PART 1

THE METABOLISM OF FOLIC ACID (PTEROYL-L-GLUTAMIC ACID)

PART 2

THE SYNTHESIS AND TUMOUR INHIBITORY PROPERTIES OF SIMPLE HYDRAZINES

by

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SUMMARY

Pteroyl-L-glutamic acid (folic acid) is a vitamin which is now known to be widely distributed in nature, being found, usually in a reduced form, in plant and mammalian tissues and in bacteria. These reduced forms and their derivatives play a vital role as co-enzymes in biochemical systems, and it as such that one of their number is an essential participant in the multiplication processes of normal cells. There is increasing evidence to suggest that folic acid is implicated in the metabolism of tumour cells also characterised as they are by uncontrolled cell multiplication. An understanding of the biosynthetic and degradative pathways of this important vitamin would therefore seem desirable. Although the biosynthesis of folic acid is now fairly well elucidated, very little is known about its biochemical degradation, and it is with the latter that the first part of this thesis is concerned.

Isoxanthopterin, was considered to be a possible ultimate degradation product of folic acid. The development of a technique for the isolation and quantitative estimation of the isoxanthopterin found to be present in human urine, is described. The results of investigations into the metabolic degradation of folic acid in the rat using the above technique to isolate the end products, are reported and discussed.

During the last four years, a series of 1,2 disubstituted hydrazine derivatives have emerged as potent tumour inhibitors. Early work on the structure-activity relationship of these compounds suggested that the group $CH_3NHNHCH_2C_6H_4$-R was a common structural feature essential to tumour inhibition.

The synthesis and chemotherapeutic estimation of a series of alkyl and aryl substituted hydrazines essentially designed to test the validity of this relationship, is described. The tumour inhibitory properties of the synthesised hydrazines are discussed in relation to the current theories as to their mode of action, including their possible reaction with folic acid derivatives.

ACKNOWLEDGEMENTS

I am greatly indebted to the Committee of the Birmingham Branch of the British Empire Cancer Campaign whose generous help and support made this work possible.

I wish to express my sincere thanks to Dr. J. A. Blair for his supervision and encouragement of the work, to Dr. D. L. Woodhouse (University of Birmingham) for carrying out the chemotherapeutical tests, to Professor W. V. Thorpe (University of Birmingham) for the provision of animal facilities, to Dr. R. Gaddie (General Hospital, Birmingham) for arranging the supply of the necessary volumes of urine, and to Dr. G. B. Briscoe for guidance in the application of radiochemical techniques. I am also grateful to Mrs. B. Taylor for performing the elemental analyses, and to Mrs. V. Shearsby, Mr. E. J. Hartland and Mr. R. Brueton for the infrared, NMR, and mass spectra which they prepared.

I am indebted to Dr. P. Zeller (F. Hoffmann-La. Roche and Co., Basle, for the generous gift of samples of 1-methyl -2-benzyl hydrazine and N-isopropyl-α-(2-methylhydrazino) -p-toluamide hydrochloride (Natulan).

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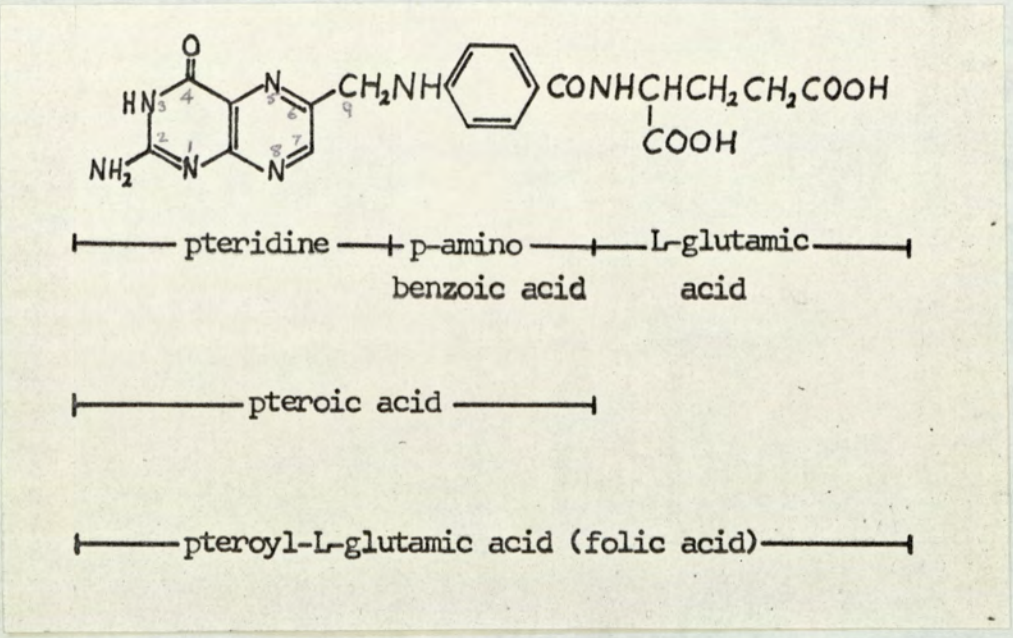
PART I

THE METABOLISM OF FOLIC ACID

(PTEROYL-L-GLUTAMIC ACID)

The Biological Role of Folic Acid and its Derivatives

Folic acid is a compound containing a substituted pteridine nucleus linked to p-aminobenzoic acid and L-glutamic acid, as shown in the following structural formulae:-

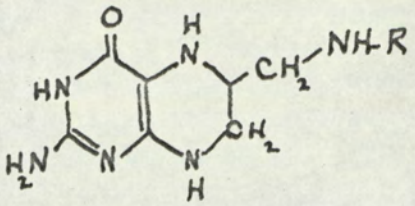


The name pteroyl-L-glutamic acid was assigned[1] because of the presence of a pteridine nucleus and a glutamic acid residue in the molecule. The name 'folic acid' is generally used however, although the name 'folacin' has been recommended by a joint nomenclature committee of the American Institute of Nutrition and the Society of Biological Chemists. The abbreviation PtGlu, recently recommended by the committee on nomenclature of the I.U.P.A.C.[2], will be used where necessary to specifically designate pteroyl-L-glutamic acid.

The biological properties of folic acid upon which attention was first focussed, were those as an anti-anemia agent or growth factor in humans, monkeys, chicks and bacteria. Since that time, and particularly since the final elucidation in 1948 of the structure of the pure pteroyl-L-glutamic acid by degradation[3] and synthesis[4], the vital role of folic acid in a considerable number of different metabolic processes has been increasingly appreciated. A number of reviews dealing with more detailed aspects of its chemistry[5,6] enzymatic activity[7-10], biological functions[6,11], clinical role[12] and metabolism[13], have now been published.

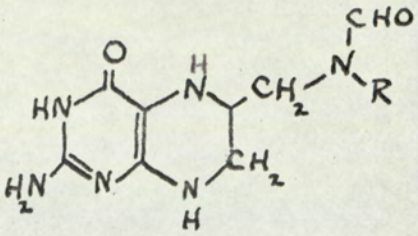
Folic acid exists in plant and animal tissues largely in the form of conjugates with more than one glutamic acid residue; in bacteria the pteroyltriglutamate (Pt Glu_3) seems to be the most prevalent form[13].

It is, however, the reduced forms of the vitamin and their derivatives, that play such a prominent part as co-enzymes in biochemical systems. The active co-enzyme forms are derivatives of tetrahydrofolic acid (formula page 3) and are involved in the biological transfer of one carbon atom units at the oxidation levels of formate, formaldehyde, and methanol, and in the interconversion of these different oxidative states[9,14]. The principal metabolically active forms which act as co-enzymes are formulated on page 3. One or other of these forms are involved in a large number of metabolic processes, involving particularly, the purine and



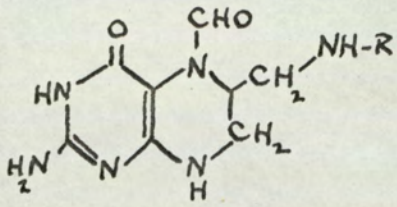
Tetrahydrofolic acid

(H_4-Pt Glu)



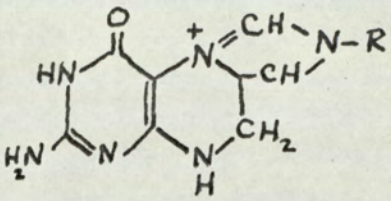
N^{10} Formyl tetrahydrofolic acid

(10-CHO-H_4 Pt Glu)



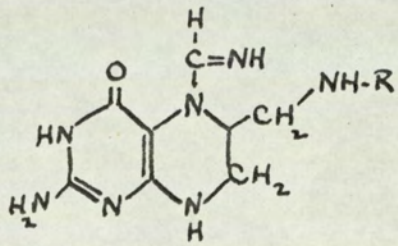
N^5 Formyl tetrahydro folic acid

or Citrovorum factor (5 CHO-H_4 Pt Glu)



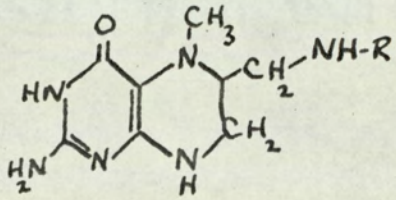
N^5, N^{10} Methenyl tetrahydro folic acid

(5,10, CH≡ Pt Glu)



N^5 Formimino tetrahydro folic acid

(5-CHNH-H_4 Pt Glu)



N^5 Methyl tetrahydro folic acid

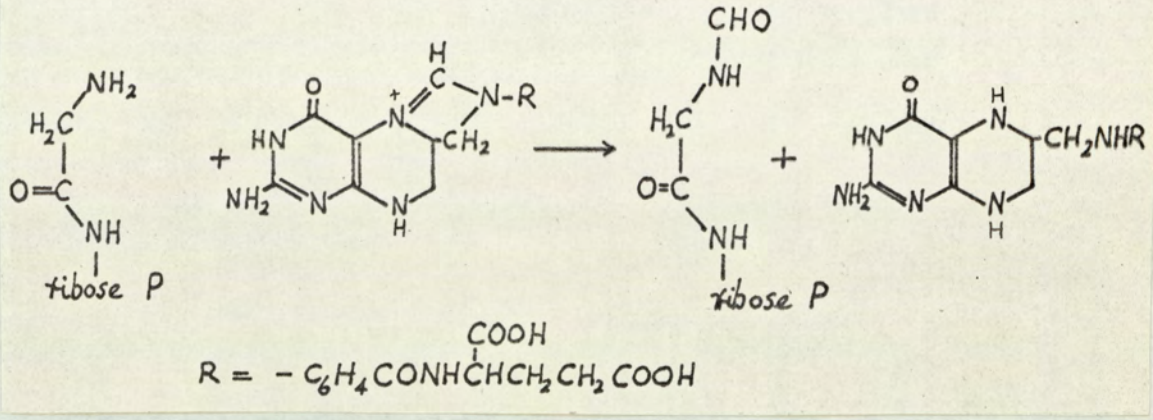
(5 CH_3-H_4 Pt Glu)

R = p-aminobenzoyl glutamic acid

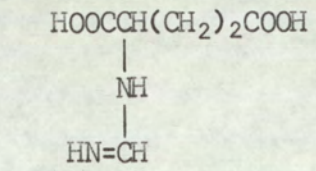
THE PRINCIPAL METABOLICALLY ACTIVE FORMS

OF FOLIC ACID

pyrimidine nucleotides eventually used in nucleic acid biosynthesis, and amino acids. For example, the introduction of the carbon atom which eventually becomes the 8-carbon of the purine ring is achieved by the formylation of glycinamide ribotide by 5,10 CH ≡ H_4Pt Glu to give formyl glycinamide ribotide[15,16]

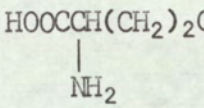


An example of a situation in which the co-enzyme becomes a one carbon unit acceptor is the reaction in which tetrahydrofolic acid is involved in the transformation of formiminoglutamic acid to glutamic acid:-[17]



formiminoglutamic acid
(FiGlu)

+ H_4Pt Glu $\xrightarrow[\text{forminotransferase}]{\text{FiGlu}}$



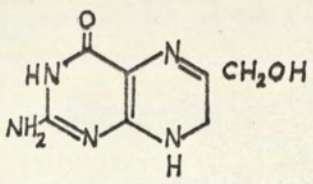
+5CHNH-H_4PtGlu

glutamic acid

The Biogenesis of Folic Acid

Woods and his co-workers[18-20] have demonstrated that p-aminobenzoic acid (PAB) and certain pteridines could increase folic acid production by resting cells of a staphylococcus aureus. Shiota[21] and more recently Wacker and co-workers[22], have obtained folate synthesis in cell-free extracts of Lactobacillus arabinosus and S. faecalis respectively by the addition of p-aminobenzoic acid and 2-amino-4-hydroxy-6-formyl pteridine. It has also been reported[23] that the combination of 2-amino-4-hydroxypteridine-6-carboxylic acid and PAB is catalysed in cell-free extracts of Myobacterium avium. From the detailed studies of cell-free extracts of Escherichia coli by Brown et. al.[24,25], and Jaenicke and Chan[26], the sequence of reactions for the synthesis of folic acid would appear to be as follows:

1.



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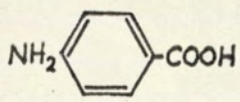
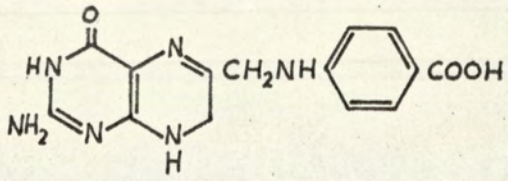


PAB

$\xrightarrow[Mg^{++}]{ATP}$

2-amino-4-hydroxy-6-hydroxydihydromethyl pteridine



Dihydropteroic acid (H_2 Pt)

2. H_2Pt + glutamate + $\xrightarrow{Mg^{++}}$ H_2Pt Glu + ADP + Pi

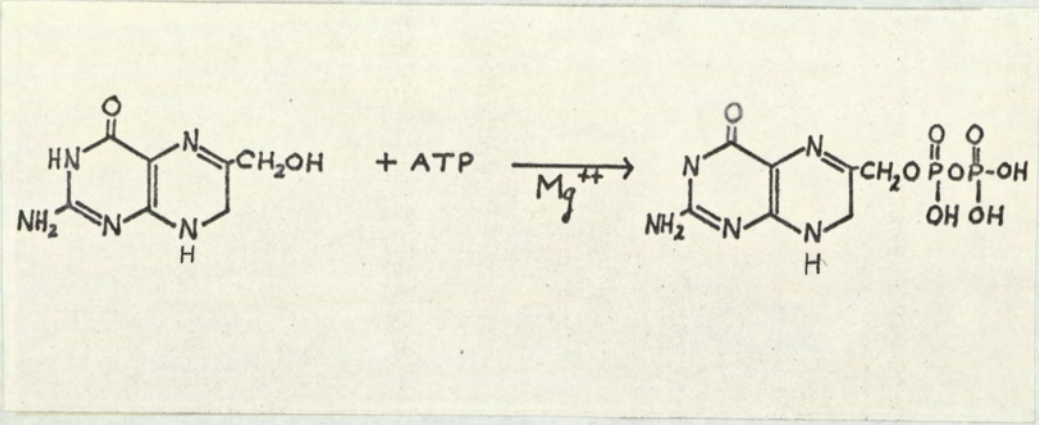
dihydropteroic acid — dihydrofolic acid

3. H_2Pt Glu + NADPH + H^+ ——→ H_4Pt Glu + $NADP^+$

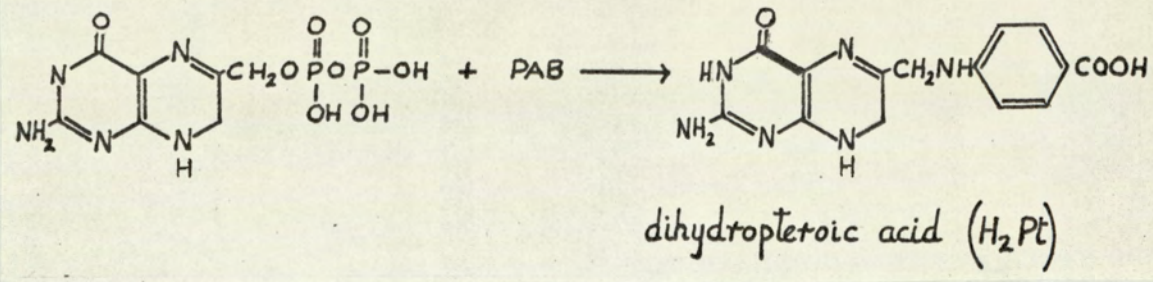
tetrahydrofolic acid

ATP	adenosine triphosphate
ADP	adenosine diphosphate
Pi	inorganic phosphate
NADP	nicotinamide-adenine dinucleotide phosphate.

The assumption that it is the dihydro form of 2-amino 4-hydroxy-6-hydroxymethyl pteridine rather than the tetrahydroform which condenses with p-amino benzoic acid, is based on the evidence that the biosynthesis of folic acid is more efficient with the former[26], and that dihydropteroate (H_2Pt) rather than tetrahydropteroate (H_4Pt) are formed as the reaction products[25]. Weisman and Brown[27] have shown that the condensation of the dihydropteridine with p-amino benzoic acid (PAB) in E. coli occurs in two distinct steps. The first involves the formation of the pyrophosphate ester of 2-amino-4-hydroxy-6-hydroxymethylpteridine, by the reaction of ATP with the pteridine in the presence of Mg^{++} ions:-



and the second involves the condensation of the pteridine-phosphate ester with PAB to produce dihydropteroic acid[28] :-



The dihydropteroic acid then condenses with glutamic acid to give dihydrofolic acid as already described. The addition of further glutamic acid residues to form folic acid conjugates only takes place after the reduction of dihydrofolic acid to tetrahydrofolic acid.

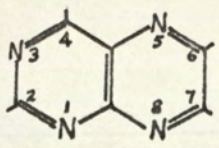
The Origin of the Pteridine Ring

The suggestion by Albert[29] that purines may be the

biological precursors of pteridines initiated a vigorous investigation into the origin of the pteridine ring itself and of any side chain attached to it.

One of the earliest investigations into the biosynthesis of pteridines was that of Weygand and Waldschmidt[30], who found that C-14 glycine and C-14 formate were weakly incorporated into leucopterin in Pieris brassicae. Although Korte and his co-workers[31] were unable to detect any incorporation of purines into folic acid in Streptococcus facaelis, purines have, in fact, been shown to be the metabolic precursors of pteridines in the insects Pieris brassicae[32] and Drosophila melanogaster[33,34], various amphibia[35-37] and bacteria[38,39]. Veira and Shaw[40] demonstrated that adenine 2-C-14 but not adenine 8-C-14 was incorporated into folic acid in Corynbacterium, although guanine was subsequently found to be a more immediate precursor.

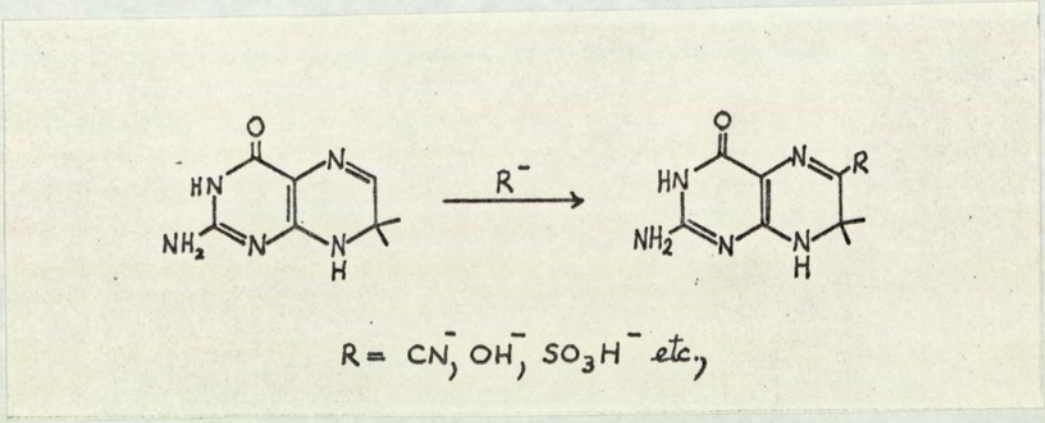
The origin of the side chain at position 6 of the pteridine ring has been a source of much controversy.



The numbering of the pteridine ring

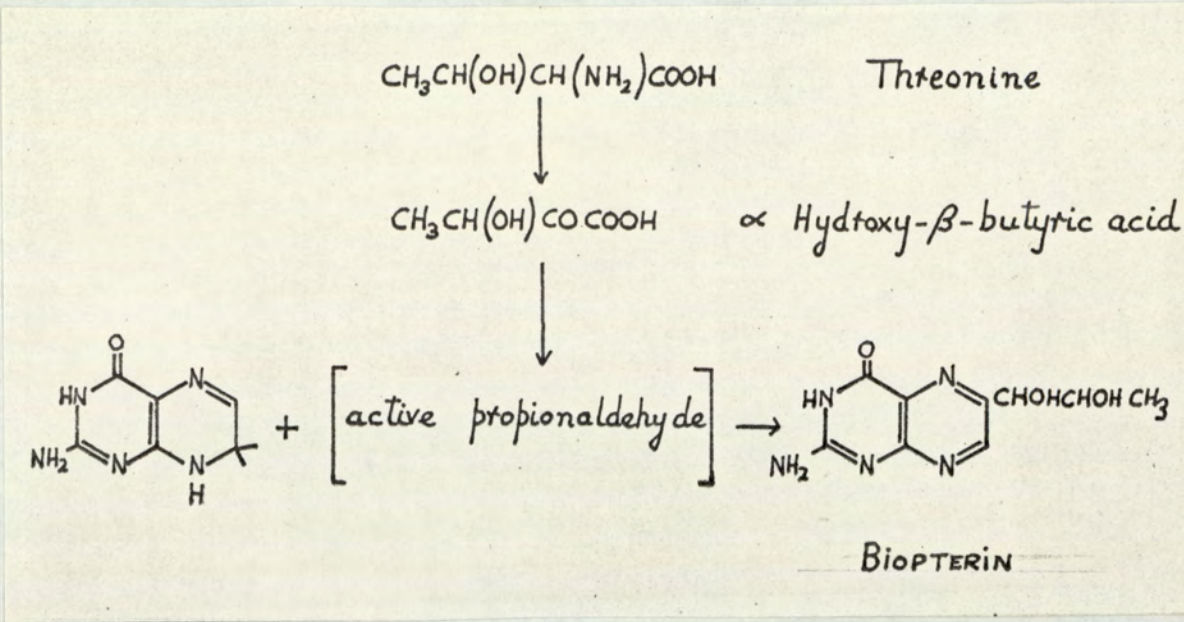
On the one hand, the demonstrated[41,42] chemical

ability of 2-amino-4-hydroxy-7,8-dihydropteridine to readily accept nucleophilic substituents at position 6, as illustrated below, has suggested to several workers that such a process could be of importance in the biosynthesis of 6-substituted pteridines [43,44].



Addition of nucleophiles to 2-amino-4-hydroxy-7,8-dihydropteridine

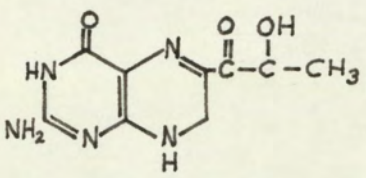
Support for such a pathway has now been claimed by MacClean, Forrest and Myers [45] on the basis of data which they interpret as showing that the amino acid, threonine, supplies the three carbon side chain at position 6 of bipterin. The pathway by which these workers suggest that this process takes place involves the deamination of threonine to yield α-keto-β-hydroxybutyric acid, which undergoes decarboxylation to active propionaldehyde, and this in turn is added to 2-amino-4-hydroxy-7,8-dihydropteridine.



A similar reaction to the latter step has been shown to occur non-enzymatically [44].

On the other hand, the fact that glucose and ribose were found [46-48] to be efficiently incorporated into the pteridine ring, led to the suggestion that the three carbon atom side chain at position 6 originated from a mono-saccharide residue, and that a pteridine with a trihydroxypropyl side chain in this position might be isolatable. Several compounds of this nature, including for example, neopterin (2-amino-4-hydroxy-6-(D-erythro-1',2',3'-trihydroxypropyl) pteridine) [49] and 2-amino-4-hydroxy-6-pteridinyl glycerol phosphate [50] have now been isolated. More recently, seemingly conclusive evidence that this side chain arises from the ribosyl group of the purine nucleotide has been

published. Krumdieck and his co-workers[39] found that, in growing cultures of Corynbacterium species, carbon atoms 6, 7, and 9 of folic acid arise from the carbon atoms 2', 1' and 3' respectively of the ribosyl moiety of guanylic acid. That this acid was efficiently incorporated into the pteridine ring confirms the work of Reynolds and Brown[38], who found that guanine only effectively played its part in the synthesis, as a nucleotide. Furthermore, observations on the biogenesis of sepiapterin

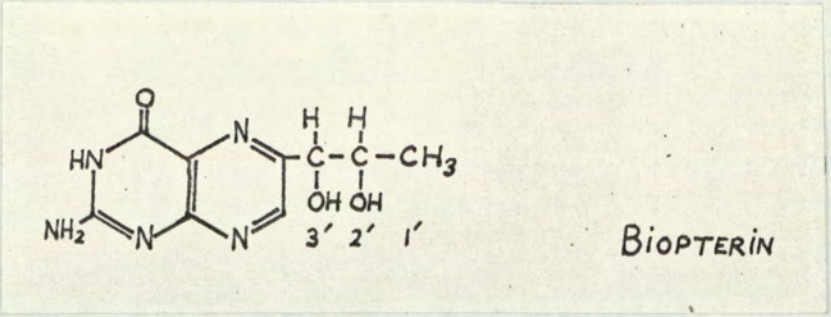


SEPIAPTERIN

in the butterfly Colias eurytheme, have indicated[51] that neither threonine nor β-hydroxybutyrate (both uniformly labelled) are incorporated into the side chain at position 6, and have confirmed that the pteridine ring and side chain arise from guanosine (or a phosphorylated derivative).

