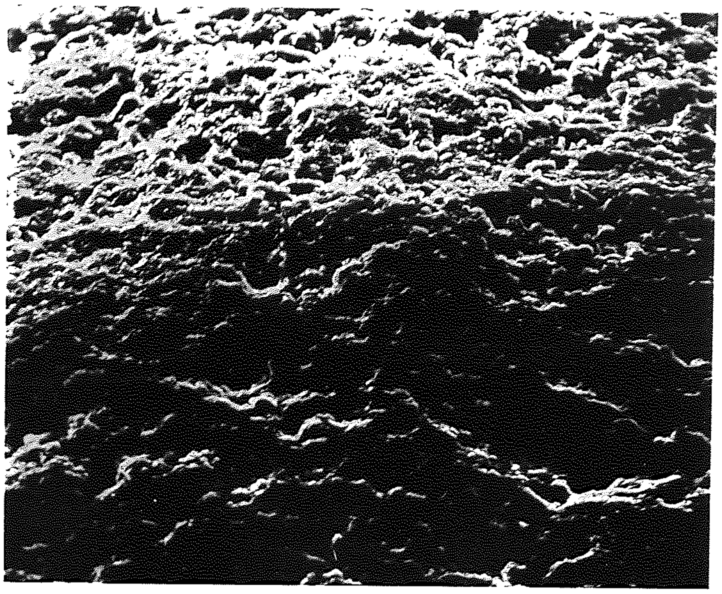
Plate 5.10



Surface of ACR/N,N'MBA (100/11.5) copolymer (Sample 4)

Magnification X 2500

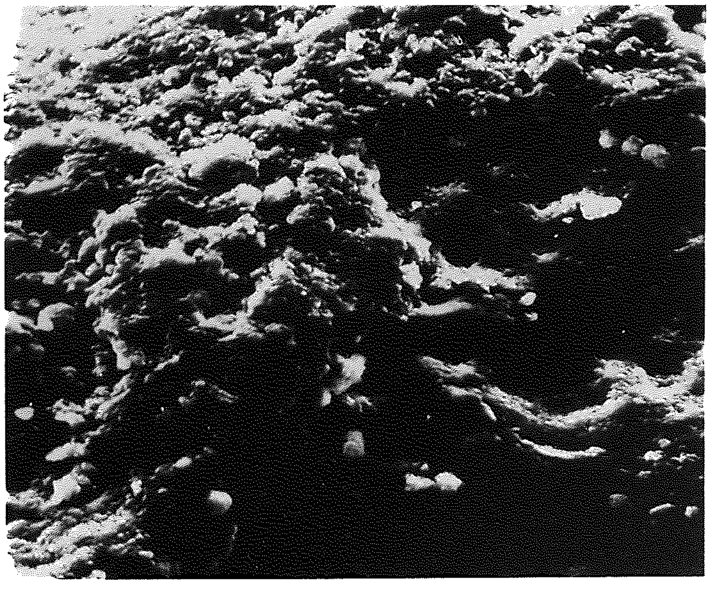
Plate 5.11



Surface of ACR/N,N'MBA (100/11.5) copolymer (Sample 5)

Magnification X 260

Plate 5.12



Surface of ACR/N,N'MBA (100/11.5) copolymer (Sample 5)

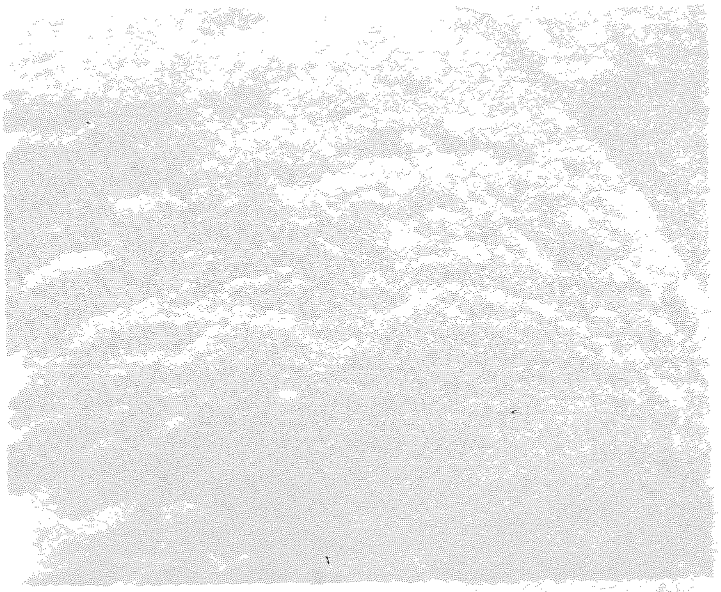
Magnification X 1600

Scanning electron microphotographs of the sufaces and interiors of MACR/N,N'MBA copolymer, and ACR/HEMA/N,N'MBA terpolymer beads are illustrated on the next few pages. Various magnifications of samples are listed in table 5.6.

Table 5.6

Key to SEM's of MACR/N,N'MBA copolymer and ACR/HEMA/N,N'MBA terpolymer Beads.

Monomer Composition Initial Molar Ratio	Plate No	Surface/ Interior	Magnification
MACR:N,N'MBA	5.13	Surface	X 250
(100:7)	5.14	Surface	X 550
	5.15	Interior	X 250
	5.16	Interior	X 1500
ACR:HEMA:EDM	5.17	Surface	X 150
(80:44:8)	5.18	Surface	X 250
	5.19	Surface	X 550
	5.20	Interior	X 150



Scanning Electron Microphotographs of MACR/N,N'MBA Copolymer and ACR/HEMA/EDM Terpolymer Beads

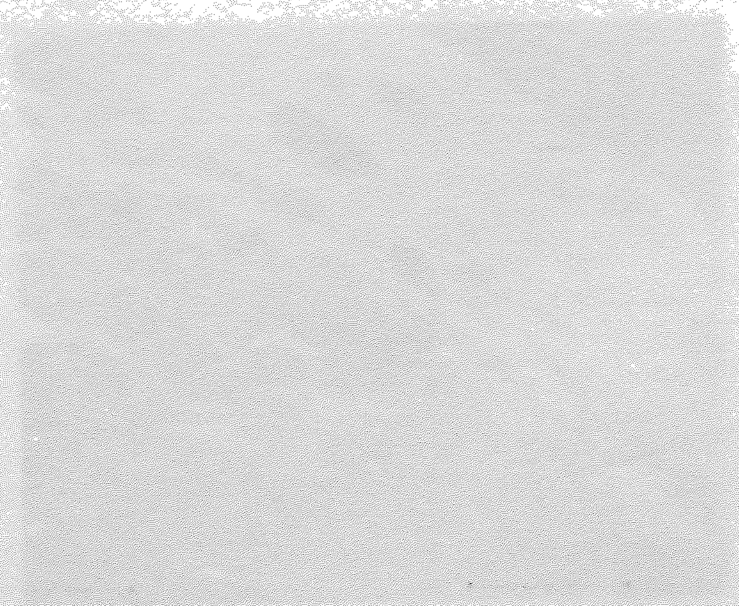
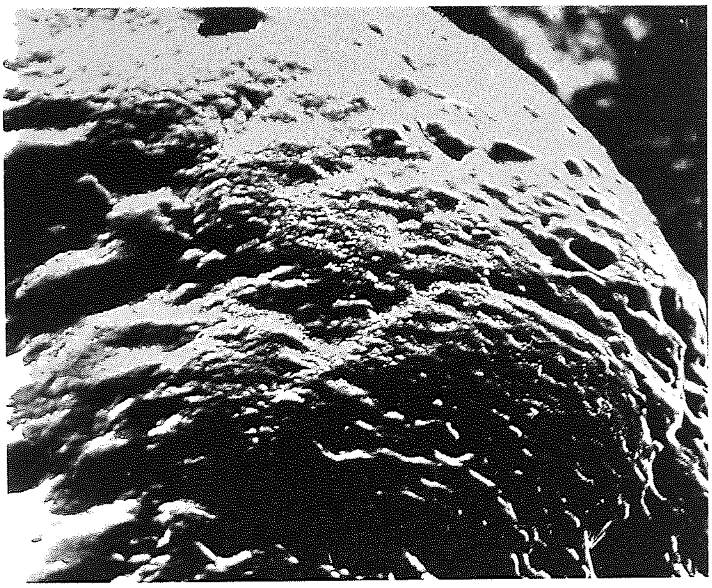


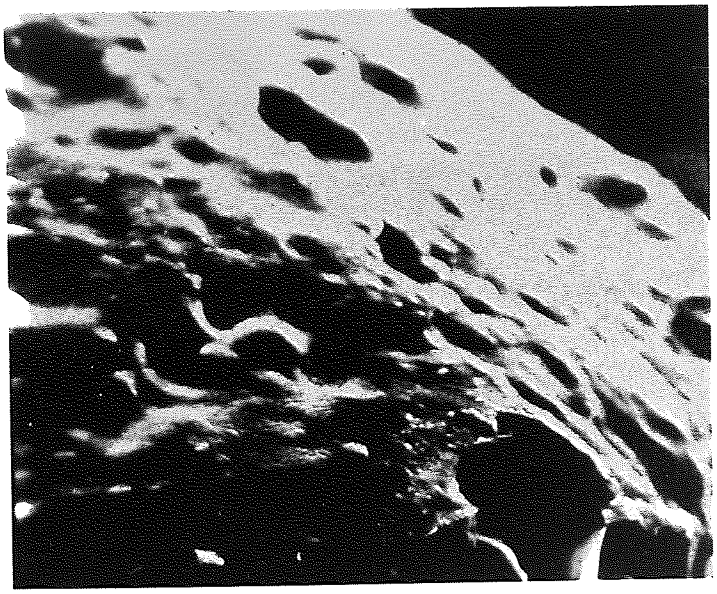
Plate 5.13



Surface of MACR/N,N'MBA copolymer

Magnification X 250

Plate 5.14



Surface of MACR/N,N'MBA copolymer

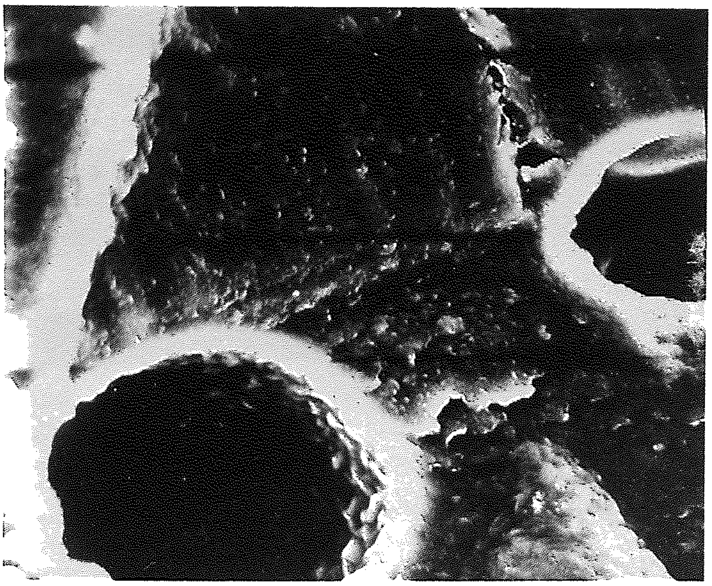
Magnification X 550

Plate 5.15

Interior of MACR/N,N'MBA copolymer

Magnification X 250

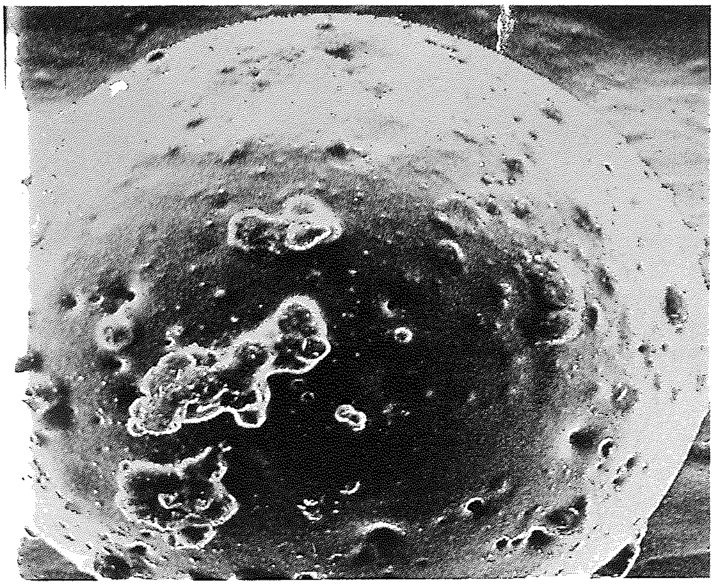
Plate 5.16



Interior of MACR/N,N'MBA copolymer

Magnification X 1500

Plate 5.17



Surface of ACR/HEMA/EDM terpolymer

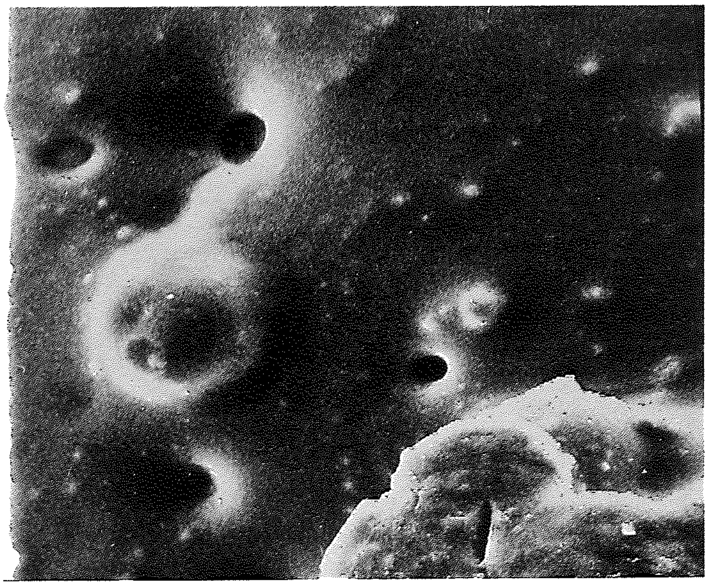
Magnification X 150

Plate 5.18

Surface of ACR/HEMA/EDM terpolymer

Magnification X 250

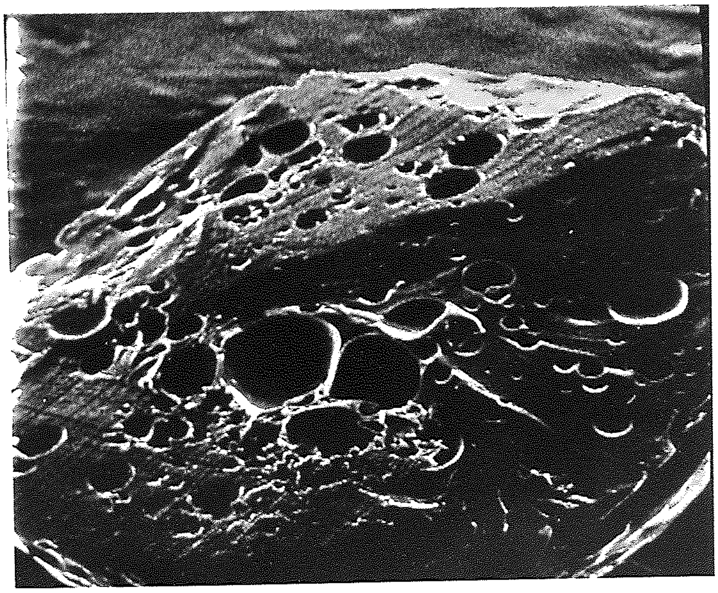
Plate 5.19



Surface of ACR/HEMA/EDM terpolymer

Magnification X 550

Plate 5.20



Interior of ACR/HEMA/EDM terpolymer

Magnification X 150

5.4 DISCUSSION

Inverse-suspension polymerisation technique was successfully adapted to produce crosslinked hydrogel beads from water soluble monomers such as acrylamide. The use of n-hexane as the hydrophobic continuous phase together with Span 20 as the suspension stabiliser led to well defined spherical beads of specific size for possible use as biocompatible adsorbents in artificial liver support systems. With this technique, for producing ACR/N,N'MBA copolymer beads, best results were obtained when following composition was used:

(i) Aqueous phase formed 5 percent (w/w) of the total polymerisation system.

(ii) Monomers formed 33 percent (w/w) of total aqueous phase.

(iii) Suspension stabilser (sorbitan monolaurate) formed 3 percent (w/w) of total monomer content, and was added to n-hexane rather than to the monomer phase.

(iv) Initiators APS/DMAPN were 2 percent each (w/w) of the monomer content.

The parameters which were found to be variable with the inverse-suspension polymerisation technique, such as monomer composition, solvent, bead size, mechanical strength, equilibrium water content and surface morphology are discussed next.

5.4.1 Monomer Composition and Solvent

Copolymer beads of ACR and MACR with N,N'MBA were synthesised in n-hexane non-solvent continuous phase using water or ethanol as the solvent. The preparation of copolymer beads of HEMA and other monomers with EDM was not very successful. Water and/or ethanol used as solvents for ACR/N,N'MBA and MACR/N,N'MBA systems prevented the partitioning of these monomers into the hydrophobic continuous phase. However, in case of HEMA and other monomers partitioning could not be prevented. The inability of certain monomers to polymerise in bead form in n-hexane was thought to be due to their hydrophobicity and their tendency to mix with the n-hexane continous phase. In order to establish this the solubility of a range of monomers in hexane was studied. The monomers considered included HEMA, DMAEMA, AA, MAA, EDM, ACR, MACR, DAA, NMACR and N,N'MBA. It was found that only monomers of acrylamide series remained insoluble in n-hexane. All other monomers were either partially or completely miscible with the dispersing phase particularly in particularly, in the presence of EDM. This solubility behaviour would result in unstable suspension of monomers in the continuous phase and would thus lead to unsuccessful bead polymerisation.

Polymerisation of HEMA with N,N'MBA, however, led to the production of beads. Due to the more hydrophilic character of N,N'MBA crosslinker than EDM, it probably reduced the tendency of the hydrophobic phase to extract HEMA from the aqueous phase. Incorporation of HEMA into ACR/N,N'MBA system also led to the synthesis of beaded polymer. This indicates that hydrogel copolymer and terpolymer beads containing HEMA and ACR/N,N'MBA can be synthesised with the inverse-suspension polymerisation in n-hexane provided the distribution co-efficient of HEMA in the comonomers and organic phase is favourable. Similarly, other monomers may be incorporated into ACR/N,N'MBA system to produce terpolymer beads.

The structure of the ACR/N,N'MBA copolymer bead matrix was affected by the type of solvent used. Polymerisation was found to proceed faster with water as the solvent than with ethanol. The beads synthesised with ethanol as the solvent were revealed by scanning electron microscopy to possess increased macroreticular character over beads prepared when water was used as the solvent. With ethanol the phase separation may have occured more easily leading to precipitation of the polymer. This type of polymerisation proceeds by the following mechanism (Barrett and Thomas, 1969). At the start of the process of polymerisation, monomer, initiator, and stabiliser are in homogeneous solution in the solvent. Initially polymer molecules formed by solution polymerisation aggregate in small groups with the stabiliser molecules to form stabilised particle nuclei of sub micron dimensions. The number of particle nuclei increases (and particle size decreases) with increasing stabiliser content. Once particles are formed they absorb monomer from the diluent phase. Within the particles the polymerisation takes place according to the law of bulk polymerisation (Barrett and Thomas, 1969).

5.4.2 Particle Size

The bead size of ACR/N,N'MBA copolymer beads obtained was in the range 2000 μm - 50 μm depending upon the type of stirrer used. Stirrers made of metal (type a, Appendix B) and polypropylene (type c, Appendix B) produced irregular particulate copolymer. These stirrers also had tendency to accumulate hydrogel on their surfaces which, at the end of polymerisation, was very difficult to remove. Anchor type glass stirrer (type b, Appendix B) in conjunction with a cylinderical resin flask provided superior stirrer action than the round bottom polymerisation vessel, and other stirrer types investigated.

The dependence of bead size upon the type of polymerisation vessel and rate of stirring for suspension polymerisation of methyl methacrylate have been investigated (Hopff et al., 1965). It was reported that the intensity of mixing as related to the diameter of the stirrer and its rate of rotation largely determined the droplet size before any polymerisation had occurred, and this in large part determined the final bead size. However, other paramters also affect bead size, such as surface tension, viscosities, and amount of suspending agent.

One general problem that was experienced in all experiments with inverse-suspenion polymerisation in n-hexane was the build up of gel on the wall of the polymerisation vessel. This was thought to be due to large temperature gradient between the reaction mixture and wall of the vessel, particularly in the upper part of the vessel. Such temperature difference was found difficult to eradicate because the upper part of vessel was constantly being cooled by the condensed solvent vapour whereas the lower part was constantly being heated by the water bath. The formation of gel in this way would reduce the yield of the polymer beads.

5.4.3 Equilibrium Water Content and Mechanical Strength

Crosslinked hydrogel beads of acrylamide series as listed in table 5.3 display a range of water content values. The EWC ranges from 52 percent in MACR/N,N'MBA copolymer to 80 percent in ACR/N,N'MBA copolymer. High hydrophilicity of poly(acrylamide) hydrogel is an important property which makes this hydrogel one of the most biocompatible synthetic materials. In general, the swelling of non-ionic hydrogels has been reported to depend on the following factors:

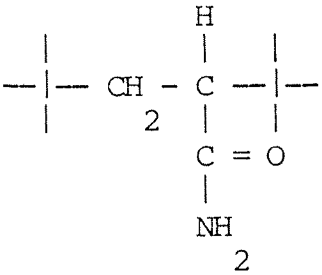
(a) the extent of crosslinking or the molecular weight of the polymer between crosslinks

(b) the interaction parameter between the polymer and solvent

(c) the relative swelling of the polymer at the time of crosslinking.

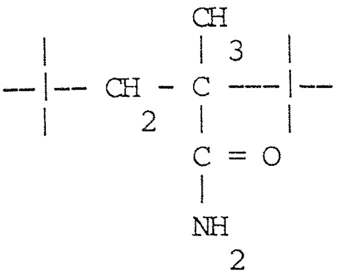
From table 5.3 it is indicated that as the percentage of the crosslinking agent in the monomer mixture increases, the EWC decreases. ACR/N,N'MBA copolymer, 100/6.4 molar ratio equilibrates with 69 percent of its weight of water, whereas the similar copolymer with 100/11.5 molar ratio absorbs only 57 percent of its weight of water. The extent of crosslinking can be an important factor in determining the water content, particularly, of high water content hydrogels such as poly(acrylamide). However, ACR/N,N'MBA copolymer with 100/10 molar ratio had an EWC of 80 percent, which is higher than the EWC (61 percent) of ACR/N,N'MBA copolymer with 100/9.2 molar ratio. This could be explained by the large solvent/monomer ratio used in the preparation of this copolymer. In this case a larger amount of water was present in the monomer solution than in other copolymers.

Replacement of ACR with MACR in MACR/N,N'MBA reduced the water content of the hydrogel. MACR/N,N'MBA copolymer's low water content may have resulted from two factors. Firstly, from low hydrophilicity resulting due to the introduction of a hydrphobic methyl group into the polymer chain, and secondly from decrease in the chain rotation caused by the methyl group. This is illustrated below by the structures of ACR and MACR:



$-[-CH_2-CH(C(=O)NH_2)-]-$

Acrylamide



$-[-CH_2-C(CH_3)(C(=O)NH_2)-]-$

Methacrylamide

With increase in crosslink density, the poly(acrylamide) beads were found to be progressively stronger, mechanically. This would be expected because with increase in crosslink density, the polymer becomes more compact and thus absorbs lesser amount of water. Since water content of the hydrogel is related to the mechanical strength, an increase in mechanical strength with decreasing water content was observed. The mechanical strength of the beads was also found to increase by the incorporation of HEMA and MACR in the copolymer. This would also be expected because HEMA and MACR are more hydrophobic monomers than ACR.

5.4.4 Scanning Electron Microscopy

Scanning electron microphotographs of the surface and interior of hydrogel bead samples show the diversity of structures that can be produced by the inverse-suspension polymerisation in n-hexane. SEM's in plates 5.3 and 5.17 show the spherical nature of the particles. Considering the SEM's on plates 5.3 to 5.12, it is observed that there is a progressive increase in porosity. This is because the samples which these microphotographs represent contained increasing amount of the crosslinking agent. SEM's in plates 5.11 and 5.12 reveal the sample to be highly porous compared with SEM's in plates 5.3 to 5.10. This demonstrates that porosity can be increased to a large degree by changing the solvent for the monomers. The porous structure is seen to be continuous from the surface to the interior of the bead (plates 5.16 and 5.20.)

In some cases, plates 5.3 and 5.17 for example, the presence of debris is observed on the surface of the beads. In the microphotograph in plate 5.17 small pieces of some kind of material seem to be attached to the surface. These act only to increase the surface rugosity. In some beads small particulates appear to be clinging to the surface which may be detachable. If the beads are to be used in haemoperfusion, such features are undesirable as they may lead to unwanted effects such as the production of micro-emboli by reacting with the blood components.

CHAPTER 6

ADSORPTION STUDIES

This chapter concentrates on the potential of hydrogel particulates, synthesised in this work, to adsorb certain selected chemical species used as markers of liver failure. The study also compares the effectiveness of hydrogel particulates as adsorbents in comparison with other materials such as ion-exchange and uncharged hydrophobic resins. On the following pages, first the phenomenon of adsorption will be introduced. Next the chemical species used as markers will be described. Results of adsorption studies will then be given. This will be followed by a discussion on the results obtained from adsorption studies.

6.1 ADSORPTION PHENOMENA

If a solid comes into contact with a gas or a liquid there is an accumulation of gas or liquid molecules at the interface; i.e. the densities of the gas or liquid at the interface are greater than their bulk densities. This phenomenon is known as adsorption and can be defined as the enrichment (i.e. positive adsorption) or depletion(i.e. negative adsorption) of one or more components in an interfacial layer. Adsorption is one of the phenomena that occur at interfaces as a result of the tendency of a system to attain the lowest state of free energy. The surfaces of solid substances, like liquids, also display a surface free energy, i.e., surface tension. However, unlike liquids solids can not reduce their surface area as the particles of solids do not undergo translational thermal movement. However, the free energy of the surfaces of solids is a most important factor in the adsorption of gases and liquids on solid adsorbents. The surface (or interfacial) free energy that arises from the residual attractive forces on the surface molecules is reduced by the interaction of these forces with those of the adsorbed molecules. Any process that tends to decrease the free surface energy of the system occurs spontaneously. Thus, it follows that all adsorption phenomena are spontaneous.

The phenomenon of adsorption was discovered more than two centuries ago. The uptake of gases by charcoal was first described by Scheele in 1773 and by Fontana in 1777. Lowitz in 1785 discovered that charcoal took the colouring matter out of solutions. The first systematic investigations were carried out by Saussure when he measured the adsorption of a variety of gases on several adsorbents (Brunauer, 1945). The term adsorption appears to have been introduced by Kayser in 1881 to connote the condensation of gases on free surfaces, in contradistinction to gaseous absorption where the molecules of the gas penetrate into the mass of the absorbing solid (Gregg and Sing, 1982).

In the majority of its practical applications the process of adsorption involves, in fact, the displacement of previously adsorbed molecules by others. For example, the adsorption of gases often involves the displacement of air by another gas, and adsorption from solution often involves the displacement of solvent molecules by a more strongly adsorbed solute. The occurrence of adsorption at the solid-gas interface is easy to perceive since it causes a decrease in the pressure of the gas. However, the effects of adsorption at the solid-liquid interface are less obvious because the concentration of molecules in the bulk of a liquid is relatively great so that the detection of this type of adsorption is more difficult. The present adsorption studies are concerned with adsorption at solid-liquid interfaces.

6.1.1 Adsorption Systems

Adsorption may occur in one of two ways, either physically or chemically. Thus, interactions between the solid adsorbent and the adsorbate molecules may range from a surface reaction, which is similar to a normal chemical reaction, to weak attractions similar to those responsible for the condensation of gases and vapours. It is therefore convenient to classify adsorption systems into chemisorption and physical adsorption on the basis of the interactions that are involved. The characteristic properties of these types of adsorption are listed in table 6.1 (Cooper and Gunn, 1972) in order to allow comparisons to be made. From the table it is clear that the two adsorptions are very different from each other; as different as ordinary condensation and chemical reaction.

Essentially, physical adsorption may be called surface condensation, chemisorption surface reaction.

Table 6.1

The characteristics of physical adsorption and chemisorption

	Physical adsorption	*Chemisorption*
1. Adsorption forces	Weak physical forces (van der Waals forces)* Heat of adsorption is usually <50 kJ/mole. May be regarded as a surface condensation	Involves transfer or sharing of electrons between adsorbent and adsorbed molecules. Heat of adsorption is usually about 60 to 420 kJ/mole. May be regarded as a surface reaction.
2. Specificity	Non-specific; i.e. will occur to some degree in any system.	Specific; i.e. only occurs when reaction is possible between adsorbent and adsorbate.
3. Reversibility	Reversible; i.e. adsorbate can be removed easily from surface in an unchanged form.	Often irreversible; i.e. adsorbate is removed with difficulty usually in a changed form: e.g. oxygen adsorbed by carbon is removed as carbon dioxide.
4. Effect of temperature	Process is exothermic; i.e. amount of adsorption decreases with rise in temperature.†	Surface reaction only proceeds above a certain temperature.‡ Reaction is usually exothermic.¶
5. Number of adsorbed layers	Monomolecular layer formed at low pressures followed by additional layers as pressure increases. Condensation of vapour in capillaries of porous solids may occur.	Restricted to formation of a monolayer.
6. Rate of adsorption	Usually rapid at all temperatures.	Usually proceeds at a finite rate which increases rapidly with rise in temperature.

* The appendix gives information on the various forces involved in physical adsorption.

† The exothermic nature of the process is indicated by the thermodynamic equation $\Delta G = \Delta H - T\Delta S$, where ΔG, ΔH, and ΔS are the changes in free energy, heat content, and entropy, respectively, that occur during a physicochemical process. For a spontaneous process such as adsorption ΔG must be negative; i.e. the process must lead to a decrease in the free energy of the system. In addition, the adsorbed molecules will have less freedom than those in the gaseous state; i.e. there is a decrease in the disorderly orientations of the gas molecules on adsorption and ΔS will be negative. Consequently, the term $-T\Delta S$, where T is the thermodynamic temperature, will be positive, and, therefore, from the above equation, H must be negative and the process is exothermic.

‡ Physical adsorption at low temperatures may give way to chemisorption at higher temperatures.

¶ It is often stated that all adsorptions are exothermic. However, some chemisorptions may be endothermic

6.1.2 Adsorption Equilibria

When an equilibrium adsorption occurs, there is a defined distribution between the solid and liquid phases, usually expressed by plotting the amount of solute adsorbed per unit weight of adsorbent against the concentration of solute in the solution, in equilibrium with the adsorbent. The resulting graph is familiarly known as the ''adsorption isotherm'' since all of the measurements are made at constant temperature. In the literature of the subject of adsorption there exists a wide variety of adsorption isotherms (Gregg and Sing, 1982). The majority of adsorption isotherms may be listed into three main types as suggested by Langmuir (1918), Brunauer, Emmett and Teller (1938), and by Freundlich (1926). These isotherms are shown in figure 6.1 (a), (b) and (c).

The Langmuir isotherm describes a situation where single layer adsorption occurs and makes the assumptions:

(a) Maximum adsorption occurs when there is a saturated monolayer of adsorbate molecules on the adsorbent surface.

(b) The heat of adsorption is constant.

(c) There is no migration of adsorbate molecules in the plane of the monolayer.

The equation for Langmuir adsorption takes the form

$$\frac{X}{M} = \frac{QbC}{(1 + bC)}$$

where X = amount of solute adsorbed.

M = amount of adsorbent used.

b = constant (related to the heat of adsorption).

C = concentration of solute in the solution in equilibrium.

Q = number of moles of solute adsorbed per unit weight of adsorbent in forming a complete monolayer.

This reduces at the extremes of adsorption, to two linear equations,

Low adsorption: $bC \ll 1$, $\frac{X}{M} = QbC$

High adsorption: $bC \gg 1$, $\frac{X}{M} = Q$

The Brunauer, Emmett and Teller (BET) isotherm describes a situation where adsorption occurs in multilayers, each layer described by a Langmuir equation. Also, that a given layer need not have completely formed prior to the start of another. The BET equation is as follows:

$$\frac{X}{M} = \frac{BCQ}{(Cs-C)1 + (B+1)(C/Cs)}$$

where B = Constant.

Cs = Saturation concentration of solute in solution.

This type of isotherm, is not widely used in liquid adsorption field, as the Langmuir and Freundlich equations.

The Freundlich isotherm has its origins in empiricism, although the relationship was later proved correct by kinetic theory. The equation for Freundlich isotherm takes the form:

$$\frac{X}{M} = K_f C^{1/n}$$

where n = Constant.

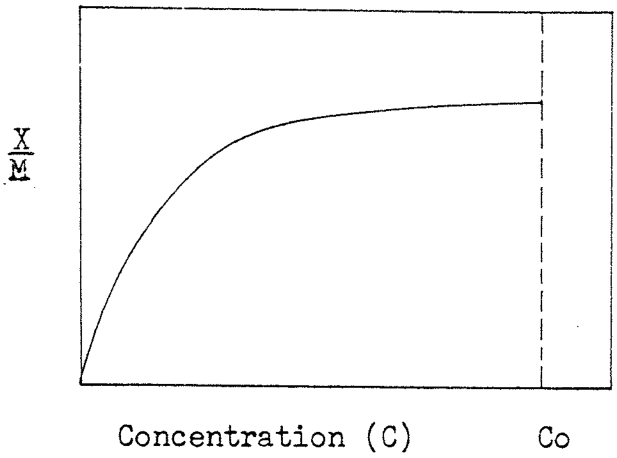
K_f = Constant.

Figure 6.1

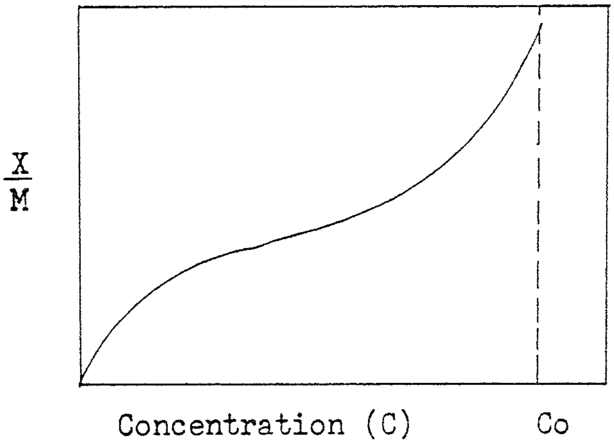
Adsorption isotherms

Co - Initial concentration

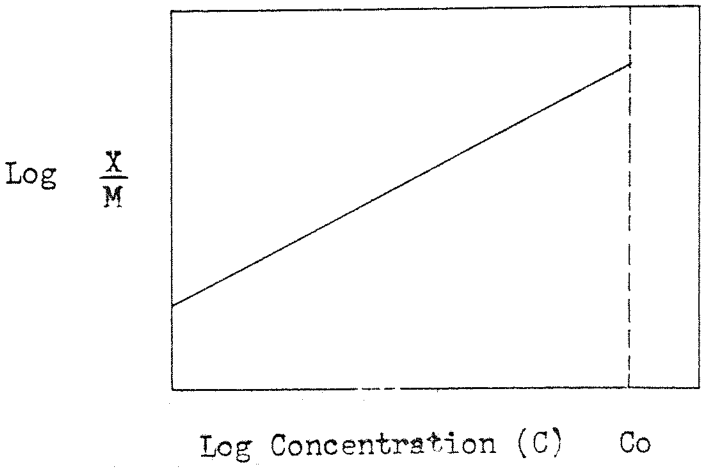
(a) Langmuir adsorption isotherm



(b) BET adsorption isotherm



(c) Freundlich adsorption isotherm



This may be fitted to a straight line logarithmic plot of,

$$\log_e \frac{X}{M} = \log_e K_f + \frac{1}{n} \log_e C_e$$

from this, the intercept gives the approximate value of the adsorption capacity, while the slope indicates the adsorption intensity.

Besides the three main types of isotherms described above there are borderline cases which, as in most classifications, are difficult to assign to one class rather than another. There are indeed a considerable number of isotherms which are difficult to fit into the classification at all.

The Langmuir, BET and Freundlich isotherms, although originally proposed for adsorptions at the solid-gas interface, have been used for adsorption from solution. Attempts have been made to find a universal equation applicable to all solution isotherms, a suitable one has not been developed due to the wide variety and complexity of the systems. Also as pointed out by Kipling (1965) 'many equations have proved inadequate because the role of the solvent has been neglected'. Also the adsorption of solutes from solution at the solid-liquid interface has been studied to a lesser extent than gas adsorption, although the phenomenon has been used practically for centuries; e.g. in the clarification of liquids. It is only in more recent years that the theories of adsorption from solution have become more developed and the importance of the solvent recognised.

6.2 POTENTIAL TOXINS AND MARKERS OF LIVER FAILURE USED IN THIS WORK TO STUDY ADSORPTION CHARACTERISTICS OF HYDROGEL PARTICULATES

It was stated in chapter 1 that several metabolites suspected of acting as toxins accumulate in the circulation of patients suffering from the syndrome of fulminant hepatic failure. The accumulation of metabolites is in part due to failure of excretion by the liver and may be responsible for some of the manifestations of the syndrome. These substances which are either water soluble or lipid soluble and therefore bound to protein in plasma, form useful markers for the evaluation of adsorbents for haemoperfusion.

In this work a range of chemical species with acidic functional groups were encompassed to study adsorption characteristics of hydrogel particulates. A complete list of the compounds used is given in table 6.2. Based on the type of acidic groups present, the marker molecules fall into three categories:
(i) that possessing sulphonic acid groups, i.e. bromosulphophthalein (BSP); (ii) those possessing carboxylic acid groups, i.e. bile acids and bilirubin; and (iii) those possessing a primary amino group in addition to a carboxylic acid group, the amino group being in the -position with respect to the carboxylic acid function, i.e. -amino acids. BSP, bilirubin and bile acids are weak acids. Amino acids are

dipolar ions and, thus, contain both a positive and a negative charge. Dipolar ions are also called zwitterions.

Table 6.2

Marker compounds used in adsorption studies

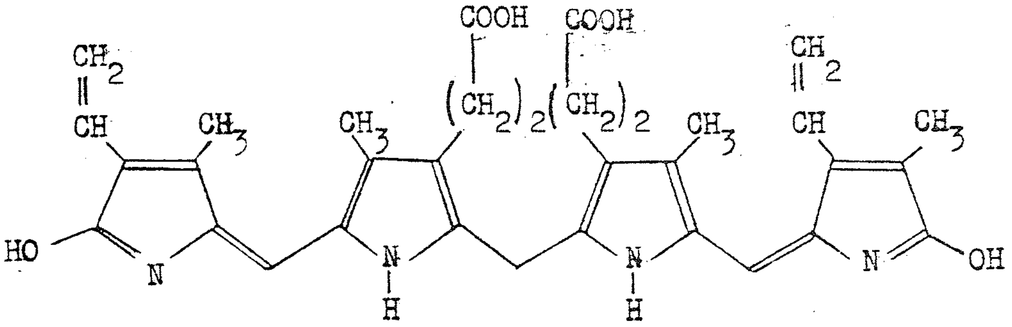
Chemical species	Molecular weight
Bromosulphophthalein, sodium salt	837.84
Bilirubin	584.70
Bile acids:	
Cholic acid, sodium salt	430.60
Deoxycholic acid, sodium salt	414.50
Amino acids:	
Phenylalanine	165.19
Methionine	149.21

Amino acids, phenylalanine and methionine, are examples of water soluble liver toxins. Bile acids, cholic acid and deoxycholic acid, are examples of liver toxins which are both water soluble and bound to plasma proteins. Bilirubin, on the other hand, is a lipid soluble toxin which is also bound to plasma proteins. Chemical structures of BSP, deoxycholic acid (sodium salt) and phenylalanine were illustrated in chapter 2 (figure 2.5). Structures of bilirubin, cholic acid (sodium salt) and methionine are shown in figure 6.2.

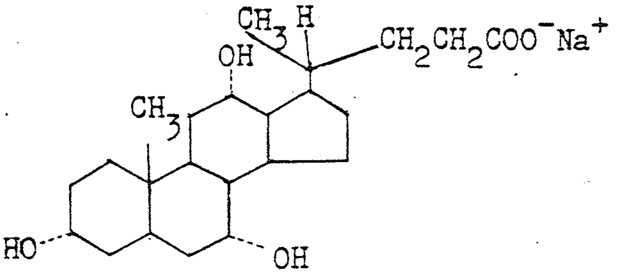
BSP is an organic dye in the form of a white powder and is used in medical laboratories as a test of liver function. The dye

Figure 6.2

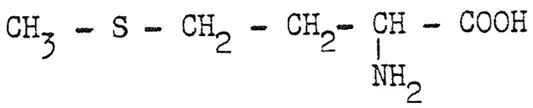
Structures of bilirubin, sodium cholate and methionine



BILIRUBIN



SODIUM CHOLATE



$CH_3 - S - CH_2 - CH_2 - \underset{NH_2}{\underset{|}{CH}} - COOH$

METHIONINE

is injected into the blood stream, is rapidly removed by the liver and excreted in the bile. A healthy liver removes the dye at a faster rate than a diseased liver. Consequently, the rate of removal can be used as a test of liver damage. In fact, its metabolism involves the main aspects of the hepato-biliary function namely sinusoidal uptake, conjugation and biliary excretion (Molino et al., 1983). BSP is a large molecule, similar in size to certain blood toxins. Previously it has been used in relation to ion-exchange and uncharged hydrophobic resins, and it is a stable compound. Bilirubin, bile acids and amino acids have previously been discussed as potential toxins in liver failure (chapter 1, section 1.5)

Adsorption studies with BSP, bile acids and amino acids were carried out by dissolving these compounds in normal saline. Adsorption of bilirubin was studied from chloroform. Saline was used as the solvent for water soluble chemical species as it eliminates certain complications which are faced when plasma is used as the solvent. The use of saline provides a simpler system by eliminating protein binding effects and partitioning of the solute which might occur between plasma and the hydrogel surface. Saline has essentially the same salt concentration and hence the same osmotic pressure as blood, and has previously been used in adsorption studies involving ion-exchange and uncharged resins. Bilirubin adsorption was studied from chloroform because the polymer adsorbent used in this case was in the dehydrated state (i.e. xerogel), and it was thought that present results could be compared with those reported in literature. Also, chloroform is a good solvent for bilirubin and enables its easy spectrophotometric determination to be carried out.

Since the chemical species used in adsorption studies are weak acids, the pH of the medium would determine the type of charge present on the species in solution. Therefore, the pH values of solutions of BSP, bile acids and amino acids in saline were determined. These were found to be as follows:

Compound	pH
Bromosulphophthalein	6.8
Cholic acid	6.7
Deoxycholic acid	6.5
Phenylalanine	5.8

The analytical methods used to assay the marker molecules were described in chapter 2.

In connection with adsorption studies it must be added that selected samples of hydrogel particulates synthesised in this work were used in a parallel research project to study the adsorptions of certain chemical species with basic functional groups (Caesar 1984). The chemical species used as markers of liver failure in that study included, amongst others, ammonia, and false neurotransmitters octopamine and phenylethanolamine. These nitrogenous substances were studied by using a Technicon autoanalyser in conjunction with the continuous flow apparatus. The continuous flow apparatus will be described in the following section.

6.3 TECHNIQUES USED TO STUDY ADSORPTION CHARACTERISTICS OF HYDROGEL PARTICULATES

Two different techniques were used for studying the adsorption of model toxins with hydrogels. The first method used was the conventional dye equilibrium adsorption isotherm technique. Most studies of adsorption of toxins from solution have been concerned with equilibrium conditions and predominantly with adsorption isotherm. In this research these studies looked at binding of the toxin to the adsorbent under both kinetic and equilibrium conditions. However, when adsorbents are used in columns in haemoperfusion, the dynamic aspect of adsorption is also important. Therefore, investigations were then extended to a continuous flow technique. These two approaches used for studying adsorptions will now be described.

6.3.1 Equilibrium Adsorption Isotherm Technique

This approach, as mentioned previously, is the most commonly used method for studying the removal of a solute from solution by an adsorbent. By using this static technique the affinity of hydrogel particulates for marker molecules was determined as follows. Adsorption of all toxins except bilirubin was studied with hydrated hydrogel particulates. To achieve this the surface water of particulates was first removed by wiping with a soft tissue paper. To study bilirubin adsorption, the particulates were first dehydrated in a vacuum oven at 60°C and adsorption with dried particulates was then studied. Aliquots of hydrogel particulates (usually 1 g) were weighed into sample bottles. A known volume of appropriate solution of the toxin (usually 10 ml) of known concentration was added to the bottle which was then tightly closed. The system was held at room temperature (either shaken or unshaken) until equilibrium was established. Bottles containing bilirubin solution were deoxygenated by filling with nitrogen atmosphere and then equilibrated at 4 °C in the dark (by wrapping in an aluminium foil) to prevent oxidation, thermal or photochemical degredation. The concentration of the solution used was usually 1 mmol/litre for BSP and deoxycholic acid, 0.5 mmol/litre for cholic acid, 2 mmol/litre for amino acids, and 0.2 mmol/litre for bilirubin. These concentrations correspond to the respective concentrations of these toxins which prevail in the blood in liver failure. Variations in concentration of toxins were made in some experiments and these have been indicated where applicable.

The amount of solute adsorbed was determined from changes in the concentration of the solution. The time taken for equilibrium to be attained was established by measuring the concentration of the solution at various time intervals. An important factor to be considered in relation to adsorption by hydrogels is the 'dilution effect'. Due to high water content of hydrogels the effective concentration of the system is diluted. Therefore, the actual concentration of the toxin in solution would be lower than the apparent concentration. The actual concentration was calculated using EWC of the hydrogel.

After equilibrium had been reached, the supernatant layer in the sample bottle was assayed for residual solute. From the solution concentration and equilibrium, and dilution by the water in the hydrogel, the amount of toxin bound was deduced at each resulting equilibrium concentration. In certain cases it was found appropriate to centrifuge the samples before assaying the supernatant layer.

Equilibrium adsorption isotherms were obtained for certain samples of hydrogel particulates. This was achieved by measuring adsorption at different concentrations of the solute by different weights of the adsorbent, and plotting amounts of the solute adsorbed against corresponding weights of the adsorbent.

The adsorptions of BSP and bilirubin by hydrogel particulates were compared with commercially available adsorbents, such as XAD-2, XAD-4, XAD-7 and Dowex 1x4, in order to obtain some idea about the effectiveness of hydrogels as adsorbents in comparison with the resin materials.

6.3.2 Dynamic Adsorption Technique

The second method used to study adsorption characteristics of hydrogel particulates was the continuous flow technique. Adsorption from solution is often carried out as a ''batch'' process, i.e. an adsorbent and solution are agitated together until equilibrium is established. In the practical use of adsorption, however, two advantages may accrue from allowing the solution to flow through a column of adsorbent. The first is that continuous operation may be maintained. The second is that a column can be very effective in the separation of a number of solutes present in the same solution or in the complete removal of a single solute from its solvent. Any preferential adsorption shown by the adsorbent can be exploited in a column which gives the effect of the summation of the separations achieved in many consecutive batch processes.

The dynamic adsorption technique was used as a model of haemoperfusion. If the weight of the adsorbent and the rate of flow of solution of the toxin used are the same as those used in haemoperfusion, the results from dynamic adsorption studies can be related to clinical situations.

The dynamic technique involved the passage of a solution of the toxin over a bed of hydrogel particulates, collection in an automatic fraction collector with subsequent analysis of the solution. To study the adsorption of toxins by using dynamic technique, the continuous flow apparatus was first set up. This apparatus was previously investigated by the author, to a limited extent, during an undergraduate project in the department of chemistry. The description of this apparatus will now be given.

6.3.3 The Continuous Flow Apparatus

The continuous flow apparatus shown in plate 6.1 and also illustrated diagramatically in figure 6.3 consisted of three parts: the reservoir into which the solution of the toxin was first added; the double walled flow cell (column) into which the adsorbent was placed; and the automatic fraction collector into which the liquid after passing through the adsorbent dropped at a known rate. The automatic fraction collector used was LKB Radirac type with the following specification:

Siphon stand type	3404 B
Tube rack type	3407 C
Tube rack holder type	3415 A
Rotator type	3401 B

The test tube rack of the fraction collector had four rows, each containing 60 test tubes. Therefore, 240 fractions could be collected automatically. The apparatus was set up by fitting the flow cell below the reservoir, and adjusting the siphon stand and the fraction collector according to instructions supplied with the instrument. Both the reservoir and the flow cell were fitted with stop cocks which enabled the rate of flow to be controlled. A piece of silicone tubing

Plate 6.1

The continuous flow apparatus

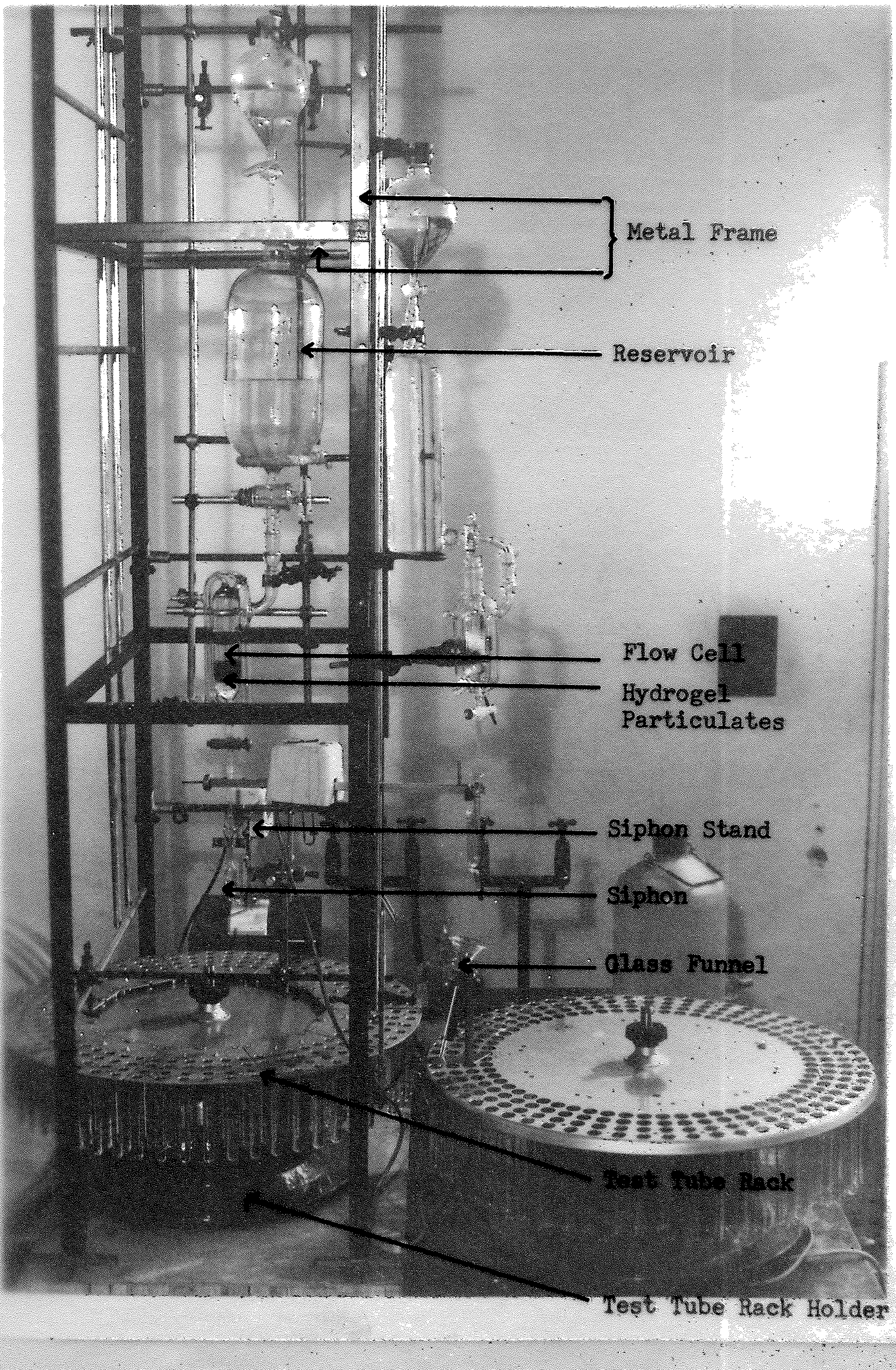


Plate 6.1

The continuous flow apparatus

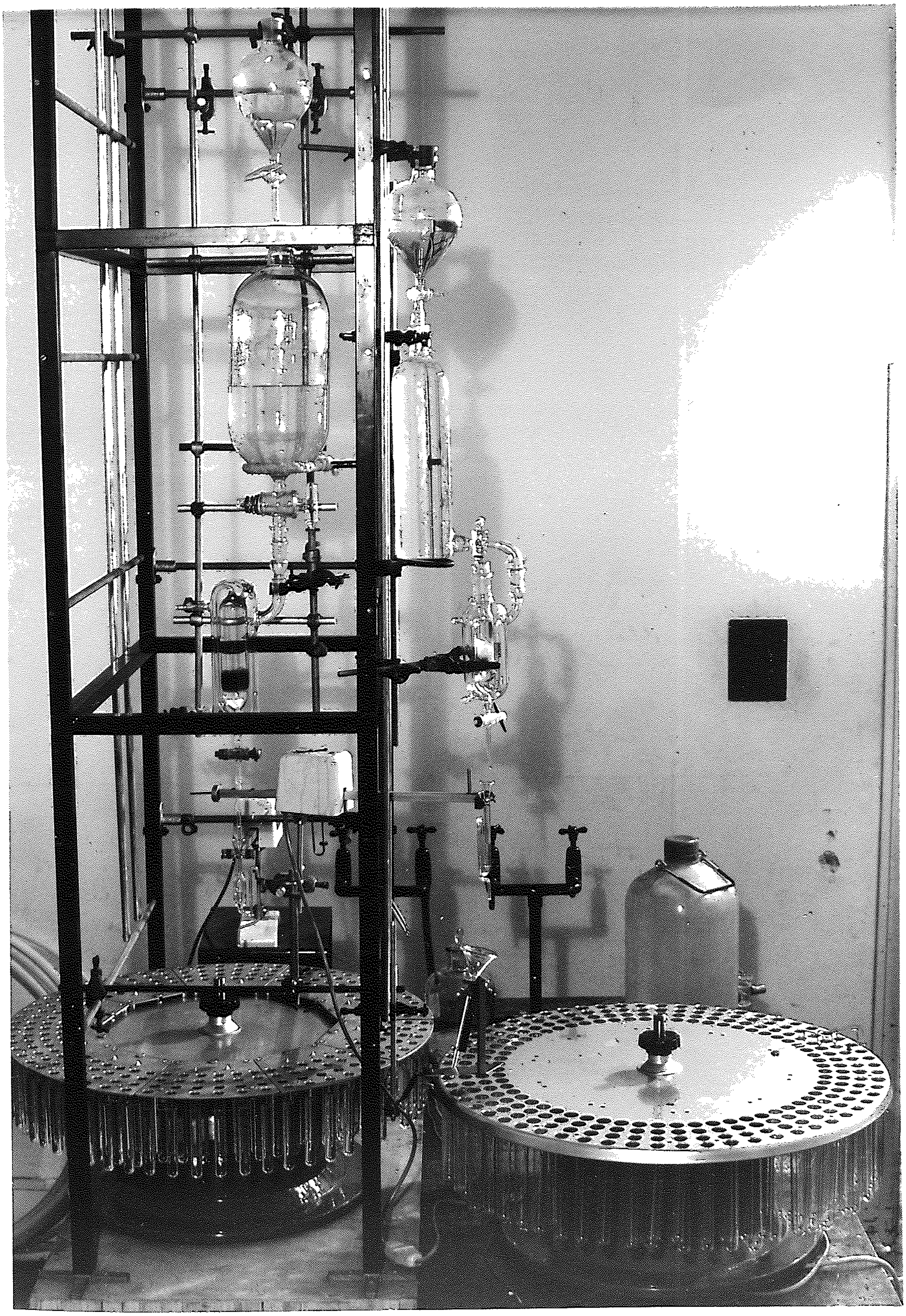
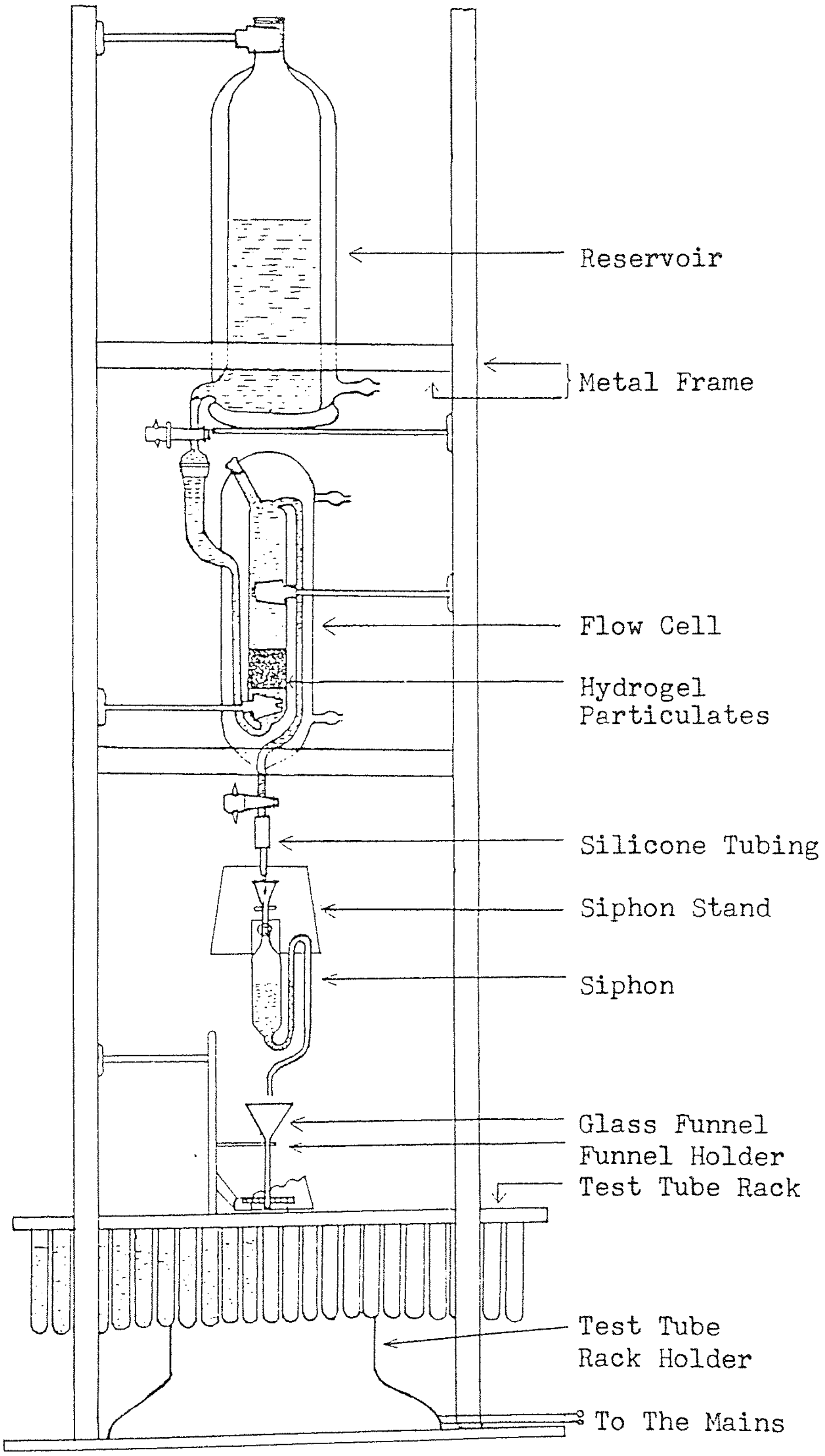


Figure 6.3

Diagram of the continuous flow apparatus



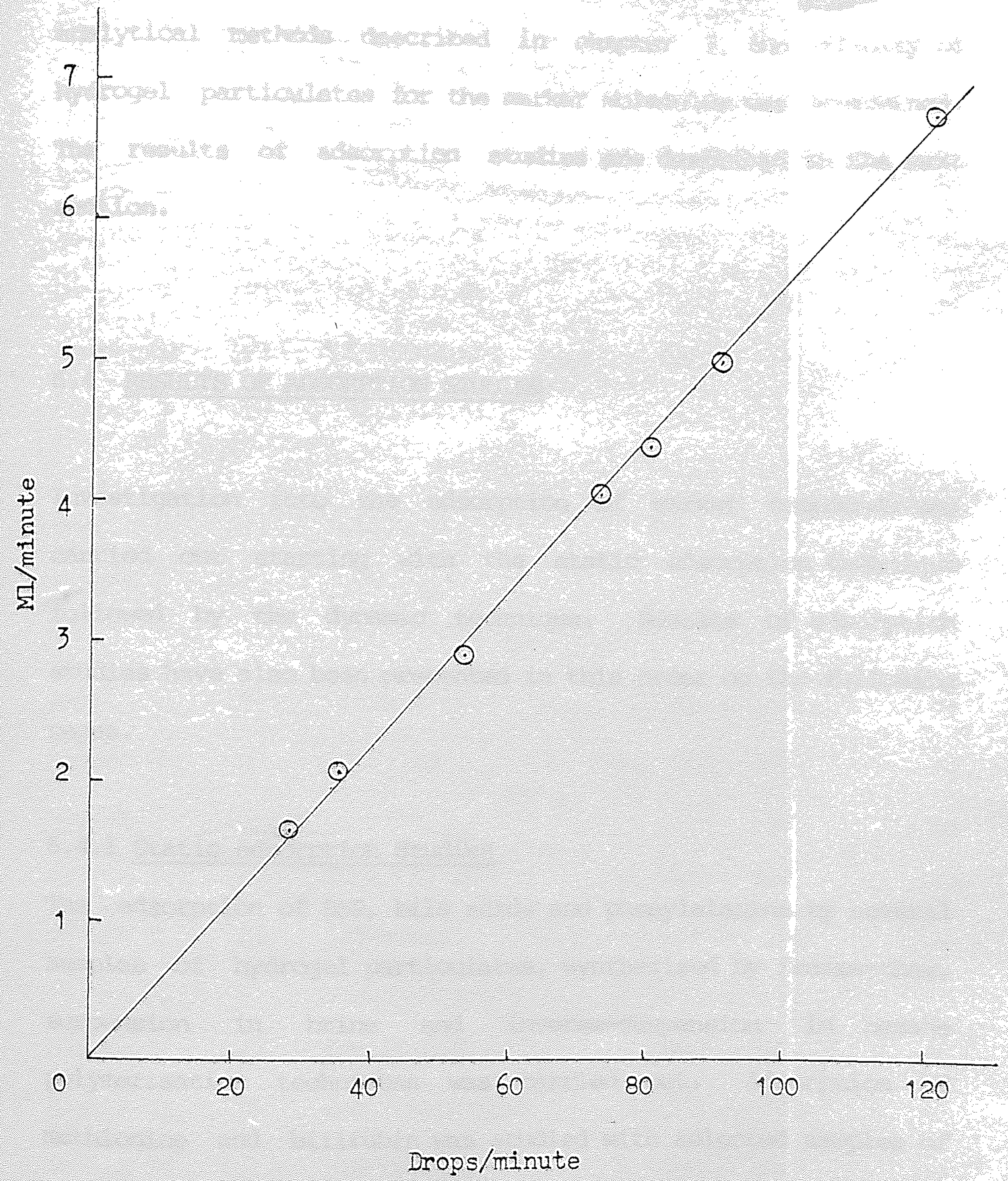
carrying a glass tube drawn out into a nozzle was connected to the outlet of the flow cell, which made the process of droplet formation more effective.

In the dynamic adsorption technique a known volume of the solution of a known concentration of the toxin in saline was allowed to flow through a known weight of the hydrogel adsorbent at a known rate. The weight of the adsorbent, the concentration of the toxin, and the rate of flow used were such that they mimicked the technique of clinical haemoperfusion. Figure 6.4 shows a graph of drops/minute plotted against ml/minute.

The method used was as follows. 20 g of hydrogel particulates, with their surface water removed with a soft tissue paper, were added to the flow cell. The solution of the toxin, usually 1 mmol/litre, was added to the reservoir. The appropriate stop cocks were opened and solution was allowed to flow through the flow cell. Particular attention was paid to remove any air-locks present in the flow system. The rate of flow was adjusted to be 90 drops/minute (300 ml/hour). After flowing through the cell, the solution accumulated in the siphon drop by drop. The siphon when full discharged liquid into the funnel which directed it to the sample tube. The process was continued until desired number of fractions had been collected (each fraction being 14.5 ml). Samples were then analysed for

Figure 6.4

Relationship between drops/minute and ml/minute for the continuous flow apparatus



any unadsorbed toxin. Results were expressed as adsorption isotherms by plotting the cumulative amount of the toxin adsorbed against the corresponding fraction number.

Using the adsorption techniques described above, and the analytical methods described in chapter 2, the affinity of hydrogel particulates for the marker molecules was determined. The results of adsorption studies are described in the next section.

6.4 RESULTS OF ADSORPTION STUDIES

Investigation into the adsorption of marker compounds was carried out starting with the static adsorption technique followed by the dynamic technique. Results of adsorption studies have also been presented in this order on the following pages.

6.4.1 Static Adsorption Studies

The adsorption of BSP, bile acids and phenylalanine by several samples of hydrogel particulates, synthesised by freeze-thaw, suspension in brine and inverse-suspension in hexane polymerisation techniques was carried out. Adsorption of methionine and bilirubin was studied with selected samples of hydrogel particulates only.