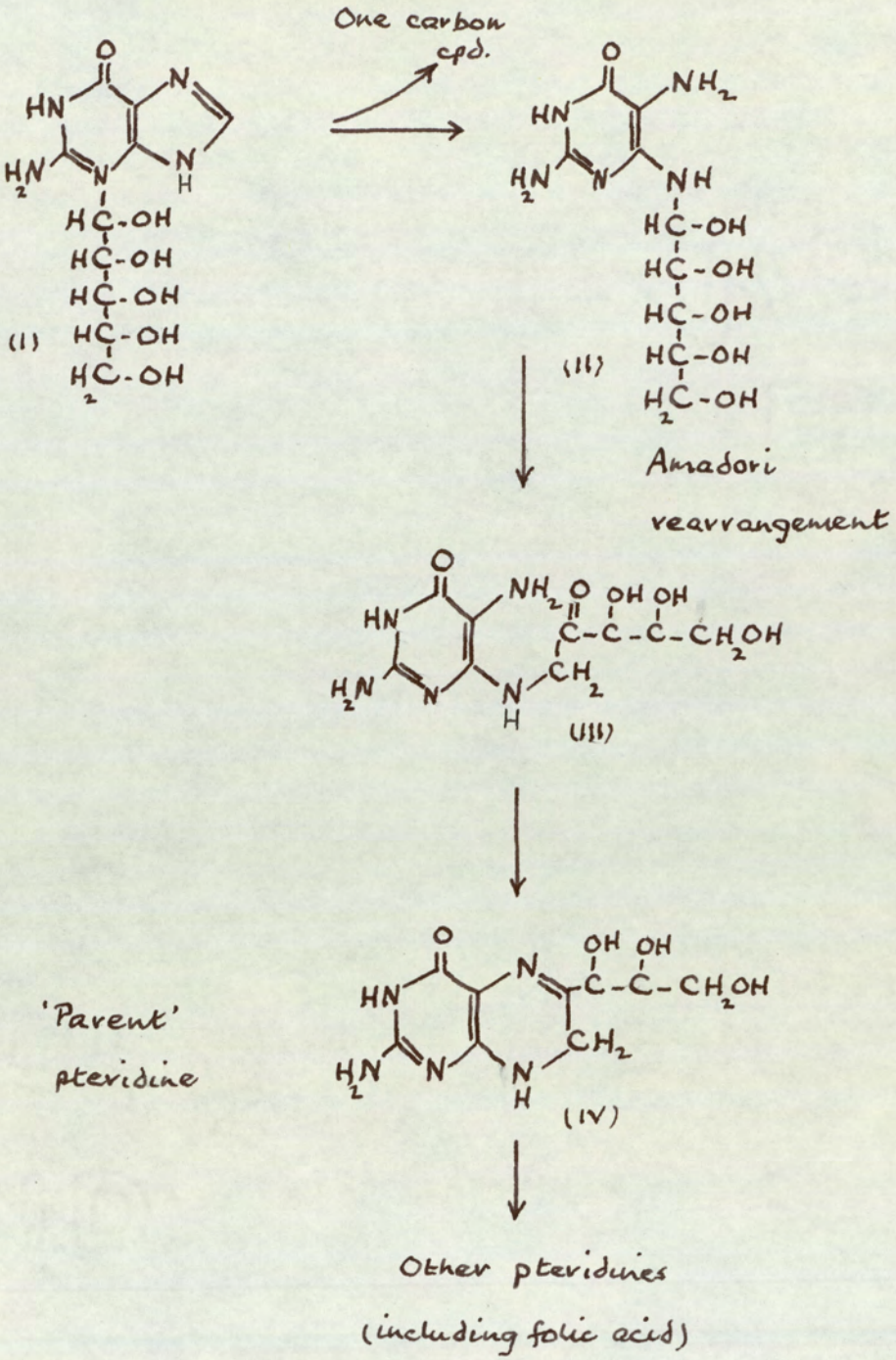
Finally, Brenner-Holzach and Leuthardt[52] have clearly demonstrated that, in biopterin extracted from Drosophila adult fruit flies to which glucose-6-C-14 and 2-C-14 had been administered, the carbon atoms 2' and 3' of the side chain are traceable to the

glucose atoms 5 and 6.



BIOPTERIN

On the evidence presently available, it would seem that the pathway of pteridine biogenesis is as formulated on page 13 . The imidazole ring of the purine (I) undergoes ring opening with the loss of the C-8 carbon to give a 5-amino-4-ribosylamino-pyrimidine (II). This glycosylamine then yields the corresponding 1-amino-1-deoxypentulose (III) by means of the Amadori rearrangement, and the latter compound cyclises to give the 6-substituted pteridine with a 3-carbon side chain. (IV). It would thus appear likely that this 2-amino-4-hydroxy-6 trihydroxy-propylpteridine probably in its reduced or phosphorylated form, is the "parent" compound from which the other 6-substituted pteridines are formed. Although, as is concluded by Watt[51], the formation of these pteridines with side chains of less than three carbon atoms, probably proceeds by the stepwise removal of the appropriate carbon atoms, it is still open to conjecture as to how a pteridine such as biopterin which contains a 6-substituted



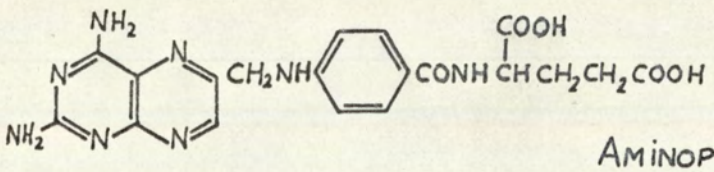
THE BIOGENESIS OF PTERIDINES

side chain of a different stereochemical configuration to the "parent", is formed.

In conclusion, it is of interest to note that a biochemical precedent for the type of rearrangement detailed above in which guanosine is converted to the "parent" pteridine has been reported by Yanofsky[53]. A chemical prototype has also been described by Stuart and Wood[54].

Folic Acid in Normal and Malignant Cells

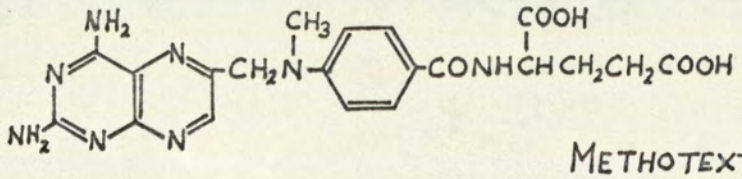
The fundamental importance of the pteridine co-enzymes in metabolic processes at the cellular level becomes apparent when cell division studies are undertaken. It is now known, that 5-formyltetrahydrofolic acid, produced in the cells of mammals by reductive formylation of folic acid, is essential to enable the chromosomes to undergo normal splitting (anaphase)[55] It is here that the dramatic effect of folate antagonists is readily seen, a small amount of aminopterin (4-amino-pteroyl-L-glutamic acid) being sufficient to completely halt cell division at the



AMINOPTERIN

previous, metaphase, stage[56].

Pteridines themselves have, for a number of years, been known to induce, in various species, a renal hypertrophy[57,58] which is characterised by increased cell division, although folic acid was initially found to be inactive in this respect[59]. Recently however, it has been observed that the injection of folic acid[60,61] and sodium folate[62] both, in fact, cause a considerable renal hypertrophy of this type in the rat, together with a marked increase in the rate of synthesis of deoxyribosenucleic acid. It is not unreasonable to expect therefore, that folates, as essential participants in the multiplication processes of 'normal' cells, may also play a prominent role in cancer, characterised as it is by uncontrolled cell division. It is significant in this respect, that it is an anti-folic agent, methotextrate (4-amino-N^{10}-methyl pteroyl-L-glutamic acid), which at present, is one of the drugs



METHOTEXTRATE

most widely used in the treatment of leukemias.

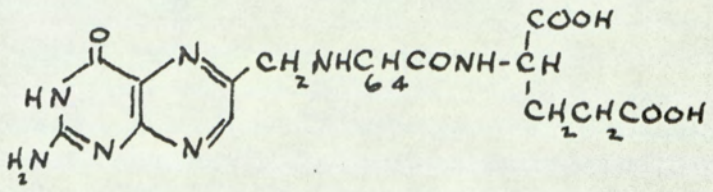
Swendseid and his co-workers[63] following up earlier work by Daft and Sebrell[64] who demonstrated that 'folic acid' was essential for leucocyte maturation in the rat, undertook an

investigation into folic acid concentrations in leucocytes of normal and leukemic patients. Their results showed that the folic acid concentration in the latter was considerably above the normal level, that it increased with the severity of the disease, and that the elevated folic acid levels could be normalised by radiotherapy. It has also been found[65] that certain neoplastic tissues are characterised by a higher level of folic acid activity than normal tissues. More recent experimental work[66-69] involving the detailed investigation of folate concentrations in patients with malignant blood diseases has indicated, for example, that most forms of leukemia are characterised by a deficiency of folic acid in the serum[66,67], and by an increase in the activity of such folate coenzymes as 5,10-methylene tetrahydrofolic acid dehydrogenase and 10-formyl-tetrahydrofolic acid synthetase in the leukemic cells[68]. In view of the apparent importance of folic acid and its derivatives in malignant diseases, it was therefore considered worthwhile to investigate the metabolism of this vitamin, with particular reference to its 'in vivo' degradation.

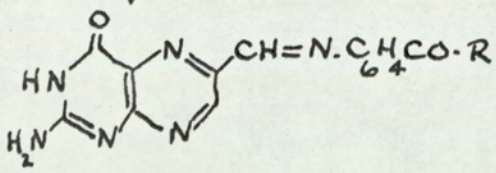
The Bio-degradation of Folic Acid

In view of the vigorous efforts which have been, and are being made, to elucidate the biosynthetic pathway to folic acid, it is surprising to find how little is known about its biochemical

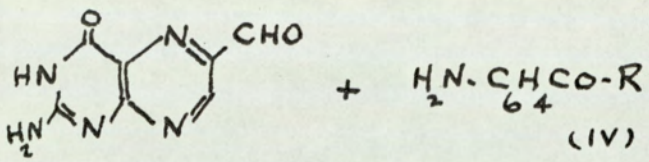
degradation. Blair[70] has shown that when pteroyl-L-glutamic acid (PtGlu) is incubated with the flavoprotein, xanthine oxidase, and methylene blue, 2-amino-4-hydroxypteridine-6-carboxylic acid is formed, as also is a much smaller amount of the corresponding 2-amino-4-hydroxypteridine-6-aldehyde. The relative proportions of these two products are reversed when the PtGlu is incubated with methylene blue alone. The pteroyl glutamic acid remains essentially unaffected when incubated with the xanthine oxidase alone. The widespread occurrence of isoxanthopterin (2-amino-4,7-dihydroxypteridine) in nature, considered together with the experimental results just described, suggest that isoxanthopterin may be a major end-product of the metabolism of substituted pteridines (including folic acid derivatives)[71,72], and that a feasible route by which this degradation could occur, would be as illustrated on page 18 . Compounds of the type (I) readily transfer hydrogen to suitable acceptors such as methylene blue and 2,6-dichlorophenol, to form anils (II), which are readily hydrolysed to the corresponding aldehyde and amine[3] . The generation from pteroyl glutamic acid (PtGlu) for example, of 2-amino-4-hydroxypteridine-6-aldehyde (III) and the corresponding carboxylic acid (V), by sulphurous acid cleavage[73] and alkaline hydrolysis[3] respectively, can be accounted for by such dehydrogenations.



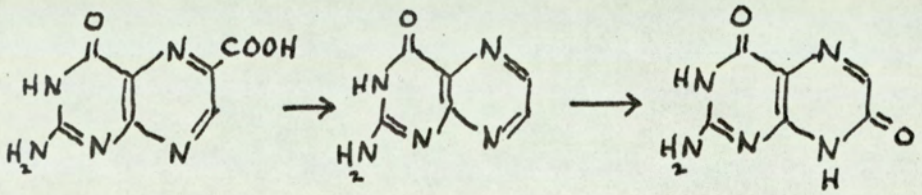
Pteroyl-L-glutamic acid (I)



Anil (II)



2-amino-4-hydroxy pteridine-6-aldehyde(III)



2-amino-4-hydroxy pteridine-6-carboxylic acid (V)

2-amino-4-hydroxy pteridine (VI)

Isoxanthopterin (VII)

$R = NH\text{-}CH(COOH)CH_2CH_2COOH$

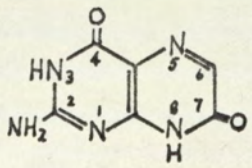
A SUGGESTED SCHEME FOR THE DEGRADATIVE METABOLISM OF FOLIC ACID

Xanthine oxidase[74,75] and liver aldehyde oxidase[76] have both been shown to be capable of effecting the oxidation of the above aldehyde to the carboxylic acid (V). The latter is readily decarboxylated by photolysis in solution[40,71] to give 2-amino-4-hydroxypteridine (VI), which in turn is efficiently converted to isoxanthopterin (VII) by the enzyme, xanthine oxidase[74,77]. That each individual stage of the suggested degradation pathway is biochemically feasible is clear from the experimental evidence detailed above. Evidence in support of the overall scheme has been published by Blair[78], who, subsequent to the incorporation of 2-C-14 pteroyl-L-glutamic acid into the food of Drosophila melanogaster, was able to isolate radioactive isoxanthopterin. The chemical degradation of folic acid to isoxanthopterin via 2-amino-4-hydroxypteridine-6-carboxylic acid, has also been demonstrated by Shaw and his co-workers[79].

As the presently available evidence would thus appear to suggest that isoxanthopterin is a major end-product of the 'in vivo' degradation of folic acid, it is clear that an investigation into the validity of this suggestion, which is the aim of the first part of this thesis, necessitates an efficient, and if possible rapid, technique for the isolation and quantitative estimation of isoxanthopterin from natural sources. It was decided to attempt the development of the necessary analytical method using human

urine which is known to contain isoxanthopterin[70], and which was readily available in large quantities. The procedures adopted for the successful detection, isolation and estimation of isoxanthopterin from the urine are discussed (page 24) and detailed (page 57).

Carbon-14 tracer studies being ethically unacceptable in the human system, it was considered feasible to follow the metabolic pathway under examination using pteridines enriched with the non-radioactive carbon-13 isotope. Tanabe and Detre[80] have recently reported the use of such isotopic techniques in biosynthetic studies. It was planned to use 6-^{13}C-pteroyl glutamic acid and to characterise any resulting 6-^{13}C enriched isoxanthopterin appearing in the urine by means of its nuclear magnetic resonance spectrum, either by identification of the ^{13}C atom itself, or the proton attached to it.



ISOXANTHOPTERIN

However, as the quantity of isoxanthopterin found to be present in urine was too small to enable such identification techniques to be utilised, the investigation of the metabolic degradation of folic acid was therefore pursued in the rat using 2-^{14}C labelled folic acid. After the administration of the

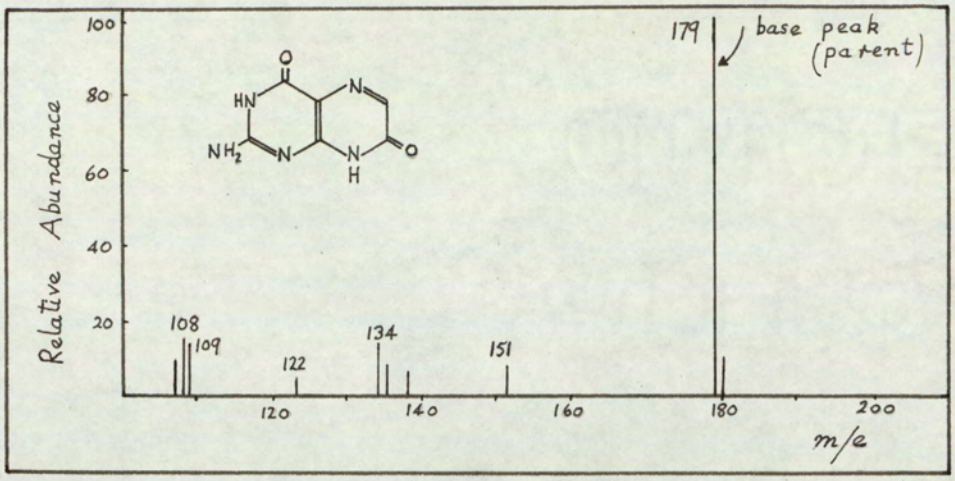
compound to the animal, the urine was analysed for radioactive isoxanthopterin using the techniques previously developed for human urine. The results so obtained and their implications with regard to the 'in vivo' degradation of folic acid, are discussed in the next section.

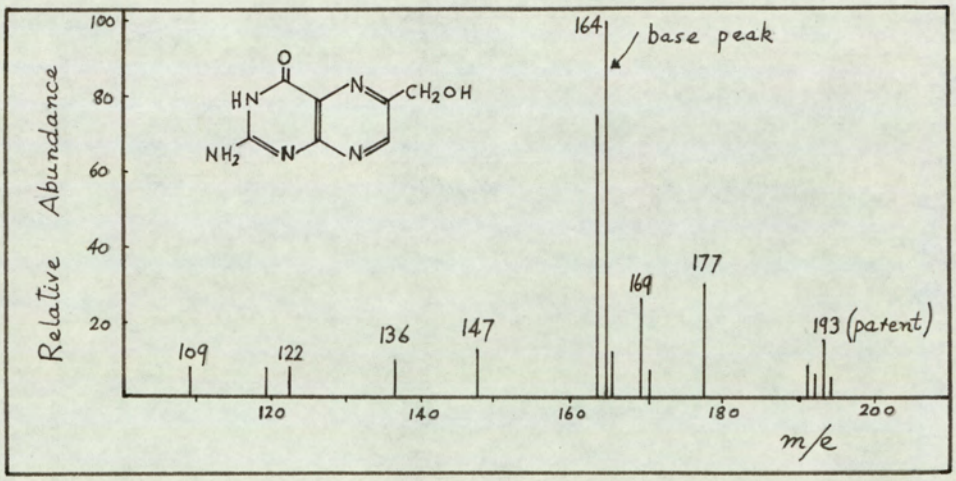
Discussion and Conclusions of Experimental Work

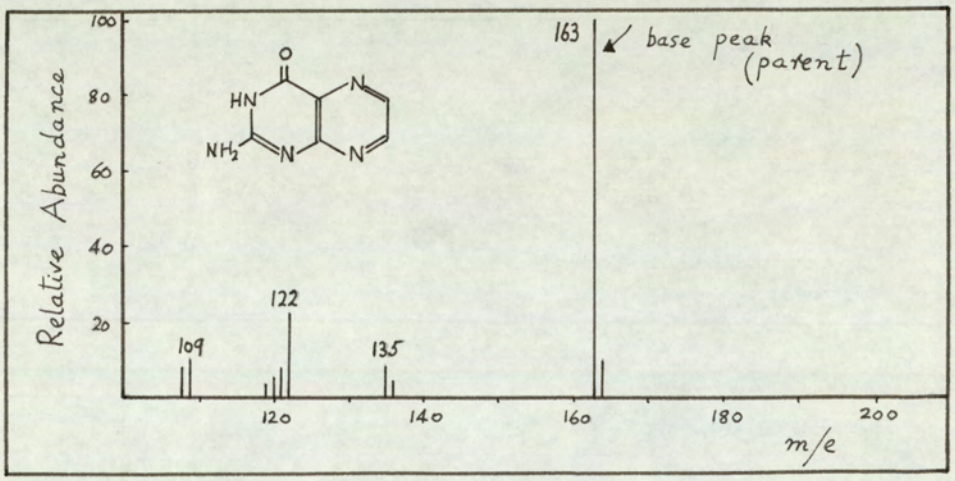
1. The Synthesis and Mass Spectra of Isoxanthopterin and Similar Pteridines

The synthesis of 2-amino-4,7-dihydroxypteridine (isoxanthopterin), 2-amino-4-hydroxy-6-hydroxymethylpteridine and 2-amino-4-hydroxypteridine using the procedures to be found in the literature are detailed on page 53 . The UV and NMR spectra, together with paper chromatographic analysis, satisfactorily established the identity and the purity of the synthesised products. In common with previous workers, it was found that the pteridines gave unsatisfactory and inconsistent elemental analysis figures.

It was observed however that the pteridines give characteristic and well defined mass spectra in which the parent peak, except in the case of 2-amino-4-hydroxy-6-hydroxymethylpteridine, is by far the most prominent feature. The major peak in the spectrum of the latter compound corresponds to the pteridine nucleus after cleavage of the hydroxymethyl group. A comparison of these mass spectra (page 23), which have not been previously recorded, reveals that the pteridines also have closely similar cracking patterns. These spectral features suggest that mass spectrometry could be a powerful new tool in the analysis and identification of the pteridine nucleus. The applications of mass spectrometry to the investigation of the metabolism of folic acid is considered later in the discussion.







Pteridines : Examples of Mass Spectra

2. The Isolation of Isoxanthopterin from Urine

The analysis of such a complex chemical mixture as urine, for compounds which are present only in minute quantities, is likely to be a very tedious and inefficient process, particularly until an adsorbent is found which is capable of preferentially adsorbing the type of compound under investigation. The choice of adsorbent is therefore of primary importance.

(a) Choice of Adsorbent:- A number of pteridines have now been isolated from urine, the separation techniques employed being very diverse. Koschara[81] succeeded in isolating 2-amino-4,6-dihydroxypteridine (xanthopterin or uropterin as it was then known) from human urine by a process which involved the treatment of acidified urine with Fullers earth, a material which adsorbs both pteridines and purines. The Fullers earth adsorbate was then subjected to repeated extraction with a variety of reagents including sodium hydroxide, pyridine and silver nitrate, until a pure sample of the xanthopterin was obtained.

A considerably improved analytical method is due to Patterson and his co-workers[82], who isolated biopterin from urine by extracting the pteridines and similar compounds onto charcoal. The charcoal was then eluted with a mixture of ethanol, water and ammonium hydroxide. The biopterin which was thus recovered (in very poor yield) was isolated and purified by the use of counter-

current solvent distribution and partition chromatography. A further technique for the isolation of urinary pteridines has been reported by Blair [83], who extracted freshly voided urine with liquid phenol, the phenol layer then being extensively washed with ether. The aqueous phase resulting from the extraction of these ethereal washings with water, was then evaporated to dryness and repeatedly extracted with dilute ammonium hydroxide. The analysis of this extract by paper chromatography yielded isoxanthopterin.

An adsorbent which is now finding increasing application in the isolation and purification of biological materials is florisil, a synthetic magnesium silicate. In the field of pteridine chemistry it was used by Hutchings and co-workers [84] to effect the purification of pteroyl-L-glutamic acid and by Dimant et. al. [85], to carry out the preliminary isolation of benzpteridine derivatives (flavins). One of the distinct advantages of florisil as an adsorbent, as was observed by Forrest and Mitchell [86], is that, although pteridines are strongly adsorbed (and particularly so) under acid conditions, they can be readily removed by even weakly alkaline eluents.

A technique which would seem to be particularly suitable for the isolation and purification of small quantities of pteridines, is that of ion exchange chromatography. Although Forrest and

Mitchell [86] found that such resins as Amberlite IR 100, IR 50 and Dowex 50 were ineffective in the purification of pteridine pigments isolated from Drosophila melanogaster, Rembold and Buschmann [87] have recently reported the development of an ion exchange technique for the reproducible separation of a considerable number of pteridine mixtures. The substances are fractionated on a Dowex column with water and a formic acid-ammonium formate gradient. The resulting fractions are then further separated using ion exchange cellulose. By this method the authors were able to efficiently separate microgram quantities of pteridines.

A combination of several of the analytical procedures already described has been employed by Sakurai and Goto [49] to isolate neopterin (2-amino-4-hydroxy-6-(D-erythro-1',2',3', trihydroxypropylpteridine) from human urine. Their technique consisted of passing the acidified urine through a florisil column, and then adsorbing the pteridines present in the column eluates onto charcoal. Leaching of the charcoal with 2% ammonium hydroxide-ethanol (3:1) followed by a further purification of the resulting eluate using a Dowex ion exchange column, resulted in the isolation of the pure neopterin.

A consideration of the various adsorbents, which as described above, have been employed in the isolation and purification of pteridines, suggested that florisil, or possibly charcoal, might

be suitable to effect the recovery of isoxanthopterin from human urine.

Preliminary experiments to evaluate the suitability of charcoal in this respect were carried out using xanthopterin (page 55). The results indicate that such pteridines are very strongly adsorbed even from alkaline solutions, and that even the use of a boiling solution of aqueous pyridine failed to re-extract any xanthopterin from the charcoal. It is significant in this respect that Forrest and Mitchell[86] were also unable to find a suitable eluent to effect the re-extraction of pteridine pigments adsorbed onto charcoal, even when the latter had been previously deactivated by impregnation with stearic acid. On the other hand, as has already been mentioned, several workers [49,82] appear to have successfully employed a mixture of ethanol and ammonia to remove pteridines such as biopterin and neopterin from charcoal. The large volumes of this eluent necessary to effect the removal however, together with the likelihood that the elution would be far from quantitative, render the use of such a solvent unattractive. It was therefore concluded that charcoal was unlikely to prove a suitable adsorbent for the isolation of isoxanthopterin from urine.

In contrast, similar preliminary experiments (page 56) to evaluate florisil as an adsorbent indicated that although isoxanthopterin was strongly adsorbed in acid conditions, elution of the

pteridine could be rapidly and efficiently effected with dilute (0.5N) ammonium hydroxide at room temperature. It was therefore decided to attempt the preliminary extraction of any isoxanthopterin present in the urine by means of florisil.

(b) Column Chromatographic Procedure:- Preliminary experiments (page 56) to ascertain the adsorption characteristics of isoxanthopterin on a florisil column under various pH conditions indicated that even after elution with 5 litres of 1N hydrochloric acid and 500 ml. of distilled water, the pteridine band remained strongly adsorbed at the top of the column, thus making it unlikely that any isoxanthopterin initially adsorbed from the acidified urine, would be eluted by the large volumes of urine subsequently passed through the column. Although further elution of the florisil test column with 11.5 litres of buffer solution (pH 7.6) caused a considerable diffusion of the intensely fluorescent isoxanthopterin band, none of the pteridine was removed from the column, as was determined by the absence of fluorescence in the eluate. Further elution of the column with dilute (0.5N) ammonium hydroxide was however found to effectively remove the isoxanthopterin from the florisil.

On the basis of this chromatographic data it was decided to attempt the isolation of isoxanthopterin from human urine by the following method, the detailed procedure of which is given on page 57 .

The acidified urine was passed through a florisil column made up in 1% hydrochloric acid and the column then eluted successively with (a) distilled water (to remove any acid left in the column) (b) buffer solution pH 7.6 and (c) dilute ammonium hydroxide. The elution of the column with borate buffer solution was designed to remove the non-pteridine materials which would otherwise emerge in the ammonium hydroxide eluate. The various eluates were then evaporated to dryness and extracted with dilute ammonium hydroxide. The paper chromatographic procedures adopted for the analysis of the resulting ammoniacal extracts for isoxanthopterin, are considered later in this discussion.

The first urine analysis (page 62) did, in fact, reveal the presence of isoxanthopterin, and as would be expected from the preliminary experiments carried out to determine the adsorption characteristics of this pteridine on florisil, the isoxanthopterin was found only in the ammonium hydroxide eluate. Although this analysis was successful in that the presence of isoxanthopterin in urine was definitely established, it also served to spotlight points at which the analysis technique could, with profit, be streamlined. One of the major problems encountered was in the concentration of the borate buffer eluate of the florisil column. The evaporation of large volumes of buffer solution resulted, as

was to be expected, in the deposition of considerable quantities of inorganic residues, which not only increased the chance that some isoxanthopterin would be lost by adsorbtion onto them, but also rendered the task of reducing the eluate to a volume which could be conveniently handled by the techniques of paper chromatographic analysis, considerably more difficult. In order therefore to separate the majority of the inorganic salts which compose the buffer solution, from any pteridines present, and thus to simplify the work-up procedure,the total buffer eluate from the florisil column was, prior to concentration, acidified to approximately pH 2 with concentrated hydrochloric acid, and then passed through a second florisil column identical to the first. By this means the inorganic salts pass straight through and any pteridines present are adsorbed onto the florisil. This second column was then eluted with

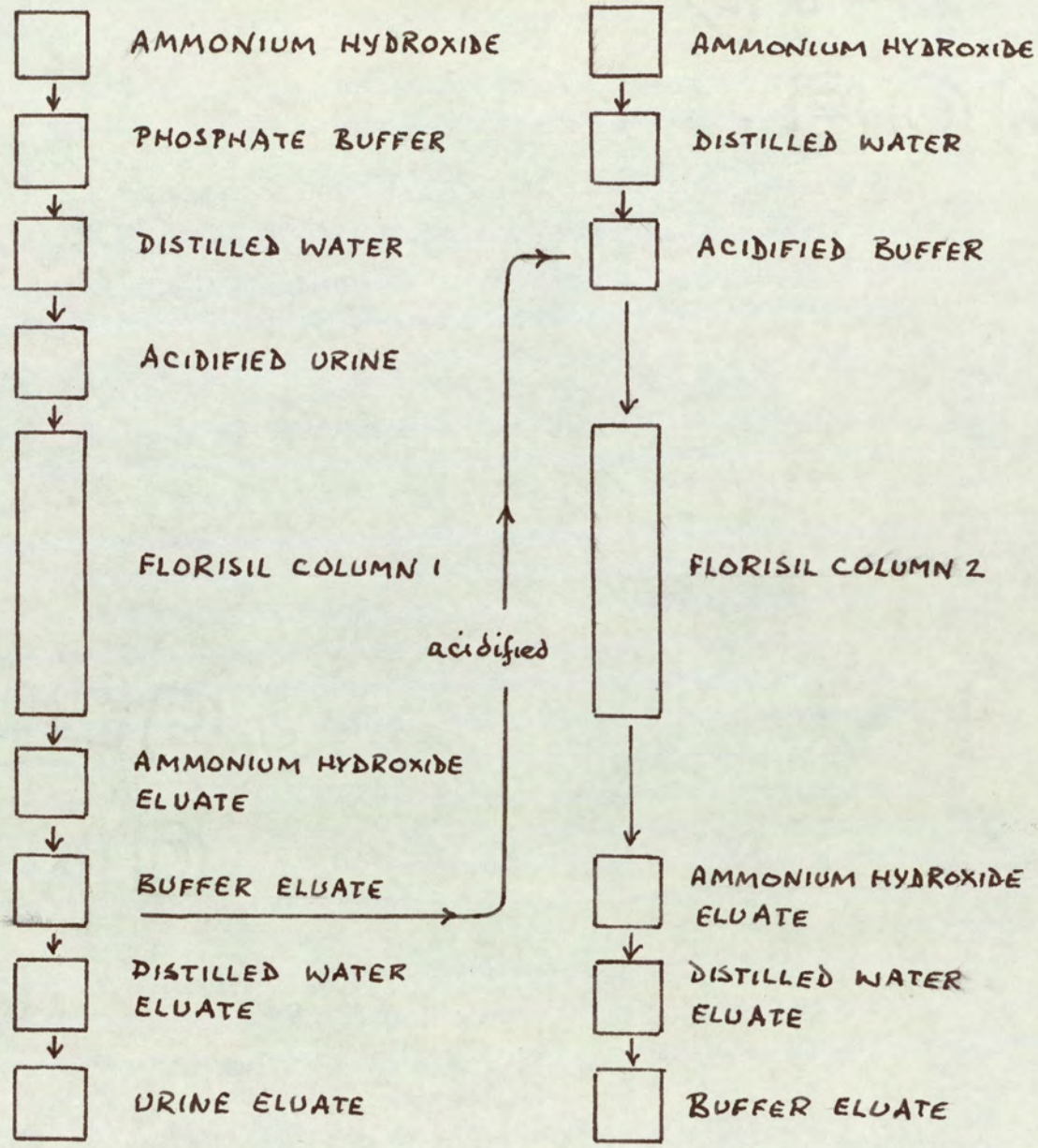
a) distilled water (to remove last traces of acid and inorganic salts

and b) dilute ammonium hydroxide (0.5N)

The ammonium hydroxide eluate was then concentrated and analysed by paper chromatography in the usual way. The use of the second florisil column as described above was found to so facilitate the work-up of the buffer eluate, that it was adopted as standard practice for subsequent urine analyses. By way of a summary, the column chromatographic procedure which was finally adopted for the analysis of volumes of urine of two litres or more,

is detailed on page 32 . The acidified urine was passed through florisil column 1, and the column then eluted with distilled water and buffer solution. The latter was then acidified, and passed through florisil column 2 which was, in turn, eluted with distilled water and ammonium hydroxide. The latter eluate was then analysed for isoxanthopterin by paper chromatography. Isoxanthopterin was found in the ammonium hydroxide eluate of the first florisil column only in the first urine analysis; in subsequent analyses the pteridine was present only in the buffer eluate and thus finally emerged in the ammonium hydroxide eluate of the second column. The ammonium hydroxide eluate of the first florisil column was therefore eliminated from the analytical procedure.