Table 6.3

Hydrogel particulates and resins used in static adsorption studies

Hydrogels:

Sample*		Composition (Initial Molar Ratio)	Particle Size (μm)	EWC (%)
Sample I	(F)	AA:EDM	1000 - 500	76
Sample 1	(S)	(100:8.5)	100 - 25	77
Sample III	(F)	AA:HEMA:EDM	1000 - 500	58
Sample 2	(S)	(60:40:8.5)	150 - 50	64
Sample IV	(F)	MAA:HEMA:EDM	1000 - 500	56
Sample 3	(S)	(50:50:4.6)	150 - 25	58
Sample V	(F)	HEMA:EDM	1000 - 500	51
Sample 4	(S)	(100:10)	500 - 250	56
Sample VI	(F)	ACR:N,N'MBA	1000 - 500	80
Sample 6	(S)	(100:10)	80 - 20	88
Sample 3	(IS)		1000 - 500	80
Sample VII	(F)	HEMA:DAA:EDM	1000 - 500	53
Sample 8	(S)	(100:25:9)	150 - 25	50
Sample VIII	(F)	HEMA:NMACR:EDM	1000 - 500	59
Sample 9	(S)	(90:10:8.5)	1000 - 500	61
Sample IX	(F)	HEMA:DMAEMA:EDM	1000 - 500	62
Sample 10	(S)	(90:10:8.5)	1000 - 500	77

Table 6.3 continued

Resins:

Sample	Composition	Particle Size
Dowex 1x4	Polystyrene-divinyl benzene (substituted with quaternary ammonium ions)	20 - 50 Mesh
Amberlite XAD-2	Polystyrene-divinyl benzene	20 - 50 Mesh
Amberlite XAD-4	Polystyrene-divinyl benzene	20 - 50 Mesh
Amberlite XAD-7	Acrylic ester	20 - 50 Mesh

* Symbols in brackets indicate the synthetic technique:

(F) = Freeze-thaw polymerisation

(S) = Suspension polymerisation in brine

(IS) = Inverse suspension polymerisation in hexane

A complete list of samples used to study the adsorption of BSP, cholic acid, deoxycholic acid and phenylalanine is given in table 6.3. Sample numbers in the table refer to the samples synthesised as described in chapters 3, 4, and 5 (tables 3.2, 4.1 and 5.1). All static adsorption experiments were carried out in duplicate, and the mean of two separate determinations was taken.

Table 6.4

Adsorption of BSP with hydrogel particulates and resins by static adsortpion technique

Hydrogels:

Monomer Composition	BSP adsorbed* (μmol/g)		
	Freeze-thaw Polymerisation	Suspension in brine	Inverse Suspension in hexane
AA:EDM	3.39	2.66	
AA:HEMA:EDM	2.45	1.76	
MAA:HEMA:EDM	2.50	1.24	
HEMA:EDM	6.86	5.60	
ACR:N,N'MBA	3.98	6.17	4.09
HEMA:DAA:EDM	8.20	6.40	
HEMA:NMACR:EDM	6.52	6.17	
HEMA:DMAEMA:EDM	10.00	9.20	

Resins:

Resin	BSP Adsorbed* (μmol/g)
Polystyrene-divinyl benzene Copolymer, Dowex 1x4	10
Macroreticular, acrylic ester, XAD-7	9.94

* Adsorption by 1g of beads from 10 ml of 1 mmol/litre solution of BSP in saline.

6.4.1.1. Adsorption of BSP

The amounts of BSP adsorbed by various samples of hydrogel particulates are shown in table 6.4. The table also shows the amount of BSP adsorbed by two commercial materials; Dowex 1x4 and XAD-7 resins.

Figure 6.5 shows the adsorption of BSP as a function of time with HEMA/DMAEMA/EDM terpolymers, sample IX and sample 10, synthesised by freeze-thaw and suspension polymerisation in brine respectively. Equilibrium adsorption isotherms of BSP with the same two samples of terpolymers are shown in figure 6.6. Figure 6.7 shows equilibrium adsorption isotherms of BSP with HEMA/DAA/EDM terpolymer, sample VII and sample 8, synthesised by freeze-thaw and suspension polymerisation in brine respectively.

The adsorption of BSP as a function of weight of the hydrogel adsorbent was studied for two samples: HEMA/DMAEMA/EDM terpolymer (sample IX) synthesised by freeze-thaw, and HEMA/DAA/EDM terpolymer (sample 8) synthesised by suspension polymerisation in brine. Figure 6.8 shows graphs of the amount of BSP adsorbed as a function of weight of the adsorbent.

Figure 6.5

Adsorption of BSP as a function of time for HEMA/DMAEMA/EDM terpolymer beads synthesised by two different techniques

Adsorption by 1g of beads from 50 ml of 2.5 mmol/litre solution in saline:

⊡ Freeze-thaw polymerisation

⊙ Suspension polymerisation in brine

Particle size: 1000 - 500 µm

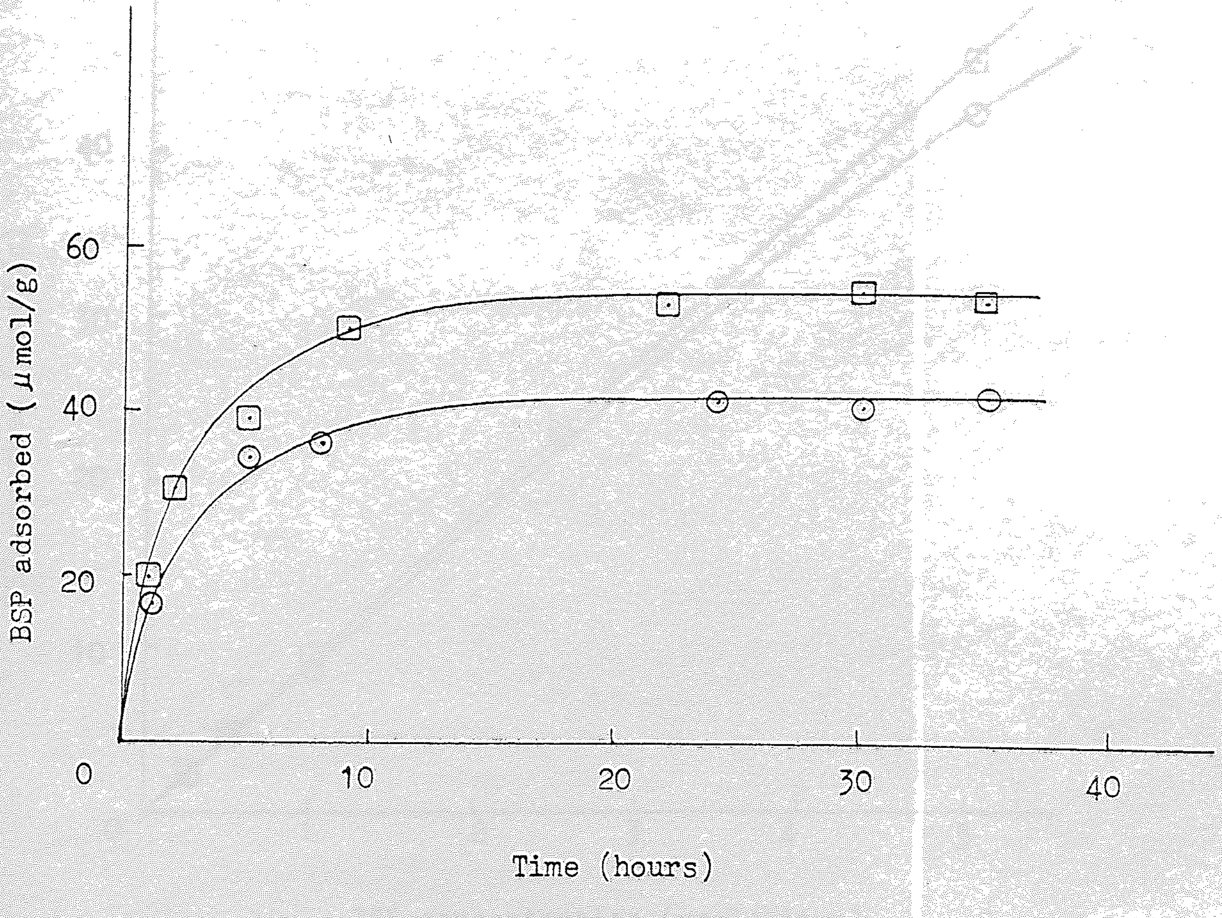


Figure 6.6

Equilibrium adsorption isotherms for BSP with HEMA/DMAEMA/EDM terpolymers

⊡ Freeze-thaw polymerisation

⊙ Suspension polymerisation in brine

Particle size: 500 - 250 μm

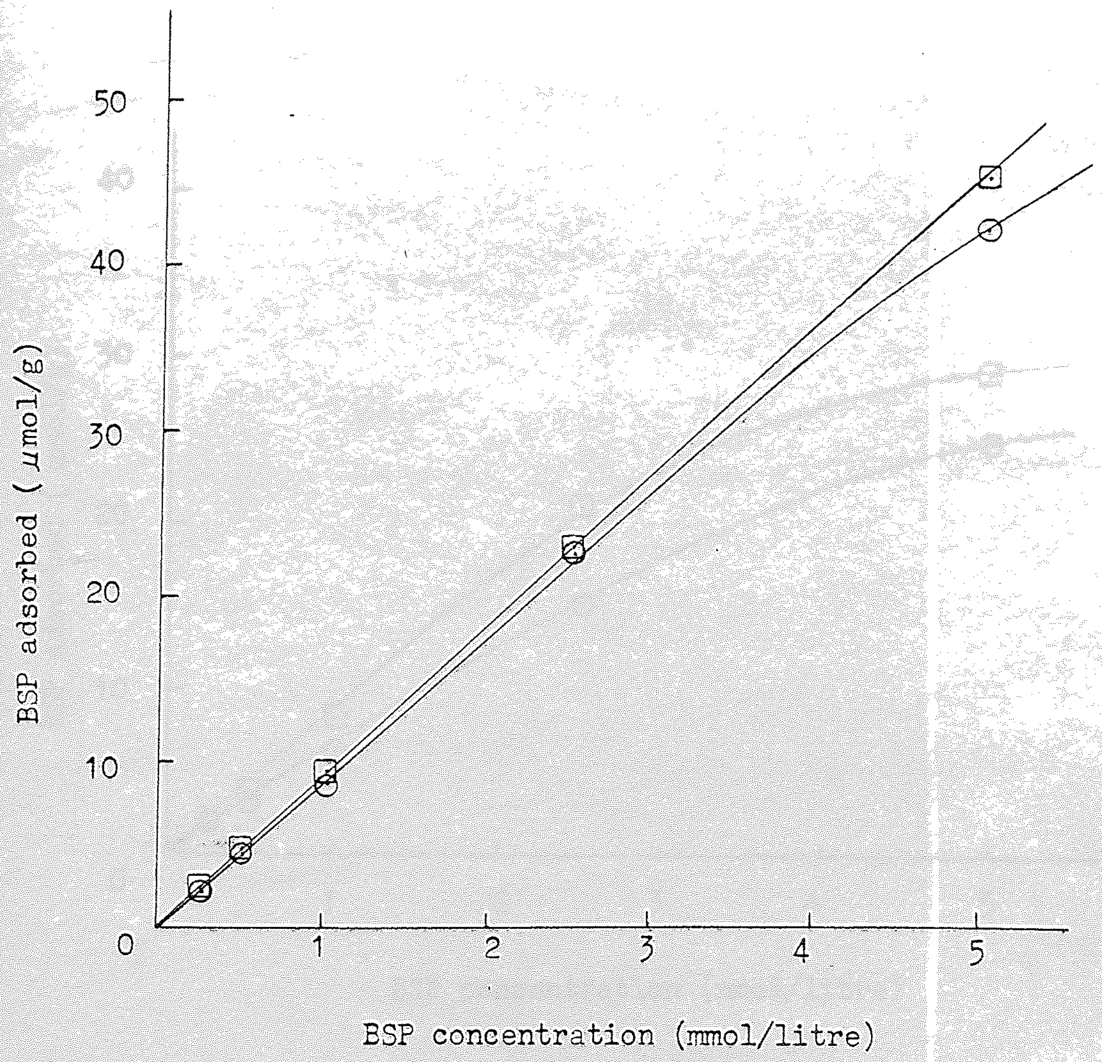


Figure 6.7

Equilibrium adsorption isotherms for BSP with HEMA/DAA/EDM terpolymer beads

⊡ Freeze-thaw polymerisation

⊙ Suspension polymerisation in brine

Particle size: 1000 - 500 μm

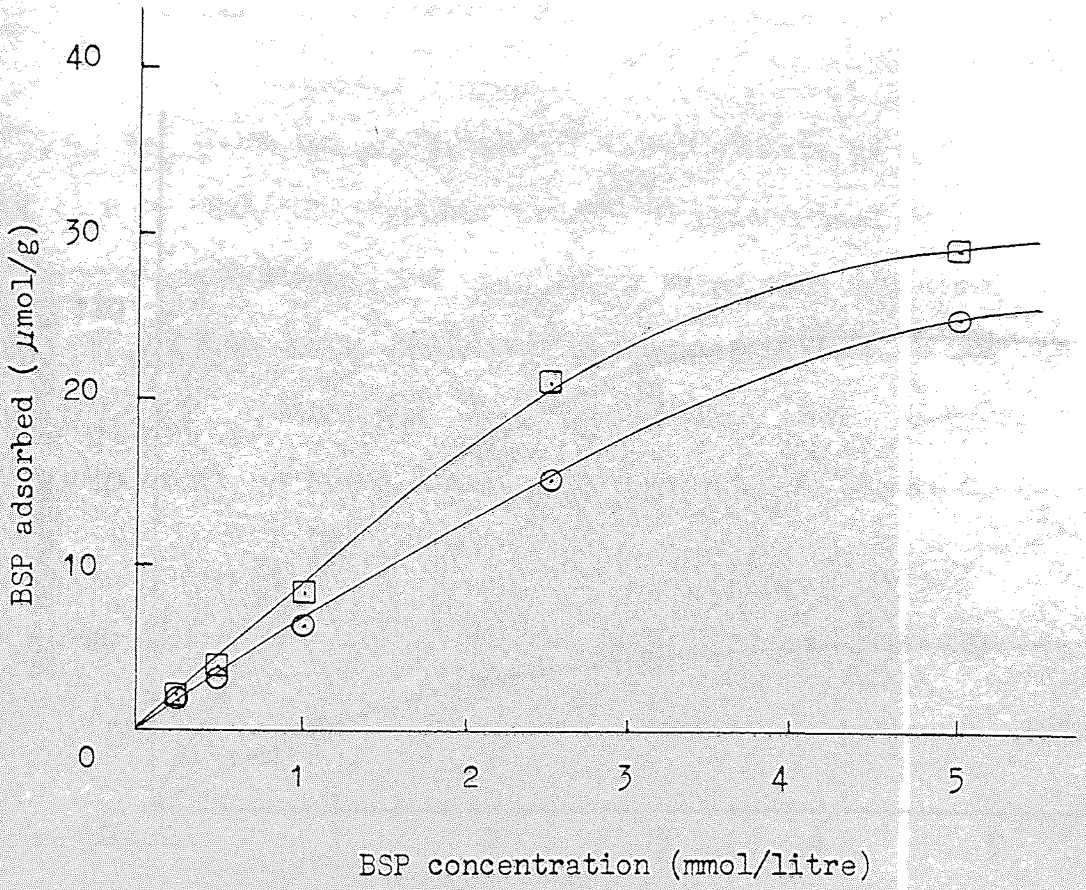
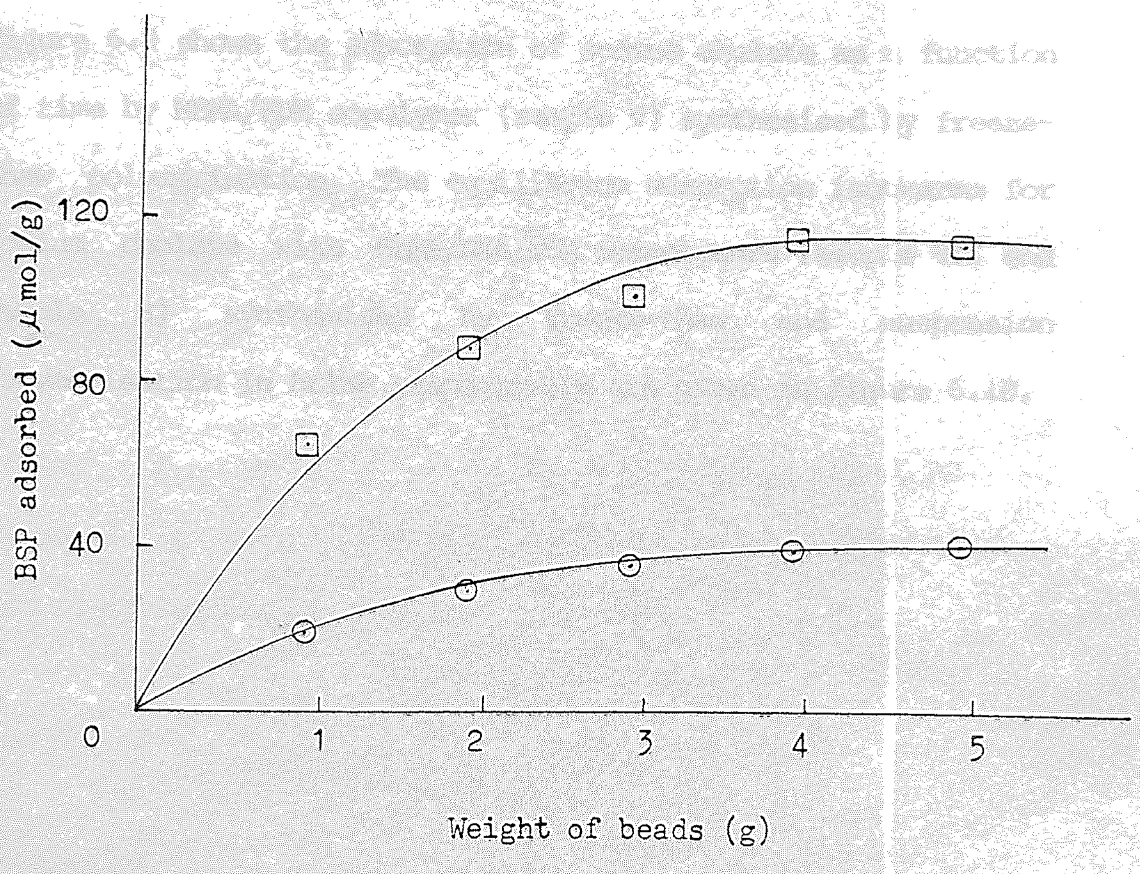


Figure 6.8

Adsorption of BSP as a function of weight for two samples of hydrogels synthesised by two different techniques

⊡ Adsorption by HEMA/DMAEMA/EDM terpolymer (Freeze-thaw polymerisation) from 50 ml of 2.5 mmol/litre BSP in saline

⊙ Adsorption by HEMA/DAA/EDM terpolymer (suspension polymerisation in brine) from 50 ml of 1 mmol/litre BSP in saline



6.4.1.2 Adsorption of Cholic Acid and Deoxycholic Acid

Adsorption of 0.5 mmol/litre solution of sodium cholate and 1 mmol/litre solution of sodium deoxycholate was studied by shaking the samples for 12 hours. All samples of hydrogel particulates listed in table 6.3 removed sodium cholate and sodium deoxycholate completely from solution. The amounts of sodium cholate and deoxycholate adsorbed by each sample of hydrogel beads were 5 μmol/g and 10 μmol/g respectively. Table 6.5 shows the amount of sodium cholate bound to hydrogel samples at the end of 6 hour and 12 hour periods.

Figure 6.9 shows the adsorption of sodium cholate as a function of time by HEMA/EDM copolymer (sample V) synthesised by freeze-thaw polymerisation. The equilibrium adsorption isotherms for sodium cholate with HEMA/DAA/EDM terpolymers (sample VII and sample 8) synthesised by freeze-thaw and suspension polymerisation in brine respectively are given in figure 6.10.

Table 6.5

Adsorption of cholic acid with hydrogel particulates by static adsorption technique

Monomer Composition	Cholic acid adsorbed (μmol/g)*			
	Freeze-thaw polymerisation		Suspension polymerisation in brine	
	(6 hour)	(12 hour)	(6 hour)	(12 hour)
AA:EDM	3.76	5	3.79	5
AA:HEMA:EDM	4.10	5	3.94	5
MAA:HEMA:EDM	3.85	5	3.90	5
HEMA:EDM	4	5	4	5
HEMA:DAA:EDM	4.30	5	4.50	5
HEMA:NMACR:EDM	4	5	4.40	5
HEMA:DMAEMA:EDM	4.95	5	4.90	5

* Adsorption by 1 g of beads from 10 ml of 500 μmol/litre solution of sodium cholate in saline. Adsorption at 12 hour period represents the maxiumum adsorption.

Figure 6.9

Adsorption of cholic acid with HEMA/EDM copolymer as a function of time

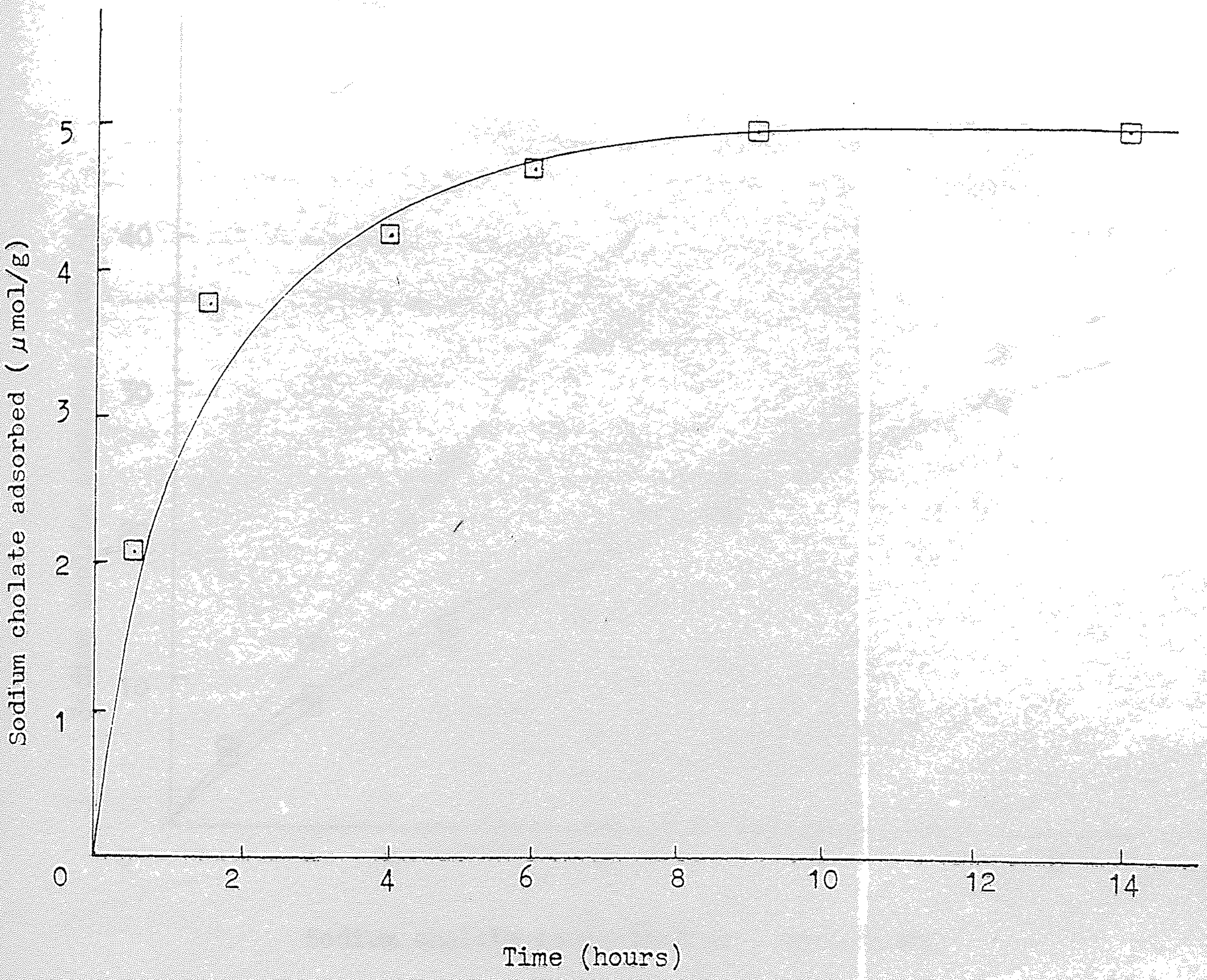


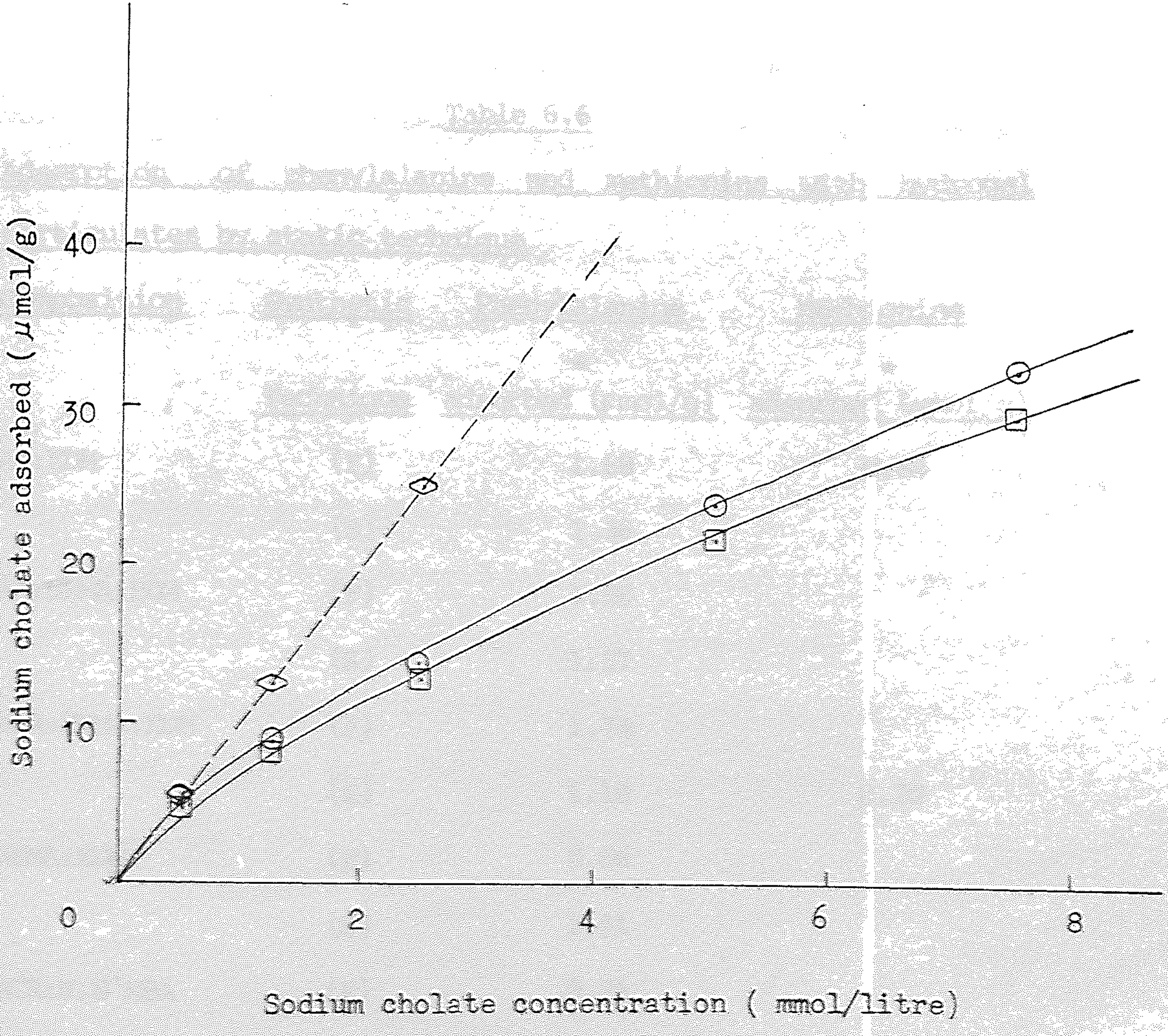
Figure 6.10

Equilibrium adsorption isotherms for cholic acid with HEMA/DAA/EDM terpolymers

⊡ Freeze-thaw polymerisation

⊙ Suspension polymerisation in brine

◇ Dotted line shows 100 percent adsorption



6.4.1.3 Adsorption of Phenylalanine and Methionine

Results of static adsorption studies of phenylalanine and methionine with hydrogel samples are tabulated in table 6.6. In this table the symbols F, S and IS refer to the synthetic techniques; freeze-thaw polymerisation, suspension polymerisation in brine and inverse suspension polymerisation in hexane respectively.

Table 6.6

Adsorption of phenylalanine and methionine with hydrogel particulates by static technique

Composition	Synthetic Technique	Phenylalanine * adsorbed (µmol/g)	Methionine * adsorbed (µmol/g)
AA:EDM	(F)	1.68	0.64
	(S)	1.36	
AA:HEMA:EDM	(F)	1.80	
	(S)	1.57	
MAA:HEMA:EDM	(F)	1.74	
	(S)	1.18	0.30
HEMA:EDM	(F)	0.66	
	(S)	1.41	
ACR:N,N'MBA	(F)	0.25	
	(S)	0.61	
	(IS)	0.79	
HEMA:DAA:EDM	(F)	1.81	
	(S)	1.57	
HEMA:NMACR:EDM	(F)	1.04	

* Adsorption by 1g of hydrogel beads from 10 ml of 0.2 mmol/litre solution of phenylalanine and methionine in saline.

Table 6.6 continued

Composition	Synthetic Technique	Phenylalanine * Adsorbed (μmol/g)	Methionine * Adsorbed (μmol/g)
HEMA:NMACR:EDM	(S)	1.27	
HEMA:DMAEMA:EDM	(F)	0.56	0.27
	(S)	0.59	

* Adsorption by 1g of hydrogel beads from 10 ml of 0.2 mmol/litre solution of phenylalanine and methionine in saline.

Symbols in brackets indicate the synthetic technique:

F = Freeze-thaw polymerisation

S = Suspension polymerisation in brine

IS = Inverse suspension polymerisation in hexane

6.4.1.4 Adsorption of Bilirubin

Amounts of bilirubin adsorbed by selected samples of hydrogels are tabulated in table 6.7. For comparison, bilirubin adsorption by three commercial adsorbents, i.e. XAD-2, XAD-4 and XAD-7 resins was also studied. The results of these adsorption are also included in table 6.7.

Table 6.7

Adsorption of bilirubin by hydrogel particulates and resin materials

Hydrogels:

Sample	Composition	Initial Molar Ratio	Bilirubin Adsorbed* (µmol/g)
Sample I	AA:EDM	100:8.5	0.40
Sample III	AA:HEMA:EDM	60:40:8.5	0.29
Sample V	HEMA:EDM	100:10	0.12

Resins:

XAD-2	Polystyrene-divinyl benzene	1.60
XAD-4	Polystyrene-divinyl benzene	1.46
XAD-7	Acrylic ester	1.48

*Equilibrium adsorption by 1 g of adsorbent particulates from 10 ml of 0.2 mmol/litre solution of bilirubin in chloroform.

6.4.2 Dynamic Adsorption Studies

Adsorption characteristics of hydrogel particulates using the continuous flow apparatus were studied for BSP and cholic acid. Results of these adsorptions are described on the following pages.

6.4.2.1 Adsorption of BSP

Table 6.8 shows the amount of BSP adsorbed by various samples of hydrogel particulates synthesised by three different polymerisation techniques.

Table 6.8

Adsorption of BSP with hydrogel particulates determined by dynamic adsorption studies

Monomer Composition (Initial Molar Ratio)	Synthetic Technique*	BSP adsorbed (μmol/20 g)
AA:EDM	(F)	360.9
(100:8.5)	(S)	112.8
AA:HEMA:EDM	(F)	55
(60:40:8.5)	(S)	66.3
MAA:HEMA:EDM	(F)	59.7
(50:50:4.6)	(S)	56
HEMA:EDM	(F)	389.8
(100:10)	(S)	213
ACR:N,N'MBA	(F)	59.6
(100:10)	(S)	127.3
	(IS)	78
HEMA:DAA:EDM	(F)	463
(100:25:9)	(S)	430
HEMA:NMACR:EDM	(F)	179.9
(90:10:8.5)	(S)	319.8
HEMA:DMAEMA:EDM	(F)	1077
(90:10:8.5)	(S)	847.2

* F = Freeze-thaw polymerisation, S = Suspension polymerisation in brine, IS = Inverse suspension polymerisation in hexane.

The graphs of cumulative amount of BSP adsorbed versus fraction number for all the samples tabulated in table 6.8 are given in Appendix (D). Apart from determining the extent of BSP adsorption by various samples of hydrogels, two additional sets of experiments were conducted. The intent of these experiments was: (i) to study the effect of varying the particle size on the rate of adsorption of BSP. This is shown in figure 6.11 which compares the adsorption of BSP by two different size fractions of the HEMA/DMAEMA/EDM terpolymer particulates synthesised by suspension polymerisation in brine. Two particle size ranges used were 1000-500 μm and 500-250 μm.
(ii) to study the effect of varying the weight of the adsorbent on BSP adsorption. Figure 6.12 shows the graphs of cumulative amount of BSP adsorbed by two different weights, 10 g and 20 g, of the HEMA/NMACR/EDM terpolymer particulates synthesised by freeze-thaw polymerisation.