As the main aim of urine analyses 1-5 was to detect and isolate a pure sample of isoxanthopterin, relatively large volumes of urine were employed, which necessitated a somewhat cumbersome column chromatographical procedure. In the final urine analysis (6), however, in which the principal object was to achieve a much more rapid analysis for isoxanthopterin, the use of a much smaller quantity of urine enabled the analytical procedure to be still further simplified. The method consisted of passing the freshly voided and acidified urine (250 ml) through a small florisil column, which was made up in 1% hydrochloric acid in the usual way. As described on page 69 , the column was then simply



COLUMN CHROMATOGRAPHIC PROCEDURE

FOR THE ANALYSIS OF HUMAN URINE.

eluted with distilled water and dilute ammonium hydroxide. The latter eluate was then concentrated and examined by paper chromatography in the normal manner, as described in the next section. As a relatively small volume of freshly voided urine was used, any interfering residues resulting from the concentration of the ammonium hydroxide eluate were minimal, thus considerably simplifying the paper chromatographic procedure also. It was found that three successive chromatograms in alternate solvent systems were sufficient to yield a chromatographically homogeneous sample of isoxanthopterin. That isoxanthopterin could be so readily isolated from urine by the above technique suggested that, by using the more rapid technique of thin layer chromatography in place of paper chromatography, the method could be of clinical use in any routine determination of urinary isoxanthopterin.

(c) Paper Chromatographic Analysis. One of the distinct advantages of dealing with pteridines is that many of these compounds are strongly fluorescent in ultra-violet light. Isoxanthopterin is no exception in this respect, being characterised by a vivid purple fluorescence. This property proved to be of vital importance in the detection and isolation of isoxanthopterin using paper chromatography, as it enabled very small quantities of the pteridine to be readily detected. The general procedure adopted for the identification and purification of the isoxanthopterin present in the various eluates of the florisil columns, is described on page 58 . In essence the method consisted of applying the

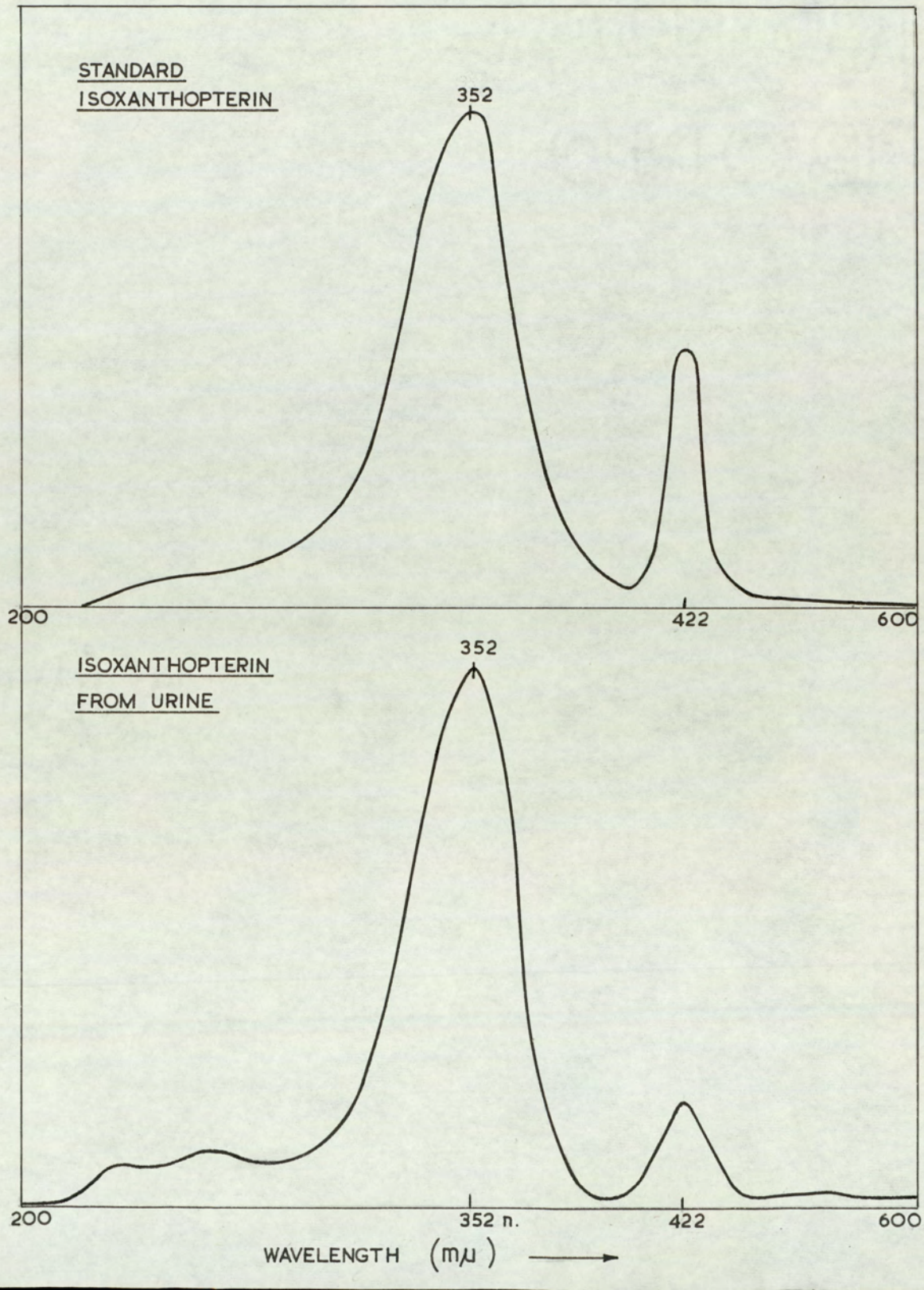
appropriate concentrated eluate to the chromatogram paper as a long (20 - 30 cm) narrow band, which was then flanked with a known sample of isoxanthopterin. After elution, the paper was air dried, and the fluorescent areas detected by UV light. Any fluorescent bands corresponding in colour and r_f value to the isoxanthopterin standard were cut out, suspended vertically and eluted directly into centrifuge tubes with dilute ammonium hydroxide. The evaporation of these eluates to dryness, followed by uptake of the residues in a small volume of dilute ammonia, gave a solution which was re-applied as a band to another chromatogram and run in a different solvent. This process was repeated, with various and alternating solvent systems until the fluorescent isoxanthopterin band was chromatographically homogeneous. A comparison in six solvent systems of the r_f values of a known sample of isoxanthopterin with those of the fluorescent material purified as above, conclusively established the identity of the latter as isoxanthopterin. The relevant data is detailed on page 72 . As was to be expected one of the major problems encountered in the paper chromatographic analysis was the masking and distortion of the fluorescent bands by tarry and often deeply coloured residues present in the concentrated eluates. Attempts to effect a preliminary separation of these residues by column chromatography on cellulose using propanol-5% acetic acid (2:1, by volume) were successful (page 63), but were

too lengthy to use as a standard procedure. The most efficient method for minimising this interference was to apply the concentrate as bands to as many as six chromatogram papers, which were then simultaneously eluted. After elution, the fluorescent bands under investigation, together with any co-running residues, were cut out and applied to further chromatograms. This process was repeated until the fluorescent bands were free of residue, and the isoxanthopterin was then obtained chromatographically pure in the normal way.

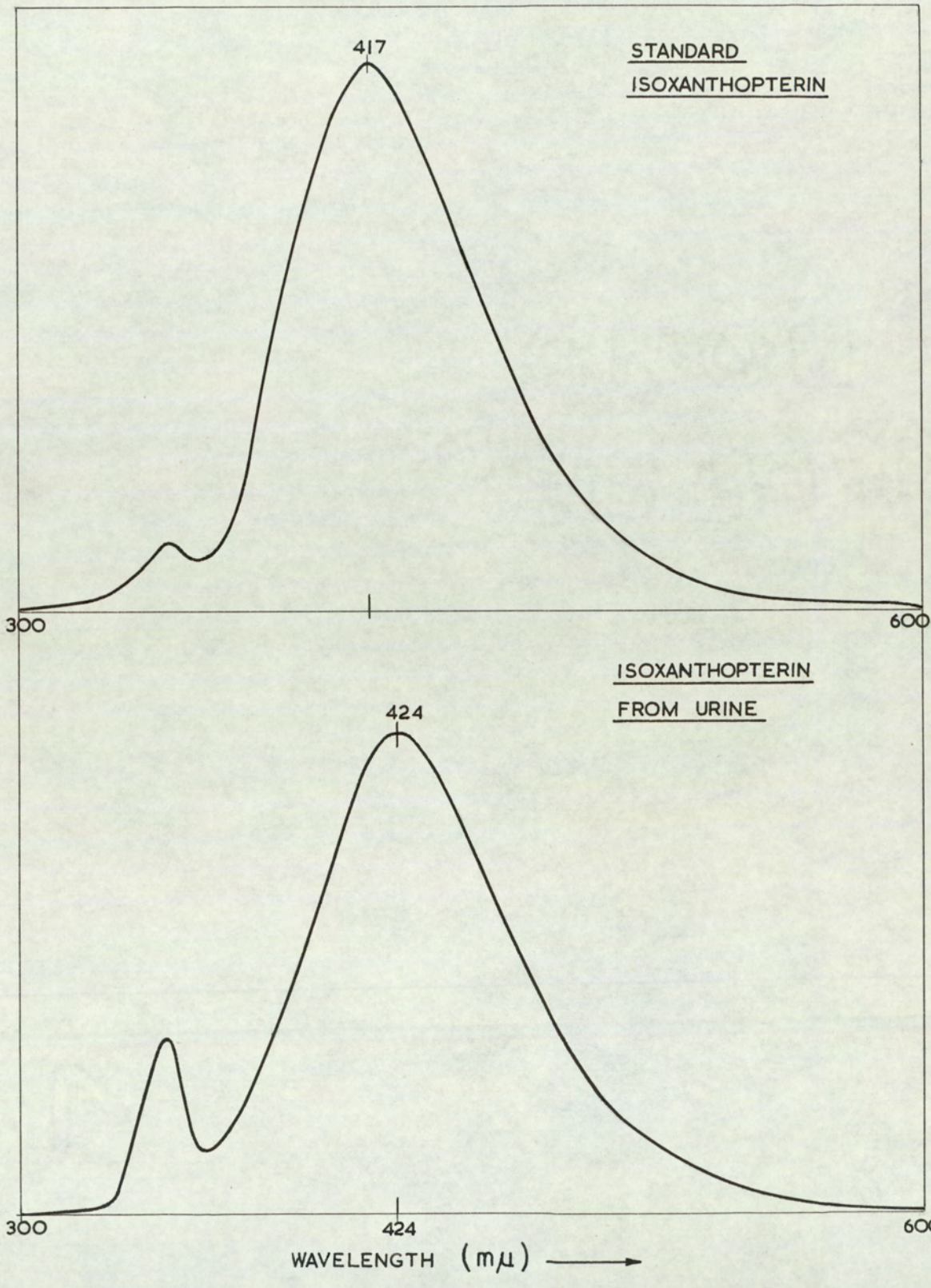
(d) Fluorimetric Determination of Isoxanthopterin

Qualitative:- Experiments (page 60) to investigate the variation with wavelength of the fluorescence intensity of isoxanthopterin, indicated that the activation wavelength at which maximum fluorescence occurs is 352 mμ; the corresponding peak emission occurs at 420 mμ. The fluorescence and activation spectra of known isoxanthopterin and that isolated from urine are compared on pages 36 and 37 . The close similarity of the spectra of these two samples, not only confirmed the identity of the urinary isoxanthopterin but also indicated that it was essentially pure from other fluorescent materials. It will be noticed however that the activation and fluorescence spectra of both the known and the urinary isoxanthopterin contain a minor peak in addition to that due to the pteridine itself. From the

ACTIVATION SPECTRA OF ISOXANTHOPTERIN

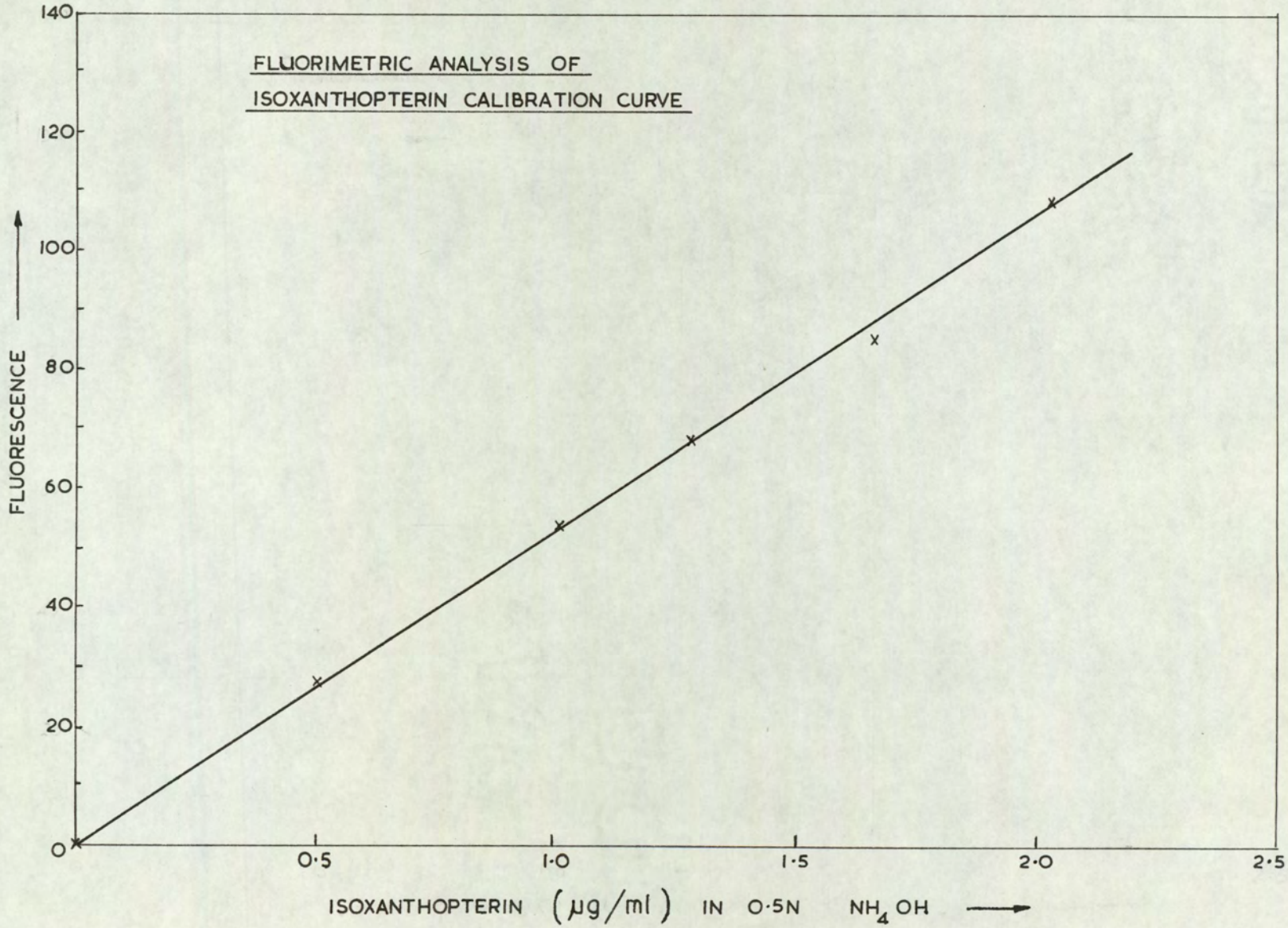


FLUORESCENCE SPECTRA OF ISOXANTHOPTERIN



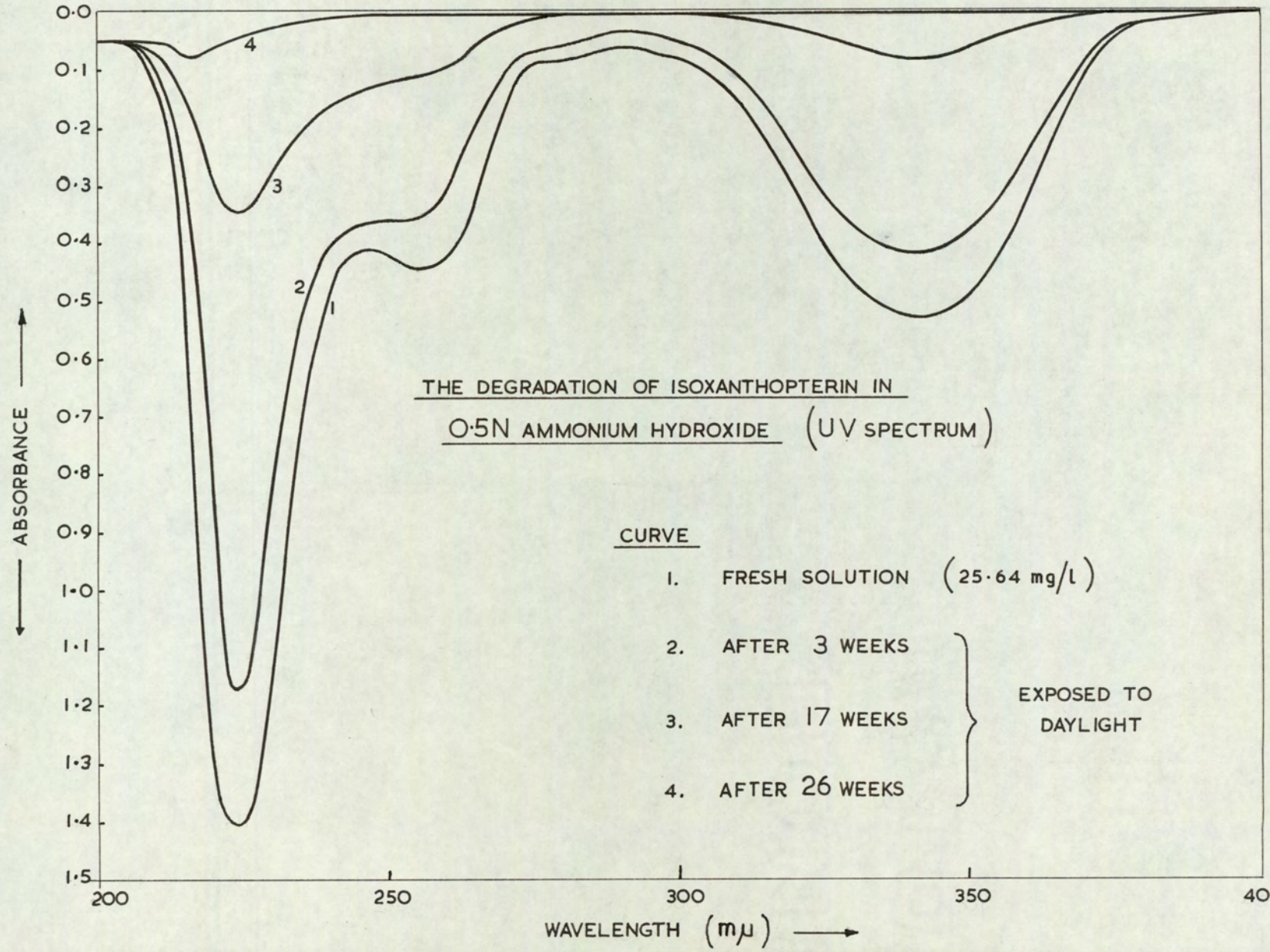
fact that the wavelength at which this peak occurred always corresponded to the wavelength of the exciting radiation, it was concluded that the peak was due to reflected light. That the reflected light peak is particularly noticeable in the spectra of urinary isoxanthopterin is due to the presence of a small amount of non-fluorescent yellow impurity which originates from the chromatogram paper itself, and which has the effect of increasing the light scattering properties of the solution. The use of chromatogram papers prewashed with 0.5N ammonium hydroxide failed to eliminate this effect.

Quantitative:- The chromatographically pure isoxanthopterin isolated from urine was quantitatively determined by fluorimetry using the general procedure described on page 61 . Standard solutions of known isoxanthopterin were made up in dilute ammonium hydroxide and used to derive a calibration graph of fluorescence v isoxanthopterin concentrations (page 39). The concentration of isoxanthopterin in the eluates resulting from the previous chromatographic purifications, was then determined by comparison of the fluorescent intensity of these solutions with the calibration curve. An examination of the fluorescence and activation spectra of isoxanthopterin indicated that in order to eliminate the effects of reflected light, it would be necessary to to cut off excitation wavelengths above 400 mμ and fluorescence wavelengths below 360 mμ. This was achieved by the use of suitable

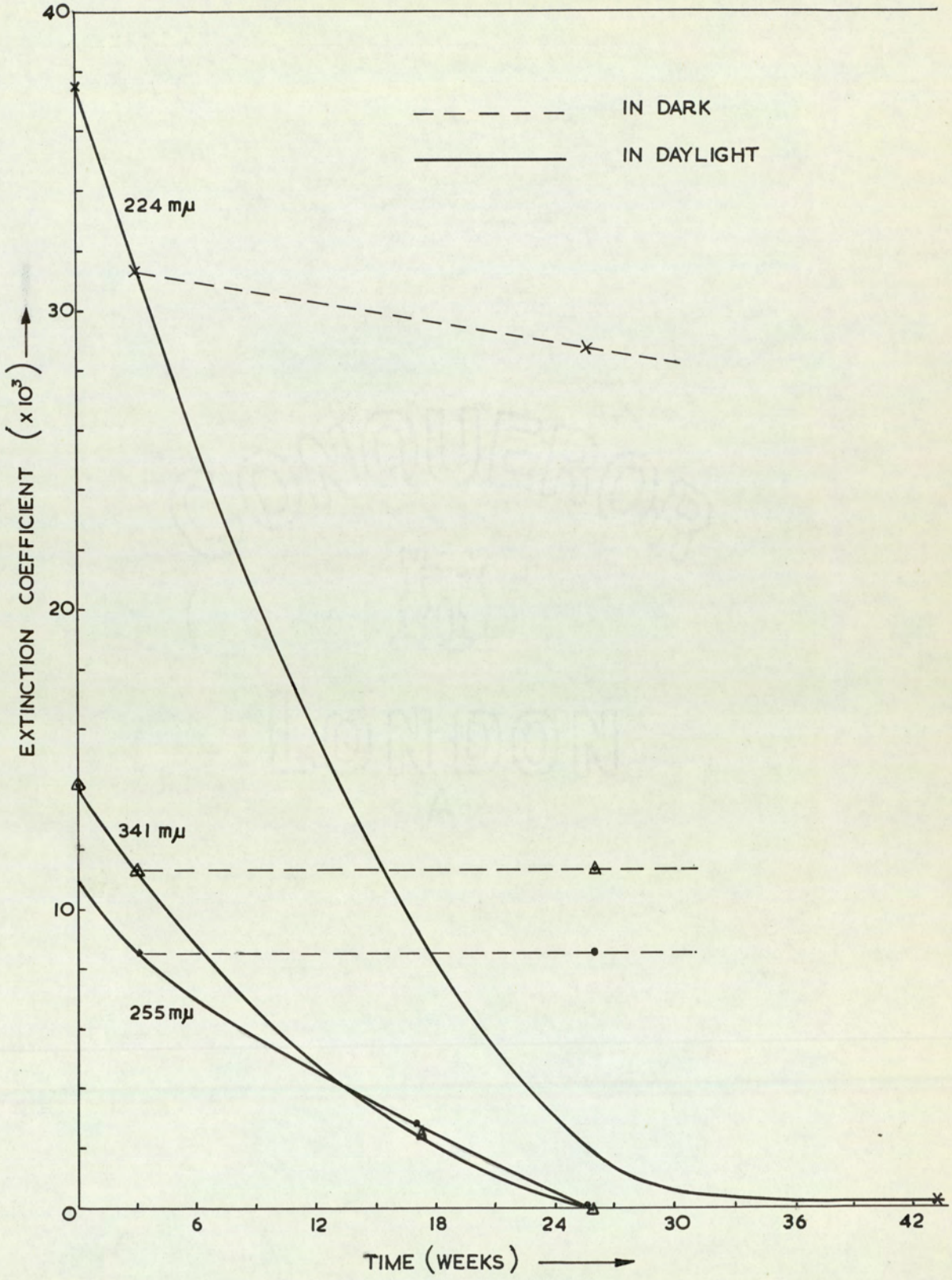

FLUORIMETRIC ANALYSIS OF
ISOXANTHOPTERIN CALIBRATION CURVE
FLUORESCENCE
140
120
100
80
60
40
20
0
0·5
1·0
1·5
2·0
2·5
ISOXANTHOPTERIN (µg/ml) IN 0·5N NH4 OH

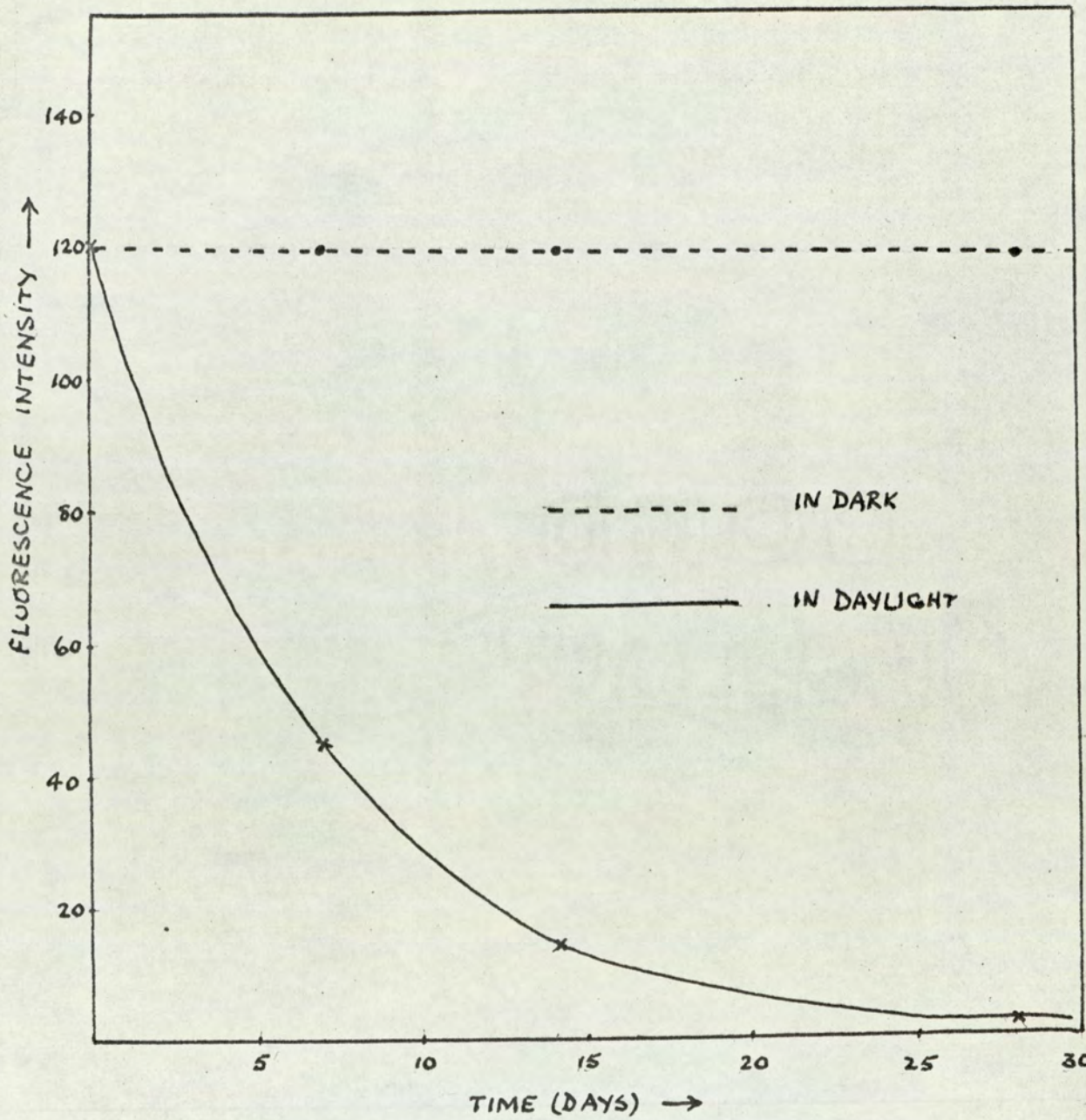
filters. The fluorimetric determination of isoxanthopterin was found to be a rapid and, owing to the intense fluorescence of isoxanthopterin, a sensitive technique, being quite capable of detecting microgram amounts of the pteridine. The quantitites of isoxanthopterin thus found to be present in the urine samples analysed, are tabled on page 71 . The validity of these results in the light of experimental observations of the stability of isoxanthopterin solutions is considered later in the discussion (page 46).

(e) Auto-degradation of Isoxanthopterin in Alkaline Solution:- During the quantitative determination of solutions of isoxanthopterin in dilute ammonium hydroxide, it was discovered that the fluorescence intensity of the standard isoxanthopterin solutions used to prepare the calibration curve, diminished by considerable and varying amounts over a relatively short period of time. In order to investigate this phenomenon further, a solution of isoxanthopterin of known concentration was made up in dilute ammonium hydroxide (0.5N) and measurements of the change with time of the UV spectrum and the fluorescence intensity under various light conditions, were made. The results of these determinations, as detailed on page 41-43 , revealed that neither the UV spectrum nor the fluorescence intensity of the isoxanthopterin solution stored in the dark showed any significant


0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·1
1·2
1·3
1·4
1·5
ABSORBANCE
200
250
300
350
400
WAVELENGTH (mμ)
4
3
2
1
THE DEGRADATION OF ISOXANTHOPTERIN IN
0·5N AMMONIUM HYDROXIDE (UV SPECTRUM)
CURVE
1. FRESH SOLUTION (25·64 mg/l)
2. AFTER 3 WEEKS
3. AFTER 17 WEEKS
4. AFTER 26 WEEKS
EXPOSED TO DAYLIGHT

THE DEGRADATION OF ISOXANTHOPTERIN IN 0·5N AMMONIUM HYDROXIDE AS MEASURED BY THE DECREASE IN EXTINCTION COEFFICIENTS OF THE UV ABSORPTIONS AT 224, 255 & 341 mμ

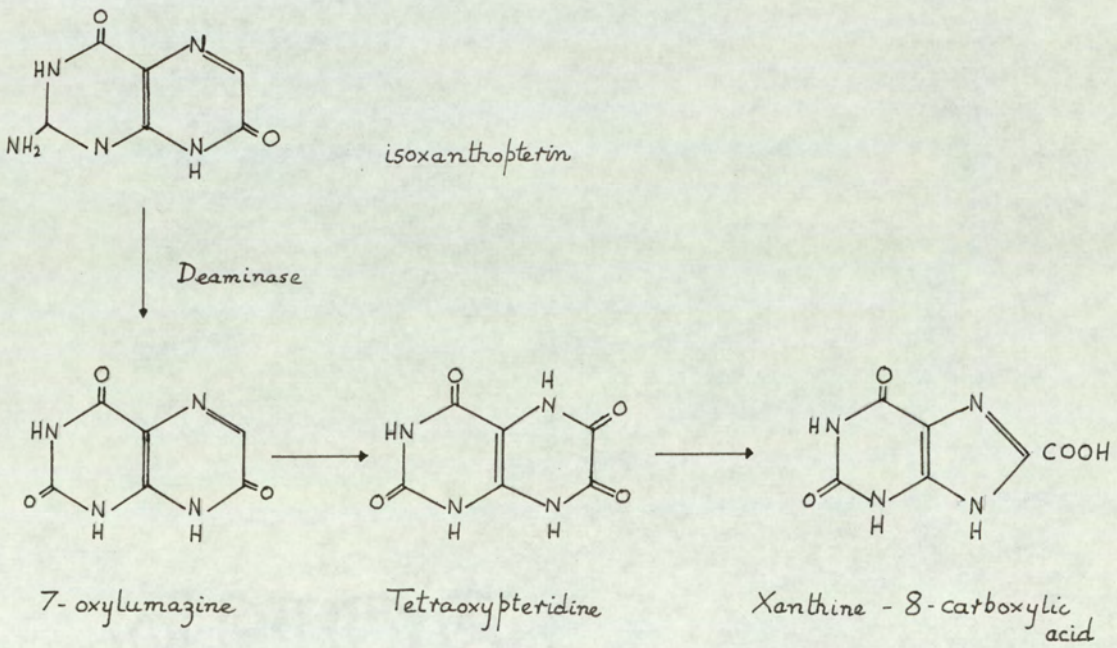




THE DEGRADATION OF ISOXANTHOPTERIN IN 0·5N AMMONIUM HYDROXIDE AS MEASURED BY THE DECREASE IN FLUORESCENCE INTENSITY

diminution. That the exposure of the solution to daylight however, causes, as the graphs indicate, an immediate and rapid weakening of both the UV spectrum and the fluorescence intensity, makes it quite clear that the degradation process is photolytic in nature. It is rather difficult to understand the mechanism of this degradation particularly as the pteridine ring is generally considered to be, chemically at least, very stable.

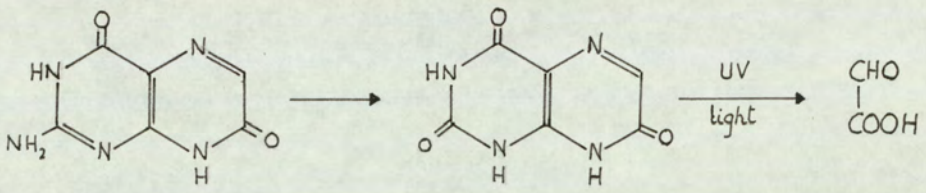
Isoxanthopterin is known [88] to be enzymatically converted to xanthine-8-carboxylic acid, probably via the following intermediates:-



The experimentally observed features of the degradation of isoxanthopterin cannot be accounted for by such a process, or its chemical equivalent however, as any xanthine-8-carboxylic

acid thus formed would be readily detectable by its UV spectrum, a prominent feature of which is a strong peak at 290 mμ. As is indicated on page 41 , no such peak appears during the degradation of isoxanthopterin.

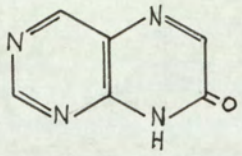
That both the fluorescence intensity and the UV spectrum of the isoxanthopterin solution are completely eliminated, suggests that the pteridine ring is destroyed. It has been shown[79] that isoxanthopterin can be chemically converted to glyoxylic acid as follows:-



isoxanthopterin — 2,4,7-trihydroxy-pteridine — glyoxylic acid

In this process, isoxanthopterin is deaminated by refluxing with hydrochloric acid to give 2,4,7-trihydroxypteridine, which is then rapidly and quantitatively converted to glyoxylic acid by irradiation with UV light. As this final product does not of course, adsorb in the ultra-violet and is non-fluorescent, it is possible that the experimentally observed degradation of isoxanthopterin could proceed by a photolytically initiated but chemically similar process. It is interesting in this respect that the action of

aqueous solutions of hydrazine and hydroxylamine on 7-hydroxypteridine



7 - hydroxypteridine

both cause extensive decomposition of the pteridine ring[89].

(f) Isoxanthopterin content of urine:- The quantities of isoxanthopterin isolated from the various urine samples analysed, are tabled on page 71. The discovered tendency of isoxanthopterin to decompose, at least in dilute ammonia solutions, together with the fact that the analytical procedure adopted for the extraction and purification of isoxanthopterin from urine involved the use of dilute ammonia as an eluent, means that the figures quoted, and in particular those for analyses 2 and 3, may be somewhat approximate. In analyses 4, 5 and 6, owing to the increased efficiency of the analytical procedure, and the precautions taken to minimise the exposure of the ammoniacal isoxanthopterin solutions to the light, the figures quoted are probably much nearer to the true urinary content. As indicated in the experimental section, the urine investigated in analysis 5 was that of a folic acid deficient patient. In order to facilitate a direct comparison of the isoxanthopterin content of this urine with the same volume of 'normal' urine (analysis 4), the experimental procedures adopted for these two analyses were identical in all respects. Although

on the basis of this single comparison, there would seem to be little difference in the isoxanthopterin content of the two urines, it would, of course, be essential to conduct a considerable number of such comparisons before any meaningful conclusions could be drawn. Although the quantities of isoxanthopterin isolated in the various analyses were considerably less than expected, being of the order of micrograms, it is interesting to note that the daily requirement of free pteroyl-L-glutamic acid in the human system is thought to be of the order of 20 - 50 µg [90,91]. Although estimates vary considerably, the total daily intake of folates from the average diet is considered to be between 100 - 150 µg [91,92]. On the basis of the postulated conversion of pteroyl-L-glutamic acid to isoxanthopterin, the quantities of the latter isolated from urine thus appear to be of a similar order, at least, to those of its possible precursor.

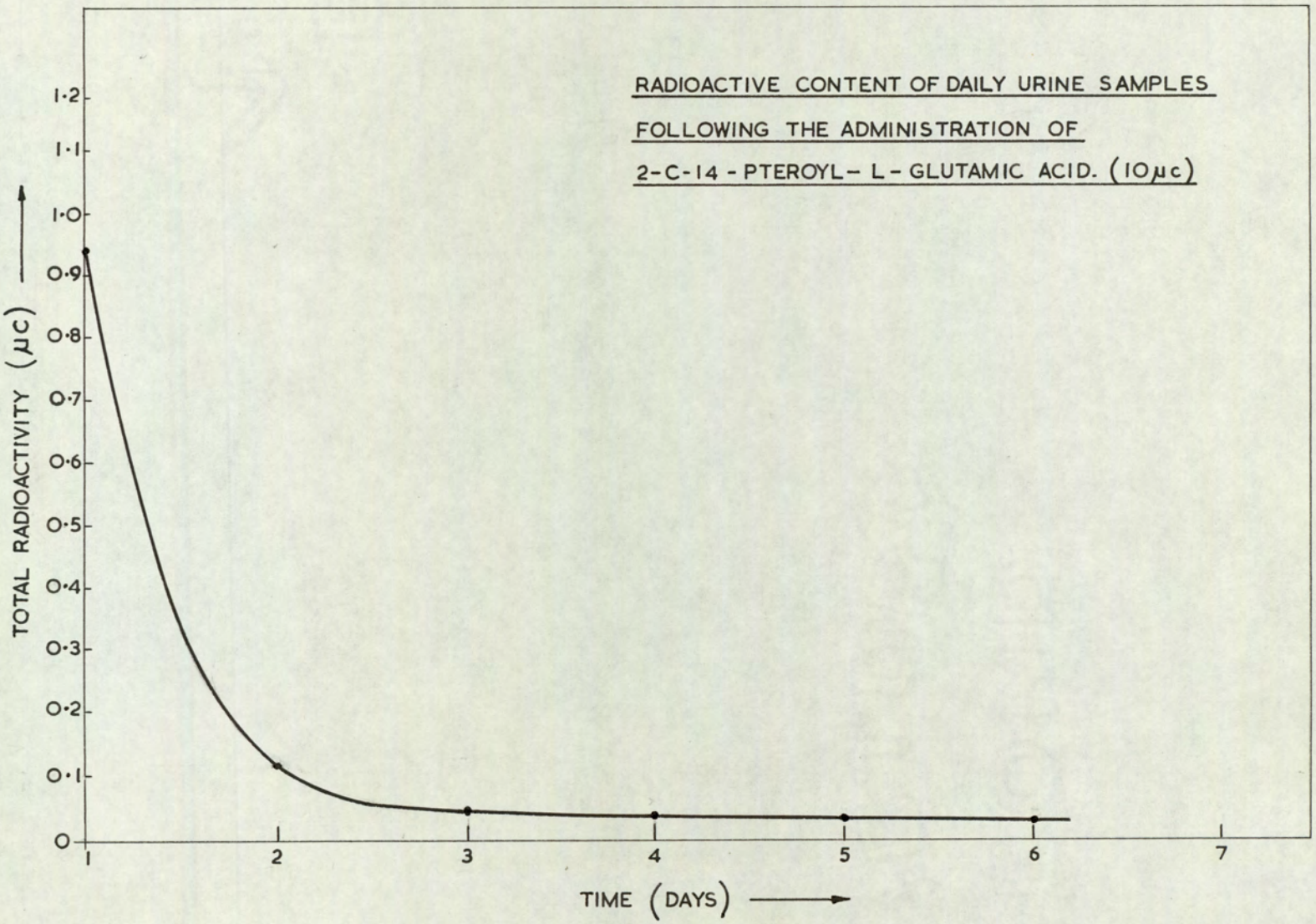
(g) Summary:- A rapid analytical procedure for the isolation of isoxanthopterin from human urine is described. Although further work on the quantitative aspects of the method is still required, it would appear to be potentially suitable for the clinical determination of urinary isoxanthopterin.

3. Tracer Experiments in the Rat Using 2-C14-Folic Acid

In a preliminary experiment to investigate the 'in vivo' metabolism of pteroyl-L-glutamic acid, the acid, labelled at position

2, was administered to a rat, and the subsequently voided urine was then analysed for radioactive metabolites and in particular for radioactive isoxanthopterin. The details of the experimental procedures adopted in this metabolic study are described on page 73. Following the administration of radioactive folic acid, each daily urine sample was made up to a known volume, and the total radioactivity measured by liquid scintillation counting. The scintillator used (NE 220) for all the counting experiments is dioxane based and is thus particularly suitable for the counting of aqueous solutions. The considerable diversity in the degree of quenching experienced due to the variation in colour of the urine samples, made it necessary to determine the efficiency of counting of each individual sample. This was carried out by counting the radioactive urine sample itself, and then recounting it after the addition of a known activity of an internal standard, C-14-n-hexadecane. The difference between these two measured activities, divided by the calculated absolute disintegration rate of the added standard gives the efficiency with which the particular urine sample was counted. Knowing this figure, the absolute activity of the sample can be calculated.

The radioactive content of the daily urine samples so obtained (pages 49,78) showed that the total radioactivity present in the urine was 1.18 μc, or 11.8% of the administered dose (10 μc),


RADIOACTIVE CONTENT OF DAILY URINE SAMPLES
FOLLOWING THE ADMINISTRATION OF
2-C-14 - PTEROYL - L - GLUTAMIC ACID. (10μc)
TOTAL RADIOACTIVITY (μc)
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
7
TIME (DAYS)

and that 90% of this activity emerged in the first 48 hours after administration.

In order, therefore to avoid unnecessary dilution in the later stages of the urine analysis, only the first two radioactive urine samples, were further analysed. The chromatographic procedure adopted (page 74) for the analysis of this radioactive urine was essentially that developed to analyse small quantities of human urine (analysis 6). The urine was passed through a small florisil column which was then eluted with 1% hydrochloric acid, and by a small volume of 14% ammonium hydroxide. The liquid scintillation counting of the urine, and ammonium hydroxide eluates of the florisil column indicated (page 79) that under the elution conditions used, nearly 80% of the radioactivity applied to the column was thus eluted, and that two-thirds of this activity was contained in the ammonium hydroxide eluate.

The paper chromatographic analysis of this eluate revealed a number of fluorescent and radioactive areas. A comparison of these areas with those of standard isoxanthopterin as detailed on page 80 , suggests that radioactive isoxanthopterin is, in fact, present in the urine of the rat following the administration of 2-C-14-pteroyl glutamic acid.

In order to confirm this and to establish the metabolic relationship between folic acid and isoxanthopterin, it will be

necessary to repeat and enlarge the scope of the experiment to include the isolation and purification of urinary isoxanthopterin to a constant specific activity.

A further technique which could well prove to be of considerable importance in the detection and determination of pteridines is that of mass spectrometry. As has already been described, even small amounts of such pteridines as isoxanthopterin give well defined mass spectra, which are characterised by a prominent parent peak. It is conceivable that the latter feature could find a useful application in the preliminary identification of the small quantities of pteridines extracted from urine.

A further and more interesting application would be the use of mass spectrometry in the elucidation of pteridine metabolism. The facility with which a well defined mass spectrum of isoxanthopterin was obtained, suggests that the detection and quantitative estimation of this compound, isotopically enriched with C^{13} or even N^{15}, should also be quite feasible. It should therefore be possible to follow the degradation of pteroyl-L-glutamic acid by administering it in a C^{13}-enriched form, and using mass spectrometry to detect any similarly enriched metabolic products. The use of such non-radioactive isotopes would, of course, enable this degradation process to be investigated in the human system.

4. Summary

The experimental work detailed in the first part of this thesis demonstrates that in confirmation of earlier work, isoxanthopterin is indeed present in human urine. The development of a rapid method for its isolation, purification, and quantitative determination, was successfully achieved and is described in detail.

A preliminary tracer experiment in the rat designed to further investigate the postulated degradation of the biologically important vitamin, pteroyl-L-glutamic acid, to isoxanthopterin, supports the suggestion that this conversion occurs in 'vivo'. Further work to conclusively confirm and to establish the extent of this possible conversion is however required, and a suggestion that this could also be carried out in the human system by the use of mass spectrometry, is made.

EXPERIMENTAL

PART 1(a) The isolation of isoxanthopterin from urine.

Apparatus and Chemicals:- Florisil (60/100 mesh) was supplied by Koch Laboratories Ltd., Colnbrook, Buckinghamshire.

The fluorescent pteridines were detected on the paper chromatograms with a UV lamp (Hanovia Ltd., Slough, Bucks.) with a peak emission of 354 mμ. The fluorescence and activation spectra of isoxanthopterin were determined using an aminco-Bowman Spectrophotofluorimeter. The quantitative estimation of isoxanthopterin was carried out on a Locarte Spectrofluorimeter. Both instruments were fitted with a high pressure Xenon Arc Lamp. An AEI MS9 spectrometer operated at 250°C and 10^{-8} mm Hg was used to obtain the mass spectra.

2-amino-4-hydroxy-6-hydroxymethylpteridine

The reaction between 6-hydroxy-2,4,5-triaminopyrimidine and dihydroxyacetone under the conditions detailed by Baugh and Shaw[93] yielded the desired pteridine in 43% yield. Paper chromatography (Whatman No.3.MM.) using a variety of solvents, indicated that the product was pure; UV, in 0.1N NaOH, maxima in mμ (log ε) at 254 (4.27) 362 (3.82), lit.[93], 253 (4.34) 362 (3.84); in 0.1N HCl, 245 (3.97) 321 (3.86), lit.[93], 247 (4.01) 322 (3.89) NMR, in TFA, singlets at 1.26τ (vinyl proton), 1.5τ (NH;broad) and 4.80τ (methylene protons). Mass spectrum, predominant

ions ($^{m}/_{e}$) at: 193 (parent), 177, 169, 164 and 163.

2-amino-4-hydroxypteridine

To a hot (70°) solution of 6-hydroxy-2,4,5-triaminopyrimidine dihydrochloride (0.47g., 23mM) in water (40 ml.) containing a trace of sodium dithionite, a solution of glyoxal monohydrate (0.21g., 26mM) in water (10 ml.) was added with stirring. The resulting mixture, which rapidly turned red, was allowed to stand at room temperature for 20hr. The condensation product (I) which had precipitated from the acid solution (pH2) as a bright red flocculent solid, was collected, washed successively with water, ethanol, and ether, and finally dried for several hours at 110°; UV in 0.1N HCl, maxima in mμ (log ε) 245 (4.2) 316 (4.1), minimum at 270; IR, KBr disc, maxima (cm^{-1}) at 3250, 3070, 1700, 860, 800; Mass spectrum, predominant ions ($^{m}/_{e}$) at 163, 135, 122 and 109.

The condensation product (I) was then dissolved in 2N sodium hydroxide, and 2N hydrochloric acid added dropwise until the mixture was just acid, at which point a pale yellow solid precipitated. By again dissolving this material in alkali and re-precipitating with acid, a pure sample of the desired 2-amino-4-hydroxypteridine was obtained (0.15g., 48%); UV, in 0.1N NaOH, maxima in mμ (log ε) 252 (4.35) 360 (3.91), lit.,[94] 252 (4.34) 358 (3.88); The r_f values of the synthesised pteridine (see table page 72) corresponded closely with the literature values.[95] Mass spectrum, predominant ions ($^{m}/_{e}$) at: 163(parent), 135, 122, and 109. The infrared and

mass spectra of the condensation product (I) were found to be identical with those of the 2-amino-4-hydroxypteridine, and it was concluded that the condensation of the glyoxal derivative to give the pteridine took place in acid solution.

2-amino-4,7-dihydroxypteridine (Isoxanthopterin)

The condensation of ethyl glyoxalate hemi-acetal,[96] and 6-hydroxy-2,4,5-triaminopyrimidine dihydrochloride by the method of Albert and Wood[97] gave an anil, ethyl-2,4-diamino-6-hydroxypyrimidine-5-azomethincarboxylate, m.p. > 300° (IR, KBr disc, maxima (cm^{-1}) at 3580, 3400, 3100 and 1680), which upon ring closure in alkaline media gave the required isoxanthopterin in 60% yield. Purification for analysis was effected by dissolving the product in dilute sodium hydroxide and re-precipitating it by the addition of acid; UV, in 0.1N NaoH, maxima in mμ (log ε): 221 (4.56) 254 (3.98) 341 (4.05); lit.,[98] 223 (4.58) 254 (4.06) 339 (4.15); IR, KBr disc, maxima (cm^{-1}) at 3120, 1660, 1400; NMR, in 0.09N NaoD, singlet at 2.0τ. Mass spectrum, predominant ions ($^{m}/_{e}$) at 179 (parent), 151, 134 and 108.

The adsorbtion of xanthopterin on animal charcoal

A solution of xanthopterin (3.0 mg.) in a phosphate buffer solution pH 7.6 (25 ml.) was vigorously shaken with animal charcoal (0.5g) for 5 min, the latter being removed from the now only faintly fluorescent solution, by filtration.

The charcoal was then extracted successively with the following solvents: a) 0.5N ammonium hydroxide (10 ml); b) 50% aqueous pyridine (20 ml) at room temperature, and c) 50% aqueous pyridine (20 ml) at the boil, the resulting individual extracts evaporated to dryness, and then taken up in a small volume of 0.5N ammonium hydroxide. Investigation of each extract by paper chromatography and UV analysis, failed to show the presence of even small quantities of xanthopterin. It was therefore concluded that animal charcoal was unsuitable for isolation procedures involving pteridines.

The behaviour of isoxanthopterin on florisil (60/100 mesh)

When an ammoniacal solution of isoxanthopterin (0.01%) was shaken with florisil (2g.) for 40 min., no appreciable adsorption of the isoxanthopterin occurred, as detected by the undiminished fluorescence of the solution in UV light.

Acidification of the solution with 4N hydrochloric acid caused the precipitation and adsorption of the isoxanthopterin on to the florisil, which itself then exhibited the characteristic deep blue fluorescence of isoxanthopterin. The latter was rapidly removed from the fluorescent adsorbent by extraction at room temperature with 0.5N ammonium hydroxide.

Chromatography of isoxanthopterin and 2-amino-4-hydroxypteridine on a florisil column

In order to ascertain the adsorption characteristics of

isoxanthopterin on a florisil column under various pH conditions, the following experiment was carried out.

A solution of isoxanthopterin (51 µg) in IN hydrochloric acid (5 litres) was applied to a florisil column (100g., 17 cm x 6 cm) made up as a slurry in 1% hydrochloric acid. The florisil was topped with a layer of acid-free sand.

The pteridine remained as a very narrow band (1mm) at the top of the column. Elution with IN hydrochloric acid (5 litres) broadened the fluorescent band to 10mm. Distilled water (500ml) caused little or no further broadening, but although the borate buffer, pH 7.6 (11.5 litres) resulted in the fluorescent area becoming more and more diffuse, no isoxanthopterin was removed from the column. Finally, 0.5N ammonium hydroxide was passed through, a relatively small volume (100ml) of which,was sufficient to remove the majority of the isoxanthopterin from the column.

The experiment was repeated under identical conditions with 2-amino-4-hydroxypteridine. It was found that this compound, in contrast to isoxanthopterin, was removed from the column relatively easily by the borate buffer, approximately 3 litres of which was sufficient to elute the main fluorescent band.

Urine Analysis (General Procedure)

Urine: The urine under investigation was supplied by the General Hospital, Birmingham, and was, unless described to the contrary, from healthy subjects.

Column Chromatography:- The column consisted of florisil (60/100 mesh) made up as a slurry in 1% hydrochloric acid. The florisil was topped with a substantial layer of acid-free sand (B.D.H.) to minimise disturbance by the large volumes of eluent.

The passage of the unfiltered urine through the column was followed by distilled water, phosphate buffer, pH 7.6, and finally 0.5N ammonium hydroxide.

Eluate Work-Up:- The intensely coloured urine and distilled water eluates, were usually discarded, as previous evidence (page 57) had shown that under acid or neutral conditions, the pteridines under investigation remained strongly attached to the florisil.

The particular eluate under examination was evaporated to dryness using a rotary evaporator, and the resulting residue vigorously agitated with fresh 0.5N ammonium hydroxide. The insoluble residue which was removed from the ammoniacal suspension by filtration through a medium grade filter, was dried and put to one side. The filtrate, which sometimes needed to be further concentrated was then examined by paper chromatography.