The graphs of cumulative BSP adsorption shown in figures 6.11 and 6.12, and also in Appendix (D) follow a similar pattern. The graphs show a rapid initial increase in adsorption which then tends to reach a plateau. The initial rapid rise is taken as corresponding to the strong tendency of the surface of the adsorbent beads to bind BSP molecules, and the levelling off can be attributed to the tendency of the surface of the adsorbent to reach equilibrium value corresponding to the time

Figure 6.11

Cumulative amount of BSP adsorbed by HEMA:DMAEMA:EDM terpolymer beads (sample 10) of two different size fractions

⊡ 500 - 250μm size fraction

⊙ 1000 - 500μm size fraction

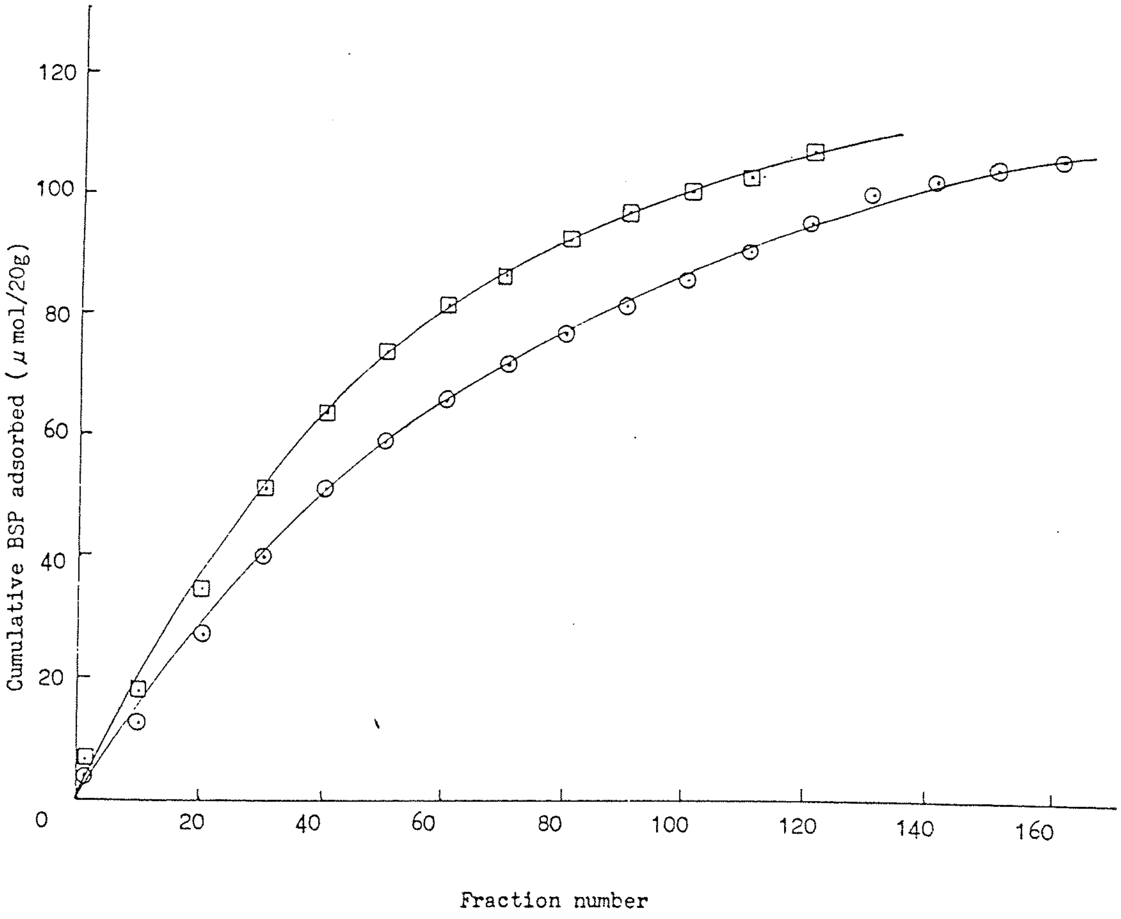
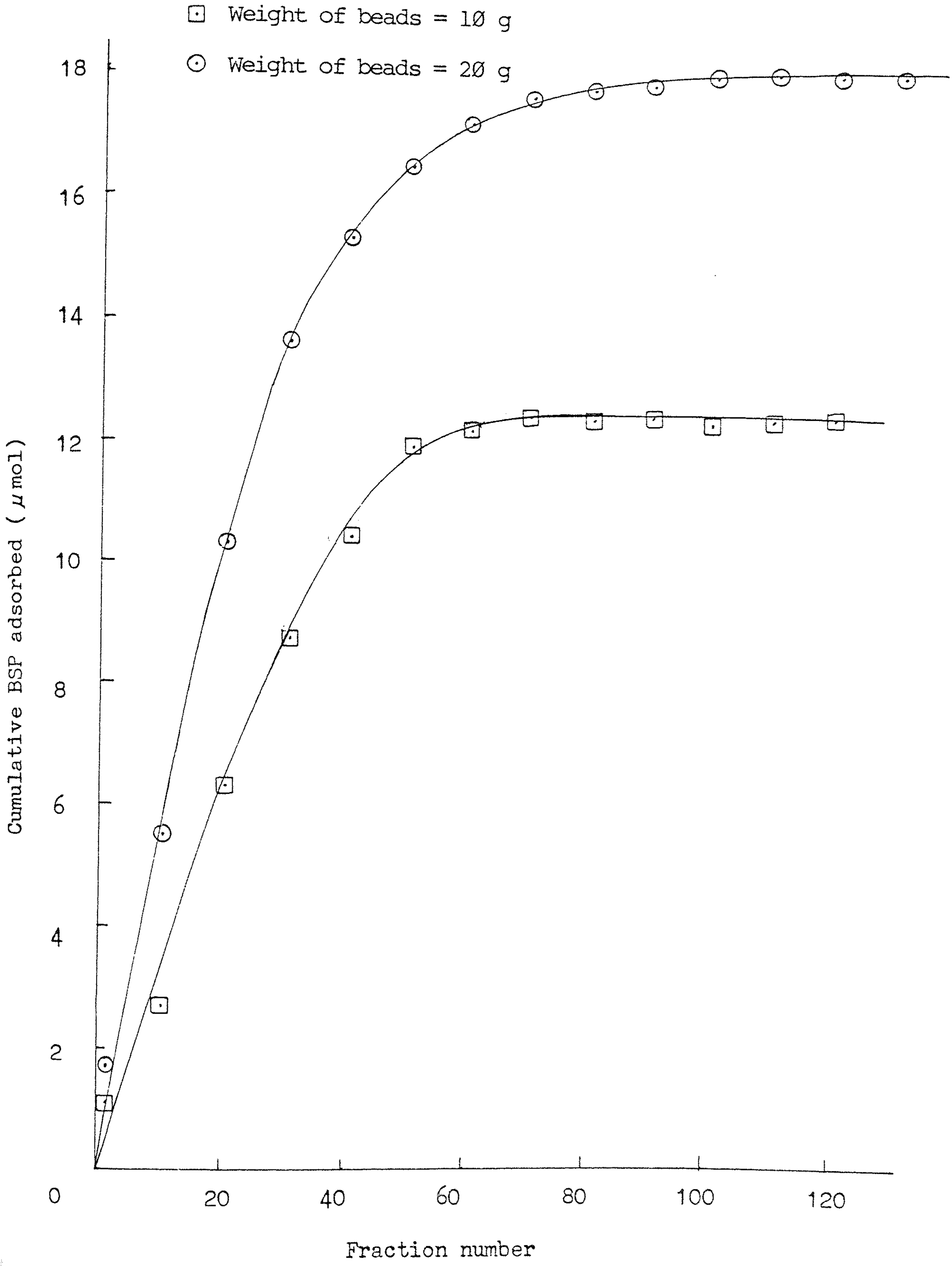


Figure 6.12

Cumulative amount of BSP adsorbed by two different weights of beads of HEMA:NMACR:EDM terpolymer (sample VIII)



when the surface tends to become saturated with BSP molecules. The amount of BSP adsorbed depends markedly upon the technique used to synthesise the hydrogel beads though no empirical relationship can be established.

6.4.2.2 Adsorption of Cholic Acid

Table 6.9 shows the extent of cholic acid removal by various samples of hydrogel beads. The amounts of cholic acid adsorbed were calculated from the graphs of cumulative amounts of the acid adsorbed versus the corresponding fraction numbers. The graphs of cumulative cholic acid adsorption are included in Appendix (E).

Table 6.9

Adsorption of cholic acid with hydrogel particulates determined by dynamic adsorption studies

Monomer Composition	Cholic Acid Adsorbed (μmol/20 g)		
	Freeze-thaw	Suspension	Inverse-suspension
AA:EDM	189.5	173.5	
AA:HEMA:EDM	342.9	706.2	
MAA:HEMA:EDM	2114	1818	
HEMA:EDM	433.7	172.5	
ACR:N,N'MBA		204.4	817.6
HEMA:DAA:EDM		1611	
HEMA:NMACR:EDM		709.8	
HEMA:DMAEMA:EDM	1404	1092	

6.5 DESORPTION EXPERIMENTS

It was thought that like adsorption, desorption of the adsorbed solute may be a useful guide to the strength of binding of the solute to the adsorbent. Therefore, a small number of experiments were conducted on selected samples of hydrogels to study the release of adsorbed species (BSP) by the solvent water. More important, these experiments were thought to give indication as to whether some solute remained on the surface of the adsorbent after the rate of desorption had fallen to zero (strictly, to a value too low to be detected).

The method used to study desorption by the static technique was as follows: At the end of the adsorption studies carried out as previously described, hydrogel beads (1 g) were removed from the solution and their surface was gently wiped with a soft tissue paper to remove excess supernatant. The beads were transfered to a clean sample bottle followed by the addition of 10 ml of distilled water. The contents of the sample bottle were mixed by shaking and allowed to re-equilibrate at room temperature. An aliquot of the supernatant was then analysed for the release of any BSP from the beads.

BSP desorption by the dynamic technique was studied as follows: After the completion of the adsorption experiment, hydrogel beads were taken out of the flow cell in order to remove excess supernatant from their surface, and to clean the flow cell.

The excess supernatant was removed by wiping the surface of beads with a soft tissue paper, and the flow cell was cleaned by washing with distilled water. Beads were then returned to the clean flow cell and distilled water was allowed to flow through the cell. Fractions collected in this manner were assayed for any released BSP, as previously described.

Table 6.10 lists the samples of hydrogel beads used in desorption experiments, and compares the amount of BSP adsorbed and desorbed by these samples. The samples used in desorption studies were synthesised by suspension polymerisation in brine.

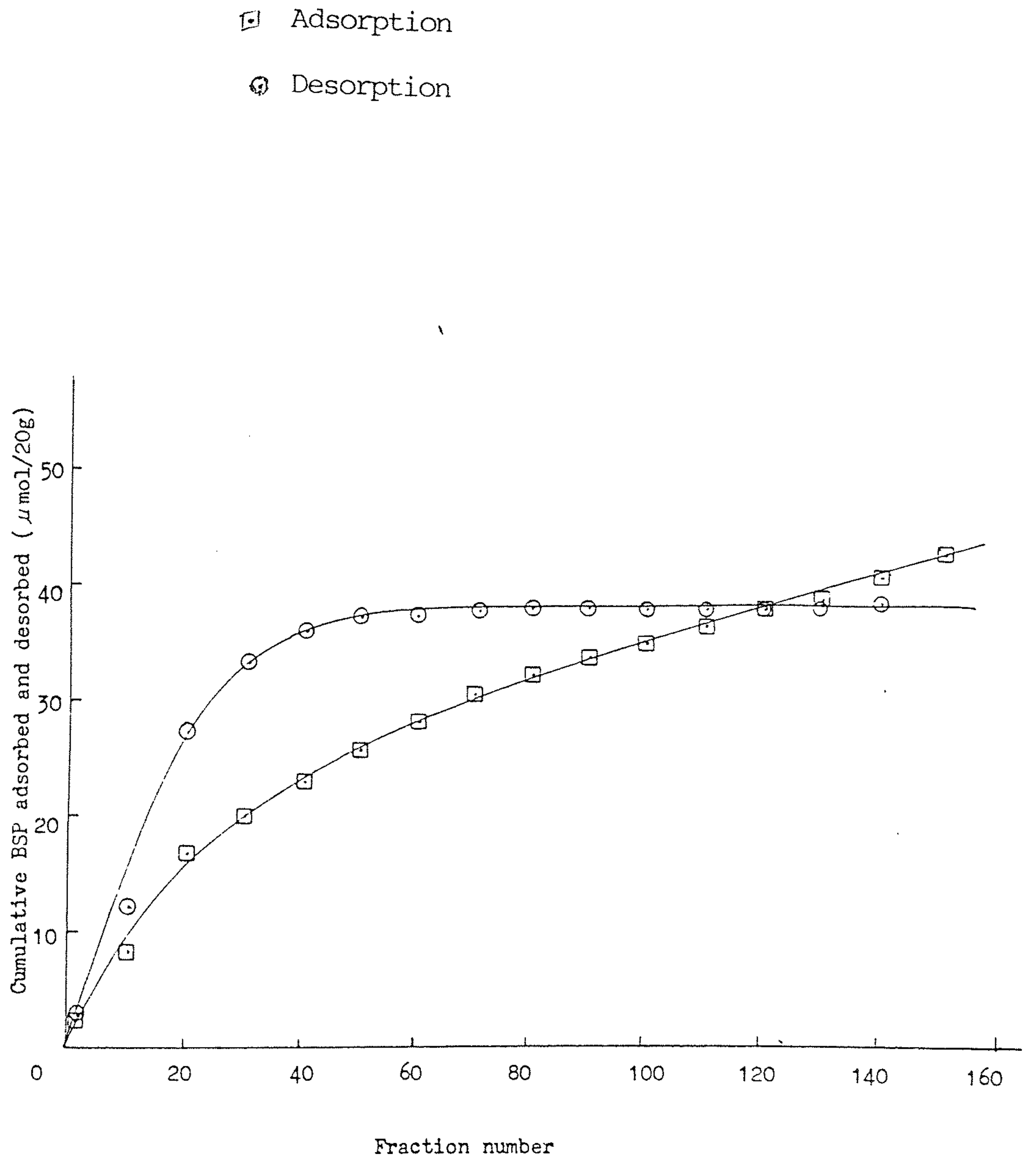
Table 6.10

Comparison of the BSP adsorption and desorption by hydrogel particulates, determined by static and dynamic techniques

Sample Number	Composition	Amount of BSP (μmol/g)	
		Adsorbed	Desorbed
Static Technique:			
D1 (=Sample 8, Table 6.3)	HEMA:DAA:EDM	6.4	5.5
D2 (=Sample 10, Table 6.3)	HEMA:DMAEMA:EDM	9.2	2.7
Dynamic Technique:			
D2 (=Sample 8, Table 6.3)	HEMA:DAA:EDM	21.5	19.2

Figure 6.13

Cumulative amount of BSP adsorbed and desorbed by the HEMA:DAA:EDM terpolymer synthesised by suspension polymerisation in brine



Graphs of cumulative amount of BSP adsorbed and desorbed (determined by dynamic technique) for the HEMA/DAA/EDM terpolymer (sample D2) are compared in figure 6.13. The amounts of BSP adsorbed and desorbed by 1 g of this sample as calculated from figure 6.13 are listed in table 6.9.

6.6 DISCUSSION

The chemical species used as markers of liver failure to study the adsorption characteristics of hydrogel particulates, synthesised in this work, were adsorbed to varying extent. A wide variation in equilibrium adsorption was displayed by different hydrogels towards the same chemical species, and by different chemical species towards the same sample of hydrogel particulates. Hydrogel adsorbent particulates used in adsorption studies were synthesised by three different techniques: freeze thaw polymerisation, suspension polymerisation in brine and inverse-suspension polymerisation in hexane.

The synthetic studies (chapters 3, 4 and 5) have demonstrated that there are several parameters which can be varied during the preparation of hydrogel particulates. Thus the chemical structure of the hydrogel, together with particle size, porosity, density and equilibrium water content can all be controlled by altering the polymerisation conditions and/or the synthetic technique. Before discussing in detail the individual hydrogel bead - liver toxin systems, the various factors which generally affect the adsorption of a solute from solution will be described.

6.6.1 Factors Influencing Adsorption From Solution

(i) Solute concentration: An increase in the concentration of the solute will cause an increase in the amount of adsorption that occurs at equilibrium until a limiting value is reached.

(ii) Temperature: It has previously been pointed out in table 6.1 that most adsorption processes are exothermic. Application of Le Chatelier's principle indicates that the amount of adsorption will decrease as the temperature rises.

(iii) Surface area of adsorbent: Since adsorption is a surface phenomenon, an increase in the surface area of the adsorbent for example by reducing the particle size or increasing the porosity, will lead to an increase in the amount of adsorption. The specific surface area is a quantitative characteristic of adsorption.

(iv) pH of the solution: In solutes that show incomplete dissociation into ions in solution, for example, weak acids a change in pH of the solution will affect the dissociation of the acid. Therefore, adsorption would be determined by whether the ionised or unionised species is the most strongly adsorbed.

(v) Adsorbent-solute interactions and solvent competition: The adsorption of a solute from dilute solutions involves breaking solute-solvent bonds and adsorbent-solvent bonds and the formation of adsorbent-solute bonds. The strength of the various interactions between the components of a system, i.e. adsorbent, solute and solvent, will depend on their mechanisms, which, in turn, depend on the structures of the components. It is quite possible that an adsorbent will have a strong affinity for a particular type of solute, which will therefore be adsorbed preferentially from a mixed solution. This gives rise to what is known as selective adsorption.

The solvent can influence the amount of adsorption in a variety of ways, the effects of which are the basis of the main difference between adsorption from solution and the adsorption of gases. For example, if the solvent is inert, i.e. has no appreciable affinity for the adsorbent or the solute, then the adsorption of the solute will be at a maximum. However, the amount of solute adsorbed will decrease if -

(a) the solvent has an affinity for the adsorbent, since there will be a competition between the solute and the solvent for the adsorbent surface. They may be adosrbed at the same or different sites on the surface. The first mechanism will involve a direct competition and the second may lead to a decrease in the number of sites available for solute adsorption because of the overlapping by solvent molecules adsorbed at adjacent sites;

(b) the solvent has an appreciable affinity for the solute, since there will be a competition between the solvent and the adsorbent for the solute. Thus, a change in solvent from one in which the solute is sparingly soluble to one in which it is appreciably soluble will lead to a decrease in the amount of adsorption; or

(c) a combination of the above two effects occurs.

(vi) Removal of adsorbed impurities: The removal of such impurities will increase the efficiency of the adsorbent. Adsorbents, the efficiencies of which are increased by these cleaning processes or by an increase in their surface area, are often termed activated adsorbents. Activated charcoal is a familiar example of this kind of adsorbent.

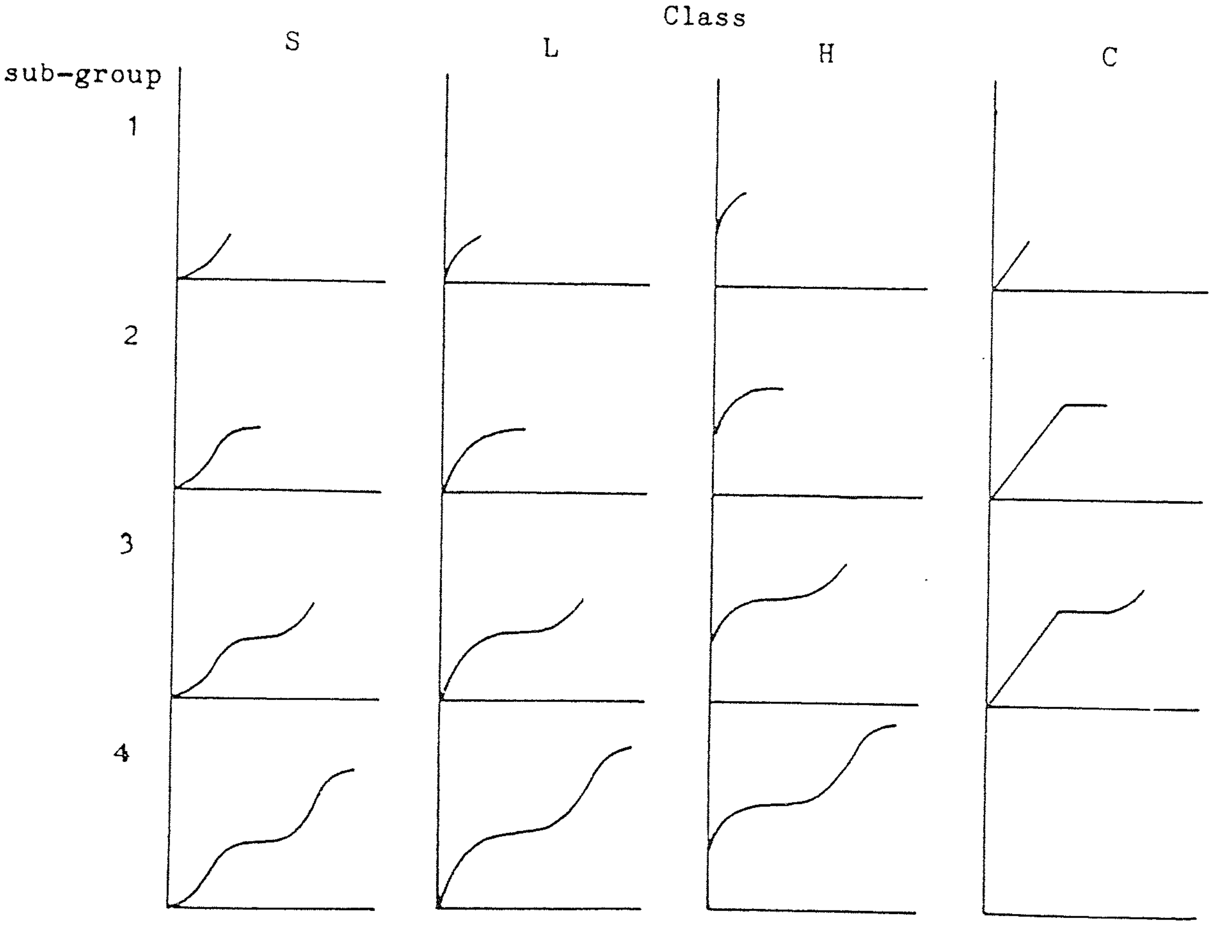
The various factors described above either individually or in combination can, therefore, affect the adsorption process from solution. Adsorption of individual chemical species will now be discussed.

6.6.2 BSP Adsorption

The results of BSP adsorption show that a great variety of adsorption isotherm shapes are possible with hydrogel particulates as adsorbents. Due to the wide variety and complexity of the systems involved in adsorption processes, it is difficult to draw an empirical relationship which may be applicable to all adsorption isotherms. However, the shapes of individual isotherms can be explained. Although the majority of the isotherms have the shape shown in figure 6.1 (a) several other shapes are observed. The isotherms for adsorption of organic solutes from soluion have been classified into four main groups depending on the shape of the initial part of the isotherm (Giles et al., 1960). Each of these groups is then sub-classified on the basis of the shape of the isotherm at higher concentrations. The classification is shown in figure 6.14.

Figure 6.14

Classification of isotherms for the adsorption of solutes from solution according to Giles et al., (1960)



Ordinates - Amount of solute adsorbed/per unit weight of adsorbent; Abscissae - Equilibrium concentration of solution

The initial curvature of the S curves indicates that adsorption becomes easier as the equilibrium concentration rises. This curve is characteristic of systems in which the solute molecule is monofunctional, i.e. the solute molecule has a single point of strong attachment onto the adsorbent, and there is an appreciable intermolecular interaction with in the adsorbed layer. These conditions usually result in the vertical orientation of adsorbed molecules.

The L curve is so-called because it is similar in shape to Langmuir isotherm shown in figure 6.1 (a).

The H or high affinity curve is characteristic of systems in which there is a strong interaction between the adsorbent and adsorbate, which results in complete removal of solute from solutions of low concentration.

The C curve represents a constant partition of the solute between the adsorbent and solution. This type of curve is given by solutes which penetrate into the solid more readily than does the solvent.

The adsorption isotherms (static technique) of BSP with HEMA/DMAEMA/EDM terpolymers (figure 6.6), and HEMA/DAA/EDM terpolymers (figure 6.7) can be explained in terms of L1 and L2 curves. Figures 6.6 and 6.7 show that the amount of adsorbed BSP increases as the concentration increases. However, in figure 6.6 the isotherm is linear upto a BSP concentration of 5 mmol/litre and only begins to reach a plateau at this concentration. However, in figure 6.7 the start of the plateau is observed at a BSP concentration of 2 mmol/litre. Similar graphs were obtained for the cumulative amount of BSP adsorbed by HEMA/DMAEMA/EDM terpolymer (figure 8D, Appendix D) and HEMA/DAA/EDM terpolymer (figure 6D, Appendix D) by dynamic studies. It has been reported that if the adsorbed solute molecules in the monolayer are so oriented that the new surface they present to the solution has low attraction for more solute molecules, the curve has a long plateau; if they are oriented so that the new surface has high attraction for more solute, the curve rises steadily and has no plateau (Giles et al., 1960). This indicates that BSP has higher affinity for the HEMA/DMAEMA/EDM terpolymer beads than the HEMA/DAA/EDM terpolymer beads since the isotherm for the former terpolymer remains linear until high BSP concentration is reached.

Results of BSP adsorption from both static technique (table 6.4) and dynamic technique (table 6.8) show that hydrogel particulates with functional groups such as tertiary amino group in DMAEMA and amide group in ACR had greater affinity than hydrogel particulates with acidic functional groups such as carboxyl group in AA and MAA, for the disulphonic acid BSP. Hydrogel particulates with HEMA/EDM monomers only, showed adsorption similar to the basic functional group containing particulates. Very strong adsorption of BSP with

HEMA/DMAEMA/EDM terpolymer indicates that ionic interaction was the possible mechanism of adsorption by this sample. The mechanism of adsorption of BSP with other hydrogel samples seems to be governed by electrostatic forces of attraction. Since the forces of attraction between BSP molecules and various hydrogel adsorbent samples seem to differ, therefore,it follows that the strength of binding should also vary. That BSP molecules are held with varying strength by different hydrogel samples is demonstrated by desorption experiments. Results of desorption studies (table 6.10) demonstrate that whereas 86 percent (static technique) and 89 percent (dynamic technique) of the adsorbed BSP was released by HEMA/DAA/EDM particulates, only 29 percent (static technique) was released by HEMA/DMAEMA/EDM particulates. Thus, in addition to adsorbing greater amount of BSP, HEMA/DMAEMA/EDM particulates bind this solute with stronger forces than HEMA/DAA/EDM particulates. Therefore, adsorption isotherms of BSP with hydrogels may be interpreted in terms of physical adsorption and chemisorption. The adsorption forces which arise depend on the chemical properties of the hydrogel adsorbent surface (in addition to the chemical and physical properties of BSP molecules) and they represent the so-called "surface affinity". Surface affinity represents the potential of a given hydrogel bead - BSP system and thus varies among different systems.

Another important parameter determining the adsorption of BSP by hydrogel particulates is the synthetic technique used for synthesising the particulates. Results of both static adsorption (table 6.4) and dynamic adsorption (table 6.8) suggest that hydrogel particulates with a certain monomer composition and synthesised by different polymerisation techniques adsorb different amounts of BSP. The explanation for this lies in the different reaction conditions used in synthetic techniques. These reaction conditions resulted in variation in the structure of the polymer matrix formed. Therefore, the nature of the porous structure and hence available surface for adsorption differs among hydrogel particulates synthesised by different methods. Different porous structure would result in variation of activity of binding sites.

It is perhaps worth emphasising that amongst hydrogel adsorbents as a whole, a wide and continuous range of pore sizes were to be found, from macropores through mesopores to micropores. The structure and volume of these pores also affects the extent of adsorption. Macroporous hydrogel particulates possess pores with molecular dimensions in excess to the size of BSP molecules. Therefore, in the case of macroporous beads BSP molecules would diffuse into the pores with greater ease than they would into the pores of smaller dimensions. This will enhance the uniformity of flow, and the rate of attainment of adsorption equilibrium will increase.

Tables 6.4 and 6.8 show that hydrogel particulates synthesised by the freeze-thaw technique generally showed slightly better adsorption than those synthesised by suspension polymerisation in brine. However, for ACR/N,N'MBA copolymer beads the order is reversed. This can be explained by the inability of this composition to polymerise in macroporous form by the freeze-thaw technique. As discussed previously in chapter 3 this inability resulted from different freezing behaviour of the monomer/solvent mixture in n-hexane. On the other hand, the same composition polymerised by the suspension polymerisation techniques displayed macroreticular nature of the polymer matrix, which therefore, would be expected to have larger surface areas than the polymer beads synthesised by the freeze-thaw technique. ACR/N,N'MBA copolymer from suspension polymerisation in hexane showed BSP adsorption which is intermediate to the other two synthetic techniques.

The adsorption of BSP by hydrogels was also affected by the weight of the particulates used. Figure 6.8 showed the amount of BSP adsorbed by various weights of HEMA/DAA/EDM and HEMA/DMAEMA/EDM terpolymer samples, determined by static technique. As observed from these graphs the amount of BSP adsorbed did not rise linearly with increase in weight of the particulates. This may be explained by either the occurrence of insufficient concentration of BSP in solution as the weight of the adsorbent is increased, or due to some type of hindrance to access for BSP to the binding sites on the adsorbent.

Similarly, adsorption study carried out by dynamic technique (figure 6.12) on two different weights of HEMA/NMACR/EDM terpolymer shows that 10g of particulates adsorbed 124 μmol BSP and 20g adsorbed 180 μmol BSP. The adsorption of BSP was, however, found to be independent of the particle size of the adsorbent used. In figure 6.11 cumulative amounts of BSP adsorbed by two different particle sizes of HEMA/DMAEMA/EDM terpolymer, determined by dynamic technique is shown. Graphs show that the rate of binding was faster for smaller particles, but binding at equilibrium was similar for both particle sizes. Therefore, one way of increasing the rate of removal of metabolites is by reducing the particle size and hence increasing the contact surface area for a given weight of adsorbent.

Table 6.4 listed the adsorption of BSP by Dowex 1x4 and XAD-7 resins. It is observed that although all samples of hydrogels adsorbed BSP only HEMA/DMAEMA/EDM terpolymer beads were capable of adsorbing this chemical species in comparable amounts to the resin materials. The lower amounts of BSP adsorption by hydrogels in comparison with resins may be related to the differences in surface areas available for adsorption. The surface areas of hydrogel beads synthesised by the freeze-thaw technique and having similar compostions as used in the present work have previously been measured (Skelly, 1979) using a BET surface area apparatus available in the department. Surface area measurements were made on dehydrated beads and were reported to be in the region of 8-11 square metres per gram. This value of surface area is low compared with commercially available resins. For example, surface area of XAD-7 resin is reported to be 450 square metres per gram (Skelly, 1979). However, it must be added that the range of surface area for hydrogel samples quoted above only represents the surface area of the macropores and does not take into account the microporous nature of the polymer matrix which is lost upon dehydration of the hydrogel.

However, BSP adsorption by HEMA/DMAEMA/EDM beads in comparable amounts to the resins suggests that such hydrogel particulates may also have surface areas similar to resins.

Figure 6.5 showed the amount of BSP adsorbed as a function of time onto HEMA/DMAEMA/EDM terpolymers, by the static adsorption technique. The figure shows rapid initial adsorption until a limiting value is reached when the BSP adsorption-time curve reaches a plateau. Similar pattern of BSP adsorption with time is shown by the graphs obtaned from dynamic studies (Appendix D, figures 1-8D). However, the adsorption of a solute from solution takes place in several stages and any of these stages may influence the rate of adsorption process. These different stages involved in adsorption process will be discussed in a later section on adsorption kinetics.

6.6.3 Bile Acid Adsorption

Results of bile acid adsorption show that all hydrogel samples were capable of adsorbing sodium salts of both cholic acid and deoxycholic acid from saline. Complete removal of these chemical species by hydrogel particulates as determined by static adsorption technique indicates their strong binding to hydrogel beads. Dynamic adsorption studies carried out for cholic acid, however, show that different hydrogel samples had different affinities for cholic acid.

Adsorption of cholic acid with HEMA/EDM copolymer from freeze-thaw polymerisation studied as a function of time (figure 6.9) shows large initial adsorption which then reaches a plateau. The flattening of the curve corresponds to the exhaustion of solute from solution as cholic acid was completely adsorbed. The equilibrium adsorption isotherms (figure 6.10) for cholic acid with HEMA/DAA/EDM terpolymers synthesised by freeze-thaw and suspension polymerisation in brine have similar shapes, although the amount of cholic acid adsorbed at higher concentrations by the samples differs. Both adsorption isotherms can be classified as being L type (figre 6.14). The shape of the curves of these isotherms shows that the amount of adsorbed cholic acid increases as the concentration increases. The curves begin to reach a limiting value although complete plateau is not reached. However, higher concentration of the bile acid than used in the present work could lead to saturation.

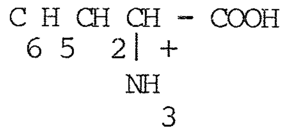
The amounts of cholic acid adsorbed by various samples of hydrogels as calculated from the cumulative graphs of dynamic studies are listed in table 6.9. Large variation in the amount of cholic acid adsorbed is observed amongst hydrogel samples. Samples with MAA, DAA and DMAEMA monomers in their composition show greater adsorption of the acid than other samples. Therefore, the presence of hydrophobic groups as in MAA and DAA, and positively charged groups such as tertiary amine group of DMAEMA seem to be important for the binding of cholic acid to hydrogels.

Although, a wide variation amongst hydrogels for the adsorption of cholic acid is observed, no definite trend can be established from the results obtained. This is because apart from the nature and structure of monomers, several other factors can affect the adsorption abilities of hydrogel particulates. Thus various parameters such as synthetic technique, particle size, porosity and water content which were discussed in relation to the adsorption of BSP would also influence the binding of cholic acid with hydrogels. The most important of these parameters , the specific surface area of particulates seems to play a major role in determining the adsorption of cholic acid too. The differences in surface areas among different hydrogel samples result from employing different polymerisation techniques which produce different porous structure in the adsorbents.