Paper Chromatography:- Unless otherwise stated, the descending technique was adopted employing Whatman No. 3 MM paper, the chromatograms being run in an atmosphere well saturated with the solvent vapour. The bottom edge of the papers were serrated to ensure an even flow of solvent.

Part or all of the appropriate concentrated eluate was applied to the paper as a long (20-30cm) narrow band, flanked by known samples of the pteridines being looked for. To give as good a separation as possible the solvent was allowed to run to within a few centimetres of the serrated edge (40cm), the paper was then air-dried, and the fluorescent areas detected by UV light. Any fluorescent bands corresponding in colour and r_f value to the standard pteridines were cut out, suspended vertically and eluted direct into centrifuge tubes with dilute ammonium hydroxide. Evaporation of these eluates to dryness, followed by uptake of the residues in a small volume of dilute ammonia, gave a solution which was re-applied as a band to another chromatogram and run in a different solvent.

This process was repeated, with various and alternating solvent systems until the fluorescent band under observation was chromatographically homogeneous. The solvents, ratios by volume, commonly employed were:-

1. Propanol - 1% ammonia (2:1)
2. n-Butanol-acetic acid-water (4:1:5)
3. Propanol-5% acetic acid (2:1)
4. 5% acetic acid
5. t-Butanol-pyridine-water (12:3:5)

Fluorimetric analysis of isoxanthopterin solutions

Qualitative:- An Aminco-Bowman Spectrophotofluorimeter employing a high pressure xenon arc lamp as the excitation source, was used to obtain fluorescence and activation spectra of isoxanthopterin (page 36,37). The instrument was operated with the smallest slit width possible in order to minimise any stray or reflected light. Preliminary experiments with a standard solution of isoxanthopterin in dilute ammonium hydroxide (0.5N) indicated that maximum fluorescence was caused by radiation of wavelength 352 mμ, and maximum emission occurred at 420 mμ. These values were found to remain unaffected by relatively large variations in solute concentration.

The activation spectrum was therefore obtained by fixing the emission wavelength at 420 mμ and varying the activation wavelength. Conversely, the fluorescence spectrum was produced by fixing the activation wavelength at 350 mμ and plotting the emission wavelength against fluorescence intensity. Both spectra showed a small peak due to reflected light, at 420 mμ and 350 mμ respectively. The corresponding spectra of the chromatographically homogeneous isoxanthopterin isolated from urine, were obtained under identical conditions. The larger reflected light peaks in these spectra are due to a small amount of a faintly yellow non-fluorescent impurity which originates from the chromatogram paper itself, and has the effect of increasing the light scattering properties of the solution under investigation.

Quantitative:- The quantitative determination of isoxanthopterin was carried out in dilute ammonium hydroxide solutions using a Locarte Fluorimeter. In order to eliminate any reflected light, it was found necessary to cut out excitation wavelengths above 400 mμ and fluorescence wavelengths below 360 mμ. This was achieved by the insertion of a Locarte LF2 filter on the excitation (primary) side, and an LF8 filter on the emission (secondary) side of the sample under investigation.

Using freshly made up solutions of isoxanthopterin of known concentrations in dilute ammonium hydroxide, a calibration curve was drawn up of fluorescence intensity v isoxanthopterin concentration. The chromatographically purified isoxanthopterin eluate from each urine analysis was made up to a known volume in dilute ammonium hydroxide, its fluorescence intensity measured, and the concentration of isoxanthopterin directly obtained from the calibration curve. The above curve is to be found on page 39 .

Auto-degradation of isoxanthopterin in alkaline solutions

During the quantitative determination of solutions of isoxanthopterin in dilute ammonium hydroxide, it was found that the fluorescence intensity of the standard isoxanthopterin solutions used to prepare the calibration curve, diminished by considerable and varying amounts over a relatively short period of time. The corresponding UV spectra of these solutions were also found to be

significantly weaker.

In order to more thoroughly examine these phenomena, a solution of isoxanthopterin in dilute ammonium hydroxide was freshly made up, and the UV spectrum recorded. After being allowed to stand in the daylight for three weeks, the UV spectrum was again noted, and the solution immediately divided into two portions, one of which remained in the daylight as before, whilst the other was kept in complete darkness. After approximately six months, the UV spectra of each portion was recorded. The spectra (page 41,42) clearly indicate that the degradation process is photolytic, and that the product or products do not absorb in the ultra-violet. In a parallel experiment under similar conditions, the diminution in the fluorescence intensity of the standard isoxanthopterin solution, was observed. The data obtained (page 43) confirmed that the degradation was light dependant, and demonstrated that, after exposure to the light for four weeks, the test solution was, in fact non-fluorescent. No attempt was made to elucidate the nature of the degradation products.

Urine Analysis I.

Unfiltered urine (10 litres) was passed through a florisil column (100g., 17cm x 6cm) and the column eluted with:-

1. Distilled water (500ml.)
2. Phosphate buffer, pH 7.6 (11.5 litres)
3. 0.5N ammonium hydroxide (8 litres)

The urine and distilled water eluates were discarded, the phosphate buffer and ammonium hydroxide eluates being worked up as previously indicated.

Phosphate buffer eluate The deeply coloured fluorescent eluate was collected in two separate fractions.

Fraction 1:- (2 litres) Paper chromatography of the concentrated eluate gave a multitude of fluorescent bands, none of which, after repeated chromatography in various solvent systems, was identified as isoxanthopterin.

Fraction 2:- (9.5 litres) Concentration of the eluate to 50 ml., precipitated a finely divided solid (2.9g) which was removed by centrifugation, the resulting supernatant liquors being rather viscous. Paper chromatography of a portion of these liquors again indicated the absence of isoxanthopterin.

Ammonium hydroxide eluate The eluate was collected in four separate fractions, the first of five litres, and the remainder of one litre each.

Fraction 1:- Concentration of this fraction, which was very deeply coloured, gave a considerable amount of tarry residue which seriously interfered with the preliminary paper chromatographic separations, some of which indicated the possible presence of the isoxanthopterin. In order to facilitate the separation of the brown residues from the fluorescent materials under investigation, the total concentrate (6ml) was chromatographed on a cellulose

(standard grade) column. The concentrate was absorbed on cellulose powder (2g), dried at 110°, and after being finely ground was then added to the top of the cellulose powder (100g) column made up in propanol - 5% acetic acid (2:1). Elution with the same solvent gave a number of fluorescent fractions essentially free from tarry residues, paper chromatography of which definitely established the presence of isoxanthopterin.

Fractions 2-4:- The concentrates of these fractions were sufficiently free from tarry residues to render the preliminary purification on a cellulose column unnecessary. Isoxanthopterin was found in much diminished amounts, in fractions 2 and 3, but was not detected in the final concentrate.

No attempt was made, in this analysis to quantitatively determine the detected isoxanthopterin, the r_f values of which corresponded closely with a known sample in various solvents. (See table page 72).

Urine Analysis II

Unfiltered urine (10 litres) was passed through a florisil column (100g., 17cm x 6cm) and eluted with a) distilled water (500 ml.) b) borate buffer, pH 7.6 (11.5 litres) and c) 0.5N ammonium hydroxide (8 litres). The urine eluate was discarded as usual.

Distilled water eluate:- The eluate was concentrated to 15 ml., and examined by paper chromatography in the usual way for isoxanthopterin and 2-amino-4-hydroxypteridine. No trace of either pteridine was found.

Borate buffer eluate:- In order to separate the majority of the inorganic salts from any pteridines possibly present in the eluate, and thus to simplify the work-up procedure, the total eluate was acidified to pH2 with concentrated hydrochloric acid, and the resulting solution passed through a secondary florisil column, identical to the primary one. Prior to its passage through the secondary column, the first two litres of buffer eluate deposited, on acidification, a small quantity (0.2g) of a purple crystalline material (I) which was collected, dried and put to one side for future investigation.

The acid liquors from the passage of the acidified buffer solution through the secondary column were discarded, and the column eluted with two separate portions (1 litre each) of 0.5N ammonium hydroxide. Neither eluate contained any isoxanthopterin or 2-amino-4-hydroxypteridine.

The purple crystalline solid (I), m.p. > 300°, which precipitated on the acidification of the first two litres of the borate buffer eluate, was ground to a fine powder and leached with hot concentrated ammonium hydroxide for ten minutes. The

yellow supernatant liquors were filtered off from the now white solid (II), concentrated, and analysed by paper chromatography. The presence of isoxanthopterin was clearly indicated. The concentrate also contained a small amount of another fluorescent material, the r_f values of which corresponded well to a known sample of 2-amino-4 hydroxypteridine.

The white solid (II) was repeatedly extracted with hot ammonium hydroxide until isoxanthopterin could no longer be detected in the concentrated extraction liquors. The total isoxanthopterin contained in these extracts was separated by paper chromatography from the fluorescent impurities also present, and quantitatively determined by fluorimetric analysis, according to the procedure outlined on page 61 . The yield of isoxanthopterin was estimated to be 25 µg. The fluorescence and activation spectra of the isoxanthopterin isolated from the urine corresponded closely with those of the known material (page 36,37) as did the r_f values in a variety of solvents. (See table page 72).

Ammonium hydroxide eluate:- The eluate, of the primary column, was concentrated to 15 ml. No isoxanthopterin or 2-amino-4-hydroxypteridine could be detected.

Extraction of the florisil columns:- The total contents of each column were extracted with hot concentrated ammonium hydroxide (250 ml.) in order to ensure that no isoxanthopterin or 2-amino-

4-hydroxypteridine remained attached to the florisil. The insoluble residues were filtered off and the filtrates each concentrated to approximately 15 ml. Neither of the above pteridines were found in either concentrate.

Conclusion of Urine Analysis II

25 µg of isoxanthopterin was isolated from 10 litres of urine. A very small amount of 2-amino-4-hydroxypteridine was possibly present also.

Urine Analysis (III)

Unfiltered urine (10 litres) was applied to a florisil column (100g., 17cm x 6cm) and eluted with the same solvents as used in the previous analysis.

Borate buffer eluate:- Repeating the procedure adopted in analysis (II), the total buffer eluate from the primary column was acidified to pH2 (no solid precipitated) and passed through the secondary florisil column, which was then eluted with two successive portions (1 litre each) of dilute ammonium hydroxide. Concentration of the first ammoniacal eluate to 10 ml, followed by paper chromatographic analysis again established the presence of isoxanthopterin, although no 2-amino-4-hydroxypteridine was found. Only a trace of isoxanthopterin was found in the second ammoniacal eluate. The yield of isoxanthopterin as determined by fluorimetric analysis was 23 µg. The fluorescence and activation spectra again differed very little from those of the known material.

Ammonium hydroxide eluate (8 litres):- The concentrate (5 ml.) of the primary column eluate contained no isoxanthopterin.

Conclusion: Isoxanthopterin (23 µg) was identified and isolated from 10 litres of urine. No 2-amino-4-hydroxypteridine was found.

Urine Analysis (IV)

Two litres of unfiltered urine, in which red brown crystals were visible, were passed through a florisil column (100 g., 17cm x 6cm), which was then eluted with a) distilled water (500 ml.) and b) borate buffer, pH 7.6 (11.5 litres). The ammonium hydroxide eluent of the primary column which normally followed, was dispensed with, as previous analyses had shown that any isoxanthopterin or similar material present in the urine had already been removed by the buffer eluent.

Borate buffer eluent:- After passage of the acidified buffer solution through the secondary florisil column, the latter was eluted with a) distilled water and b) dilute ammonium hydroxide. The distilled water eluent, the eluate from which was discarded, was designed to remove any remaining inorganic buffer residues which would otherwise be eluted by the ammonium hydroxide eluent.

Paper chromatography of the total concentrated ammoniacal eluate (5 ml.), revealed an intensely fluorescent material, which was identified as isoxanthopterin. Further purification of the fluorescent band on successive chromatograms alternately run in

propanol - 5% acetic acid (2:1) and propanol - 1% ammonium hydroxide (2:1) gave a pure sample of isoxanthopterin in dilute ammonium hydroxide, which was estimated by fluorimetric analysis to contain 87 µg of the pteridine.

Urine Analysis V

Urine:- The urine was that of a patient admitted to hospital suffering from folic acid deficiency.

Two litres of this urine were analysed under exactly identical conditions to those employed in analysis (IV). The ammonium hydroxide eluate of the secondary florisil column was again found to contain isoxanthopterin, which was purified and isolated by the normal procedure, and then estimated by fluorimetric analysis. The total quantity of isoxanthopterin isolated was found to be 102 µg.

Urine Analysis VI

In an attempt to streamline the hitherto somewhat tedious procedure for the detection and isolation of isoxanthopterin from urine, a modified analysis method was adopted, and found to be successful.

In this method, the unfiltered urine (250 ml.) was passed through a small florisil column. (13g., 4cm x 3.5cm) made up in the normal manner, and the column simply eluted with a) distilled water (100 ml.) and b) dilute ammonium hydroxide (150 ml.) The distilled water eluate was discarded.

The ammonium hydroxide eluate was evaporated to dryness and taken up as usual in a small volume of fresh ammonium hydroxide. Investigation by paper chromatography revealed the possible presence of 2-amino-4-hydroxypteridine and definitely established that of isoxanthopterin. The yield of the latter was estimated by fluorimetric analysis to be 24 µg.

Isoxanthopterin from Urine: Summary of Results

The total isoxanthopterin isolated from each urine analysis is summarised on page 71 . As the isoxanthopterin resulting from analysis 2 and 3 was estimated prior to the discovery that the calibration standards degraded so rapidly, the yields of isoxanthopterin quoted for these analyses must be regarded as approximate only. The yields for the remaining analyses were estimated with freshly made up standards however, and can be considered to be accurate to within ± 5%.

Isoxanthopterin Isolated from Urine

Urine Analysis	Urine Volume (litres)	Isoxanthopterin Isolated (µg)
1	10	Qualitative Only
2	10	25
3	10	23
4	2	87
5	2	102
6	0.25	24

R_f Values of Isoxanthopterin and 2-Amino-4-Hydroxy Pteridine

SOLVENT	1	2	3	4	5	6	7
Synthesised Isoxanthopterin	0.46	0.36	0.39	0.33	0.28	0.28	
Isoxanthopterin Isolated from Urine	0.47	0.36	0.40	0.33	0.26	0.28	
Synthesised 2-Amino-4-Hydroxy Pteridine	0.68	0.55				0.46	0.46
Literature Values for 2-Amino 4-Hydroxypteridine	0.66	0.55				0.44	0.45

1. 5% Acetic Acid
2. 3% Ammonium Chloride
3. Propanol - 5% Acetic Acid (2:1)
4. Propanol-Water-15N Ammonium Hydroxide (2:1:0.05)
5. t-Butanol-Pyridine-Water (12:3:5)
6. Propanol - 1% Ammonium Hydroxide (2:1)
7. n-Butanol-Acetic Acid-Water (4:1:5)

EXPERIMENTAL

PART 1(b): The metabolism of folic acid in the rat.

Apparatus and Chemicals:- Folic acid - 2-C-14 (specific activity 31.4 mc/mM) as its dipotassium salt, and C-14 n-hexadecane (0.781μc), were both supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

The subject of the experiment, a healthy 20 week old female Wistar rat, was provided by the Department of Physiological Chemistry, University of Birmingham.

The liquid scintillator, NE 220, designed for use with aqueous solutions, was supplied by Nuclear Enterprises, (G.B.) Ltd., Sighthill, Edinburgh 11. The counting of the urine samples was carried out on a Nuclear Enterprises Automatic Liquid Scintillation Spectrometer, and that of the paper chromatograms on a Packard Radiochromatogram Scanner R.7200 (Packard Instrument Co. Ltd., Wembley, Middlesex.)

EXPERIMENTAL

Administration of C-14 labelled folic acid

Folic acid-2-C-14 (10μc) as its dipotassium salt, was dissolved in distilled water (1 ml.), and orally administered to a healthy 20 week old female Wistar rat, which had not previously been used for any experimental work.

Urine collection:- The urine samples were collected for one day prior to, and daily for 6 days after the administration of the radioactive folic acid. The 24 hr. samples (each approximately 10 ml.) were collected over a small volume (0.5 ml.) of concentrated hydrochloric acid, and although faece free, were deeply coloured. The final two urine samples were collected together over 48 hr.

Liquid Scintillation Counting of Samples:- Each urine sample was made up to a known volume with distilled water, and 0.1 ml. of the diluted solution added to a counting vial, the volume being made up to a total of 5 ml. by the addition of oxygen scavenged scintillator. All the samples were counted at - 5°C on two channels, the mean counting rate being taken as the average of a minimum of 4 separate measurements, each of 10,000 accumulated counts. Because of the variations in the degree of quenching encountered, all the samples were recounted after the addition of an internal standard, C-14 n-hexadecane. The background activity was determined using the non-radioactive urine sample (1). From the data (page 78) obtained as above, the total radioactivity recovered in the urine, 90% of which emerged in the first 48 hrs, was found to be 1.18 μc, or 11.8% of the radioactivity administered.

Column chromatography of the radioactive urine:- Urine samples 2 and 3 (1.06 μc) were combined (total volume 45 ml.) and passed through a small (4g.) florisil column made up as a slurry in 1% hydrochloric acid. The column was then eluted with the same solvent (15 ml.)

followed by 14% ammonium hydroxide (11 ml.) The urine, hydrochloric acid and ammonium hydroxide eluates all possessed a blue fluorescence in UV light. The first two were made up to 50 ml. and 25 ml. with distilled water and 1% hydrochloric acid respectively. In order to preserve the ammonium hydroxide eluate in its concentrated form, the total volume was weighed (11.28 g) without further dilution.

Liquid scintillation counting of all three eluates was carried out under the same conditions as were used for the counting of the original urine samples. The total radioactivity recovered from the column, as shown in the data (page 79) was 0.83µc (78%), 0.55 µc of which was present in the ammonium hydroxide eluate.

Analysis of the ammonium hydroxide eluate for radioactive isoxanthopterin:-

A known amount (0.673g) of this eluate was applied as band to a Whatman No. 3 MM paper and the chromatogram run in propanol - 1% ammonium hydroxide (2:1), with an accompanying isoxanthopterin standard. The paper was then dried at 105° for 1 hr., and any fluorescent areas detected in the usual way.

The radioactive areas of the chromatogram were detected using a Packard Radiochromatogram Scanner, as follows:-

A longitudinal strip (width $1\frac{7}{8}$") was cut from the paper chromatogram, and a relatively strong radioactive marker was added, to enable any active areas found during the scanning

process, to be accurately pin-pointed. The positions of these areas were denoted by r_f values in the normal way.

Any fluorescent band, corresponding approximately with the isoxanthopterin standard and with a radioactive area, was eluted from the chromatogram with dilute ammonium hydroxide, evaporated to dryness, taken up in a small volume of fresh ammonium hydroxide and re-run as a band on another chromatogram in a different solvent. The dried paper was examined for fluorescent and radioactive areas as before. The data regarding these areas and the relationship between them, in the four different and successive solvent systems employed,is given on page 80 . Owing to the considerable effect of the ammonium hydroxide eluate on the r_f values of the isoxanthopterin standard, the values quoted in the table are those in which the latter was run in conjunction with a portion of the eluate itself.

The first chromatogram, (R 1), showed coincident fluorescent and radioactive areas at an r_f value closely approximating to that of isoxanthopterin. Re-application of the fluorescent area on to a second chromatogram (R2) run in propanol - 5% acetic acid (2:1), split the band into two distinct fluorescent areas(a) and (b) each corresponding to a well defnned radioactive area of similar r_f values, as the table indicates. Each fluorescent area was eluted in the normal way, and re-chromatographed ($R3_A$ and $R3_B$ respectively) in t-butanol-pyridine-water (12:5:3). Chromatogram R3A showed

one radioactive and fluorescent band at or near the origin, the identity of which was not further investigated, whilst $R3_B$ gave two fluorescent areas of r values 0 - 0.05 and 0.12 - 0.17 (weak) respectively. Scanning of this chromatogram showed up radioactive areas at 0 - 0.05 and 0.08 - 0.26, the r_f of the isoxanthopterin standard being 0.18.

Finally, the radioactive area (r_f 0.12 - 0.17) corresponding to the isoxanthopterin standard was eluted from $R3_B$ and applied to a fourth chromatogram (R4) and run in isopropanol - 1% ammonium hydroxide (1:1). Although barely detectable, a fluorescent area was probably present at r_f 0.34 - 0.38, as was a radioactive one at r_f 0.32 - 0.37. The r_f of the isoxanthopterin standard was 0.35.

From the results obtained from the above chromatographic experiments it is distinctly possible that radioactive isoxanthopterin may be present in the ammoniacal eluate.

The Recovery of Radioactivity from Rat Urine Following the Oral Administration of C-14 Folic Acid

ADMINISTRATION

URINE SAMPLE NUMBER →	1	2	3	4	5	6 + 7
Volume (ml)	20	20	25	25	25	25
Counting Efficiency (%)	69.2	63.8	72.9	74.0	74.6	68.1
Counting Rate (cps)	1.20	110.90	14.58	4.02	3.05	4.67
Total radioactivity (μc)	-	0.94	0.12	0.05	0.04	0.05
% of recovered activity	-	79.6	10.2	3.4	2.5	4.2

Total radioactivity administered 10μc

Total radioactivity recovered 1.18 μ c

The Distribution of Radioactivity between the Eluates of the Florisil Column, on to which Urine Samples 2 &3 had been placed

Eluate	Urine	1% hydrochloric acid	14% ammonium hydroxide
Volume (ml)	50	25	11.28g
Counting Efficiency (%)	80.7	83.5	75.9
Counting Rate (cps)	11.19	11.32	137.70
Total radioactivity (µc)	0.19	0.09	0.55
% of recovered activity	22.8	10.9	66.3

Radioactivity applied to column ... 1.06

Radioactivity recovered from column ... 0.83

% recovery 78.3%

CHROMATOGRAM ——→	R1	R2	R3A	R3B	R4
Solvent	Propanol-1% Ammonium Hydroxide (2:1)	Propanol-5% Acetic Acid (2:1)	t-butanol-pyridine-water (12:5:3)		Isopropanol-1% Ammonium Hydroxide (1:1)
r_f of Isoxanthopterin Standard	0.45	0.31	0.21	0.18	0.35
r_f of Fluorescent Areas	0.36 - 0.50	0.12 - 0.17 and 0.23 - 0.43 →	0 - 0.05	0 - 0.05 and 0.12 - 0.17 →	0.34 - 0.38 ?
r_f of Radioactive Areas	0.36 - 0.48	0.11 - 0.19 and 0.22 - 0.32 →	0 - 0.05	0 - 0.05 and 0.08 - 0.26 →	0.33 - 0.37 ?

Radiochromatographic Analysis of the Ammonium Hydroxide Eluate for Radioactive Isoxanthopterin: The Correlation between the Fluorescent and Radioactive Areas of each Paper Chromatogram

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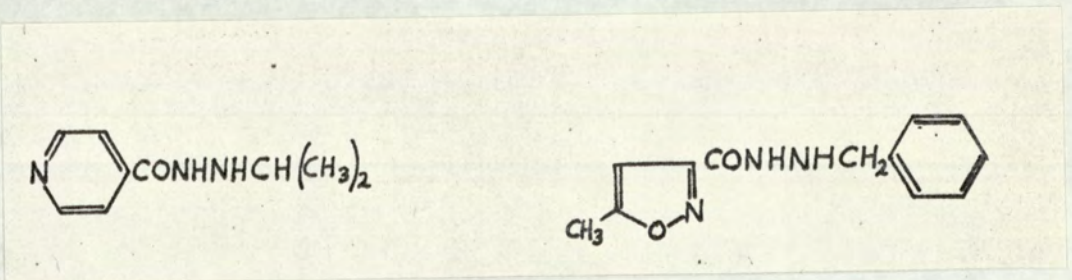
PART 2

THE SYNTHESIS AND TUMOUR INHIBITORY PROPERTIES OF SIMPLE HYDRAZINES

The Pharmacological Activity of Hydrazines

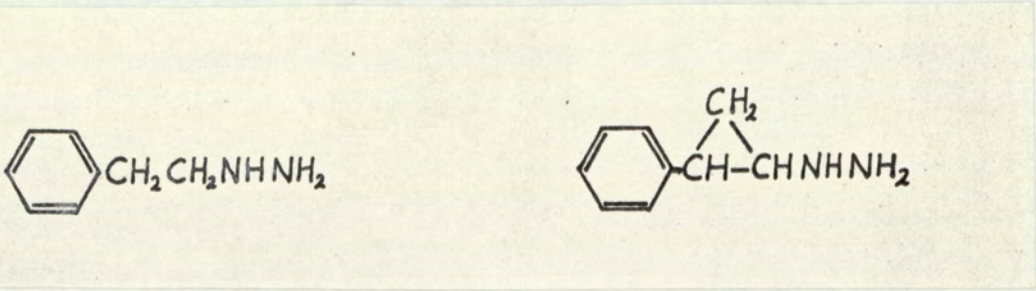
Apart from the introduction of a few cyclic hydrazines such as antipyrine and aminopyrine as pharmaceuticals in the late nineteenth century, hydrazine and its derivatives appeared at one time to be of little biological interest. The discovery[1-3] in 1952 however, of the potent tuberculostatic action of isonicotinic acid hydrazide, and the antiphlogistic activity of phenylbutazone, and their subsequent development to become two of the most widely used hydrazine pharmaceuticals, has firmly established the, now rapidly increasing, pharmacological importance of this group of compounds.

Further investigation of isonicotinic acid hydrazide and similar tuberculostatics by Zeller and Barsky[4] led to the discovery that certain hydrazine derivatives exert a strong stimulant and anti-depressant activity on patients suffering from nervous disorders. These compounds are characterised by a strong inhibition of the enzyme mono-amine oxidase, and are therefore summarised under the collective term, mono-amine oxidase inhibitors. A number of these compounds, such as those formulated below, are now used in the chemotherapy of nervous diseases.



Iproniazide

Isocarboxazide



Phenelzine

Tranylcypromine

Further experimental work over the last ten to fifteen years has demonstrated that, in addition to their tuberculostatic and monoamine oxidase inhibitory properties, both cyclic and acyclic hydrazine derivatives possess a very broad spectrum of pharmacological activity[5-8], including carcinogenicity.

This spectrum was recently further enlarged when Bollag and Grunberg[9], whilst testing a series of hydrazines for another purpose discovered that 1-methyl-2-benzyl hydrazine displays a powerful inhibitory effect on the growth of transplantable tumours in mice and rats. As this compound failed to show a satisfactory chemotherapeutic index, and as it led, furthermore, to severe liver damage, Zeller and co-workers[10] synthesised a large series of derivatives of similar structure in order to find substances having better chemotherapeutic properties combined with a lower toxicity. The screening of these compounds revealed some forty to be efficient tumour inhibitors, among which N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride (Natulan) and 1-methyl-2-p-allophanylbenzyl

hydrazine hydrobromide (II), were found to be the most promising and have undergone extensive biological and chemical trials.

$CH_3NHNHCH_2C_6H_4CONHCH(CH_3)_2.HCl$ Natulan

$CH_3NHNHCH_2C_6H_4CONHCONH_2.HBr$ (II)

These workers found that only compounds containing the group $CH_3NHNHCH_2C_6H_4$-R displayed appreciable cytotoxic activity.

It was therefore considered worthwhile to synthesise and evaluate as tumour inhibitors, a series of simple aryl and alkyl hydrazines, in order to further elucidate, if possible, this structure-activity relationship and the mode of action of these compounds. The inhibitory activity of the synthesised hydrazines will be discussed in the light of present day theories of its origin.

The Metabolism of Hydrazine Derivatives

Following the discovery by Zeller[10], Bollag and their co-workers[9] that a number of hydrazine derivatives possessed considerable tumour inhibitory properties, clinical trials of the most promising one, N-isopropyl-α-(2 methylhydrazino)-p-toluamide hydrochloride (Natulan), were rapidly initiated. Martz[11] found that Natulan was particularly effective in the treatment of patients suffering from Hodgkin's disease, even though the previous chemotherapy of most of the cases had included treatment with alkylating agents (vinca alkaloids and corticosteroids). The

fact that no cross resistance occurred between these drugs and Natulan suggested that the latter might function by a different biochemical mechanism.