For comparison with commercial materials, it is difficult to find references in literatures for the adsorption of bile acids with resins from saline. Most of the existing data refer to adsorption from plasma. Horak (1981) studied in-vitro binding characteristics of uncharged resins for bile acids from plasma. Amounts of cholic acid adsorbed were reported to be 1200 μmol/20g for XAD-2, 3200 μmol/20g for XAD-4, and 800 μmol/20g for XAD-7. Similarly, Dunlop et al. (1978) reported adsorption of cholic acid from plasma to be 760 μmol/20g for XAD-2, 2800 μmol/20g for XAD-4, and 1500 μmol/20g for XAD-7. Present adsorption studies with cholic acid from saline show that concentrations of cholic acid (table 6.9) comparable to those adsorbed by commercial materials can be removed by certain samples of hydrogel particulates. However, it should be pointed out that there will always be differences in the adsorption process taking place when plasma is used and when saline is used as the solvent. These differences arise from differences in the polarity of the bile acid when present in plasma and in saline. Also in plasma there will be a tendency on the part of the bile acid to bind to the proteins. Therefore, the bile acid would tend to partition between plasma and the adsorbent. The pH of water soluble acidic species in saline is solely determined by the dissociation constant of the acid, whereas in plasma the pH would be governed by the natural buffers present in plasma.

6.6.4 Amino Acid Adsorption

Adsorption of amino acids phenylalanine and methionine was studied with static technique only. Results of these studies (table 6.6) underline the variation in phenylalanine adsorption caused by polymerising a certain monomer composition by different synthetic techniques. Various parameters (discussed previously) could influence the amino acid adsorption. However, pH of the solution was thought to have great effect in determining the extent of adsorption of the amino acids. This is because the pH of solutions of BSP and bile acids was approximately neutral whereas, the pH of phenylalanine solution in saline was determined to be 5.8. When a crystal of an amino acid (which is in the dipolar ionic form) is dissolved in water, the solution formed is not neutral. The ammonium ion portion of the amino acid can release a proton to the water, while the carboxylate ion portion can accept a proton from the water. However, since the pH of phenylalanine solution was 5.8 (slightly acidic) the phenylalanine would be in the following form and would be adsorbed in this ionic form:



$$C_6H_5CH_2\underset{NH_3^{+}}{\underset{|}{CH}}-COOH$$

In general for amino acids, Ka the acid dissociation constant measures the acidity of the ammonium ion, and Kb the base dissociation constant measures the basicity of the carboxylate ion. Thus, in amino acids, contrary to what one might expect at first glance, the acidic group is the ammonium ion and the basic group is the carboxylate ion. Table 6.6 shows that greater binding of phenylalanine and methionine took place with hydrogel samples possessing acidic functional groups, whereas lesser amounts of these acids were bound by samples containing basic functional groups. HEMA/DMAEMA/EDM terpolymers adsorbed least amount of amino acids. This may be explained by repulsion between the positive charges present both on the amino acid and the terpolymer. The hydrogen bonding between the carboxyl groups of hydrogels and amino acids could be responsible for greater binding of the amino acids to hydrogel samples containing acidic functional groups.

6.6.5 Bilirubin Adsorption

Adsorption of bilirubin by hydrogel particulates can be treated separately. Firstly, because bilirubin in comparison with other chemical species used in the present studies, is a lipid soluble toxin. Secondly, the solvent used for this lipid soluble or hydrophobic compound was chloroform. Thirdly, adsorption studies using bilirubin were carried out with dehydrated hydrogel particulates. Compared with other chemical species, bilirubin is an exception since it is not water soluble and is therefore neutral.

Results of bilirubin adsorption (table 6.7) with hydrogel particulates indicate that AA/EDM copolymer adsorbed the most bilirubin and HEMA/EDM copolymer the least. AA/HEMA/EDM terpolymer showed bilirubin binding intermediate to the other two samples. Thus, in the hydrogel series, bilirubin was strongly bound to the beads containing carboxylic acid groups and the binding varied with the acrylic acid content of the polymer. These results indicate that the binding of this toxin is determined by the interaction between the carboxylic acid groups present both on acrylic acid and bilirubin molecules. This suggests the mechanism of adsorption to be hydrogen bonding. In comparison, the commercially available adsorbents bound greater amounts of bilirubin than the hydrogel samples, the XAD-2 resin beads adsorbing the most. Greater binding of bilirubin by the resins may be related to the hydrophobic nature of the bilirubin molecules and the adsorbent. The lower binding of bilirubin to hydrogels than resins can be explained on the basis of hydrogels presenting more polar surfaces than resins to the bilirubin molecules.

The criterion for good adsorption has been reported to be polarisability of the molecule as distinct from the possession of a permanent dipole (Sing, 1973). Polarisability usually arises in an organic molecule by the possession of electrons as in double bonds, and is particularly high in alternating double bonds which, in turn, usually confer colour on the compound. As bilirubin has two sets of six alternating double bonds it should have a high polarisability, it is highly coloured and should be strongly adsorbed. However, lower binding of this chemical species by hydrogels suggests that the carboxylic acid groups of bilirubin contribute little to its adsorption with hydrogels. This is because being polar in nature carboxyl group can not dissociate into ions in non-polar solvent chloroform. Therefore, bilirubin molecules show no acidic or basic character when dissolved in chloroform.

It should be added that the adsorption studies of bilirubin were carried out on the dehydrated hydrogel (xerogel). Upon dehydration the porous structure of the particulates is altered and microporosity is destroyed. Therefore, the size of pores and the surface area of hydrogel beads available for the adsorption of bilirubin would also differ when the beads are in the hydrated and the dehydrated state. Therefore, when used in the dehydrated state the hydrogels may not exhibit their true adsorption ability. However, if the property of good biocompatibility of hydrogels is to be retained they should be used in the hydrated state. Therefore, the next step with regard to the adsorption of bilirubin with hydrogel particulates is to study their adsorption by hydrated bead samples from plasma.

In literature the adsorption studies of bilirubin with resins have been reported mainly from plasma, although adsorptions with charcoal have been studied by using chloroform as the solvent. As there will always be differences in polarity and other physical properties between plasma and chloroform, quantitative conclusions are not possible.

From the results of adsorption of various chemical species with hydrogel particulates it is indicated that these adsorbents have different affinities for different species. It was mentioned earlier in this chapter that the amount of solute adsorbed is dependent on several factors. The rate of attainment of equilibrium is also determined by various parameters. Figures 6.5 and 6.9 illustrated graphs of BSP adsorption by HEMA/DMAEMA/EDM terpolymer and cholic acid adsorption by HEMA/EDM terpolymer respectively, as a function of time, obtained from static adsorption technique. These figures and graphs of cumulative amount adsorbed for BSP (Appendix D) and cholic acid (Appendix E) illustrate that the time of attainment of equilibrium between the solute and the adsorbent varies among different solutes and different adsorbents. It is therefore, important, in the design of a column for haemoperfusion, to identify various stages involved in the adsorption process. Kinetics of adsorption is thus important and will now be discussed.

6.6.6 Kinetics of Adsorption

The kinetics of adsorption involves the prediction of the rate-limiting step, that is the step which most prevents the passage of a solute molecule to the active sites of the adsorbent particulates. The rate-limiting step is, hence, the most significant step, since it describes the step which controls the speed at which the reaction proceeds. Any of the following stages involved in the adsorption process could represent the limiting stage:

(a) Flow of solution brings the solute into the vicinity of the adsorbent

(b) It must then diffuse through the relatively stagnant layer of fluid adjacent to the adsorbent particle and then:

(c) be adsorbed on the external surface and simultaneously diffuse through the pores in the adsorbent itself to:

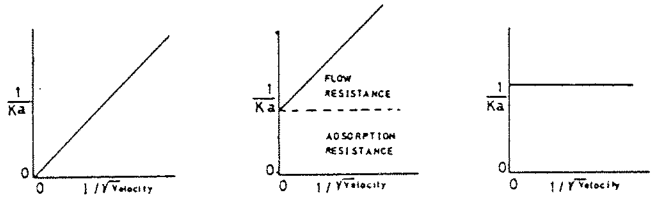
(d) the surface where most of the binding takes place.

A displaced substance, for example water, must undergo the reverse journey. Once an adsorbent has been selected the only step in the adsorption process that can be significantly altered by for example column design is step (b). It is thus appropriate to determine the significance of this step relative to all other steps combined. The thickness of this layer can be decreased by increasing the velocity of flow, and at sufficiently high velocity its contribution will become negligible. By measuring the removal of substances at various flow rates, an index of effectiveness can be calculated, the mass transfer coefficient. This is defined as the number of moles of substances removed per unit time per unit surface area for a unit concentration gradient.

The process of dynamic adsorption is, in fact, analogous to the technique of adsorption chromatography at the liquid-solid interface (Kipling 1965). For moderate flow rates, the distribution of solute on the column can be calculated by assuming that a condition close to equilibrium is maintained. For this purpose the column is considered in terms of a series of small elements and the equilibrium between solution and adsorbate for each element is calculated for successive small intervals of time in terms of mass transfer through the column (Kipling 1965).

Dunlop et al. (1975) reported the importance of boundary layer diffusion in relation to the adsorption of paracetamol and certain amino acids from plasma by charcoal, and the adsorption of bile acids and bilirubin from plasma by XAD-2 and XAD-7 resins. This is shown in figure 6.15. The figure illustrates that the removal of paracetamol and most amino acids (except tryptophan) from plasma by charcoal is almost entirely controlled by the thickness of the boundary layer. For the

Figure 6.15 (Dunlop et al., 1975)



Case 1

Process entirely flow contolled.

Case 2

Both flow and other resistances important.

Case 3

Flow unimportant.

Determination of importance of boundary layer diffusion as a rate-limiting step by extrapolation to infinite blood velocity: (i.e. velocity $^{-0\cdot5}$ *= 0). Ka is the mass transfer coefficient.*

(*a*) *extreme case where process entirely controlled by boundary layer diffusion. This state is approached for removal of paracetamol and most amino-acids (except tryptophan) from plasma by charcoal and bile acids from plasma by XAD-2 and XAD-7.*

(*b*) *both flow and other resistances (e.g. pore diffusion) important —found for bilirubin removal from plasma XAD-7.*

(*c*) *extreme case where boundary layer diffusion is unimportant, e.g. removal of tryptophan from plasma by charcoal.*

adsorption of tryptophan, on the other hand, steps other than thickness of the boundary layer round the particle are important. It may be relevant that tryptophan is the only amino acid that is significantly bound to protein. Adsorption of several bile acids by XAD-7 is also controlled by boundary layer. The thickness of boundary layer may also be applicable to the adsorption by hydrogel particulates of various solutes in the dynamic technique. These may be responsible for variation in the time taken by various solutes to attain equilibrium with hydrogel beads.

The overall results from adsorption studies show that hydrogel particulates are good adsorbents for certain water soluble chemical species possessing acidic functional groups, and which are found either free in plasma or bound to the plasma proteins. Hydrogels also showed some adsorption of a lipid soluble liver toxin, bilirubin. Adsorption studies demonstrate that the adsorption of acidic chemical species with hydrogels involves several types of forces which range from weak Van der Waals forces to strong ionic attachment. Although the interaction between liver toxins and hydrogel adsorbents can be modified and varied over a wide range by employing different synthetic techniques, the detailed adsorption behaviour between a certain toxin-adsorbent system may not be predictable. Nevertheless, the use of different synthetic techniques enables to control various parameters such as chemical composition, particle size distribution, porosity, water content which confer adsorptive properties on hydrogel particulates.

The pattern of adsorption from static and dynamic adsorption techniques was similar. The greater amounts of adsorption obtained in the dynamic technique can be related to the larger concentration of the solute passing continuously over the adsorbent bed. Thus, an indication to the adsorption behaviour of an adsorbent for a certain toxin can be obtained by the static technique. The continuous flow technique can then be used to determine the adsorption ability under dynamic conditions. This shows the usefulness of the dynamic technique which can simulate the technique of haemoperfusion.

This research project concentrated on investigating the adsorption of acidic hepatic toxins. However, the adsorbent materials synthesised in this work can be used as potential adsorbents for other toxic chemical species. In fact, the work carried out by Caesar (1984) in a parallel research project has demonstrated that hydrogel particulates are also useful adsorbents for the removal of certain Nitrogen-based hepatic toxins, for example ammonia and amines. Thus, toxic chemical species having molecular weights ranging from that of ammonia to bromosulphophthalein can be removed with hydrogel particulates. This wide range of adsorptive ability exhibited by hydrogels is made possible by the fact that various types of acidic, neutral and basic functional groups can be incorporated into the bead structure during the polymerisation. Thus, acidic monomers such as AA and MAA present in the initial composition lead to the synthesis of beads which are useful for the binding of basic chemical species. Basic monomers such as DAA, NMACR and DMAEMA present in the initial monomer composition result in the formation of beads which act as good adsorbents for the removal of acidic toxins. In this way cationic or anionic exchanging ability can be confered on hydrogels.

The commercial adsorbent materials for haemoperfusion which are currently available, for example, charcoal, ion-exchange resins and hydrophobic resins possess low biocompatibility and low-specificity for hepatic toxins. The property of inherent good compatibilty of hydrogels with blood and tissues, and the manner in which the structure of macroporous hydrogel particulates can be modified in much the same way as ion-exchange resins may provide alternative future potential adsorbents for haemoperfusion systems.

CHAPTER 7

ENZYMATIC DETOXIFICATION SYSTEMS

In the previous chapters various aspects of adsorbent haemoperfusion were considered. Biocompatible hydrogel particulates were synthesised, and their potential to adsorb certain toxic compounds was demonstrated. This chapter describes an alternative therapeutic approach for the treatment of intoxications. The approach described here involves the use of enzymes and exploits the specificity of enzymatic reactions for the removal of toxins by a route which is close to the physiological pathway.

In this chapter first the basic approach of enzymatic detoxification is introduced, and various enzymes that are involved in the detoxification of endogenous and exogenous toxins are described. Various techniques that are available for the immobilisation of enzymes have been discussed, and the immobilisation and characterisation of urease on hydrogel beads synthesised in the present research have been described.

7.1 INTRODUCTION

Enzymes are one of the most important and specialised class of proteins. They act as the essential catalyst of a wide number of chemical reactions linked to the intermediate metabolism of cells. The majority of the functions of the liver are catalysed by enzymes. Enzymes in the liver cell convert many lipophilic metabolites and toxins into more hydrophilic compounds allowing their excretion into bile and urine. Today we know a number of toxic compounds which accumulate in patients with hepatic failure and which inhibit liver regeneration and metabolism of liver and brain. A synergistic effect in the production of coma in the experimental animals has been demonstrated for many of them.

Intoxication is thus the prevailing symptom in hepatic failure and detoxification processes can not be supported or replaced adequately without dangerous side effects. Impairment of synthetic function of the liver, on the other hand, can be corrected to a certain extent by substitution of the products of synthesis which are commercially available in large numbers, although due to membrane barriers not all of them can be placed where they are needed. The management of intoxication by the approach of enzymatic detoxification is an obvious starting point in liver support.

Enzymes are usually administered in the form of a solution, where in nature, they usually act in an intracellular environment and therefore are stabilised and protected. However, the conventional use of enzymes in soluble form has its disadvantages which limit their therapeutic use in medicine (Sin Chong and Chang, 1977). These disadvantages are: instability and short duration of action in the body because the enzyme is rapidly removed as a foreign protein; possible allergic reactions; possible toxic contamination from the cell extract; and development of immunological reaction and the production of antibodies with consequent enzymatic inactivity. A better way of using the enzymatic activity would be the immobilisation of enzymes on solid supports thus circumventing the above mentioned limitations. Also a column packed with matrix-bound enzymes can be used repeatedly, and the product that emerges is uncontaminated. A further advantage is that enzymes have been reported in a number of cases to be more stable when they are bound to a matrix.

The interactions of highly specific enzymatic reactions are optimal in the environment and integrity of the intact liver cell. It has been demonstrated however that many enzymes retain excellent activities outside their natural environment and allow effective application in a purified and immobilised form, even in blood and serum as shown in-vitro and in-vivo. Efforts are therefore being directed towards developing immobilised enzyme preparations and devices which can be used in an extracorporeal liver support system. The development of a bioreactor (Chibata et al., 1977) in this way would, therefore, enable the use of the enzymatic activity with minimal collateral negative effects.

To carry out detoxification with immobilised enzymes several procedures are possible, but fundamentally, immobilised enzymes are placed in an enclosed system through which blood is allowed to circulate continuously to come in contct with immobilised enzymes before returning to the body. Thus, the recirculating blood would carry the substrates to the immobilised enzymes to be acted on by the enzymes. The first extracorporeal immobilised enzyme system tested in-vivo was in the form of a microencapsulated urease system to remove blood urea (Chang, 1966). Since this initial demonstration that microencapsulated enzymes can be used for detoxification, many other immobilised enzyme systems have been reported for haemoperfusion (Chang, 1972, 1977, 1980). More recently, artificial cells have been used successfully in haemoperfusions to lower tyrosine and phenols in hepatic coma rats (Shu and Chang, 1980, 1981). With the development of artificial cells containing multienzyme systems (Chang, 1977) especially the recent development of lipid-polymer membrane systems to retain substrates and cofactors (Yu and Chang, 1981), more complex metabolic haemoperfusion systems will be available on the horizon. One of the latest advances in this field has been the design of a lipophilic hollow fibre module which greatly facilitates long term enzymatic detoxification reactions of exogenous and endogenous toxins (Brunner et al., 1983). In this technique native enzymes together with their co-factors are circulated on the outside of the lipophilic fibre while the toxin containing media (blood or serum) are circulated inside the hollow fibres. Lipophilic toxins (phenols, fatty acids, mercaptans) penetrate the lipid membrane and are converted by the corresponding enzymes to hydrophilic compounds which then can not rediffuse into the blood. This technique offers the possibility of using highly active soluble enzyme preparations and a fully utilisable cofactor supply. The lipid membrane assures the absence of immunological hazards while mass transfer of the toxins is not impaired (Brunner et al., 1983).

Immobilised enzyme systems have been demonstrated to enhance the removal of drugs from poisoned experimental animals (Brunner and Loesgen, 1977). Further studies are required to determine whether it is possible to adapt these methods to the clinical situation, in particular whether it is possible to immobilise enzymes on biocompatible support materials. The present work explores the possibility of immobilising enzymes on biocompatible hydrogel beads synthesised specifically for haemoperfusion systems. This study investigates a new approach where an enzyme can be combined with hydrogel adsorbent to form a haemoperfusion system where both enzymatic functions and adsorbent functions can be carried out simultaneously. Here urease is used as a model enzyme for testing the feasibility of this approach. Various enzymes involved in detoxification reactions are now described.

7.2 IMPORTANT ENZYMES REQUIRING IMMOBILISATION

It is well known that the biological system responsible for detoxification of a variety of drugs and, in general, for xenobiotic compounds, is located in the smooth endoplasmic reticulum of hepatic cells and can be isolated as microsomes by cell fractionation. Microsomes contain a great variety of enzymes which modify toxins by increasing their polarity and consequently their solubility in water, thus decreasing their permeability into tissues and promoting excretion. Among these enzymatic biotransformations, oxidations, reductions, dealkylations and deesterifications are the most relevant reactions. Some enzymes responsible for these reactions are cytochrome-p-450, NADPH-cytochrome-p-450 reductase and epoxide hydratase (Guengerich et al., 1979).

A second group of enzymes, conjugative enzymes, convert hydroxylic compounds to their inactive forms, which are suitable for removal from the body by renal excretion. In conjugation, products are synthesised by the coupling of compounds with available endogenous substrates which are usually a carbohydrate (glucuronic acid) or a peptide (glutathione). Important enzymes involved in detoxification of endotoxins and exotoxins are described next.

7.2.1 UDP-glucuronyltransferase

This is a key enzyme in detoxification. 80 percent of exogenous drugs and toxins and a comparable proportion of endogenous substances are excreted from the body as glucuronide conjugates (Williams, 1959). The enzyme catalyses the transfer of the glucuronic residue from uridine diphospho-glucuronic acid (UDPGA) to endogenous and xenobiotic compounds such as bilirubin, amines, phenols, aromatic thiols, and other substances possessing suitable functional groups. The enzyme can be immobilised by various methods to be described in section 7.3. The first method applied was binding to agarose in beaded form (Brunner, 1975). Agarose beads have been used for in-vitro and in-vivo glucuronidation (Brunner, 1975). The potential use in hepatic failure apart from phenols includes the conjugation of amines, bile acids, and bilirubin. The activity of the enzyme immobilised in liquid lipid membranes is much higher be it on a weight or a protein basis (Halwachs et al., 1981). The enzyme has been solubilised and purified by various methods.

7.2.2 Alkane Thiol-Methyltransferase

This enzyme reaction was found during the search for a detoxification process of the powerful group of toxic mercaptans which accumulate in the liver failure patient (Holloway et al., 1979). The enzyme has been found to catalyse the methylation of alkane thiols by transfer of methyl group from S-adenosylmethionine to the sulph-hydryl group of the thiol. For example, methyl mercaptan is methylated by this enzyme, and dimethylsulphide is formed. It is known that dimethylsulphide is 14,000 times less effective in producing coma in experimental animals than methyl mercaptan (Zieve et al., 1974) and it is a far less potent inhibitor of important hepatic and cerebral enzymes. The possibility of immobilisation of the enzyme on Sepharose, with high retention of activity has been demonstrated (Tegtmeier et al., 1981). Efforts to examine the feasibility of the clinical application of this system are continuing. The enzyme is located in a membrane-bound state in the endoplasmic reticulum, but its removal from this environment by detergent solubilisation has already been successfully executed. The enzyme is extremely stable.

7.2.3 Glutathione Transferase (Ligandin)

Glutathione transferase catalyses another important conjugation reaction in the pathways of detoxification. Glutathione conjugates have the requisite physicochemcial properties of biliary excretion and, together with their catabolites, are present in the bile in relatively high concentrations. Their metabolites, the mercapturic acids are found in the urine.

In hepatic failure highly reactive intermediates of oxidation reactions, such as superoxides and peroxides, are formed in high concentrations. The detoxification of these highly reactive toxins by glutathione conjugation is of great importance. Routine measurements of these peroxides however do not exist. Investigations into the enzymatic process of glutathione conjugation are therefore at present carried out with nonphysiological substances. The enzyme can be prepared with no major difficulties from the cytosol of rabbit, rat, pig or human liver.

The enzyme has been immobilised, for example on hollow fibre reactors (Callegaro et al., 1981) with some success. Due to great affinity of the nonphysiological substrates towards the carrier material, the investigations with immobilised enzymes are made extremely difficult. A large number of different ligandins have been described and not until all of them have been investigated can a final judgement in this respect be made. Since glutathione is a bifunctional protein and apart from its enzymatic qualities is an important carrier protein for drugs and physiological metabolites in the liver, it is hoped that the immobilisation of ligandin can provide the clinician with a "specific adsorbent" for physiological substrates such as bilirubin and bile acids.

7.2.4 Sulphate Transferase

This enzyme is capable of conjugating sulphate to many of the acceptor substrates of glucuronidation, and it is well established that the organism can exchange sulphation for glucuronidation and vice versa with many substrates if one pathway is blocked or damaged (Williams, 1959). Experiments in laboratory animals have demonstrated that sulphation normally plays a subordinate role compared with glucuronidation. Sulphate transferase has been successfully immobilised on agarose (Anhalt et al., 1981) with a high retention activity. However, the enzyme has not been used much for detoxification purposes because the necessary co-factor adenosine 3'-phosphate 5'-phosphosulphate (PAPS) is extremely expensive and very unstable.

7.2.5 Cytochrome-p-450

The enzyme cytochrome-p-450 together with NADPH-cytochrome-p-450 reductase is available for hydroxylation reactions as it has been prepared in soluble stable form (Van der Hoeven and Coon, 1974). Most of the work with this enzyme has been done on drug detoxification and little is known about hydroxylation reactions of endogenous toxins. One group of endogenous toxins, however, that is eligible for hydroxylation reactions with cytochrome-p-450 is free fatty acids. Free fatty acids which find their levels elevated in the blood of patients in hepatic failure are rendered more hydrophilic by this enzyme which facilitates their excretion from the body.

Cytochrome-p-450 has been successfully immobilised on agarose and acrylamide, and encapsulated into liquid lipid vesicles (Brunner, 1981). While cytochrome-p-450 in the soluble form is immediately converted into cytochrome-p-420 when it comes into contact with blood or serum, it is very stable in the immobilised form (Brunner, 1981).

7.2.6 Urease

All the previously described enzymes carry out their detoxification functions in the liver cell. However, another important enzyme involved in detoxification is urease which is produced by organisms of the intestinal flora. Urease catalyses the conversion of urea into ammonia. The hydrolysis of urea by bacterial urease is a quantitatively significant source of hepatic ammonia; at least 25 percent of the urea produced is degraded by bacterial urease (Powers and Meister, 1982). It appears that urea diffuses from the liver into the circulating blood, is hydrolysed by bacteria in the colon and elsewhere in the gastrointestinal tract, and the ammonia thus generated diffuses into the portal blood and is transported to the liver, where it is reconverted to urea. Urea formation in the liver takes place through a cyclical system of enzyme reactions called the Krebs urea cycle (Powers and Meister, 1982). The urea cycle operates in a tightly controlled manner to dispose of approximately 90 percent of the surplus nitrogen.

The average normal value for urea excretion is 20-35 g/24 hour. Excretion is diminished in case of renal insufficiency and liver damage. Consequently, in uraemic patients large amounts of urea accumulate in the circulation. Although the toxicity of urea is low compared with that of ammonia, its removal from patients suffering from chronic renal failure is required. Urea is a very unreactive compound and, thus, it is not easily bound directly to adsorbents. Due to several similarities between the roles and functions of the liver and kidneys, combined haemodialysis/haemoperfusion have been used for removing toxins from uraemic patients. However, haemoperfusion through adsorbents does not remove urea of necessity (Winchester, 1982). Therefore, efforts have been made to remove urea enzymatically (Cousineau and Chang, 1978). In the present research, the possibility of immobilising urease on biocompatible hydrogel beads has been investigated. This will be described in section 7.4. Various techniques available for the immobilisation of enzymes will first be discussed.

7.3 IMMOBILISATION TECHNIQUES

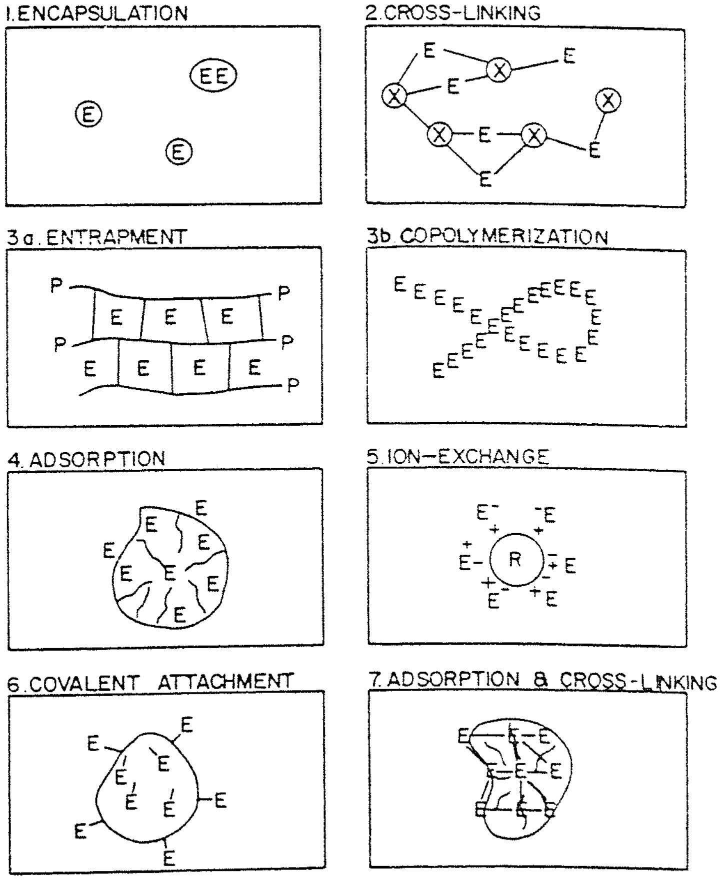
In literatures numerous methods for the immobilisation of enzymes have been reported and many classifications of immobilisation methods have been proposed. The various techniques can be classified into six basic approaches and their combinations. These approaches are: encapsulation; crosslinking; entrapment/copolymerisation; adsorption; ion-exchange; and covalent attachment. These approaches are schematically depicted in figure 7.1. Methods for enzyme immobilisation have recently been reviewed (Attiyat and Christian, 1984).

In addition to the six basic approaches, several miscellaneous methods for the immobilisation of enzymes are available which include photochemical immobilisation and physical immobilisation by hollow-fibre membranes (Mosbach, 1976).

Chemical coupling of enzymes with reactive supports is the most frequently used method of enzyme immobilisation. In general, the covalent binding procedure involves the formation of an activated support, followed by the reaction of the activated support with an enzyme to form a composite. A single reaction may be involved, or several steps may be required in preparing the activated support and coupling with an enzyme. Enzymes can be covalently bound to water-insoluble synthetic polymers like poly(acrylamide), methacrylic acid polymers, and polypeptides. Enzymes can be bound to a water-insoluble natural carriers like agarose, cellulose, and sephadex. Various hydrophilic polymer supports were reviewed by Mosbach (1971). Synthetic hydrophilic polymers based on acrylates and acrylamides to which enzymes have been covalently bound have been described (Mosbach, 1976).

Figure 7.1

Methods of immobilising enzymes, schematic representations



Although a large number of techniques are available for enzyme immobilisation, from the viewpoint of the application of haemoperfusion, three methods have been reported to exhibit acceptable characteristics with regard to their carrier material (Brunner, 1981). These three methods of immobilisation will now be described.

7.3.1 Binding of Enzymes to Agarose by the Cyanogen Bromide (CNBr) Method

Coupling of enzymes to agarose through the CNBr activation procedure is a classical and commonly used method of enzyme immobilisation (Lynn, 1975). Agarose becomes a suitable carrier material for in-vivo application only in the form of large beads (Brunner, 1981). The beaded form guarantees excellent haemocompatibility and flow characteristics in a column. Another important advantage of agarose beads is the possibility of including, when necessary, cofactors into the beads, preferably adsorbed to adsorbent materials for release over long time intervals. After depletion of the cofactor supply the beads can be reloaded with cofactor by incubation in concentrated cofactor solutions. Since the CNBr reaction can also be carried out with monohydroxy supports, it provides a potential technique for immobilising enzymes on biocompatible support materials such as crosslinked poly (HEMA).

7.3.2 Copolymerisation of Enzymes with Acrylamide and Acrylic Acid

Immobilisation of enzymes with acrylamide and acrylic acid copolymer often gives a slightly better yield in enzymatic activity, otherwise this preparation has similar characteristics to those of agarose beads (Brunner, 1981).

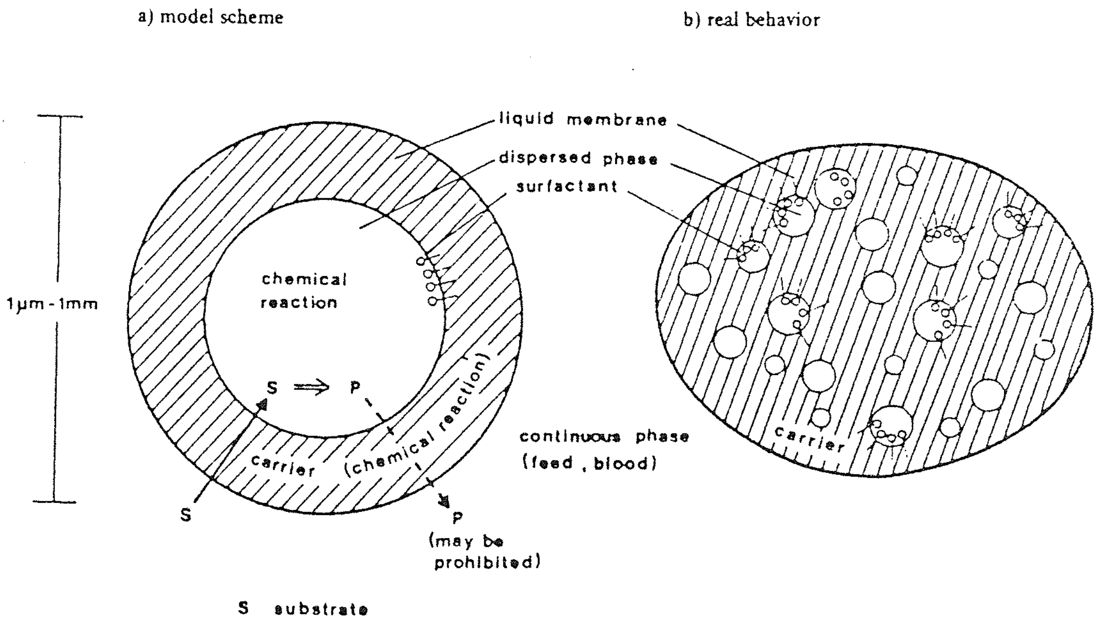
7.3.3 Encapsulation into Liquid Lipid Membranes

Liquid membrane technique is a simultaneous extraction/stripping process which uses a dispersed emulsion instead of a pure solvent as the receiving phase (Halwachs et al., 1981). The liquid membrane means a liquid component of a multi-emulsion which separates two other miscible liquids; the continuous phase - plasma and blood - from which the solute is to be removed and the dipersed phase - the enzyme solution - which receives the solute. The use of the enzyme UDP-glucuronyltransferase in combination with the extractant prepared from paraffin oil has been demonstrated in-vitro to remove phenols (Halwachs et al., 1981). A scheme for liquid surfactant membrane is given in figure 7.2.

The composition of the lipid phase has to be adjusted for the individual type of enzymatic processes employed. In hydroxylation reactions with cytochrome-p-450, for example, the presence of even trace amounts of lecithin completely inhibits the reaction. Haemocompatibility and enzymatic activities of this technique are greater than in the other two methods of enzyme immobilisation. This is shown in table 7.1 which compares enzyme activities after different immobilisation procedures (Brunner, 1981).