Our present knowledge of the metabolism of cytotoxic hydrazine derivatives, which is still far from complete, stems mainly from the efforts which have been made by a number of workers to elucidate the degradation pathway of Natulan itself. Raaflub and Schwarz[12] demonstrated that, following the intravenous administration of Natulan, the concentration of the hydrazine in the blood diminishes very rapidly, the 'half-life' being a matter of a few minutes only. This rapid disappearance has been attributed[9,12,13] to the facility with which Natulan and similar compounds, particularly those containing benzyl groups, undergo autoxidation to the corresponding azo compound, with the liberation of hydrogen peroxide[14,15]. The oxidation has been found to occur via a free radical process . The auto-oxidation of Natulan itself, which proceeds much more rapidly 'in vivo' (in blood plasma, for example) than 'in vitro', yields N-isopropyl-α-(methylazo)-p-toluamide[12]. ('Azo-Natulan').

It is well known that such azo compounds are capable of tautomerism to a hydrazone or hydrazones[16], and that such rearrangements can be catalysed by bases and free radicals[17,18]. Under the right conditions simple alkyl hydrazones can be readily converted into the constituent aldehyde and hydrazine[19], as was

confirmed by Berneis[14], who was able to show that formaldehyde resulted from the autoxidation of 1-methyl-2-benzyl hydrazine phosphate. Although the expected aldehyde product from Natulan, N-isopropyl-4-formyl benzamide has yet to be detected either

$$(CH_3)_2CHNHCO.C_6H_4.CHO$$

'in vivo' or 'in vitro', Oliviero and Kelly[13] have reported the isolation of the corresponding acid, N-isopropyl-terephthalamic acid from the urine of man, and various animals, following the administration of a single parenteral dose of Natulan. In a similar experiment Raaflub and Schwarz[12], using radioactive Natulan, were able to recover 42% of the active sample as the above acid derivative. On the basis of the experimental work detailed above, these authors postulated the following scheme for the metabolism of Natulan:-

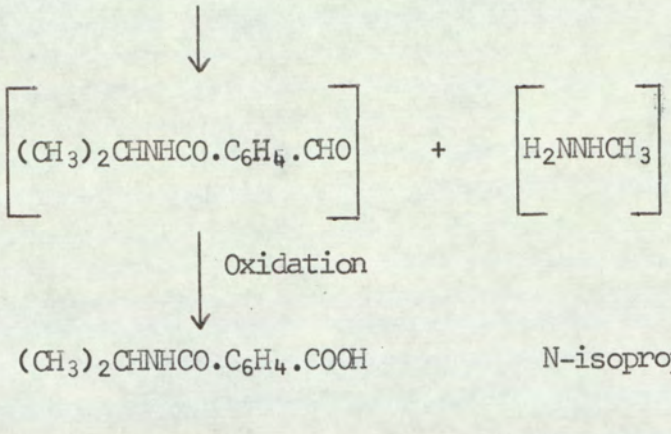
$(CH_3)_2CHNHCO.C_6H_4.CH_2NHNHCH_3$ — Natulan

$\downarrow$ (o) $\longrightarrow H_2O_2$

$(CH_3)\ CHNHCO.C_6H_4.CH_2N{=}NCH_3$ — N-isopropyl-(2-methylazo)-p-toluamide

$\downarrow$

$(CH_3)_2CHNHCO.C_6H_4.CH{=}NNHCH_3$ — Hydrazo derivative



It is now clear, however, that this scheme is by no means the only degradation pathway. N-demethylation was postulated by Weitzel and co-workers [20,21] in 1964. These authors were able to show that 'in vitro' oxidation of Natulan with potassium hexacyanoferrate gave formaldehyde, and it has recently been demonstrated that the liberation of formaldehyde under these mild conditions is a characteristic of methyl hydrazines only [22]. In parallel 'in vivo' experiments with liver microsomes, Natulan has been observed to yield formaldehyde [23], as has N-isopropyl-α-(methylazo)-p-toluamide in the presence of ascites cancer cells [22]. Further evidence for the demethylation of alkyl hydrazines has been reported by Dost and co-workers [24] who observed that C-14 unsymmetrical dimethyl hydrazine was metabolised in the rat to respiratory C-14 CO_2, and monomethylhydrazine, to C-14 CO_2 and C-14 methane. Using a low dose of the monomethylhydrazine, as much as 80% of the administered radioactivity was recovered as carbon dioxide and methane. The same two gaseous products have been identified as metabolites of Natulan itself [25]. The mechanism involved in the cleavage of these one carbon atom fragments

from hydrazines or their metabolites, is not yet ascertained. Eberson and Perrson[26] have studied the oxidation of β-phenylisopropylhydrazine when it was catalysed by cupric ion under conditions resembling those in biological systems. Their data provide evidence for a free radical mechanism leading to oxidation products which include isopropyl benzene, propenyl benzene, phenyl acetone, 1-phenyl-2-propanol and β-phenyl-2-propanol. Neuman and Nadeau[27] have reported the formation of methane, nitrogen, and small quantities of carbon monoxide from a dilute aqueous solution of monomethylhydrazine upon oxidation by dilute sodium hypochlorite. Beaven and White[28] have shown that oxyhemoglobin can produce benzene and molecular nitrogen from phenylhydrazine in the presence of oxygen. It therefore appears likely that the dealkylation and dearylation of hydrazines by these relatively weak oxidising systems, proceeds via a free radical process. It can be reasonably assumed that the demethylation of Natulan and its derivatives, is no exception.

It has now become apparent, that not only is Natulan cleaved on either side of the hydrazine group, as the simultaneous liberation of the N-methyl group, and the production of N-isopropyl terephthalamic acid indicate, but that the N-N bond of the hydrazine group is itself broken. This was demonstrated by Schwarz[29], who identified C-14 methylamine as a urinary metabolite of

1-methyl-C-14-labelled Natulan and C-14 methylhydrazine, in rats. This observation indicates that metabolic reductive cleavage of this bond is not, in fact, confined to aromatic compounds [30,31].

A possible scheme for the diverse metabolism of Natulan is formulated on page 102.

As can be seen from this scheme there are a number of points at which demethylation of Natulan can take place. The three major alternatives would appear to be as follows:-

1. Cleavage of the N-methyl group from Natulan itself, or one of its oxidation products, e.g. the azo compound, or one of the hydrazones.
2. Cleavage of the N-N bond to give methylamine and subsequent demethylation.
3. The splitting-off of methylhydrazine, and demethylation either direct or via methylamine.

Considering the third alternative, it is unlikely that methyl hydrazine is the substrate of demethylation for the following reasons:-

a. Equimolar doses of C-14 monomethylhydrazine undergo considerably slower demethylation than Natulan in rats [32].

b. Steady state expiration of C-14 CO_2 occurs only ten

minutes after administration of Natulan[33]. In that time the azo-compound must have accumulated in large amounts[12], whereas the formation of methylhydrazine still requires further metabolic steps. Moreover, methylhydrazine has not yet been detected, although it might be expected to accumulate, because of its low demethylation rate.

That methylamine (Alternative 2) is not an intermediate in the production of carbon dioxide from either Natulan or methyl hydrazine was shown by Baggidini and his co-workers[34]. It therefore seems likely that the first alternative, i.e. the cleavage of the N-methyl group from Natulan, or one of its oxidation products, is the most feasible. It is improbable that Natulan itself is demethylated because of its very rapid conversion to the azo-compound, which, of course, retains the methyl group. It thus seems not unreasonable to assume, at least until proved otherwise, that the site of demethylation in the metabolism of Natulan is the N-isopropyl-(methylazo)-p-toluamide. From the foregoing, it is evident that the metabolism of Natulan and similar hydrazines is quite complex, and it is therefore to be expected that the possible mechanisms of cytotoxic activity of these compounds will be equally diverse. These mechanisms, together with the tumour inhibitory properties of the hydrazines synthesised by the author, will be discussed in the next section.

Cytotoxic Mechanisms of Hydrazines

The Synthesis of Hydrogen Peroxide

One of the earliest indications of the mode of action of the tumour-inhibitory methylhydrazine compounds was the work of Rustihauser and Bollag[35], who demonstrated that the intraperitaneal injection of 1-methyl-2-benzylhydrazine phosphate into Albino mice, previously inoculated with Ehrlich ascites cells, resulted in a marked depression of mitosis, and considerable chromosomal abnormalities. As the chromosomes contain a large amount of deoxyribosenucleic acid (DNA), the action of these hydrazines on DNA became of considerable interest. In vitro investigations by Berneis and co-workers[14,15] showed that, in the presence of molecular oxygen, N-isopropyl-(2-methylhydrazino)-p-toluamide hydrochloride (Natulan), causes a marked degradation of DNA, as indicated by viscosity measurements. The viscosity of the DNA is unaffected when oxygen is replaced by an inert gas. An equimolar quantity of hydrogen peroxide leads to a quantitatively equivalent effect on the DNA, whereas, in contrast, the addition of peroxidase or catalase to a solution of DNA containing hydrogen peroxide and Natulan, rapidly and completely arrests the degradation of the nucleic acid. These, and other observations led the authors[14,15] to assume that the oxidation of Natulan by molecular oxygen yields hydrogen peroxide by a process involving

free radicals as intermediates. The autoxidation of monomethylhydrazine, 1-methyl-2-benzylhydrazine phosphate[14], acetylphenylhydrazine[36] and phenylhydrazine[36] also liberates hydrogen peroxide.

It is generally accepted that the action of hydrogen peroxide on DNA proceeds via hydroxyl radicals[37-39]. An analogy of the effect of methylhydrazine derivatives on DNA with the effect of X-rays is therefore evident, as the latter is thought to be mainly due to the action of such radicals also[40,41]. This analogy has recently been emphasised experimentally by Berneis and co-workers[42] who observed that the combined effect of Natulan and ionising radiation upon the nucleic acid is considerably greater than the aggregate of the separate effects, and that the most severe degradation occurs when Natulan is added to the DNA solution immediately after irradiation. The authors[42] consider that this sensitising effect on DNA subjected to the action of Natulan, can be attributed to the formation of unstable peroxides during the irradiation[43], which yield free radicals as a result of their decomposition[44]. The radicals formed during this decay can then act as initiators for the autoxidation of the hydrazines, the resulting higher autoxidation rate of which increases the rate of formation of hydrogen peroxide, which in turn will accelerate the degradation of the DNA. It is also possible that the autoxidation of hydrazines may liberate strong reducing agents[14], such as methyl hydrazine (see page 102) which are capable of

reacting with the nascent hydrogen peroxide[45] to yield hydroxyl radicals.

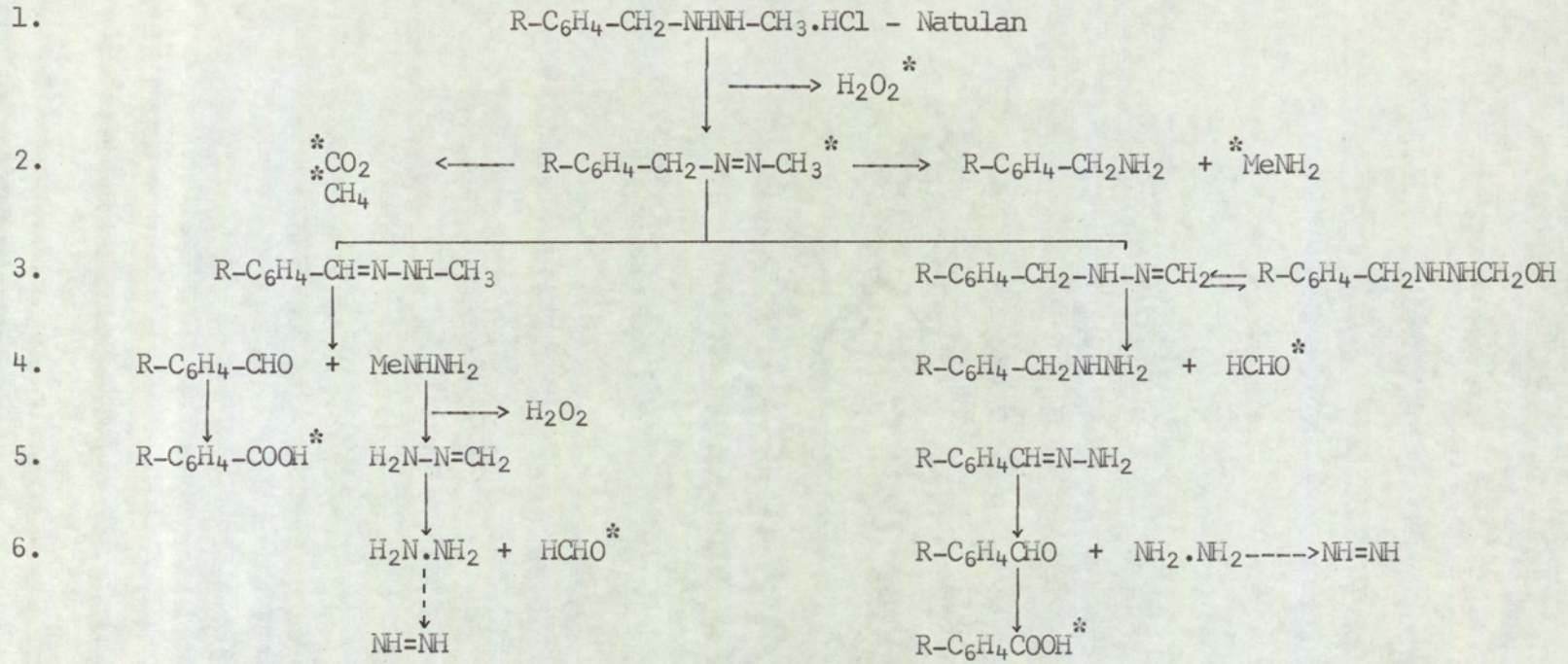
$$CH_3NHNH_2 + H_2O_2 \longrightarrow CH_3N = NH + 2OH. + H_2$$

Although the rapidity with which methylhydrazines are oxidised 'in vivo' to the corresponding azo-compounds implies that only a certain proportion of the hydrogen peroxide thus liberated may actually reach the tumour cell, it is feasible that a part, at least, of the cytotoxic activity of these hydrazines is traceable to the degradation of deoxyribosenucleic acid (DNA) by this hydrogen peroxide.

On the basis of this theory however, it is rather difficult to understand why methylhydrazine compounds are active anti-tumour agents, whereas the corresponding ethylhydrazines with the same tendency to oxidise are relatively inactive[46]. According to Bollag[46] this may be explained by the fact that ethylhydrazines are much more readily oxidised than methyl-hydrazines. This could result in the ethyl derivative being oxidised too rapidly to retain any inhibitory activity upon arrival at the site of the tumour cell.

The Formation and Tumour-Inhibitory Action of Formaldehyde

A characteristic of methylhydrazine derivatives is the liberation of formaldehyde both 'in vitro'[22,23] and 'in vivo'[21] during their degradation under mild oxidising



A POSSIBLE SCHEME FOR THE METABOLISM OF NATULAN

conditions. Compounds such as Natulan, azo-Natulan, azoxy-Natulan and 1-methyl-2-benzylhydrazine split off formaldehyde, whereas those lacking an N-methyl group, such as benzylhydrazine and p-(N-isopropylcarbamoyl)-benzylhydrazine do not.

$(CH_3)_2CHNHCO.C_6H_4.CH_2NHNHCH_3.HCl$	Natulan
$(CH_3)_2CHNHCO.C_6H_4.CH_2N{=}NCH_3$	Azo-Natulan
$(CH_3)_2CHNHCO.C_6H_4.CH_2N{=}NCH_3$ (with $\downarrow$ O on N)	Azoxy-Natulan
$(CH_3)_2CHNHCO.C_6H_4.CH_2NHNH_2$	p-(N-isopropylcarbamoyl)-benzylhydrazine

That formaldehyde can feasibly be formed from these methylhydrazine derivatives and related compounds in several ways can be seen from the postulated degradation scheme of Natulan itself (page 102). As indicated, azo-Natulan, formed by the autoxidation of Natulan[12], is capable of isomerisation in both directions to give two different hydrazones, which are in turn each hydrolysed to further degradation products.

Following the "left-hand" pathway the benzylidene hydrazone can hydrolyse to N-isopropyl-4-formylbenzamide and methylhydrazine (step 4). The oxidation decomposition of the latter could then lead to formalhydrazine ($NH_2N{=}CH_2$), which will, in turn, yield hydrazine itself, and formaldehyde, whilst the oxidation of

N-isopropyl-4-formylbenzamide will give the corresponding acid, N-isopropylterephthalamic acid (step 5). Weitzel and his co-workers[23] have shown that mild oxidation of formalhydrazine with hexacyanoferrate (III) does, in fact readily liberate formaldehyde - in 55% yield.

Alternatively, or more likely, additionally, the re-arrangement of the azo-compound could give the formal hydrazone (step 3 - "right hand pathway"):

$$(CH_3)_2CHNHCO\text{-}C_6H_4\text{-}CH_2NH\text{-}N{=}CH_2$$

which in aqueous solution would be expected to be in equilibrium with the corresponding hydroxymethyl compound; N-isopropyl-α-(2-hydroxymethylhydrazino)-p-toluamide:

$$(CH_3)_2CHNHCO.C_6H_4.CH_2NHNHCH_2OH.$$

Such compounds are unstable in aqueous solution, and decompose to yield formaldehyde and the free hydrazine[21]. The remainder of the pathway (R.H.S.) leads to the substituted benzalhydrazone (step 5), the hydrolysis of which will give N-isopropyl-4-formylbenzamide, hydrazine and N-isopropyl-terphthalamic acid (steps 6 and 7).

It is possible that formaldehyde is formed via <u>both</u> pathways. That azo-Natulan and similar compounds degrade by both routes is supported by the fact that the autoxidation of 1-benzyl-2-methyl-hydrazine yields benzaldehyde and formaldehyde simultaneously[22]. The fact that many methylhydrazines show considerable anti-tumour

activity, and are also capable of readily liberating formaldehyde upon degradation, suggests that formaldehyde itself may possess tumour-inhibitory properties. That this is, in fact, the case, was confirmed by Weitzel et. al.,[47] who demonstrated that formaldehyde completely inhibited the growth of mouse ascites-carcinoma 'in vivo'.

The origin of the inhibitory activity of such a chemically simple compound is the source of much speculation. It is feasible that it could combine with the nascent hydrogen peroxide deriving from the autoxidation of the original methylhydrazine, to yield organic peroxides[21], such as hydroxymethyl peroxide, and bis-hydroxymethyl peroxide, as follows:-

$$CH_2O + H_2O_2 \longrightarrow HOO.CH_2.OH \xrightarrow{CH_2O} HO.CH_2.OO.CH_2OH$$

Formaldehyde hydroxymethyl peroxide bis-hydroxy-methyl peroxide

That such peroxides are potent tumour-inhibitors is already well established[48]; their mode of action is thought to be intracellular alkylation reactions[47], the mechanisms of which will be discussed in the following section. Formaldehyde has also been observed to markedly inhibit the incorporation of thymidine, uridine and thymine into ascites cancer cells.[22] Recent experimental work 'in vitro', using suspensions of ascites cells, by Weitzel and co-workers[23] has succeeded in more accurately pin-pointing the

site of action of formaldehyde. From their observations it appears that a major point of attack is to prevent the formation of the nucleoside di- and tri-phosphates. If insufficient formaldehyde is added to the ascites cell suspension to completely inhibit nucleoside phosphorylation, the incorporation of the constituent bases of the nucleic acid returns to normal. It therefore seems probable that formaldehyde is also instrumental in inhibiting the final stages of the DNA or RNA biosynthesis. This could occur, for example, by attack on the polymerases, or on the bases of the already formed nucleic acids.

The reported enhanced cytotoxic activity of hydrazines bearing an N-methyl group may therefore be attributable to the fact that the degradation of such compounds is known to yield the tumour inhibitory agent, formaldehyde, a compound not liberated by the corresponding unmethylated compounds.

The Formation and Action of Alkylating Agents

The family of compounds known as alkylating agents has members drawn from a number of different chemical classes, such as epoxides, ethylene imines, alkane sulphonates and sulphur or nitrogen mustards. Such groups of compounds examples of which are formulated below, have been employed in the chemotherapy of cancer for some years.

SULPHUR MUSTARDS	$S(CH_2.CH_2.Cl)_2$	ETHYLENEIMINES	$O{<}\!-\!P\left(N\begin{smallmatrix}CH_2\\|\\CH_2\end{smallmatrix}\right)_3$
NITROGEN MUSTARDS	$RN(CH_2.CH_2.Cl)_2$	METHANESULPHONATES	$MeSO_2O(CH_2)_4OO_2SMe$
EPOXIDES	$CH_2.CH.CH.CH_2$ (O, O)	β-LACTONES	$CH_2.CH_2$ (O, -CO)

Examples of various classes of biological alkylating agents

It is generally agreed that such compounds exert their effect by alkylation of nucleophilic sites in the biological material, probably via a positively charged alkyl radical [49].

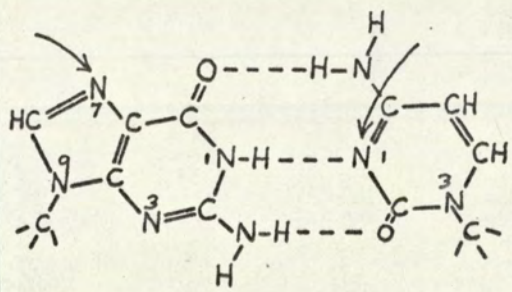
It seems not unreasonable to expect, that such potent tumour inhibitors as hydrazines and in particular alkylhydrazines, should function as alkylating agents, at least to some degree. Consideration of the postulated degradation pathway of Natulan (page 102) indicates that such compounds could feasibly include:-

1. Formalhydrazine ($NH_2.N{=}CH_2$) and its substituted derivative, ($R\text{-}NH\text{-}N{=}CH_2$) $R=(CH_3)_2CHNHCO.C_6H_4.CH_2-$
2. N-isopropyl-α-(2-hydroxymethylhydrazino)-p-toluamide $(CH_3)_2CHNHCO.C_6H_4.CH_2NHNHCH_2OH$

and,

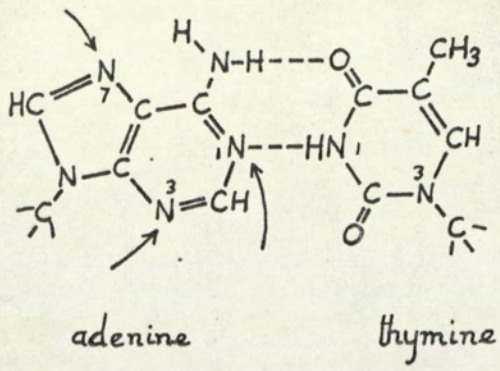
3. Formaldehyde, together with the organic peroxides hydroxy and bis-hydroxymethyl peroxide, $HOO.CH_2OH$ and $HO.CH_2OOCH_2.OH$ respectively.

Compared to many clinically useful drugs, the mode of action of alkylating agents is relatively well understood. In addition to cytotoxicity, the alkylating agents are known to be powerful mutagens[50], and to inhibit the replication of DNA-containing viruses[51]. These facts suggested that the reaction of alkylating agents with DNA might be of considerable biological significance. As one might expect from the nature of the reagent, the sites in the nucleic acids which are reactive towards alkylating agents in neutral solution are the basic ring N atoms[52]. It has been observed that the most reactive site in DNA and RNA (ribosenucleic acid) is the N-7 position of guanine,[52] an observation which is in agreement with the theoretical work of Pullman and Pullman[53]. The next most susceptible site in DNA is the N-3 position of adenine[54], although in RNA it appears to be the N-1 position[55]. Alkylation also occurs at the N-1 position of cytosine and at N-7 in adenine, but to a relatively, very small extent. The normal base pairs as they occur in DNA, and the positions at which alkylation takes place is illustrated below:-



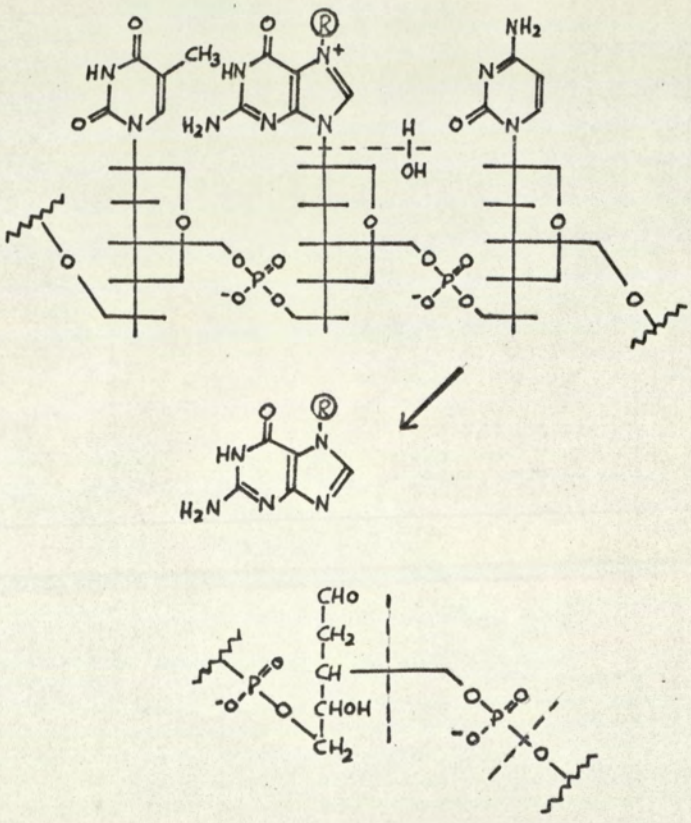
guanine cytosine

N- nitrogen atoms reactive to alkylating agents.



N - nitrogen atoms reactive to alkylating agents.

The initial alkylation of guanine at N-7 and adenine at N-3 causes the slow fission, at pH7 and 37°, of the deoxyribose bond joining these alkylated bases to the sugar-phosphate backbone chain of the DNA[56,57], as shown below:-



Lawley and Brooks[55] found that the rate of loss of the 3-alkyladenine is much greater than that of the corresponding 7-alkylguanine under the same conditions. A further result of this depurination process is the slow fission of the sugar phosphate chain itself[58], as also indicated in the above diagram. This depurination, under physiological conditions, following alkylation, occurs only for DNA. In the case of RNA, the 7-alkylguanine moiety is stable under these conditions[59].

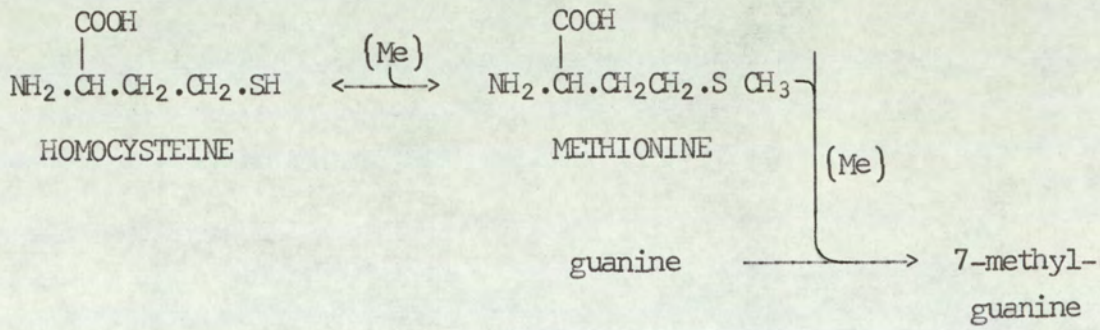
That formaldehyde itself might act as an alkylating agent, was first suggested by Weitzel and his co-workers[20]. Although formaldehyde, in vivo, might be expected to contribute to the formaldehyde pool for the de novo synthesis of the purine bases, adenine and guanine (C_2 and C_8 atoms), and to the methyl group of thymine, it is possible that it might also alkylate any of the purine or pyrimidine bases of DNA and RNA in the manner first discussed. It is known that methylhydrazines, including Natulan itself, liberate formaldehyde readily 'in vitro'[22] and 'in vivo'[23]. Recent experimental work has lent considerable support to the suggestion that this liberated formaldehyde does, in fact, act as an alkylating agent. Kreis and Yen[60], using C-14-Natulan

$$*CH_3.NHNH.CH_2.C_6H_4.CONHCH(CH_3)_2.HCl$$

*Labelled at this atom

were able to demonstrate that, at least part of the Natulan

molecule is incorporated into DNA and RNA 'in vivo'. More specifically they found that the N-methyl group was oxidised, and incorporated into adenine, guanine and the methyl group of thymine. The same radioactive bases were identified from parallel experiments using C-14 formate. Strong evidence that the C_1 unit derived from the N-methyl group of Natulan acts as an alkylating agent, has been obtained by Kreis et. al [61], who after the intraperitoneal injection of C-14 Natulan (labelled at the N-Me group) into mice inoculated with P815 leukemic cells were able to identify radioactive guanine and 7-methylhypoxanthine. The distribution of radioactivity between the purines and their corresponding methyl derivatives indicated that the methyl group of Natulan not only contributes to the formate pool, but is also transferred by circumventing the pool, either by a direct transmethylation, or indirectly, via homocysteine and methionine:-



Very similar results were obtained using C-14 formate instead of C-14 Natulan. The indirect pathway via homocysteine and methionine is one which is of considerable importance in general

biological transmethylation reactions. It may be significant in this connection that Mandel and co-workers [62], whilst investigating the metabolic fate of the C-14 labelled methyl group of methionine, were able to identify the same unmethylated and methylated bases in the urine of mice carrying mammary carcinoma, as those reported by Kreis [61].