Figure 7.2

Schematic representation of a liquid surfactant membrane



However, this method of immobilisation has not been investigated in-vivo in the experimental animal, but only in-vitro. This is because separation of liquid lipid vesicles from blood in a continuous flow device has not been completely solved. The use of continuous flow plasmapheresis may overcome this problem.

Table 7.1

Enzyme activities after different immobilisation procedures

Enzyme	Soluble enzyme % activity	Bound to agarose beads % activity	Copolymerized with acrylamide % activity	Encapsulated into liquid lipid vesicles % activity
UDP-glucuronyltransferase 17.6 nmol phenol/min/mg protein	100	20	22	100
Methyltransferase 0.62 nmol ethane thiol/min/mg protein	100	18	21	not measurable
Cytochrome-p-450 9.9 nmol/mg protein	100	12	16	100
NADPH-cytochrome-p-450 reductase 196.0 nmol cytochrome-c/min mg protein	100	20	25	105

7.4 IMMOBILISATION OF UREASE ON HYDROGEL BEADS

In this study urease was used as a model for investigating the feasibility of immobilising enzymes on biocompatible hydrogel beads. It was thought that this enzyme could be used as a model since it is a classical 'marker' of uraemia and since its substrate, urea, is a major endogenous constituent of the body. The enzyme is easily available and can be easily assayed by established analytical methods. The enzyme derived from Jack Beans and obtained from Sigma Ltd. (Type IX) was used.

Urease was immobilised on hydrogel beads synthesised in the present research by suspension polymerisation techniques. Immobilisation was achieved by covalent coupling carried out in two ways depending upon the nature of functional groups present on the support used. To poly(HEMA) beads covalent coupling of urease was effected by CNBr activation method. Covalent binding to poly(ACR) beads was carried out by inter molecular crosslinking using a bifunctional reagent glutaraldehyde as the activating agent. The details of the two preparative procedures for the combined enzyme-hydrogel systems will now be described.

7.4.1 Covalent Binding of Urease to Glutaraldehyde-treated Poly (acrylamide) Beads

Poly(ACR) beads crosslinked with N,N'MBA were used as the support. The method used was as follows: Poly(ACR) beads (1 g, 500-250 μm size range) were activated for 16 hours at 4 °C using glutaraldehyde (25 ml of a 6% solution in 0.2 M phosphate buffer, pH 7.4). The glutaraldehyde-treated beads were thoroughly washed with distilled water, at 4-8 hour intervals for one day at 4 °C. The activated beads were filtered on a sintered glass funnel and transferred to a glass bottle with screw on top. 25 mg of urease dissolved in 25 ml of 0.1 M phosphate buffer, pH 7.4 were added to the beads and coupling was allowed to proceed for 16 hours at 4 °C. Urease-containing beads were washed with 0.1 M sodium bicarbonate, 0.5 M sodium chloride, and finally with 0.2 M phosphate buffer, pH

8.0. All washing solutions contained 1 mM mercaptoethanol as the stabilising agent for urease. After washing, beads were stored in the buffer at 4°C.

7.4.2 Covalent Coupling of Urease to Cyanogen Bromide-treated Poly (hydroxyethyl methacrylate) Beads

Poly(HEMA) beads crosslinked with EDM had urease immobilised on them in the following way: 5 g of beads (500-250 μm size range) were activated in 80 ml distilled water containing 200 mg CNBr. The activation step was allowed to proceed for 10 minutes at 4°C while the pH was maintained at 10.8 by addition of 2 M sodium hydroxide. The activated beads were thoroughly washed on a sintered glass funnel with ice-cold 0.1 M sodium bicarbonate. Beads were then added to the enzyme solution (40 mg protein in 40 ml 0.1 M sodium bicarbonate containing 1 mM mercaptoethanol). The coupling was allowed to take place for 12 hours at 4°C. Urease containing beads were finally washed with 0.1 M sodium bicarbonate, 0.5 M sodium chloride, and 0.1 M Tris-HCl buffer, pH 8.0. All washing solutions were 1 mM in mercaptoethanol. The beads were then stored in buffer at 4°C.

A complete list of samples of hydrogel beads used for the coupling of urease is given in table 7.2.

Table 7.2

Samples of hydrogel beads used as supports for the immobilisation of urease

Monomer Composition (Initial molar ratio)	Method of Synthesis	Particle Size (μm)	Activating Reagent
ACR:N,N'MBA (100:10)	Inverse-suspension in n-hexane	500-250	Glutar-aldehyde
HEMA:EDM (100:10)	Suspension in brine	500-250	CNBr
HEMA:NMACR:EDM (90:10:8.5)	Suspension in brine	500-250	CNBr

After immobilisation of urease on hydrogel bead samples, the evaluation of immobilised enzyme was carried out by reacting the enzyme with its substrate. This will now be described.

7.5 CHARACTERISATION OF IMMOBILISED UREASE

Characterisation of the combined urease-hydrogel system was carried out by measuring the activity of the bound enzyme and comparing it with the activity of free enzyme in solution. Urease activity was determined by its reaction on urea by a modification of the method of Van Slyke and Archibald (1944), using a pH meter. The procedure is based on the law of inverse proportionality between enzyme concentration and the time required for a given amount of product to form. This inverse proportionality is a general law of enzyme action, which appears to hold when an enzyme is stable for the observation period used. This has been demonstrated to be the case with urease (Van Slyke and Archibald, 1944). In the present timing method the urease acts on urea in a phosphate buffer solution. The ammonium carbonate formed reacts with the acid phosphate in the buffer.

$$CO_3^{2-} + H_2PO_4^{-} = HCO_3^{-} + HPO_4^{2-}$$

The result is an increase in the ratio, $HPO_4^{2-} : H_2PO_4^{-}$, and in pH, which depends on the amount of ammonium carbonate formed. Consequently, the timing principle can be applied by observing how long it takes the pH in a given buffer solution to rise to a definite point. In the present procedure a buffer solution is used which sets the initial mixture at a pH of 6.7, and the time is measured which is required for the pH to rise to 7.7. The method for determining the efficiency of immobilised and free urease to hydrolyse urea was as follows.

7.5.1 Method Used for Determining the Activity of Urease

100 ml of buffered urea solution were prepared by dissolving 3 g of urea in phosphate buffer (1M, pH 6.7). Enzyme leakage was analysed by adding 1 g of hydrogel beads with coupled urease to 10 ml of buffered urea solution. The increase in pH was followed at different time intervals using a pH meter until the pH reached 7.7. Similarly, activity of free urease in solution

was assayed by adding 1 ml of enzyme solution to 10 ml buffered urea solution, and the pH rise was followed. A blank assay was carried out by adding 10 ml of distilled water to 1 g of urease containing beads and following any change in pH over the same period of time that was taken by the test sample to increase the pH to 7.7. Results of characterisation of urease-hydrogel bead systems are described on the following pages.

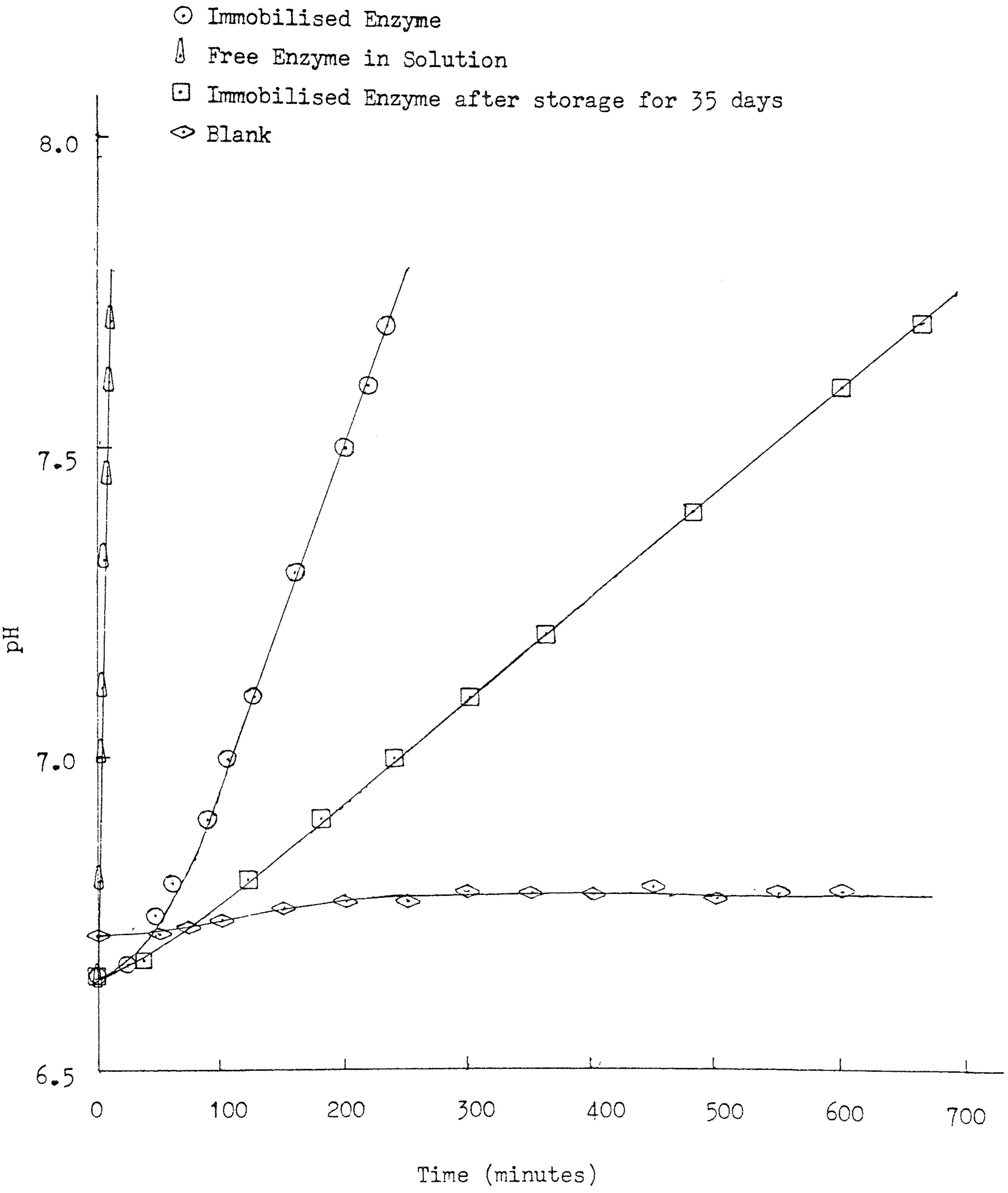
7.5.2 Characterisation of Urease Immobilised on ACR/N,N'MBA Copolymer Beads.

Figure 7.3 shows the efficiency of immobilised urease, and free urease in solution. This is represented by the graph of increase in pH against time. The change in pH over comparable time period for the blank is also shown. The immobilised enzyme is seen to be 27 times less active than free enzyme in solution.

The storage stability of immobilised urease was also tested for the urease-ACR/N,N'MBA bead system. This test was carried out as follows. The immobilised urease system in phosphate buffer, pH 6.7, was tested for enzyme leakage at 23°C. These beads were discarded and a new sample after storage at 4°C for 35 days was tested for the release of the enzyme. The pH-time graph for this test is also shown in figure 7.3. After storage the activity of immobilised urease was reduced by approximately one-third.

Figure 7.3

The efficiency of urease immobilised on ACR/N,N'MBA copolymer beads, and free urease in solution to hydrolyse urea



7.5.3 Characterisation of Urease Immobilised on HEMA/EDM Copolymer Beads.

The increase in pH with time for the hydrolysis of urea by the urease-HEMA/EDM system is illustrated in figure 7.4. The change in pH of the blank is also shown. The bound enzyme is seen to be 5 times more stable than the free enzyme. Storage stability for the urease-HEMA/EDM system was also tested as described in section 7.5.2. The pH-time graph for the test carried out after storing the system for 35 days at 4 C is shown in figure 7.5. After storage the efficiency of urease was reduced by 12 times.

In addition to the storage stability test for urease immobilised on HEMA/EDM copolymer beads, other tests for operational stability of the immobilised enzyme were carried out. In this case the urease-hydrogel bead system previously stored at 4 °C for 35 days was assayed for urease activity. Beads were washed with phosphate buffer and stored in the buffer for a further 12 day period. Beads were retested for urease activity to investigate the possibility of repeated use of the sample for hydrolysing urea. Results of one such experiment are shown in figure 7.5. The immobilised enzyme is seen to be still active even though it had previously been reacted once with urea. The efficiency of the enzyme was, however, reduced.

Figure 7.4

The efficiency of urease immobilised on HEMA/EDM copolymer beads, and free urease in solution to hydrolyse urea.

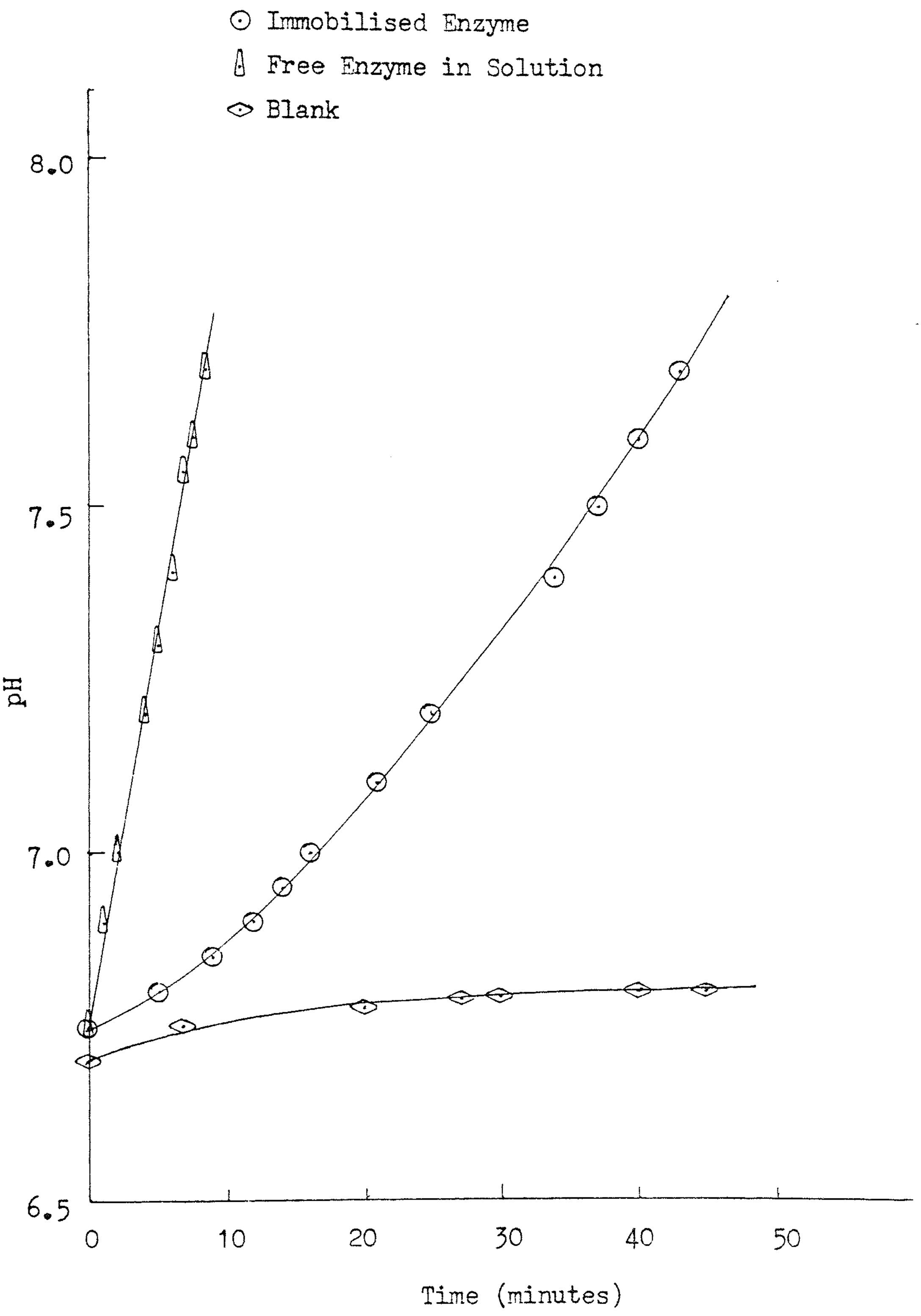
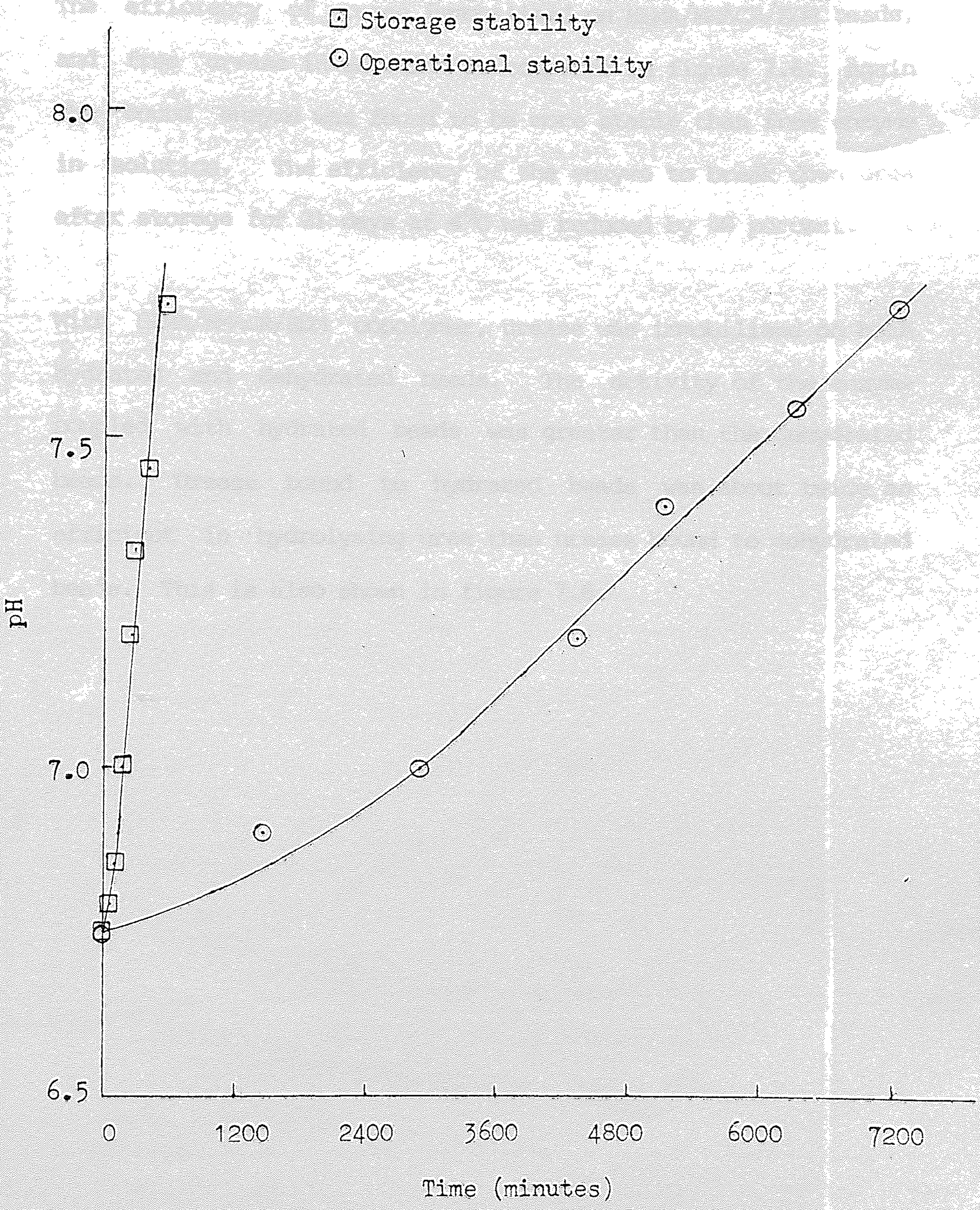


Figure 7.5

Storage stability and operational stability of urease immobilised on HEMA/EDM copolymer beads.



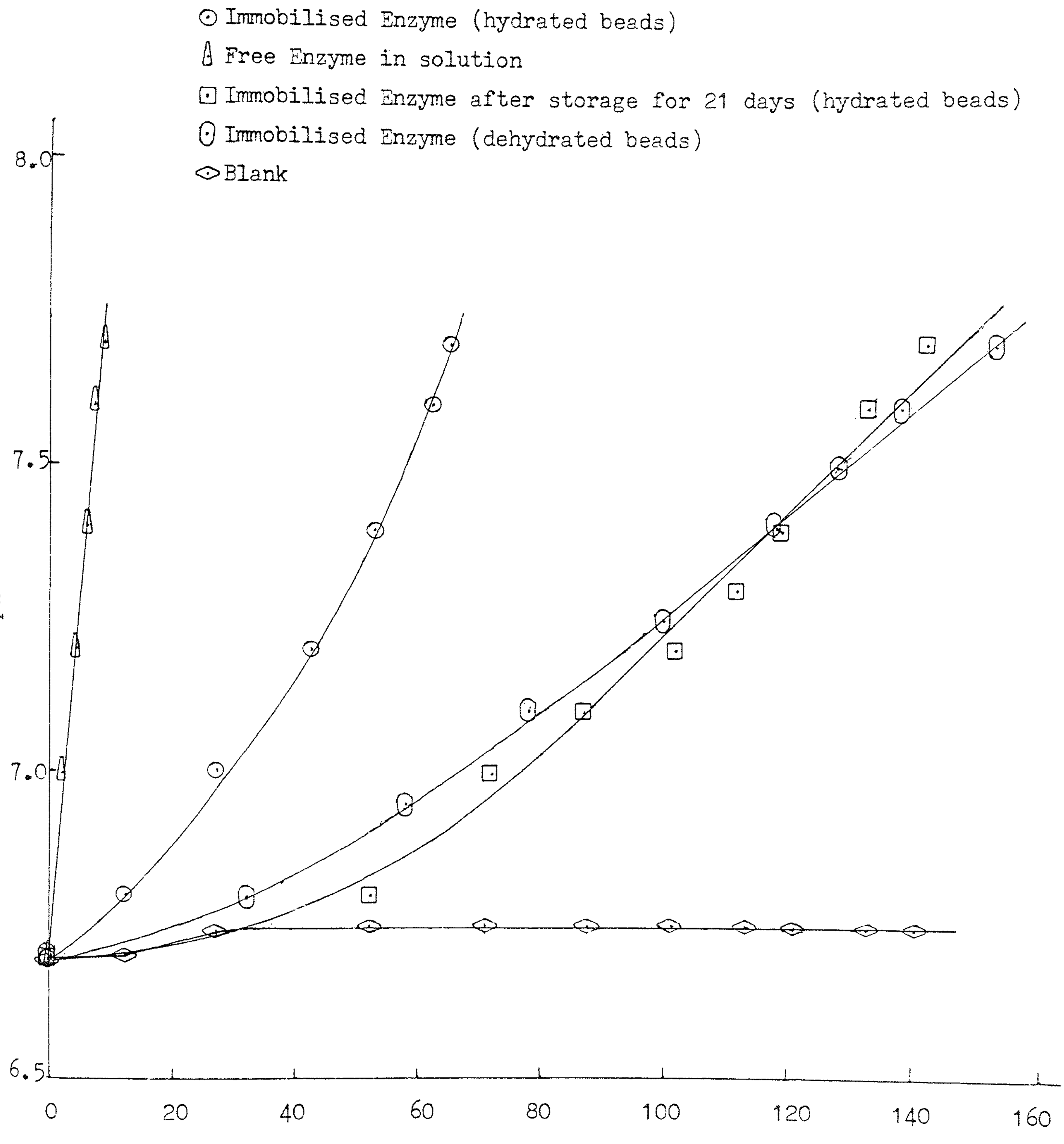
7.5.4 Characterisation of Urease Immobilised on HEMA/NMACR/EDM Terpolymer Beads.

The efficiency of urease immobilised on HEMA/NMACR/EDM beads, and free urease in solution is compared in figure 7.6. Again the bound enzyme was found to be more stable than free enzyme in solution. The efficiency of the enzyme to break down urea after storage for 21 days at 4°C was reduced by 50 percent.

With HEMA/NMACR/EDM copolymer, urease was immobilised on both hydrated and dehydrated beads. The activity of the enzyme coupled with hydrated beads was greater than the dehydrated beads. Urease bound to hydrated beads was about twice as efficient in hydrolysing urea than urease bound to dehydrated beads. This is also shown in figure 7.6.

Figure 7.6

The efficiency of urease immobilised on HEMA/NMACR/EDM terpolymer beads, and free urease in solution to hydrolyse urea



7.6 DISCUSSION

Immobilisation of urease on various hydrogel beads, synthesised for haemoperfusion, was successfully carried out by the covalent coupling technique. Derivatisation of poly(ACR) with glutaraldehyde gave preparation well suited for the binding of urease. The widely used CNBr method (Axen et al., 1967) usually applied to agarose gels was adapted to immobilise urease on HEMA/EDM copolymer and HEMA/NMACR/EDM terpolymer hydrogel beads. Due to the importance of biocompatible hydrophilic support matrices for immobilisation of detoxifying enzymes, consideration will first be given to such support matrices.

7.6.1 Hydrogel Support Matrices for Enzyme Immobilisation

When selecting supports suitable for immobilisation of enzymes one has to consider the following factors:

(i) Mechanical properties, such as rigidity and durability, while the gel product is subjected to mechanical agitation, compression etc.

(ii) Physical form (granules, sheets etc.).

(iii) Resistance to chemical and microbial attacks.

(iv) Hydrophilicity as manifested by the tendency to incorporate water into its structure.

(v) Permeability.

(vi) Ability to be derivatised with satisfactory retension of factors (i)-(v).

(vii) Price and availability.

It appears that hydrophilicity is a very important factor for the preservation of enzymes in a highly active state after their immobilisation to the solid support (Mosbach, 1976). This fact places hydrophilic gels in a very favourable position among immobilised enzyme matrices. Among synthetic hydrophilic supports, crosslinked poly(ACR) polymers occupy the most important position, while the polysaccharides and some of their derivatives dominate as hydroxylic supports for enzymes (Mosbach, 1976). Crosslinked poly(acrylic) polymers are of great interest because they show good mechanical and chemical stability and are inert to microbial degradation. In one respect they are outstanding:that is, in the ease of preparing various polymers 'tailor made' for a particular enzyme application, for example, with regard to stability and specific activity. Parameters that can be easily varied to this end include: (a) the degree of porosity and (b) the chemical composition, which can be achieved by either copolymerisation of different monomers or by chemical modification of preformed polymers.

Macroporous hydrogel polymers containing acidic, neutral and basic groups with varying physical and chemical properties were synthesised in the present research. These polymers can be used as new type of carriers for the immobilisation of detoxifying enzymes. They were prepared in the form of spherical particles, which even in the dry state exhibit high porosity and, therefore, high specific surface values. The internal structure of these crosslinked gel beads has a heterogeneous character; the particles consist of submicroscopic spheroids glued together. Pores of the beads are interconnected, and their distribution can be controlled by suitably adjusting the reaction conditions during copolymerisation.

It has been reported that the various agarose polymers most widely used as matrices for enzymes appear ideal for affinity chromatography of enzymes due to their high degree of porosity (Johansson and Mosbach, 1974). Similarly, hydrogel beads synthesised in this research provide porous and biocompatible support matrices for the immobilisation of enzymes. It must be pointed out, however, that probably no one optimal immobilisation technique and no single ideal support system can be found for one particular enzyme. The immobilisation of urease on hydrogel beads will now be discussed.

7.6.2 Urease-Hydrogel Bead Detoxification Systems

All urease-hydrogel bead systems prepared and tested in this study gave active preparations. Results of characterisation studies on immobilised urease, illustrated in figures 7.3-7.6 show that the bound urease was several fold more stable than the free enzyme in solution. Urease bound to ACR/N,N'MBA copolymer beads was 27 times more stable than the free enzyme.

Urease bound to HEMA/EDM copolymer and HEMA/NMACR/EDM terpolymer beads was 5 times and 7.5 times respectively more stable than the free enzyme in solution. The urease-hydrogel system carried out hydrolysis of urea even after storage for several weeks. The results of tests on operational stability of immobilised urease show that it is possible to use these preparations repeatedly over a period of time.

Results from this study indicate that activity of immobilised urease varied with the type of hydrogel matrix to which it was immobilised. The activity of an enzyme on immobilisation has been reported to be affected by several factors. These factors include the coupling procedure, the enzyme site involved in coupling, the orientation of the enzyme on the support surface, and the support material used (Lynn, 1975). Examples given for the immobilisation of urease to various hydrogel matrices indicate the potential of these preparations. This shows the possibility of binding certain hepatic enzymes to hydrogel beads, synthesised in this research, for use in artificial liver support systems. The development of immobilised enzymes for an extracorporeal detoxification demands efforts in two areas of research: (a) detoxifying enzymes have to be prepared which exhibit suitable activities and stability, (b) immobilisation materials and techniques must be found which display good enzymatic activity and good haemocompatibility as well as sufficient and economic co-factor supply for those enzymes where co-factors are needed. Successful immobilisation of the model enzyme urease on biocompatible hydrogel beads fulfils a primary pre-requisite for use in a detoxification device. From the data obtained, no general statement can be made as to what coupling procedure and what hydrogel matrix is ideal for immobilising any one enzyme. It has been reported that several parameters influence the binding ability of the carrier for the enzymes (Manecke and Schlunsen, 1976). These parameters will now be discussed.

7.6.3 Parameters Influencing the Binding of Enzymes to the Carrier.

Higher binding abilities and a higher relative activity (activity of the bound enzyme compared with the activity of the native enzyme) can be achieved by using smaller particle sizes and macroreticular carriers. These provide better accessability of the matrix for the enzymes and the substrates. Also the number of reactive groups influences the binding ability of the support. An increased number of reactive groups binds larger amounts of proteins provided the parallel decrease in the number of hydrophilic groups in the support does not cause extensive deterioration in the swellability. Too high concentrations of reactive groups may cause multiple binding of the enzyme molecules. Increased crosslinking of the polymeric carrier decreases swellability, and thus the amount of bound enzyme. In case of ionic matrices, binding ability is also affected by pH. This is because the swellability of the ionic matrices is pH dependent. The binding ability for enzymes depends also on the concentration of the enzyme solution during the immobilisation procedure. Higher enzyme concentrations lead to greater amounts of bound enzyme. However, using lower concentrations improves the activity yield, that is, the enzyme activity is better utilised (Manecke and Schlunsen, 1976).

The knowledge of various parameters which control the binding of enzymes to the carrier, and the availability of biocompatible hydrogel beads with varying physical and chemical properties synthesised in this research together with the different immobilisation techniques known can provide enough flexibility to obtain a 'tailor made' immobilised enzyme preparation for use in haemoperfusion.

ACR/N,N'MBA and HEMA/EDM copolymer beads synthesised in this work were investigated in a parallel research project for the adsorption of ammonia (Caesar, 1984). These copolymers removed ammonia from solution and were demonstrated to be good adsorbents for this hepatotoxin. With the use of combined urease-hydrogel bead system, urease can convert urea into ammonia which can, therefore, be subsequently adsorbed by the hydrogel adsorbent. These results demonstrate the feasibility of a combined enzyme-adsorbent haemoperfusion system for use in liver failure patients. Here the enzyme can carry out enzymatic action on specific substrates. The adsorbent can remove other wanted toxins, metabolites or even the products of the enzymatic reactions.

CHAPTER 8

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

8.1 CONCLUSIONS

From the work carried out on the synthesis and evaluation of hydrogel particulates, and immobilisation of enzymes the following conclusions were derived.

8.1.1 Synthetic Studies

(i) In the freeze-thaw polymerisation technique the use of automatic syringe injector, in comparison with burette, provides greater versatility and efficiency in varying the particle size over a wide range. With this method of polymerisation by trapping monomers in frozen solvent crystals, the polymerisation process can be carried out 'in situ' which further increases the efficiency of the technique. Although, a range of monomers can be polymerised in the particulate form, the macroporosity results only in those monomer compositions which allow freezing to take place rapidly.

(ii) Hydrophilic monomers can be polymerised by suspension polymerisation in an aqueous continuous phase by limiting their solubility in water with the use of saturated sodium chloride solution containing xanthan gum (viscosity modifier) and hydroxypropylmethyl cellulose (interfacial tension modifier). Although, the technique can be applied to a wide range of monomers, the production of large particulates (diameter > 125 μm) is limited to less hydrophilic monomers. More hydrophilic monomers such as ACR and AA polymerise in particulate size ranges lower than this. This is because of the tendency of more hydrophilic monomers to dissolve in the aqueous phase even in the presence of large concentrations of salt. Heterogeneously crosslinked macroreticular structure in the hydrogel particulate matrix can be created if polymerisation is carried out in the presence of an inert compound which acts as solvent for the monomer but only as a swelling agent for the polymer. In the absence of such a compound suspension polymerisation of hydrophilic monomers in brine results in homogeneous particulates.

(iii) ACR/N,N'MBA monomer combination, the particulates of which: from freeze-thaw polymerisation are not macroporous, and from suspension polymerisation in brine can not be synthesised in a size greater than about 80 μm in diameter, can be synthesised in higher size range and possessing macroporosity by inverse-suspension polymerisation in n-hexane. This technique is unsuitable for synthesising hydrogel particulates from more hydrophobic monomer combinations such as HEMA/EDM system. However, HEMA can be incorporated into the ACR/N,N'MBA combination to produce terpolymer particulates. Variation in the porous structure of ACR/N,N'MBA can be made by altering polymerisation conditions such as solvent for the monomers.