In addition to formaldehyde, of the other potential alkylating agents suggested in the previous discussion (page 107) as arising from the degradation of hydrazines, formalhydrazine (NH_2-N=CH_2) is known to be a potent tumour inhibitor, both 'in vivo' and 'in vitro' [22]. It is capable of inhibiting the incorporation of thymidine, uridine and leucine into ascites cancer cells in a similar way to that of formaldehyde.[22] As, in addition, formalhydrazine is considered to be the precursor of formaldehyde , yielding as it does a 55% yield of the aldehyde on mild oxidation, it seems logical to attribute the tumour inhibitory properties of the formalhydrazine to the liberated formaldehyde. According to Weitzel et. al.[22] , this, does not, in fact, seem to be the case, as an 'in vitro' comparison of the two compounds shows that a) formaldehyde inhibits the incorporation of thymidine, uridine and leucine into ascites cells far more rapidly than formalhydrazine and b) to a far greater extent (mole for mole).

That such a difference in behaviour between the two compounds

exists, is taken by the authors to indicate:-

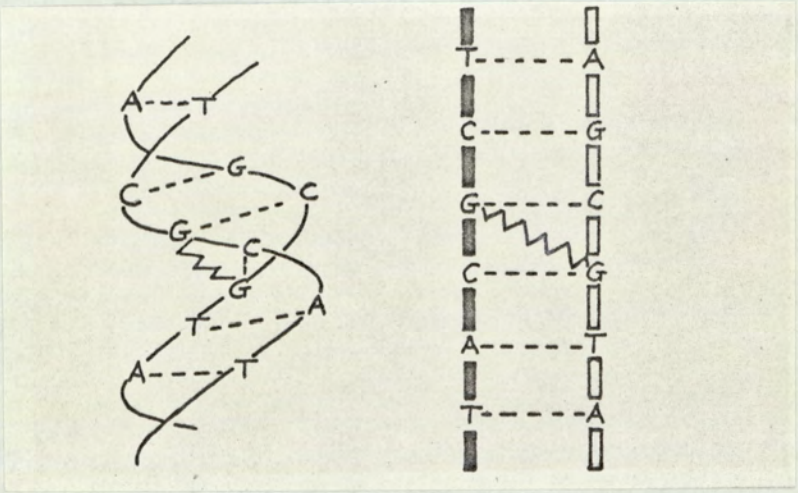
(a) that formaldehyde is not released from formalhydrazine in any great proportion under these conditions and

(b) that formalhydrazine therefore probably acts as an intact molecule on the numerous sites of cell metabolism.

That formalhydrazine may well act in the way suggested (b), seems quite likely in view of its structural similarity to known alkylating agents such as ethyleneimines. With regard to the release of formaldehyde from formalhydrazine however, their experimental results would appear capable of an alternative explanation to the one given. The fact that formalhydrazine is a slower inhibitor of uridine, thymine and leucine than formaldehyde may also be interpreted as being due to the degradation of formalhydrazine being a relatively slow process, with the consequent gradual release of formaldehyde.

As already mentioned (page 105) it is feasible that formaldehyde could also combine with any nascent hydrogen peroxide liberated during the autoxidation of the original hydrazine, to form bis-hydroxymethyl peroxide ($HO.CH_2.OO.CH_2OH$) a potential di-functional alkylating agent. The possible importance of such a compound lies in the fact that such peroxides are potent tumour

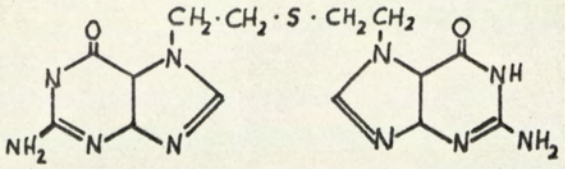
inhibitors[48], and that di-functional alkylating agents are much more powerful cytotoxic agents than the corresponding mono-functional compounds[63]. The greater cytotoxic action of such di-functional agents has been attributed to their ability to form interstrand cross-linkages between the purine bases of the DNA, and in particular between the guanine bases, as illustrated below:-



Interstrand cross-linking of DNA by a difunctional alkylating agent

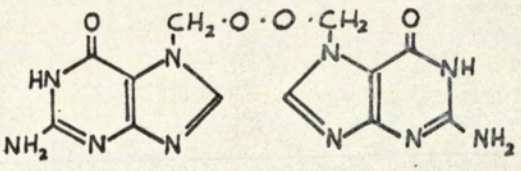
This cross-linking process is thought to interfere with DNA replication, a process which is considered to require the separation of the twin strands of the nucleic acid helix[64]. Di-(guanin-7-yl) derivatives have, in fact, been isolated from,

for example, the reaction of di-(2-chloroethyl) sulphide with either guanine nucleotides [65], or DNA itself [66]:-



The product from the di-functional alkylation of guanine at N-7 by sulphur mustard di-(2-chloroethyl) sulphide

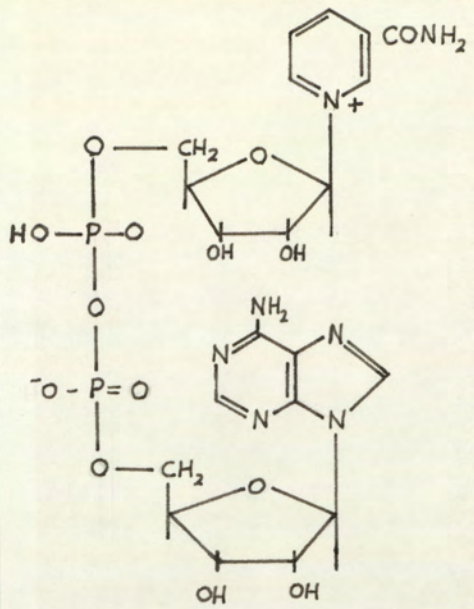
It would seem probable that any bis-hydroxymethyl peroxide formed from hydrogen peroxide, could cause alkylation of the guanine bases of DNA in a similar manner, to give the corresponding alkylated derivative:-



In summary of this section, it would seem likely that hydrazine derivatives and, in particular methylhydrazines owe at least part of their anti-tumour activity to their degradation to such potential alkylating agents as formaldehyde, formalhydrazine, and their derivatives. The prime target of these agents is the DNA itself, with the consequent interference with its role as a template for replication being responsible for the inhibition of cell multiplication.

The Inhibition of Aerobic Glycolysis of Tumour Cells

In 1923, Warburg[67] discovered that the cells of malignant tumours are characterised by a high rate of aerobic glycolysis. It is now known that alkylating agents, such as sulphur and nitrogen mustards, which are active against tumours, also inhibit this glycolysis[68]. That the tumour inhibitory hydrazines under discussion could also interfere with this glycolysis mechanism was suggested by Rustihauser and Bollag[35]. Roilt[69] and Holzer[70,71] found that inhibition of glycolysis by ethyleneimines, for example, is due to a decrease in nicotinamide-adenine dinucleotide (NAD), formerly known as diphosphopyridine nucleotide (DPN).



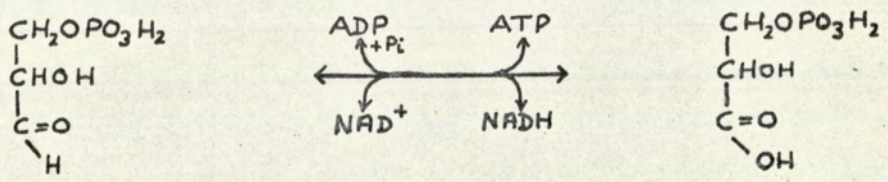
nicotinamide-adenine dinucleotide (NAD)

It has also been demonstrated that the decrease in the concentration of NAD preceeds the reduced rate of glycolysis,[72] and that inhibition of glyceraldehyde-3-phosphate dehydrogenase by the alkylation of the highly reactive SH groups of this enzyme[73,74] is caused by alkylating agents only in higher concentrations or longer interactions[75].

Investigations carried out by Witzel and his co-workers[21] into the effect of methylhydrazines on the concentrations of nucleotides and sugar-phosphates in mouse-ascites tumours, have yielded interesting and in many ways, parallel results to those mentioned above. The injection of Natulan (N-isopropy-α-

(2-methylhydrazino)-p-toluamide) and 1-methyl-2-(p-allophanyl-benzyl)-hydrazine hydrobromide (see page 92 for formulae) into these tumours causes an increase in the concentrations of nicotinamide, fructose diphosphate and triose phosphate (glyceraldehyde-3-phosphate), and a decrease in nicotinamide-adenine dinucleotide (NAD). Glyceraldehyde-3-phosphate is completely inhibited 'in vitro' by both of these methylhydrazines. These observations indicate that the two hydrazines tested do, in fact, seriously interfere with the tumour cell glycolysis mechanism, and in a similar manner to other potential alkylating agents.

Glycolysis is that mechanism whereby the living cell obtains energy from carbohydrates. Essentially this process involves the conversion of glucose to pyruvic acid via fructose and glyceraldehyde (see page 119). The main energy yielding reaction in this glycolysis process is the conversion of glyceraldehyde-3-phosphate to the corresponding carboxylic acid, a step which is catalysed by the enzyme glyceraldehyde-3-phosphate dehydrogenase. The reaction also requires, among other compounds, a supply of the nicotinamide-adenine dinucleotide (NAD).



ADP and ATP are adenosine di- and tri-phosphates respectively.

The Process of Aerobic Glycolysis

$POCH_2(CHOH)_4.CHO$	Glucose-6-phosphate
↕	
$POCH_2(CHOH)_3COCH_2OH$	Fructose-6-phosphate
↕	
$POCH_2(CHOH_3)_3COCH_2OP$	Fructose-1,6-diphosphate
↕	
$POCH_2.CHOH.CHO$	Glyceraldehyde-3-phosphate
↕	
$POCH_2.CHOH.COOP$	1,3-Diphosphoglycerate
↕	
$POCH_2CHOH.COOH$	3-Phosphoglycerate
↕	
$HOCH_2.CHOP.COOH$	2-Phosphoglycerate
↕	
$CH_2=C(OP)COOH$	Phosphoenolpyruvate
↕	
$CH_3COCOOH$	Pyruvic acid
↕	

Citric Acid Cycle

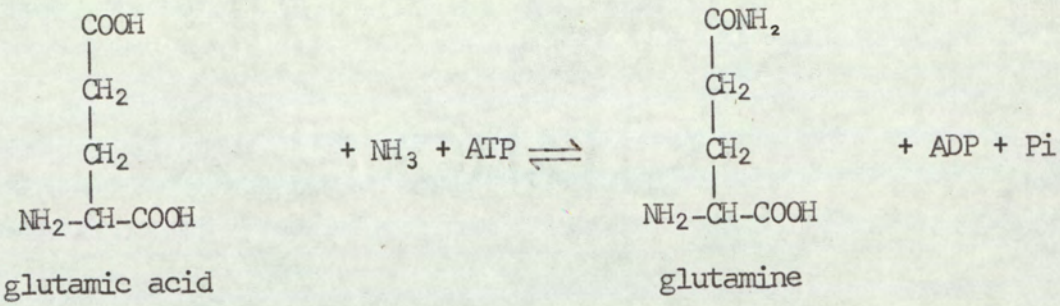
As has already been mentioned, it is these two compounds, glyceraldehyde-3-phosphate dehydrogenase and NAD, that are inhibited by Natulan and 1-methyl-2-(p-allophanylbenzyl)-hydrazine hydrobromide. Blockage of this vital reaction will result in a very serious loss of energy available to the cell, not only from the damage to the glycolysis mechanism itself, but also because any blockage of the latter will almost completely cut cut the energy normally available from the following citric acid cycle. This cycle is effective in converting the end-product of the normal glycolysis process, pyruvic acid, to carbon dioxide and water with the liberation of a considerable amount of energy.

Cell division is a process needing a relatively large amount of energy compared to other cell metabolism processes. The rapid proliferation of tumour cells will create an abnormal demand for energy, and therefore any blockage of the glycolysis mechanism by which much of this energy is made available, is likely to result in the marked inhibition of cell multiplication. Hydrazines may well, therefore, owe some of their cytotoxic activity to the inhibition of an enzyme system or systems vital to basic cell division processes.

The Substitution of Hydrazines for Ammonia in the Glutamine Synthetase System

The glutamine synthetase system essentially involves the

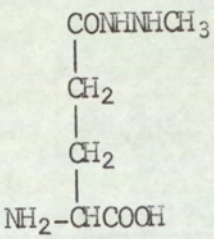
amination of glutamic acid to yield glutamine, by the following reaction[76]:-



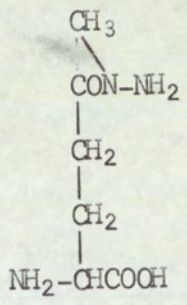
glutamic acid — glutamine

Pi = inorganic phosphate

The importance of glutamine is as a storage form of ammonia, which is toxic to animal tissues[77], and also as an intermediate in the removal of ammonia from the animal organism[78]. It has recently been observed[79] that in addition to hydrazine[80], methylhydrazine and 1,1-dimethylhydrazine are both capable of acting as substrates for glutamic acid. The probable products of the reaction with methylhydrazine are[79]:-



1-methyl-2-(5-glutamyl) hydrazine



1-methyl-1-(5-glutamyl) hydrazine

The fact that these hydrazines can substitute for ammonia to form an analogue of glutamine could therefore result in an increase of ammonia in the organism. It is possible that the build up of such a chemical, toxic as it is to animal tissues, may contribute to the tumour-inhibitory activity of such hydrazines.

The Inhibition of Protein Biosynthesis

It has been observed[23] that a number of hydrazines, and their derivatives, among them Natulan, azo-Natulan, hydrazine, methylhydrazine and N-isopropyl-4-formyl benzamide, markedly inhibit the incorporation of the amino acid, leucine, into ascites tumour cells, both 'in vitro' and 'in vivo'. Natulan is also known to inhibit the 'in vitro' incorporation of C-14 glycine into the protein of ascites cells. That hydrazine and its derivatives are capable of reacting with amino-acids in this manner, suggests that the overall biosynthesis of proteins may thus be impaired. Comparison of the extent of leucine inhibition brought about by N-isopropyl-4-hydroxymethylbenzamide (I) and N-isopropyl-4-formyl benzamide (II).

$(CH_3)_2CHNHCOC_6H_4CH_2OH$ (I)

$(CH_3)_2CHNHCOC_6H_4CHO$ (II)

reveals that the aldehyde is highly active in this respect, whereas the hydroxymethyl derivative has essentially no effect. Bearing in mind the structural similarity of these two compounds, this difference in activity points to the aldehyde group as being

responsible for the inhibition of the incorporation of the amino acid. That this may, in fact, be the case is emphasised by the finding that both formaldehyde[22] and benzaldehyde[23] cause a similar inhibition.

It therefore seems most likely that this effect is attributable to the direct reaction of the aldehyde with the amino-acid to give the Schiff base, a reaction which in the case of N-isopropyl-4-formylbenzamide would presumably proceed as follows:-

$(CH_3)_2CHNHCOC_6H_4CHO + NH_2-CH(COOH)-CH_2-CH(CH_3)_2$

N-isopropyl-4-formylbenzamide leucine

$\longrightarrow (CH_3)_2CHNHCOC_6H_4CH=N-CH(COOH)-CH_2-CH(CH_3)_2$

Schiff base

The Schiff bases arising from the 'in vitro' condensation of N-isopropyl-4-formylbenzamide with the amino-acids, leucine, glycine, lysine and cystine, have all been isolated[23] and identified, their formation proceeding rapidly in aqueous solution at room temperature. Similar bases formed by the reaction between amino-acids and other aromatic aldehydes have been known for some time[81,82].

The mild conditions under which such condensations can take place, lends support to the hypothesis, that the inhibition of

leucine incorporation into ascites cells by N-isopropyl-4-formyl-benzamide is attributable to the reduction of the free amino-acid concentration by the formation of a Schiff base, with the consequent inhibition of protein biosynthesis. This hypothesis is only tenable however for those hydrazines whose decomposition leads to aromatic aldehydes, or as in the case of methylhydrazines, to formaldehyde itself. For other hydrazines, including hydrazine itself, it is feasible that amino-acid inhibition could take place by the formation of amino-acid hydrazides, as follows:-

$$(CH_3)_2CHCH_2CHCOOH + NH_2NHR \longrightarrow (CH_3)_2CHCH_2CHCONHNHR$$

$$\qquad\qquad\quad | \qquad\qquad\qquad\qquad\qquad\qquad\quad |$$

$$\qquad\qquad NH_2 \qquad\qquad\qquad\qquad\qquad\quad NH_2$$

leucine

The synthesis of similar hydrazides of amino-acids has been reported by Khorova [83].

Whilst it is true that certain malignant tumours have been observed to continue to grow in animals given protein-free diets [84], the vital importance of an adequate supply of proteins to the metabolism of normal cells (e.g. in the supply of amino-acids essential to the synthesis of enzymes) nevertheless makes it likely that any interference with this supply, as discussed above, will have at least an indirect effect upon cell multiplication processes.

The Tumour Inhibitory Activity of Unsubstituted Hydrazine

Hydrazine itself, has very recently been shown to possess considerable tumour inhibitory properties[22], in that it causes a marked inhibition of the growth of mouse-ascites carcinoma, the mouse sarcoma 180, and the Walker carcinoma of the rat.

That hydrazine is thus active, emphasises the importance of the conjecture that hydrazine itself is a likely end product of the degradative metabolism of substituted hydrazines, as is indicated in the suggested scheme (page 102) for the degradation of Natulan (N-isopropyl-α(2-methylhydrazino)-p-toluamide hydrochloride). Furthermore, that a number of monosubstituted alkyl and aryl hydrazines such as methylhydrazine[24], 1,1-dimethyl-hydrazine[24],β-phenylisopropylhydrazine[26] and phenylhydrazine[28], can undergo dealkylation under physiological conditions or the 'in vitro' equivalent, accompanied in many cases by the detection of the alkyl group as the corresponding hydrocarbon, implies that hydrazine itself is also liberated.

The cytotoxic mechanism of hydrazine is, as yet, little understood. It is known to inhibit the incorporation of leucine and thymidine into ascites cells both 'in vitro'[22] and also 'in vivo'[23], whilst uridine is essentially unaffected at the same dose rate. It is significant that both methylhydrazine and benzyl-hydrazine show[22] a similar inhibitory pattern to that of hydrazine

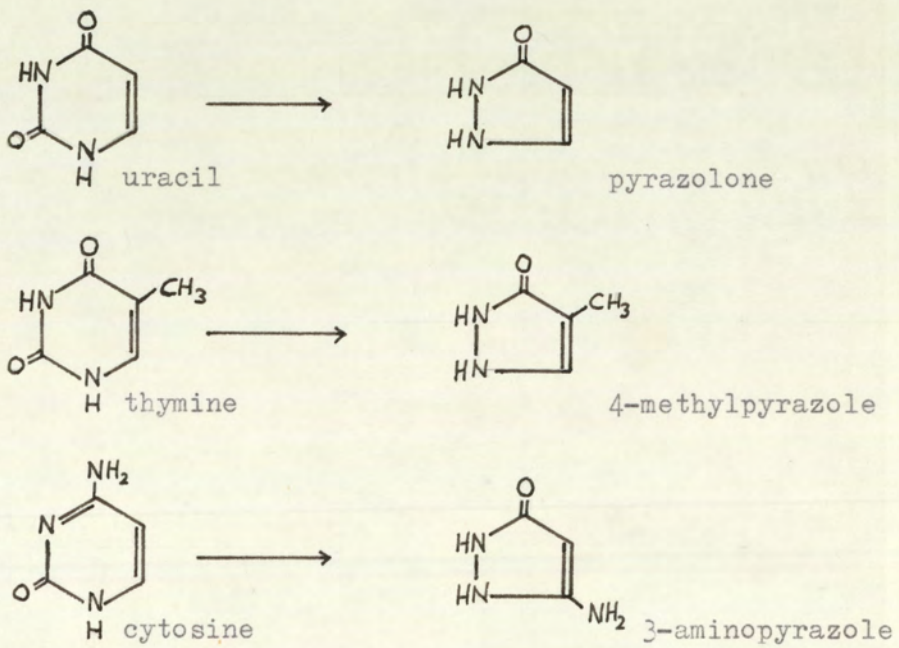
itself, thus lending further weight to the suggestion that hydrazine is a degradation product of alkyl and aryl substituted hydrazines. That hydrazine interferes with the metabolism of such compounds as uridine and thymidine led Weitzel and his co-workers [22] to postulate that hydrazine functions as a tumour inhibitor by interfering with the biosynthesis of DNA and RNA. In more detailed experiments designed to pin-point the site of attack of hydrazine on DNA, it was observed [23] that full restoration of the synthesis of this nucleic acid, previously inhibited by hydrazine, was only restored when both guanine and adenine ribosides were added, thus suggesting that hydrazine, in some way, is capable of inhibiting the deoxypurineriboside synthesis and hence the synthesis of DNA.

That hydrazine may also inhibit the synthesis of proteins, as is suggested by the fact that it is capable of inhibiting the incorporation of leucine into ascites cells, has been discussed in the previous section (page 122).

Hydrazine has also been shown to act as a mutagen to bacteria [85-87], and bacteriophages [88]. Although no satisfactory explanation for its action has yet been offered, it is observed that, although many of the mutational events are lethal, a considerable proportion are of the transitional type [89]. Hydrazine is also recognised as possessing pronounced carcinogenic properties [90-92]

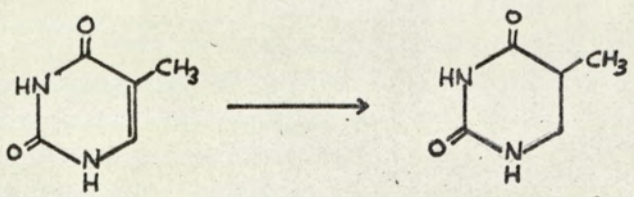
in addition to the tumour-inhibitory characteristics already described. It is not inconceivable that the former could be related to the transitional mutagenic effects, and that the tumour inhibitory activity is associated with lethal mutagenesis. The evidence for the latter only, will be discussed here.

It is known that purine bases are essentially unaffected by hydrazine hydrate, or the anhydrous reagent[89]. Pyrimidine bases do react with hydrazine, however[89], uracil being converted by ring-cleavage to pyrazolone, thymine to 4-methylpyrazolone and cytosine to 3-aminopyrazole:-



These reaction products are also formed from the corresponding bases in nucleotide linkage, the reaction rates being in the order uracil > cytosine > thymine [93,94]. In more dilute solutions, however, Brown and his co-workers [89] observed that the reactivity order of hydrazine with pyrimidine bases is thymine > uridine > 5-methylcytosine > cytosine which confirms the earlier work of Freese [88].

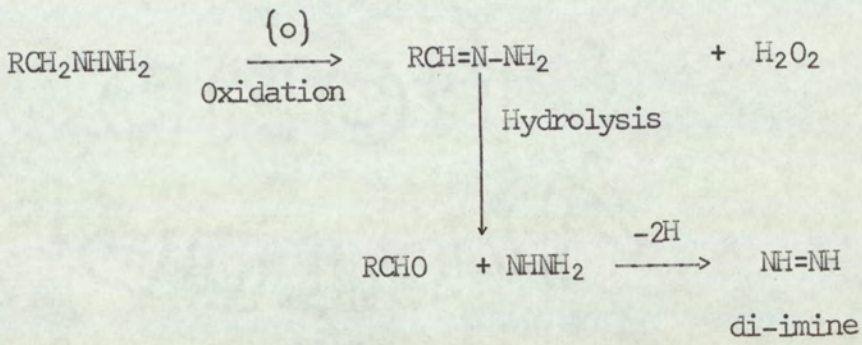
Under definitely aerobic conditions, however, the reaction of hydrazine with these bases results in the reduction of the 5,6-double bond of the pyrimidines [89]. No pyrazolone formation was observed. For example:-



thymine 5,6-dihydrothymine

The reduction of the 5,6-double bond in these pyrimidines is not surprising since the conditions Brown and his co-workers [89] used are those which are known to give rise to the powerful reducing agent diimine [95] (NH=NH). Hydrazine is known to form diimine (or diimide as it is sometimes called) in the presence of oxidising agents such as atmospheric oxygen [96,97], peroxides [96] (especially with traces of copper, Cu^{2+}), and particularly, hexacyanoferrate (III) [98],

the last named reagent being capable of the oxidation of substituted hydrazines, as has already been seen (page 95). The overall formation of diimine from mono-substituted hydrazines via hydrazine itself could feasibly take place as follows:-



where R can equal C_6H_5, CH_3, H etc.,

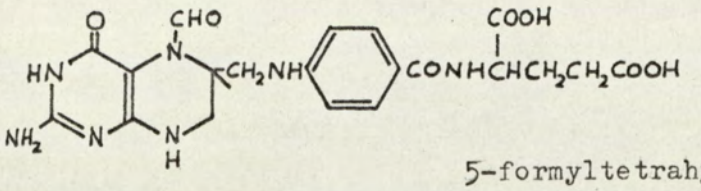
The possible routes by which diimine can be formed from disubstituted derivatives would presumably be those indicated for the degradation of Natulan (page 102).

That hydrazine reacts so readily with such essential components of nucleic acids as the pyrimidine bases to yield the corresponding 5,6 dihydropyrimidines, or even the pyrazole derivatives, suggests that hydrazine (and thus its alkyl and aryl substituted derivatives) may act as a tumour inhibitor by seriously interfering with the biosynthesis of the nucleic acids themselves. The attractiveness of this possible explanation for the cytotoxic activity of hydrazine derivatives viz., their degradation to hydrazine and its subsequent conversion to diimine, lies in the fact that this mechanism is

feasible for hydrazine itself as well as for alkyl and aryl hydrazines whether they be mono- or disubstituted.

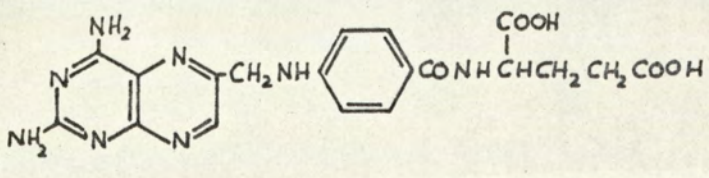
The Possible Reaction of Hydrazines with 5-Formyltetrahydrofolic Acid

As has already been mentioned in the discussion on the role of folic acid and its derivatives in normal and malignant cells (page 14), 5-formyltetrahydrofolic acid is essential in cell division.