8.1.2 Enzyme Immobilisation Studies

(iv) From the enzyme immobilisation studies it was concluded that hydrogel particulates synthesised by different techniques form suitable porous matrices for the coupling of detoxifying enzyme urease. The enzyme bound to hydrogel bead matrices by the covalent coupling method is several fold more stable than the free enzyme in solution. However, the activity of immobilised urease varies with the type of hydrogel matrix to which it is bound. The urease-hydrogel bead systems can be used repeatedly over a period of time for detoxification, since they are shown to hydrolyse urea even after storage for several weeks.

8.1.3 Adsorption Studies

(v) Adsorption studies with water soluble acidic chemical species show that hydrogel particulates are good adsorbents for the removal of such toxins. Hydrogels are also capable of binding a hydrophobic liver toxin bilirubin. The adsorption of acidic chemical species involves interactions ranging from weak Van der Waals forces to strong ionic attachment. While hydrogels in certain cases adsorb acidic toxins in amounts comparable to those adsorbed by commercial adsorbents, samples with lower adsorptive ability are capable of removing clinically useful amounts of toxins. Macroporous hydrogel particulates show greater adsorptions than their non-macroporous counterparts. In addition to macroporosity, factors such as chemical composition, particle size and water content of particulates, pH of the solution, and interfacial tension between the solution and the adsorbent all contribute to adsorption characteristics of hydrogels.

(vi) Adsorption studies of water soluble acidic compounds from saline using both static and dynamic adsorption techniques demonstrate that the use of saline provides a simple medium to carry out in-vitro evaluation of hydrogel adsorbents for haemoperfusion. The use of continuous flow apparatus for dynamic studies provides a useful model for haemoperfusion, and results from this technique can be related to clinical situations.

8.2 SUGGESTIONS FOR FURTHER WORK

(a) Although the biocompatibility of such hydrogel polymers as synthesised in this research is known to be good, studies should, however, be carried out to establish the exact nature of this biocompatibility. Three main classes of tests are available for determining blood compatibility: in-vivo, ex-vivo and in-vitro. The ex-vivo tests involve passage of blood from an experimental animal through a test device outside the organism. These tests, therefore, resemble haemoperfusion systems and results from such tests may be especially applicable to artificial liver support systems.

(b) In the technique of suspension polymerisation in brine there is need to find an alternative solvent to 1,1,2,2-tetrachloroethane (TCE) for the monomer phase. This is to avoid the risk of toxic effects which might occur with the long term use of TCE.

(c) Further experimental work is necessary with the technique of inverse-suspension polymerisation to extend the range of monomers which can be polymerised in particulate form using this technique. The use of suspension stabilisers other than Span 20, and hydrophobic continuous phases other than n-hexane may provide a solution to this problem.

(d) Adsorption studies should be extended to investigate the removal of hepatic toxins by hydrogel particulates from plasma. It is critical that devices intended for ultimate use in a clinical setting be tested using blood or plasma. Also the range of chemical species used as marker molecules may be increased. Bile acid-amino acid conjugates such as cholylglycine may form further useful markers for endotoxins. The removal of paracetamol may form a useful model for exotoxins and drugs.

(e) The effectiveness of the technique of dynamic adsorption studies to remove a mixture of several chemical species from solution by hydrogels should be investigated. This would yield information regarding any preferential adsorption shown by a sample of adsorbent towards chemical species.

(f) The immobilisation of hepatic enzymes, particularly, UDP-glucuronyltransferase (the key enzyme ivolved in detoxification reactions) to hydrogel beads should be investigated. The combination of immobilised detoxifying enzymes with the continuous flow apparatus may provide a powerful technique in the field of detoxifications. The technique if used in conjunction with the autoanalyser would have the advantages of sensitivity, selectivity, rapidity, multicomponent analysis, and the possibility of interfacing with data acquisition systems.

APPENDIX A

DERIVATION OF THE EQUATION CORRELATING EQUILIBRIUM WATER CONTENT AND DENSITY OF A HYDROGEL

If Wg = The weight of a polymer in the hydrated state

dg = The density of a polymer in the hydrated state

Vg = The volume of a polymer in the hydrated state

Wp = The weight of a polymer in the dehydrated state

dp = The density of a polymer in the dehydrated state

Vp = The volume of a polymer in the dehydrated state

and Ww, dw and Vw = The weight, density and volume of water in hydrogel

then,

$$Wg = dg \cdot Vg$$

$$= dg\ (Vp + Vw)$$

$$= dg \quad \frac{Wp}{dp} + \frac{Ww}{dw}$$

i.e.

$$\frac{dg}{dw} \cdot \frac{Ww}{Wg} = 1 - \frac{dg}{dp} \cdot \frac{Wp}{Wg}$$

$$= 1 - \frac{dg}{dp} + \frac{dg}{dp} \cdot \frac{Ww}{Wg}$$

Therefore,

$$\frac{Ww}{Wg} \quad \frac{dg}{dw} - \frac{dg}{dp} = 1 - \frac{dg}{dp}$$

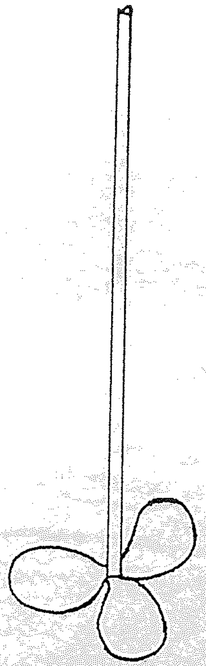
Hence,

$$\frac{Ww}{Wg} = \frac{1 - \dfrac{dg}{dp}}{dg \quad \dfrac{1}{dw} - \dfrac{1}{dp}} \quad X \quad 100\ \%$$

APPENDIX B

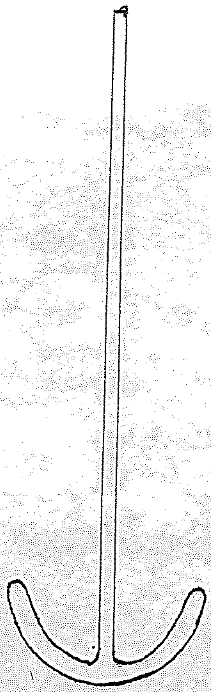
THE TYPES OF STIRRERS USED TO SYNTHESISE HYDROGEL PARTICULATES

(a)



←4.3 cm→
Stainless steel

(b)

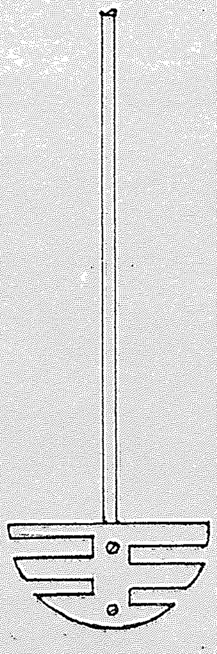


←9 cm→
Glass

(c)

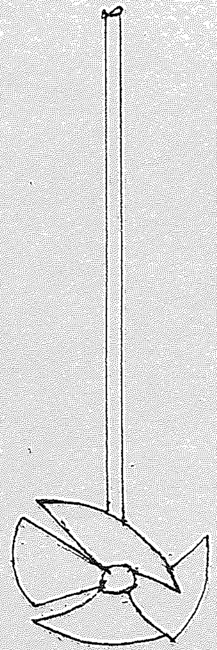
←9 cm→
Stainless steel rod;
Polypropylene paddle

(d)



←9 cm→
Stainless steel rod;
Polypropylene paddle

(e)

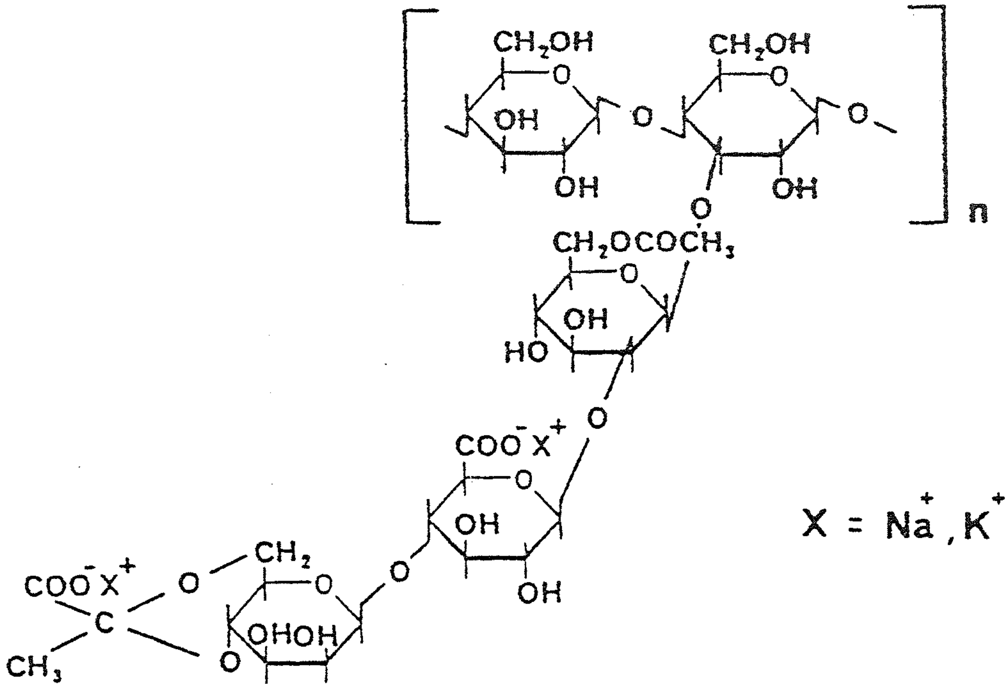


←4.5 cm→
Stainless steel

APPENDIX C

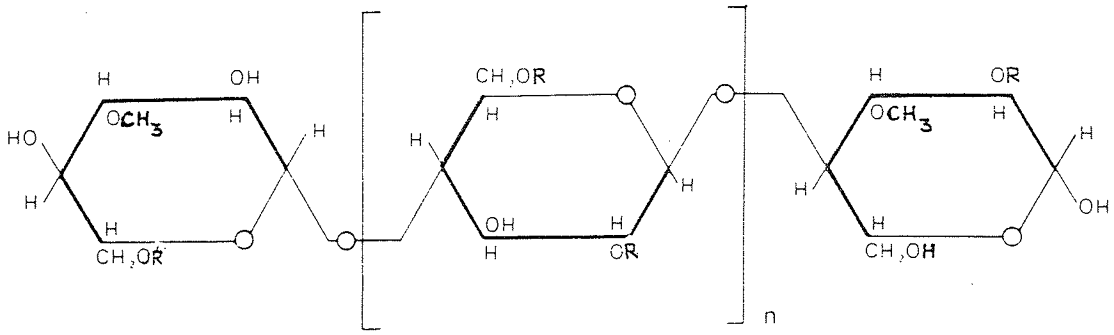
STRUCTURE OF XANTHAN GUM AND GENERAL FORMULA OF CELLULOSE GUMS

Structure of xanthan gum as proposed by Jansson et al. (1975)



$X = Na^+, K^+$ or $\frac{1}{2}Ca^{2+}$.

General formula of cellulose gums (Hamann et al., 1975)



Where R = an alkyl or hydroxyalkyl residue usually methyl, ethyl, hydroxyethyl, hydroxypropyl, or hydroxybutyl or a mixture of these groups.
Where n is any whole number within the range of about 50 to about 900

APPENDIX D

GRAPHS OF CUMULATIVE BSP ADSORPTION WITH HYDROGEL PARTICULATES

Figure 1D: Cumulative amount of BSP adsorbed by AA/EDM copolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine

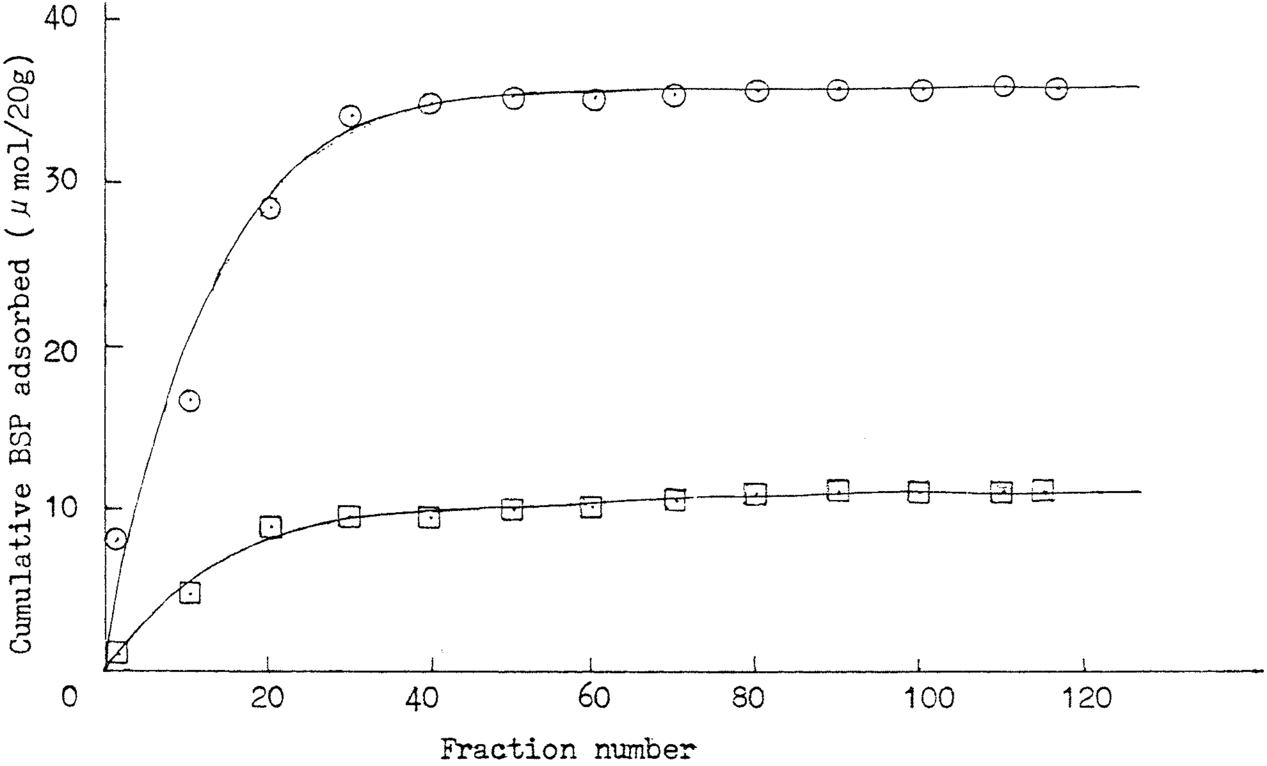


Figure 2D: Cumulative amount of BSP adsorbed by AA/HEMA/EDM terpolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine

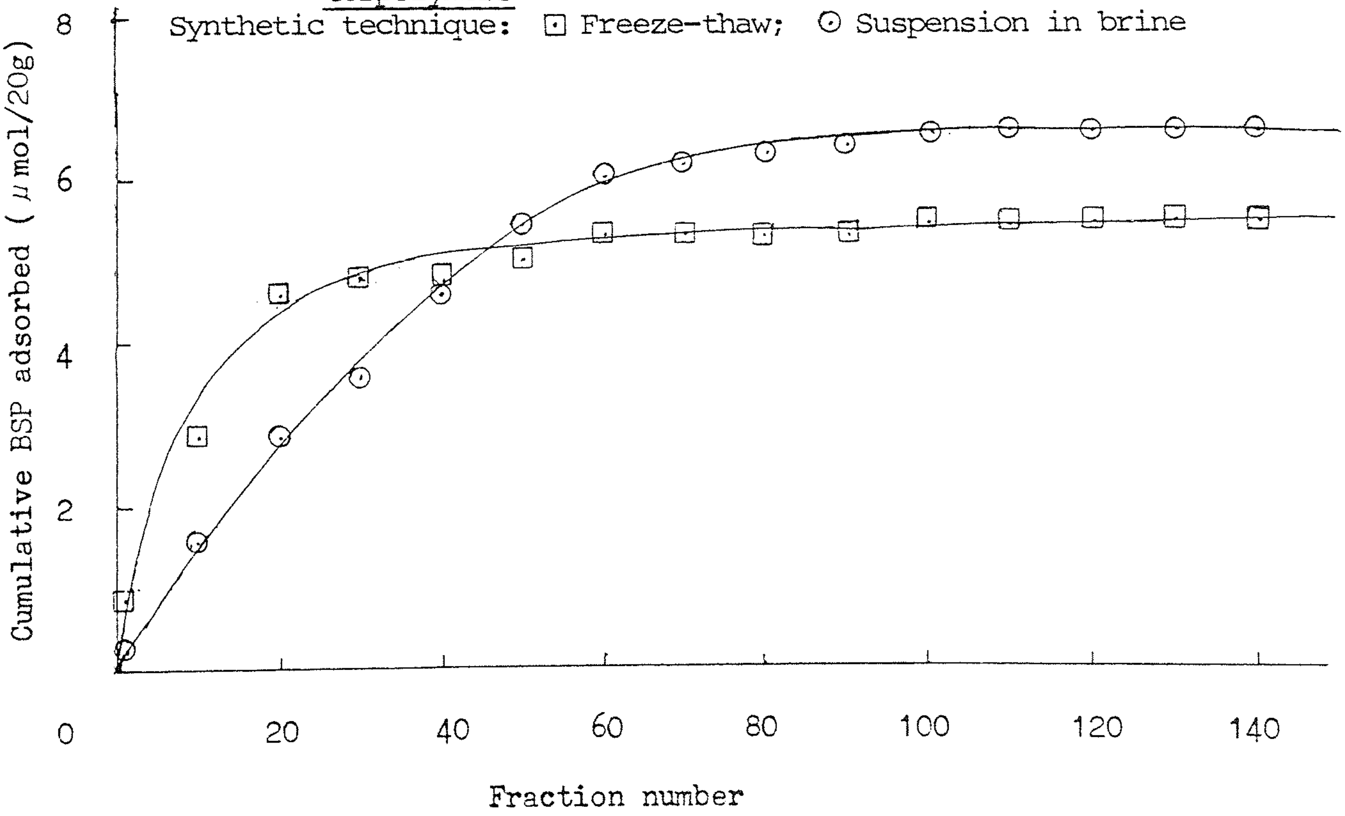


Figure 3D: Cumulative amount of BSP adsorbed by MAA/HEMA/EDM terpolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine

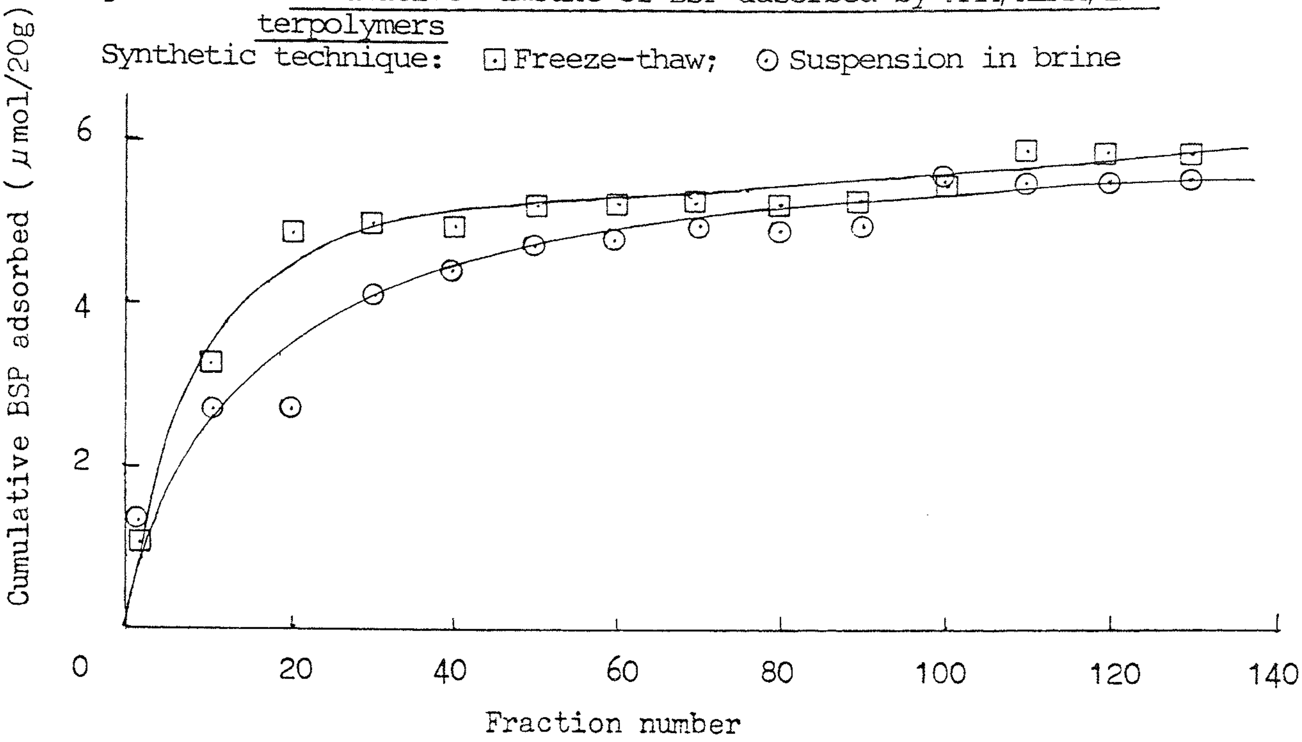
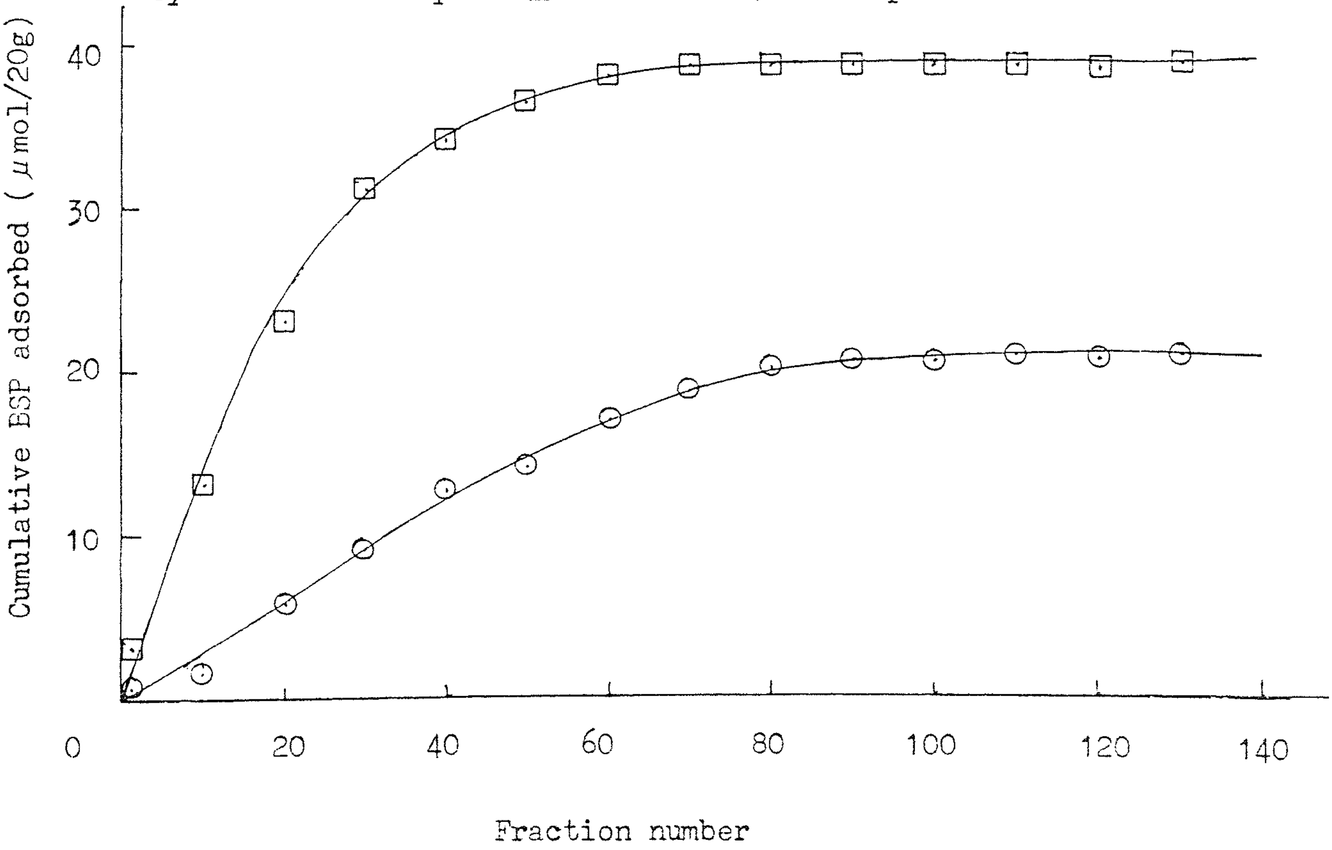


Figure 4D: Cumulative amount of BSP adsorbed by HEMA/EDM copolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine



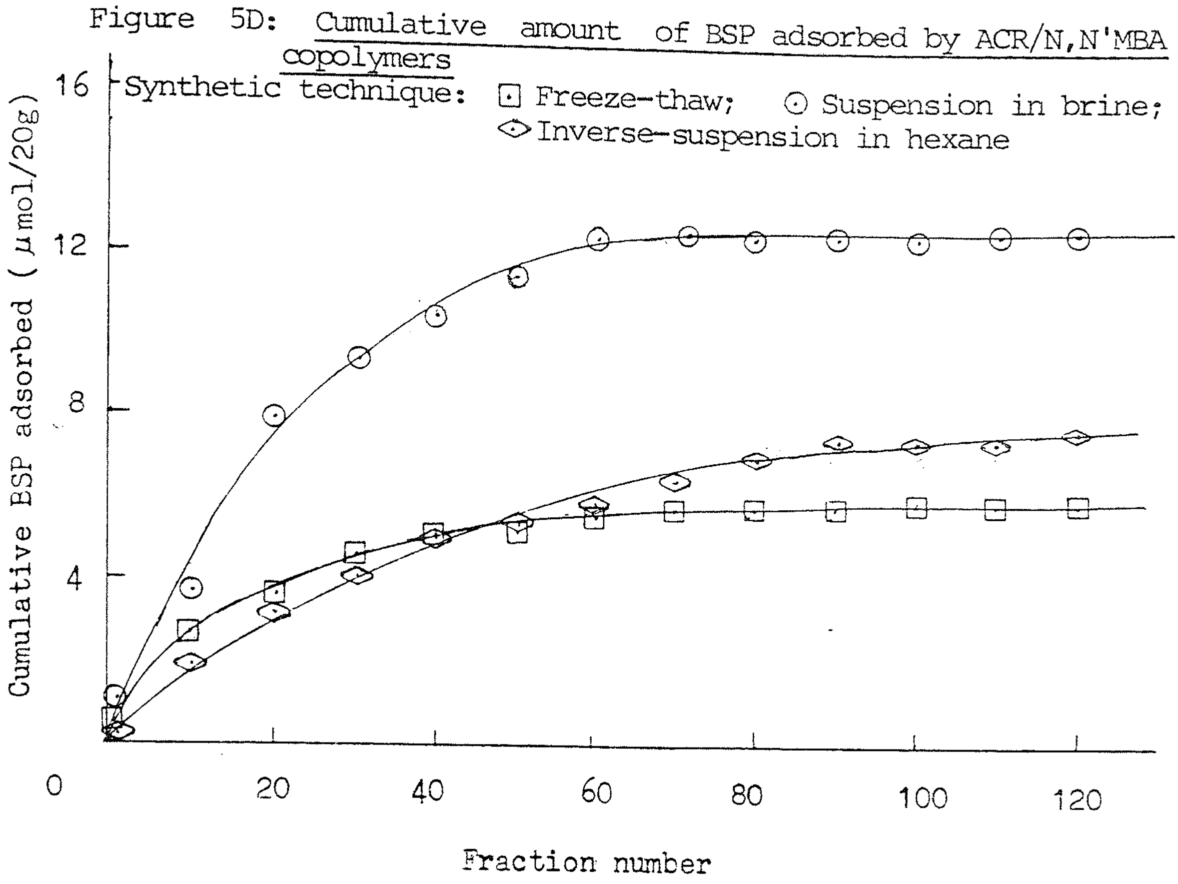


Figure 5D: Cumulative amount of BSP adsorbed by ACR/N,N'MBA copolymers
Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine; ◇ Inverse-suspension in hexane

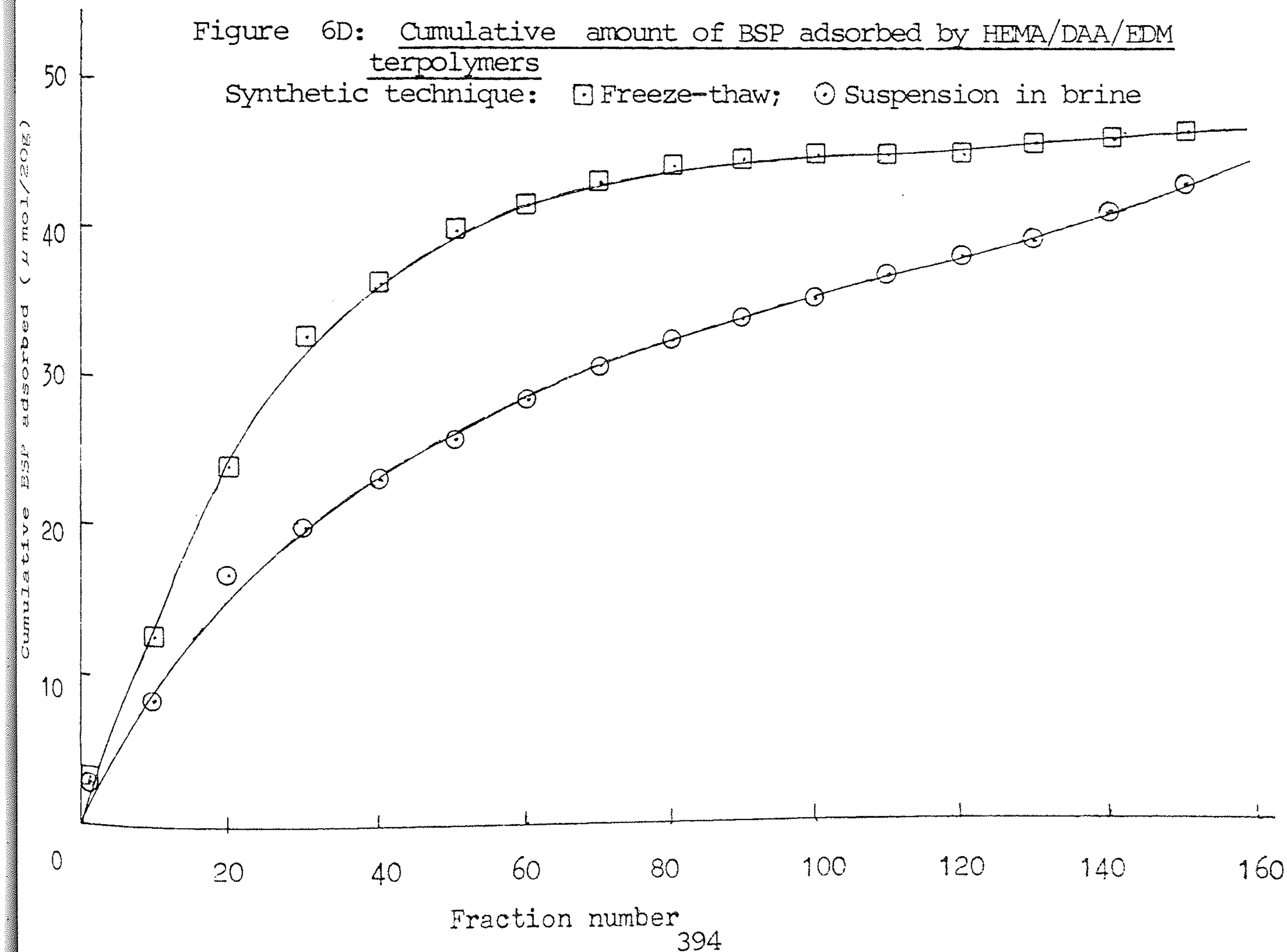


Figure 6D: Cumulative amount of BSP adsorbed by HEMA/DAA/EDM terpolymers
Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine

Figure 7D: Cumulative amount of BSP bound by HEMA/NMACR/EDM terpolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine

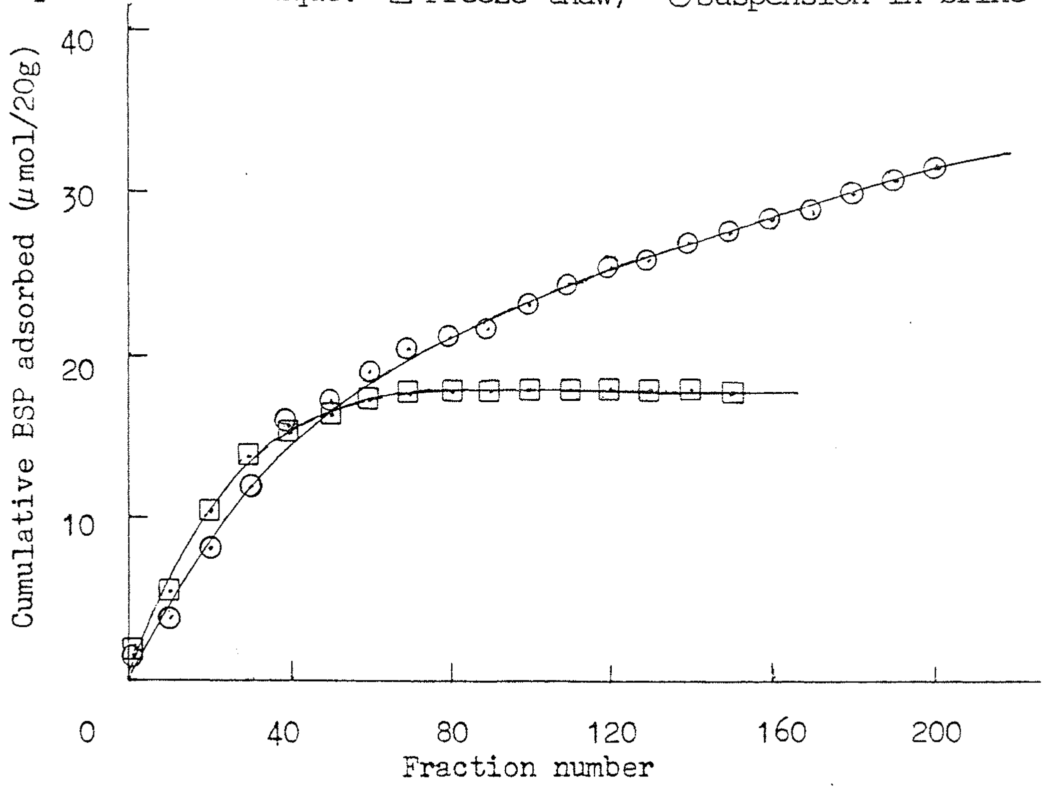
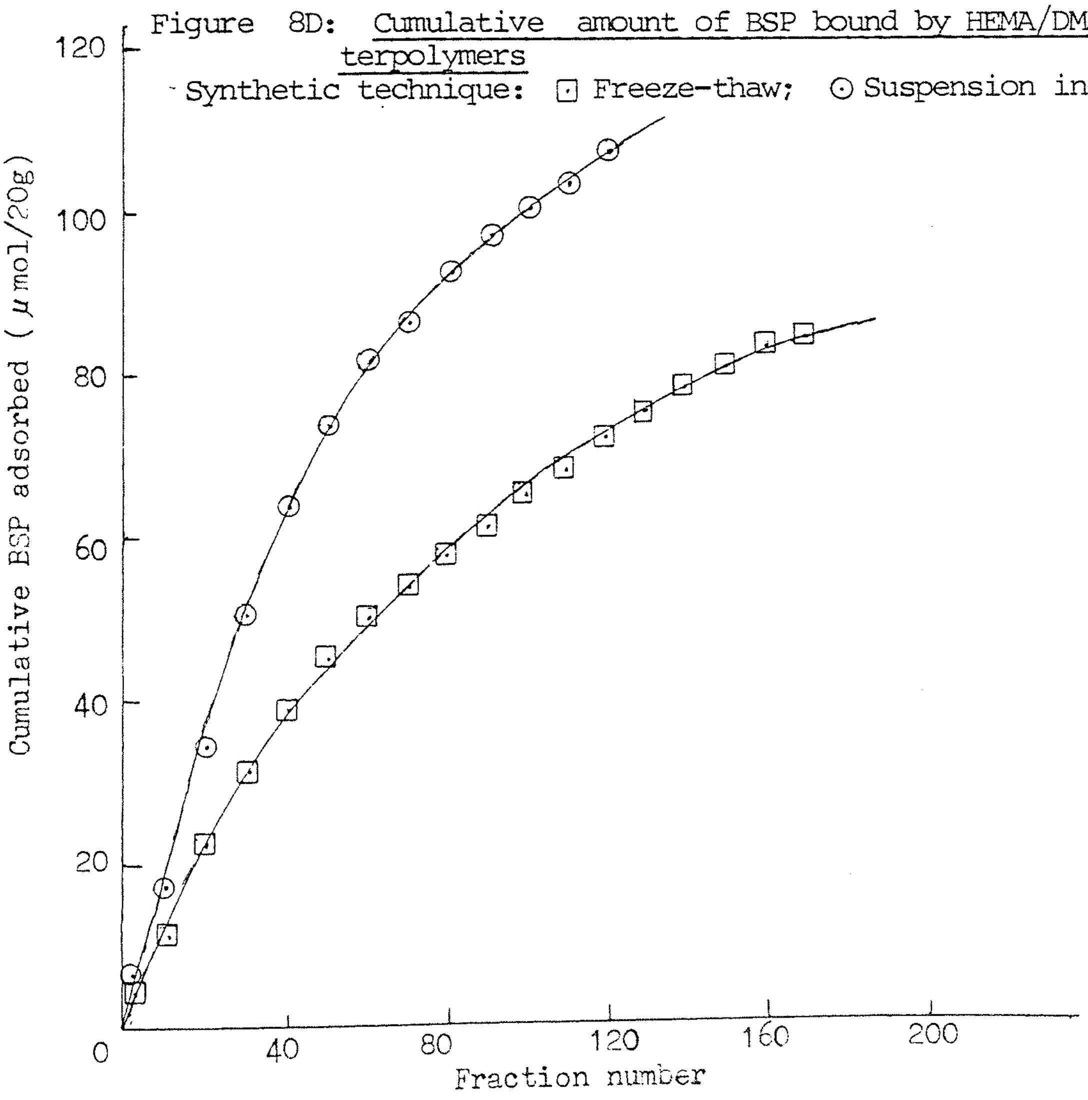


Figure 8D: Cumulative amount of BSP bound by HEMA/DMAEMA/EDM terpolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine



APPENDIX E

GRAPHS OF CUMULATIVE CHOLIC ACID ADSORPTION WITH HYDROGEL PARTICULATES

Figure 1E: Cumulative amount of cholic acid adsorbed by AA/EDM copolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine

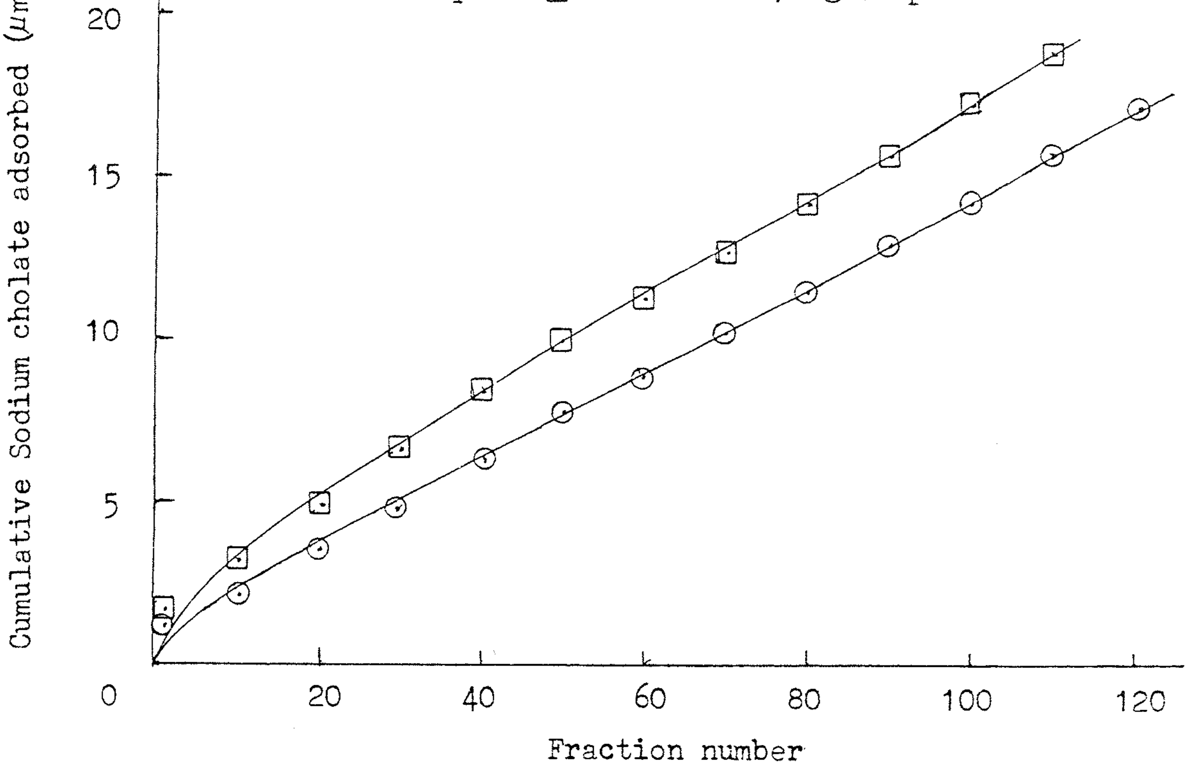


Figure 2E: Cumulative amount of cholic acid adsorbed by AA/HEMA/EDM terpolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine

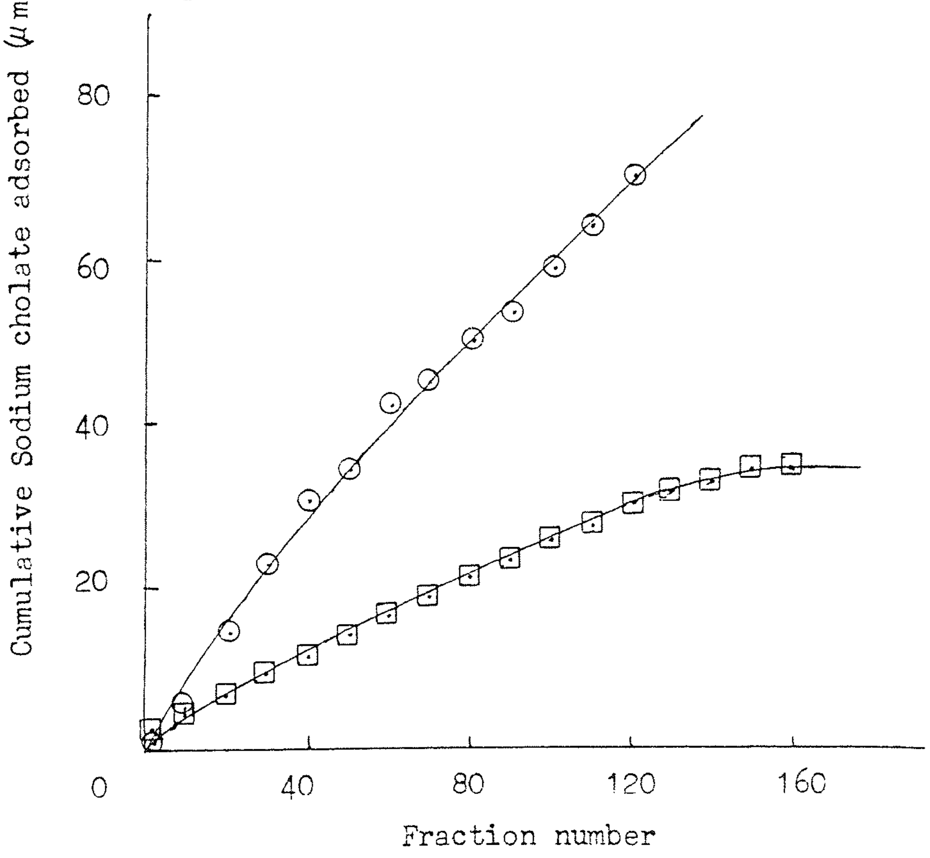


Figure 3E: Cumulative amount of cholic acid adsorbed by MAA/HEMA/EDM terpolymers

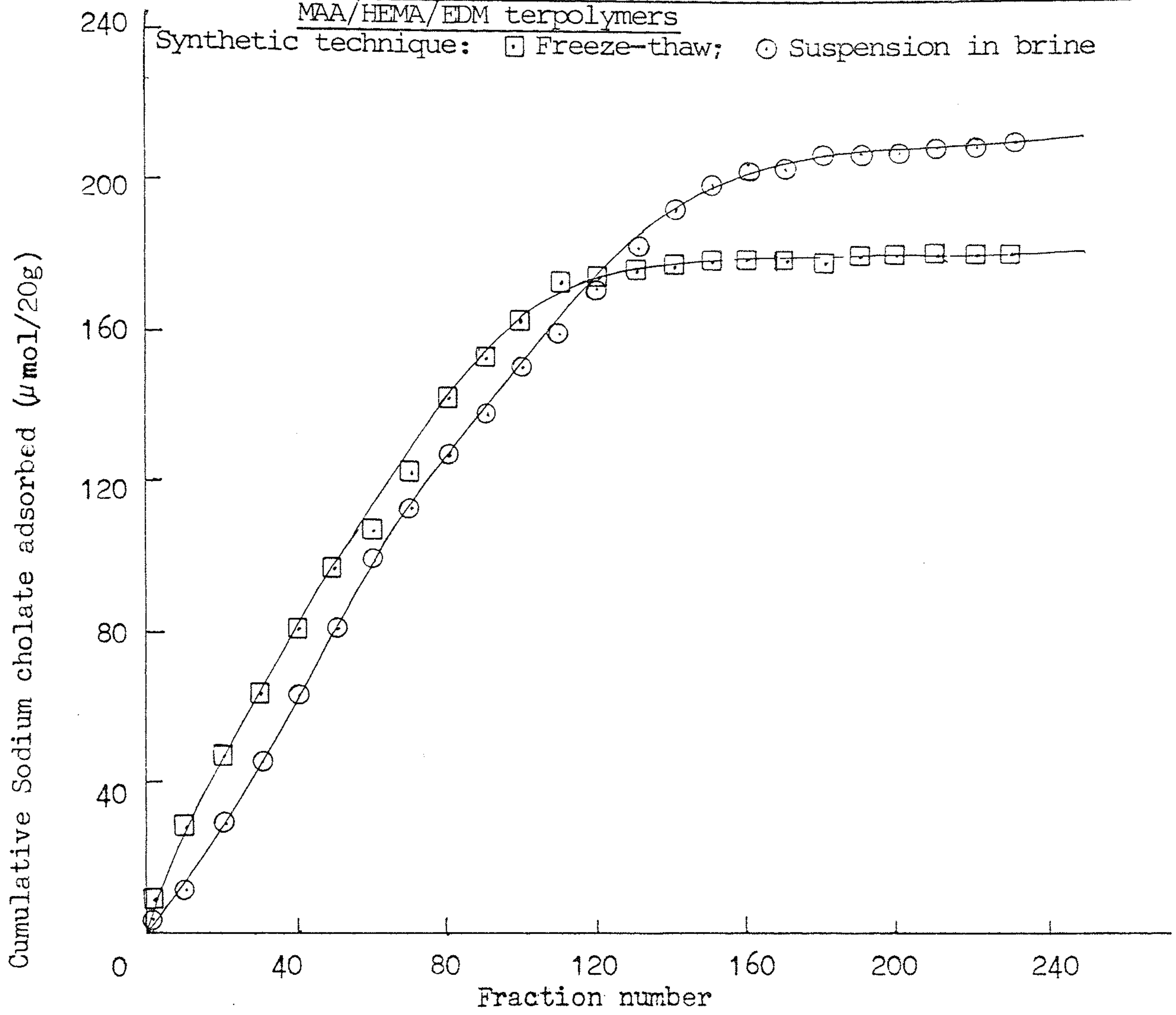
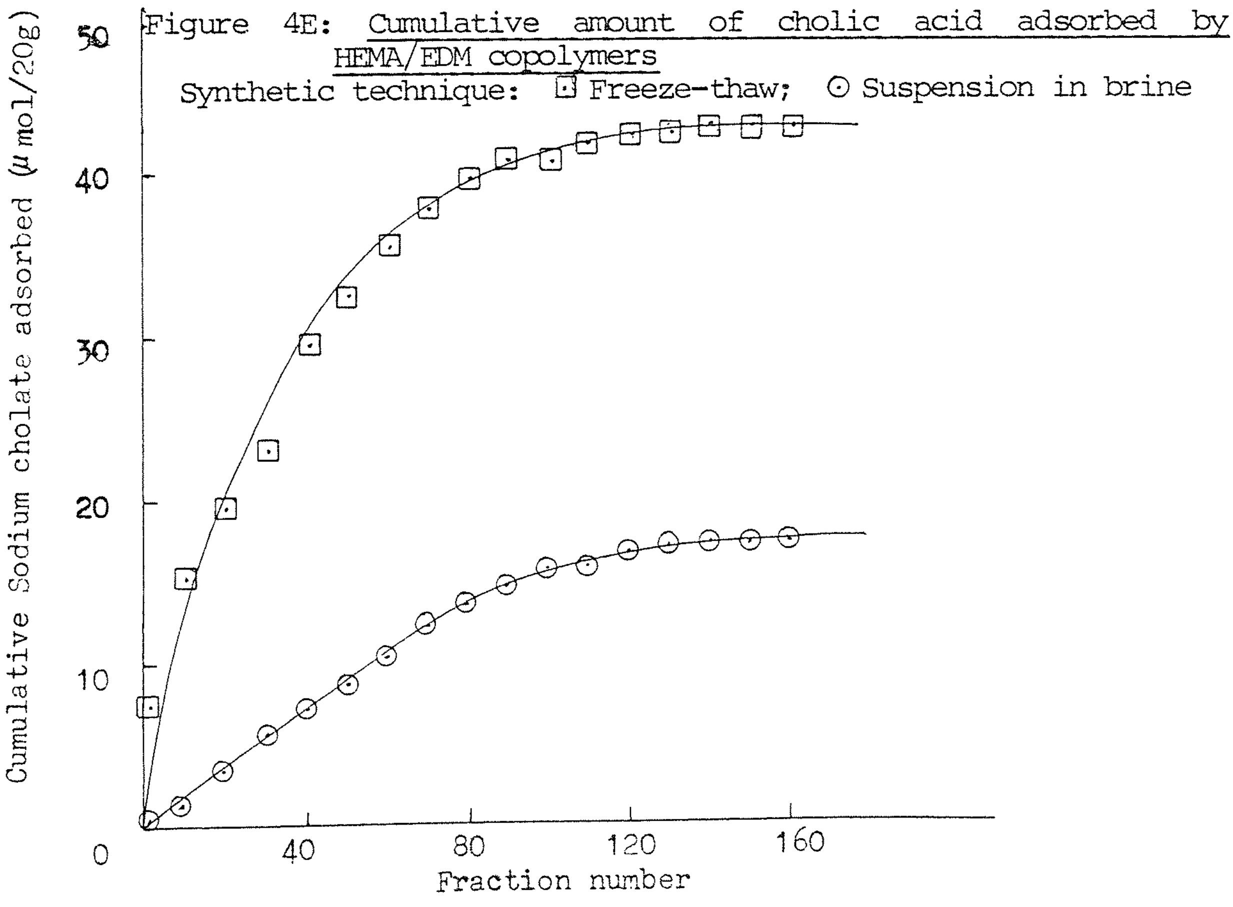


Figure 4E: Cumulative amount of cholic acid adsorbed by HEMA/EDM copolymers



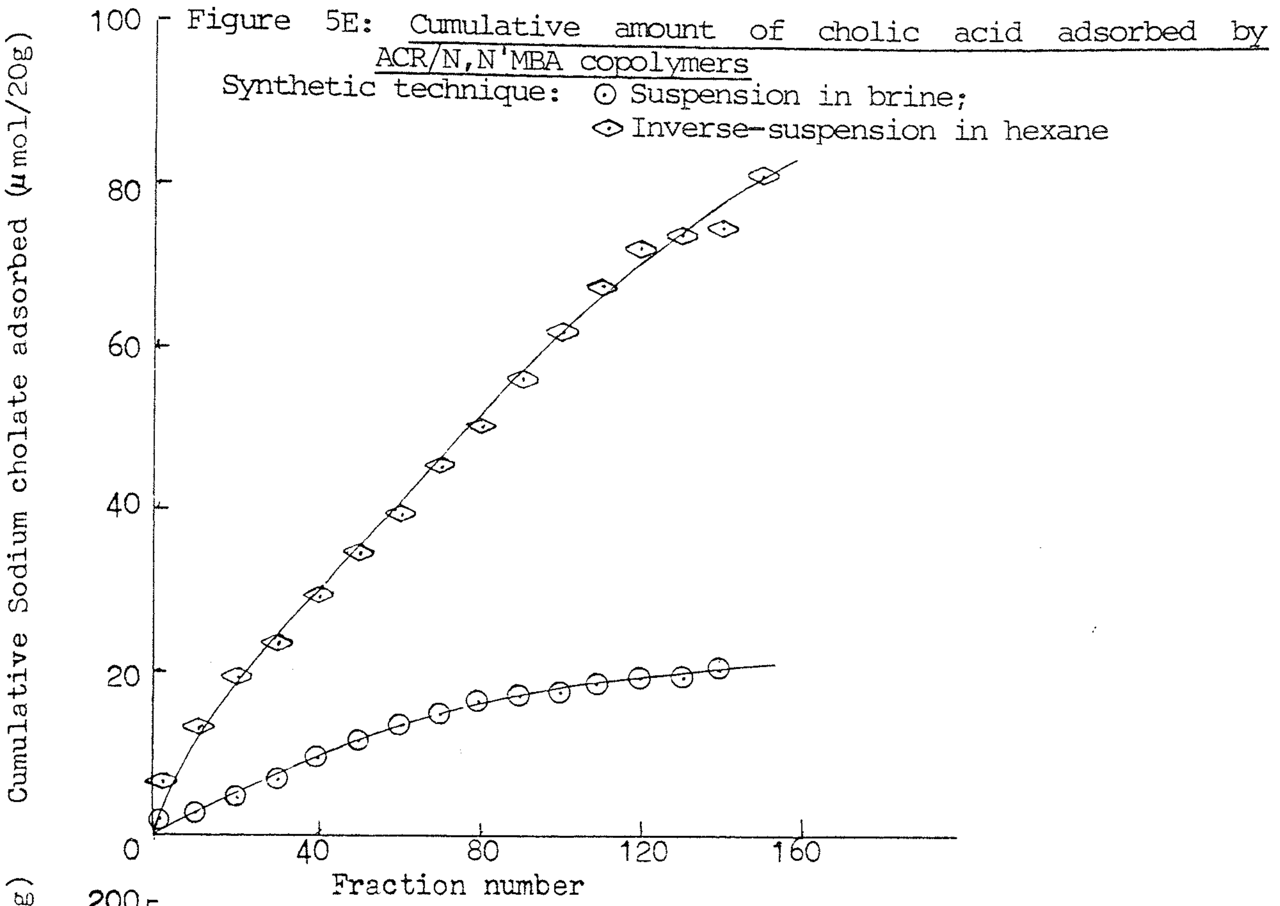


Figure 5E: Cumulative amount of cholic acid adsorbed by ACR/N,N'MBA copolymers

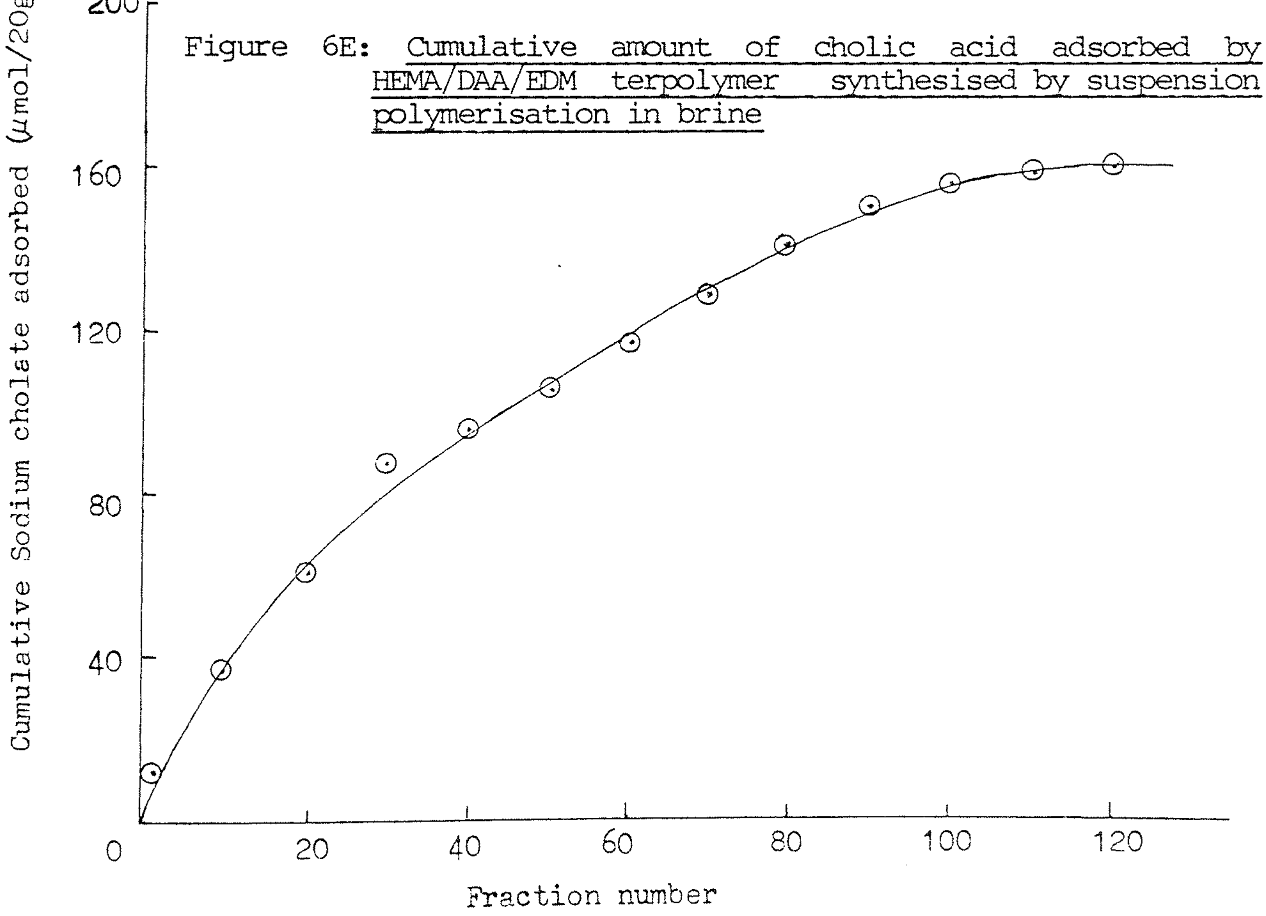


Figure 6E: Cumulative amount of cholic acid adsorbed by HEMA/DAA/EDM terpolymer synthesised by suspension polymerisation in brine

Figure 7E: Cumulative amount of cholic acid adsorbed by HEMA/NMACR/EDM terpolymer synthesised by suspension polymerisation in brine

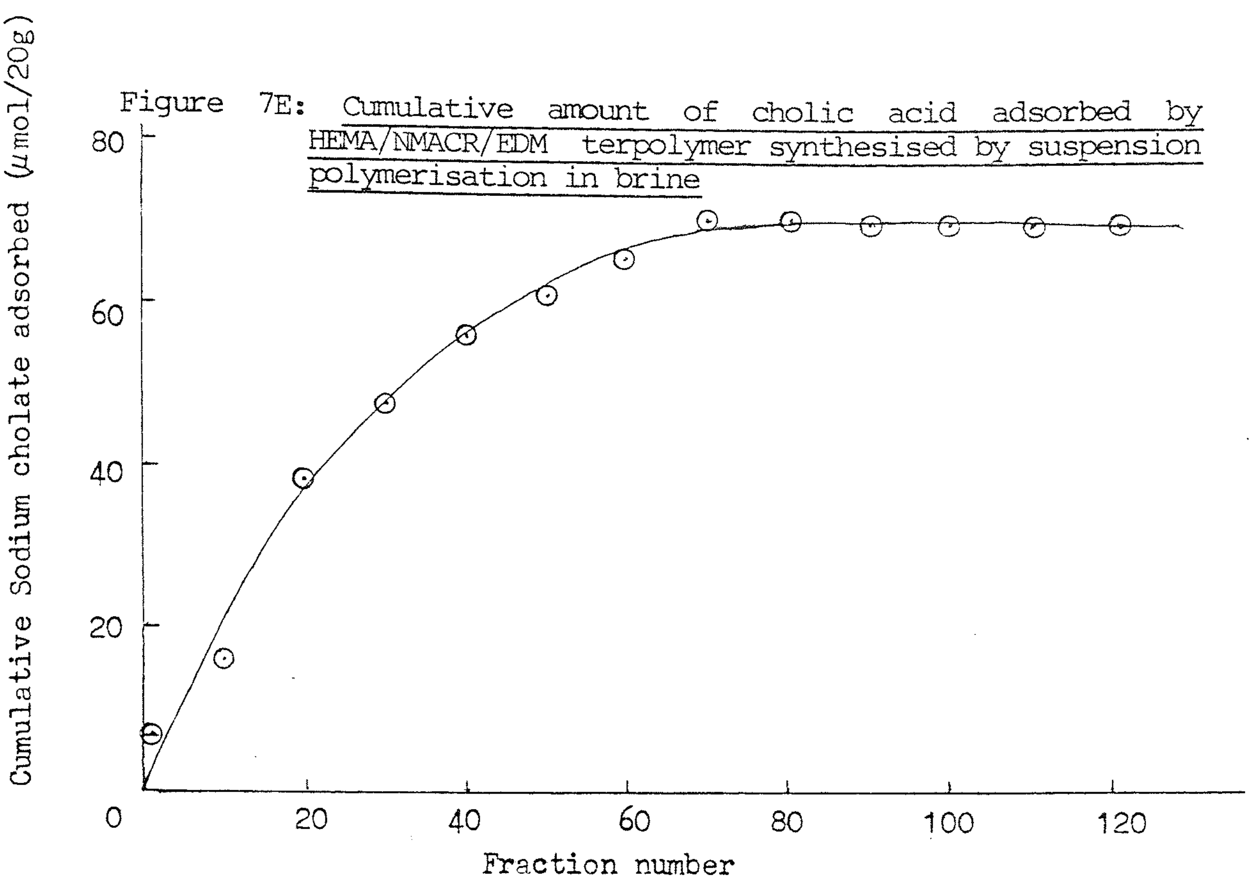
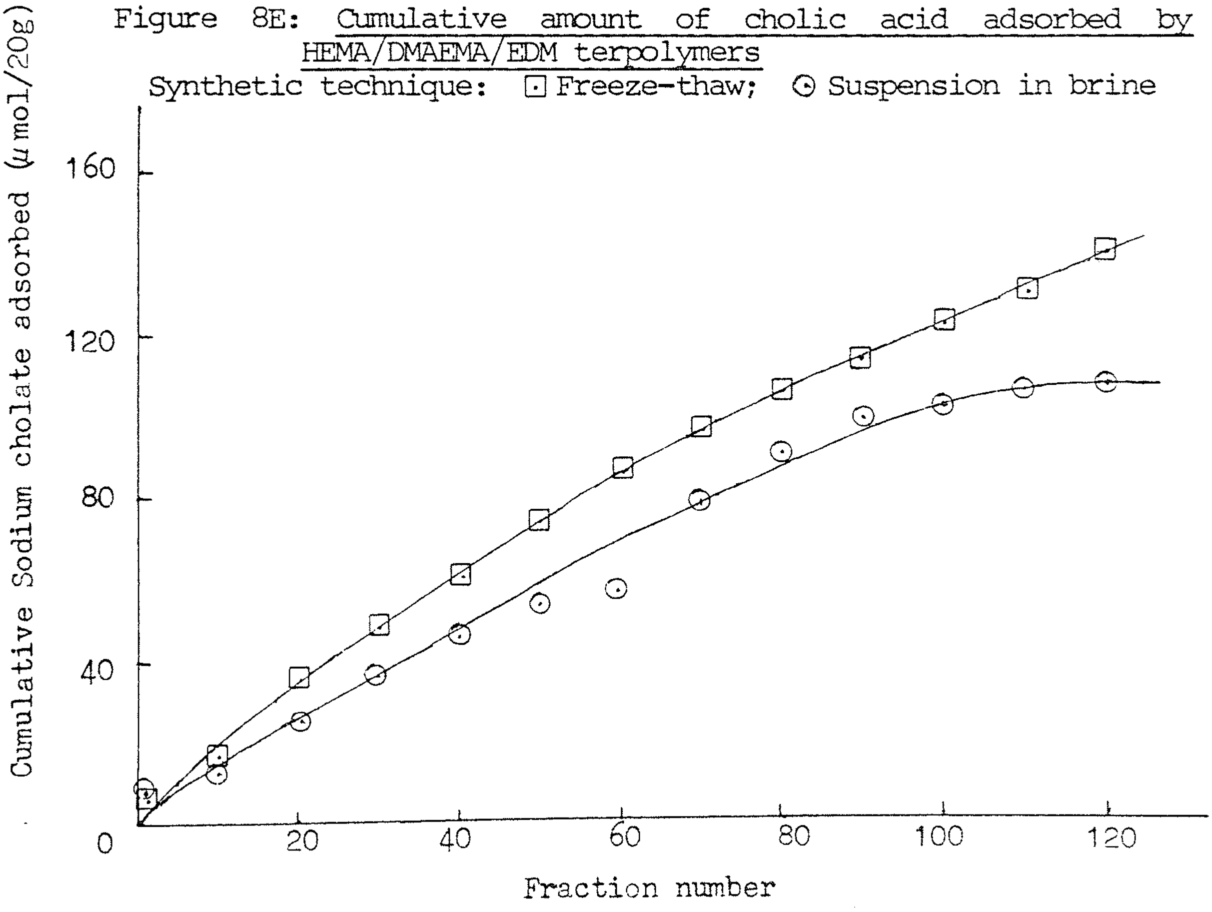


Figure 8E: Cumulative amount of cholic acid adsorbed by HEMA/DMAEMA/EDM terpolymers

Synthetic technique: ⊡ Freeze-thaw; ⊙ Suspension in brine



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