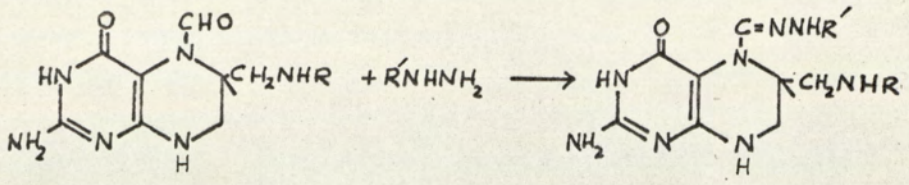
5-formyltetrahydrofolic acid

In its absence, the chromosomes are unable to undergo normal splitting (anaphase) [99] , being arrested at the previous, mataphase, stage. Whereas a tumour inhibitor such as aminopterin (4-aminopteroylglutamic acid),



inhibits the rapid proliferation of tumour cells by blocking the synthesis from folic acid of the essential 5-formyltetrahydrofolic acid, [99] , it is feasible that hydrazine and its derivatives could cause a similar reduction in the effective concentration of the formyl derivative by converting it to the corresponding hydrazone,

as follows:-



hydrazone derivative

R = p-aminobenzoic acid

Similar hydrazones of reduced pteridines have recently been synthesised by Viscontini and Nasini[100], and shown to be reasonably stable.

Whilst this particular suggested explanation for the cytotoxicity of hydrazine derivatives is as yet rather tenuous, it has the merit that it is feasible for a wide variety of such compounds, including hydrazine itself.

Tumour Inhibitory Activity of the Synthesised Hydrazines: Experimental

1. Testing Procedure:-

The tests were conducted by Dr. Woodhouse, Director of the British Empire Cancer Campaign Research Unit, University of Birmingham.

(a) Toxicity Tests:- It was established that normal mice of 25g. weight could be injected subcutaneously or intra-peritoneally with 0.5mg. of any of the hydrazine compounds, and the injections continued daily for at least ten days. Larger doses resulted in illness or death of some of the mice.

(b) Tumour Growth Tests:- The hydrazine compounds under test were administered to groups of mice bearing two types of transplanted tumours:

1. A sarcoma originally induced in F1 hybrids of the pure line strains IF and C57B1. This takes in 100% of the hybrids, and grows moderately slowly with relatively little necrosis. Untreated tumour bearing mice survive for up to one month before ulceration necessitates that they be killed. It is now in the 15th transplant generation and is very stable in growth properties.
2. A mammary adenocarcinoma transplanted into C3H pure line mice, also with 100% takes. This grows somewhat faster and the animals survive about three weeks after inoculation. It has been through over 140 passages.

Both tumours are propogated by the usual subcutaneous inoculation technique, the new growths being visible after 4-5 days. The measurement of size is practicable at about 6 days.

Injections of the hydrazines in a suitable medium (0.2 ml), were commenced at this stage. Those soluble in water were administered as an aqueous solution, those slightly soluble as an aqueous suspension, whilst for those compounds insoluble in water, a medium of olive oil was used. Unless otherwise stated the total administered dose of each hydrazine consisted of 6 daily injections, each of 0.5mg. Measurements of the size of the tumours were then again recorded.

The animals were houses in plastic boxes, 5 per box. For each separate test 5 animals were utilised, with a similar number injected with vehicle only, as controls. The tests were repeated when a possible effect of the injected compounds was indicated by the rate of tumour development.

(c) Assessment of Tumour Growth:- The animals were weighed before and after the series of injections. This was carried out to ensure that any depression of the development of the tumour, resulting from a loss or poor gain in body weight caused by the toxicity of the hydrazine, could be distinguished from any intrinsic anti-cancer activity of the injected compound.

At the beginning and conclusion of the series of injections, the

tumours were measured with callipers and the volume was estimated by the product of length x breadth, the depth being taken as equal to the breadth (the lesser of these two measurements). The weights of the tumours were also determined at the end of the tests. The ratios of original weight to final weight and original volume to final volume, were calculated for all tumours, and compared with the corresponding parameters for the untreated controls. The results are tabulated in Tables 2-5, the figures in all cases being the average for the various batches of animals. Tables 3 and 4 indicate the tumour inhibitory activity of a number of selected hydrazine derivatives at various dose levels.

TABLE 1: Hydrazines Tested for Tumour Inhibitory Activity

COMPOUND TEST NO.	NAME	STRUCTURAL FORMULA
1	Phenylhydrazine hydrochloride	$C_6H_5NHNH_2.HCl$
2	Semi-carbazide hydrochloride	$NH_2NHCONH_2.HCl$
3	Hydroxylamine sulphate	$NH_2OH.\frac{1}{2}H_2SO_4$
4	Hydrazine dihydrochloride	$NH_2NH_2.2HCl$
5	Methylhydrazine sulphate	$CH_3NHNH_2.H_2SO_4$
6	1,2-Dibenzoyl hydrazine	$C_6H_5CONHNHCOC_6H_5$
7	Benzalazine	$C_6H_5CH{=}N{-}N{=}CHC_6H_5$
8	Benzylhydrazine sulphate	$C_6H_5CH_2NHNH_2.H_2SO_4$
9	1,2-Dibenzoyl-t-butyl hydrazine	$(CH_3)_3C$, C_6H_5CO $\diagdown\diagup$ $N{-}NHCOC_6H_5$
10	1,2-Dibenzoyl-1-Methyl hydrazine	CH_3, C_6H_5CO $\diagdown\diagup$ $N{-}NHCOC_6H_5$

TABLE 1 continued:

COMPOUND TEST NO.	NAME	STRUCTURAL FORMULA
11	t-Butylhydrazine oxalate	$(CH_3)_3CNHNH_2.(COOH)_2$
12	1-Methyl-2-benzoyl hydrazine hydrochloride	$CH_3NHNHCOC_6H_5.HCl$
13	1-t-Butyl-1-benzyl-2-benzoyl hydrazine	$(CH_3)_3C$ / $C_6H_5CH_2$ > $N-NHCOC_6H_5$
14	1-Methyl-2-benzyl hydrazine hydrochloride	$CH_3NHNHCH_2C_6H_5.\ HCl$
15	N-isopropyl-α-(2-methyl hydrazino-p-toluamide hydrochloride	$CH_3NHNHCH_2C_6H_4CONHCH(CH_3)_2.$ HCl
16	Compound G	
17	Compound K;	
18	Hydrazine dihydrochloride	$NH_2NH_2.2HCl$
19	1-t-Butyl-2-benzoyl hydrazine hydrochloride	$(CH_3)_3CNHNHCOC_6H_5.HCl$
20	1-t-Butyl-1-benzyl hydrazine hydrochloride	$(CH_3)_3C$ / $C_6H_5CH_2$ > $N-NH_2.\ HCl$
21	Benzaldehyde 1-t-butyl-1-benzyl hydrazone	$(CH_3)_3C$ / $C_6H_5CH_2$ > $N-N=CHC_6H_5$
22	Benzaldehyde t-butyl hydrazone hydrochloride	$(CH_3)_3CNHN=CHC_6H_5.HCl$

Compounds G and K (Nos. 16 and 17) are both reaction products of the attempted alkylation of t - butylhydrazine with benzyl chloride, as described on page 223-224

TABLE 2: % Inhibition of Tumours by Hydrazine Compounds

COMPOUND TEST NO.	FORMULA	SARCOMA		CARCINOMA	
		size	weight	size	weight
1	$C_6H_5NHNH_2.HCl$	50	30	0	0
2	$NH_2NHCONH_2.HCl$	20	10	0	0
3	$NH_2OH.\frac{1}{2}H_2SO_4$	35	20	0	0
4	$NH_2NH_2.2HCl$ low dose rate	0	0	0	0
5	$MeNHNH_2.H_2SO_4$	50	20	0	25
6	$C_6H_5CONHNHCOC_6H_5$	0	0	0	0
7	$C_6H_5CH=N-N=CHC_6H_5$	0	0	0	0
8	$C_6H_5CH_2NHNH_2.H_2SO_4$	0	0	0	30
9	$(CH_3)_3C$, C_6H_5CO > $N-NHCOC_6H_5$	0	0	0	0
10	CH_3, C_6H_5CO > $N-NHCOC_6H_5$	50	0	39	14
11	$(CH_3)_3CNHNH_2(COOH)_2$	46	0	20	0
12	$CH_3NHNHCOC_6H_5.HCl$	0	0	42	40

TABLE 2 continued:

COMPOUND TEST NO.	FORMULA	SARCOMA		CARCINOMA	
		size	weight	size	weight
13	$(CH_3)_3C$ / $C_6H_5CH_2$ > N-$NHCOC_6H_5$	0	0	39	0
14	$CH_3NHNHCH_2C_6H_5$.CHl	64	44	68	50
15	$CH_3NHNHCH_2C_6H_4$ $CONHCH(CH_3)_2$.HCl	74	41	76	53
16	Compound G	46	20	0	0
17	Compound K	59	54	0	0
18	$NH_2NH_2$2HCl	65	40	74	13
19	$(CH_3)_3CNHNHCOC_6H_5$.HCl	0	18	52	16
20	$(CH_3)_3C$ / $C_6H_5CH_2$ > N-NH_2.HCl	55	27	53	18
21	$(CH_3)_3C$ / $C_6H_5CH_2$ > N-N=CHC_6H_5	0	23	34	0
22	$(CH_3)_3CNHN=CHC_6H_5$.HCl	0	0	26	0

Dose level - 6 daily injections of 0.5mg each except Compound Test No. 18 (6 injections of 2mg each).

TABLE 3: % Inhibition of Tumours (Sarcoma) by Selected Hydrazines at Various Dose Levels

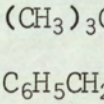
COMPOUND TEST NO.	FORMULA	INHIBITION BY:	DOSE LEVEL 6 @ 0.5mg	1 @ 2.5mg 5 @ 1.25mg	6 @ 1.25mg
5	$CH_3NHNH_2.H_2SO_4$	size weight	47 18		75 43
8	$C_6H_5CH_2NHNH_2.H_2SO_4$	s w	0 20	45 44	54 40
11	$(CH_3)_3CNHNH_2(COOH)_2$	s w	47 0		77 50
13	$(CH_3)_3C$  $N-NHCOC_6H_5$ $C_6H_5CH_2$	s w	0 13		45 13
14	$CH_3NHNHCH_2C_6H_5.HCl$	s w	64 44	47 38	49 20
15	$CH_3NHNHCH_2C_6H_4CONHCH(CH_3)_2.HCl$	s w	74 41	78 38	46 37

TABLE 4: % Inhibition of Tumours (Carcinoma) by Selected Hydrazines at Various Dose Levels

COMPOUND TEST NO.	FORMULA	INHIBITION BY SIZE	DOSE LEVEL 6 @ 0.5mg	1 @ 2.5mg 5 @ 1.25mg	6 @ 1.25mg
5	$CH_3NHNH_2.H_2SO_4$	size weight	25 26		
8	$C_6H_5CH_2NHNH_2.H_2SO_4$	s w	0 20	5 25	50 14
11	$(CH_3)_3CNHNH_2.(COOH)_2$	s	21 0		26 0
13	$(CH_3)_3C$ \ N-$NHCOC_6H_5$ / $C_6H_5CH_2$	s w	46 0		66 28
14	$CH_3NHNHCH_2C_6H_5.HCl$	s w	68 50	47 26	50 17
15	$CH_3NHNHCH_2C_6H_4CONHCH(CH_3)_2.HCl$	s w	76 53	83 36	70 20

TABLE 5: The Effect of the Introduction of an N-Methyl Group on the Cytotoxic Activity of Hydrazines

COMPOUND	SARCOMA		CARCINOMA	
	SIZE	WEIGHT	SIZE	WEIGHT
$NH_2.NH_2.HCl$	0	0	0	0
$MeNHNH_2.HCl$	50	20	0	25
$C_6H_5CONHNHCOC_6H_5$	0	0	0	0
$C_6H_5CON(Me)NHCOC_6H_5$	50	0	39	14
$C_6H_5CH_2NHNH_2.H_2SO_4$	0	20	0	30
$C_6H_5CH_2NHNHMe.HCl$	64	44	68	50
$C_6H_5CON(t\text{-}Bu)NHCOC_6H_5$	0	0	0	0
$C_6H_5CON(CH_3)NHCOC_6H_5$	50	0	39	14

Dose level: Standard (6 daily doses, each of 0.5mg)

Tumour Inhibitory Activities of the Synthesised Hydrazines:

Discussion and Conclusions

One of the most significant features of the experimental results of the tumour growth tests, which are detailed in Tables 2 - 5, pages 135 to 142 , is the wide variety of hydrazine derivatives that display inhibitory activity. As is indicated (Table 2), such compounds as phenylhydrazine, methylhydrazine, benzylhydrazine, 1, 2 - dibenzoyl - 1 - methylhydrazine, t - butylhydrazine and 1 - t - butyl - 1 - benzyl hydrazine were all found to cause definite inhibitions at the normal dose rate (6 daily injections of 0.5 mg each).

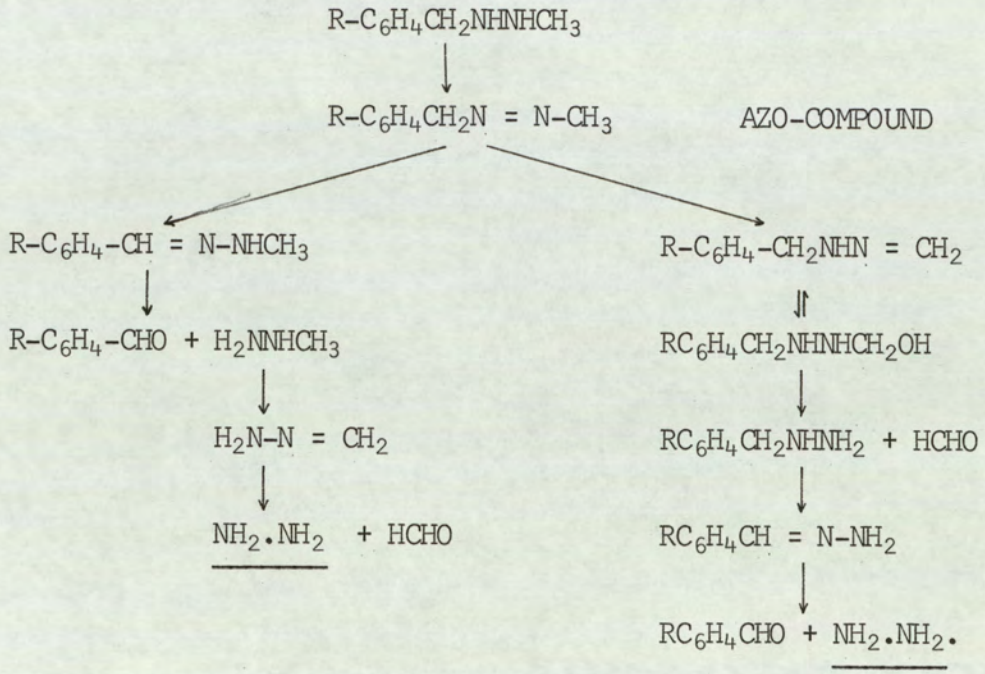
That this is so indicates that the group $CH_3NHNHCH_2C_6H_4$-R, considered by Zeller and his co-workers [10] to be essential to the display by hydrazines of any appreciable cytotoxic activity, is in fact, not indispensable, an observation which has recently been confirmed by the work of Weitzel and co-workers,[22] who found that methylhydrazine, benzylhydrazine, and formalhydrazine (CH_2=N-NH_2) all possess significant anti-tumour properties. Whilst it would now seem that the diversity of hydrazine derivatives which display a definite cytotoxic activity is greater than at first thought, it would nevertheless appear from the experimental results which are compared in Table 5 (page 142), that the introduction of an N-methyl group into the hydrazine molecule often results in the introduction of tumour inhibitory activity or the enhancement of that already present.

Recent work by Weitzel and his co-workers[22] has also spotlighted this phenomenon, an explanation for which may be connected with the fact that, in addition to potential cytotoxic products such as azo-compounds, hydrogen peroxide and hydrazine itself, which are thought to be formed during the degradation of unmethylated hydrazines, methylhydrazines would seem to be capable of liberating hydroxymethyl derivatives, formalhydrazines and formaldehyde also, all of which, as is discussed in detail earlier in the thesis (page 107*f*), could function as cytotoxic alkylating agents.

Although hydrazine itself (compound 4; Table 2) is inactive at the standard dose level, upon retesting (compound 18; Table 2) at the higher rate of 6 daily injections, each of 2 mg., it was observed to cause very pronounced inhibitions of the growth rate of both the sarcoma and carcinoma tumours. Very similar results have subsequently been reported by Weitzel.[22]

Although no very obvious pattern of inhibition is easily discernible upon examination of the tumour inhibitory data of the 21 different compounds tested (Table 2), it is possible to suggest a hypothesis capable of accounting for many of the observed activities. As has already been discussed (page 125) it is probable that hydrazine itself is an end product of the degradative metabolism of many substituted hydrazines. That hydrazine is now known to possess marked cytotoxic properties suggests that the observed tumour inhibitory activities of these substituted hydrazines may be attributable to the liberation of hydrazine itself.

The formation of hydrazine from disubstituted aralkyl hydrazine derivatives such as 1 - methyl - 2 - benzyl hydrazine (compound No. 14) and N - isopropyl - ∝ - (2 methylhydrazino) - p - toluamide hydrochloride (compound No. 15) could feasibly proceed by the pathways suggested and formulated in the earlier discussion on the metabolism of these compounds (pages 102-104). For convenience, the essential features of this possible degradation scheme are detailed below:-

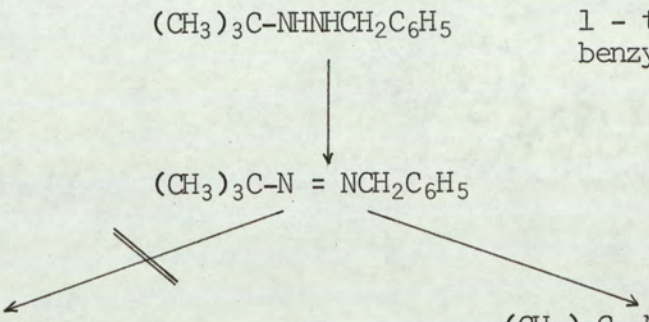


Natulan ---------- R = $(CH_3)_2CHNHCO-$

1 - Methyl - 2 - benzyl hydrazine ------- R = H

The azo-compound formed by the oxidation of the original hydrazine is capable of rearranging in both directions to give the two hydrazones indicated, both of which could, in turn, yield hydrazine itself by the steps outlined. That such 1, 2 - disubstituted hydrazines are capable

of degrading via both of these possible routes, renders more difficult any investigation designed to pin-point the origin of their cytotoxic activity. It was, therefore, considered worthwhile to attempt to synthesise and chemotherapeutically estimate a compound such as 1 - t - butyl - 2 - benzyl hydrazine, which by virtue of its structure can only participate in one of these pathways:-



However, despite numerous attempts using the various methods discussed on page 189 f the desired hydrazine proved to be unobtainable, even although the product which one might expect to be formed as a result of its oxidation, namely, benzaldehyde t - butylhydrazone, was successfully synthesised and tested for tumour inhibitory properties.

The formation of hydrazine from mono-substituted alkyl and aryl hydrazines, such as methylhydrazine and benzylhydrazine, could well be expected to proceed by a very similar pathway to that suggested for the disubstituted compound, viz., oxidation to the corresponding hydrazone, which is then hydrolysed to the unsubstituted hydrazine.

For example:

$$CH_3NHNH_2 \xrightarrow{[O]} \underset{\text{FORMAL HYDRAZINE}}{CH_2 = N - NH_2} \longrightarrow HCHO + \underline{NH_2.NH_2}$$

and,

$$C_6H_5CH_2NHNH_2 \xrightarrow{[O]} C_6H_5CH = N\text{-}NH_2 \longrightarrow C_6H_5CHO + \underline{NH_2NH_2}$$

The degradation pathway for methylhydrazine suggested above, will be seen to involve formalhydrazine as an intermediate, a compound which is known to be a potent tumour inhibitor.[22] That the latter compound can be oxidised to formaldehyde in yields of over 50% even under mild conditions,[22] strongly suggests that hydrazine might be liberated simultaneously according to the following equation:-

$$CH_2 = N\text{-}NH_2 \xrightarrow{H_2O} HCHO + NH_2NH_2$$

and in a proportion which could account for the inhibitory properties of the formalhydrazine.

Although t - butylhydrazine might also be expected to dealkylate to give hydrazine, it is clear that its structure precludes this process from occurring via the formation of a hydrazone, as in the cases of methyl and benzyl hydrazines. It is possible however that t - butyl hydrazine is dealkylated by undergoing protonation 'in vivo', a process which is known [101] to facilitate the elimination of the t - butyl group, presumably as a carbonium ion, thus liberating hydrazine itself.

$$(CH_3)_3C\text{-}NHNH_2 \xrightarrow{H^+} (CH_3)_3C\text{-}\overset{H^+}{N}HNH_2 \longrightarrow (CH_3)_3C^+ + \underline{NH_2NH_2}$$

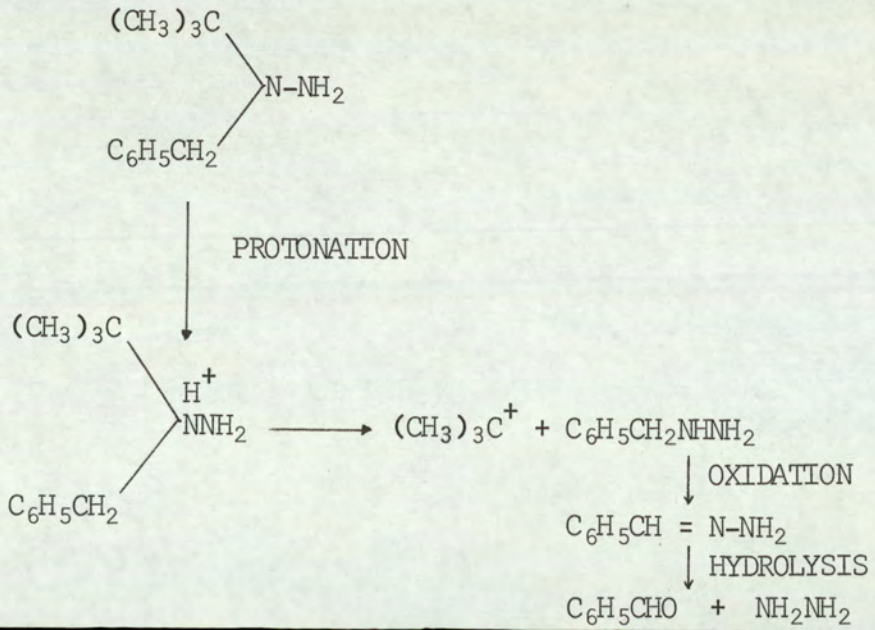
A comparison of the tumour inhibitory activities of methylhydrazine, benzylhydrazine t - butylhydrazine, and hydrazine itself at the elevated dose rates, reveals that not only do these simple hydrazines possess very similar activities to each other, but that they also display inhibitions comparable to those produced by Natulan and 1 - methyl - 2 - benzyl hydrazine. The appropriate data are listed below:-

COMPOUND	DOSE	SARCOMA		CARCINOMA	
	6x	SIZE	WEIGHT	SIZE	WEIGHT
Hydrazine dihydrochloride	2mg	65	40	74	13
Methylhydrazine sulphate	1.25mg	75	43		
Benzylhydrazine hydrochloride	1.25mg	54	40	50	14
t - Butylhydrazine hydrochloride	1.25 "	77	50	26	0
1 - Methyl - 2 - benzyl hydrazine hydrochloride	1.25 "	49	20	50	17
Natulan	1.25 "	46	37	70	20

With regard to benzaldehyde t - butyl hydrazone (compound 22; Table 2), it might be expected, in view of the previously discussed facility with which such compounds decompose to the constituent aldehyde and hydrazine, that the products of its 'in vivo' metabolism would be t - butylhydrazine and benzaldehyde, a conjecture which is

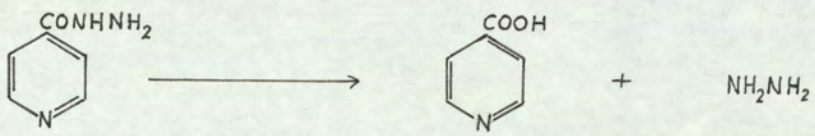
supported by the fact that, after the suspension of the benzaldehyde t - butyl hydrazone in water for a few minutes at room temperature, the odour of benzaldehyde is readily discernible. Any t - butylhydrazine liberated could then be dealkylated to yield hydrazine as described above. If benzaldehyde t - butyl hydrazone is, in fact, metabolised to t - butylhydrazine to any appreciable extent, one would expect the tumour inhibitory activities of both compounds to be, at least, comparable. Both show a weak inhibition at the normal dose rate.

The majority, if not all, of the hydrazine derivatives whose testing for tumour inhibitory activity has been reported in the literature are either mono-substituted or 1, 2 - disubstituted compounds. The results listed in Table 2 (page 138) indicate that 1 - t - butyl - 1 - benzyl hydrazine shows an inhibition on tumour growth rate which is inferior only to that of Natulan and 1 - methyl - 2 - benzyl hydrazine. This 1, 1 - disubstituted hydrazine could well liberate hydrazine by a combination of the processes postulated for methyl and benzyl hydrazines, as follows:-



That Benzaldehyde 1 - t - butyl - 1 - benzyl hydrazone (compound No. 21; Table 2) shows a somewhat less pronounced cytotoxic activity than 1 - t - butyl - 1 benzyl hydrazine itself, may indicate that the hydrolysis of the hydrazone to the hydrazine is, in this case, a relatively slow process.

The remaining group of hydrazines for which inhibitory data were obtained, are the acyl hydrazines (hydrazides). In general, it would appear that such compounds are relatively poor inhibitors, at least at the normal dose rate, as an examination of the data for such derivatives as 1, 2 - dibenzoylhydrazine, 1, 2 - dibenzoyl t - butylhydrazine, and 1 - t - butyl - 1 - benzyl - 2 - benzoyl hydrazine (Table 2, compound numbers 6, 9 and 13) indicates. That this is so, does not detract from the validity of the hypothesis that hydrazine itself, is, in fact, a major underlying cause of the cytotoxic behaviour of hydrazine derivatives, as hydrazides are relatively stable compounds, and do not readily deacylate to yield the free hydrazine.[102] Such deacylations do however occur, as was demonstrated by both Toida,[103] and Porcellati[104] and his co-workers, who, whilst investigating the metabolism of isonicotinic hydrazide, a potent tuberculostatic, observed that it broke down 'in vivo' into isonicotinic acid and hydrazine:-



That such 'in vivo' deacylations to give hydrazine are likely to be rather slow processes could account for the relatively weak inhibitory powers displayed by acyl hydrazines.

Although in the foregoing discussion on the tumour inhibitory data of the tested hydrazines, it is suggested that hydrazine itself is the underlying cytotoxic agent, it should be born in mind that hydrazine may so function, as its 'oxidised form', diimine (NH = NH), a compound which is a very strong reducing agent. Owing to the highly transitory nature of diimine however, the experimental evidence for such a possibility will, of necessity, remain indirect. Further support, or otherwise, for this suggestion could nevertheless be obtained by testing for tumour inhibitory activity such compounds as azodicarboxylates, arenesulphonyl hydrazides, chloracetyl hydrazide and hydroxylamine - O - sulphonic acid, all of which are known[95] to release diimine during their decomposition, as is illustrated below:-

$$\bar{O}_2C\text{-}N = N\text{-}CO_2^- + 2H^+ \longrightarrow 2CO_2 + \underline{HN = NH}$$

Azodicarboxylate — diimine

$$C_6H_5SO_2\text{-}NH\text{-}NH_2 \longrightarrow C_6H_5SO_2H + \underline{HN = NH}$$

benzene sulphonyl hydrazide

$$ClCH_2\text{-}CO\text{-}NH\text{-}NH_2HCl \xrightarrow{\text{alkali}} 2NaCl + 2H_2O + H_2C{=}C{=}O + \underline{HN = NH}$$

chloracetyl hydrazide — ketene

$$2H_2NO\text{-}SO_3H \xrightarrow{-2H^+} \underline{HN = NH} + 2SO_4^{2-}$$

Hydroxylamine - O - sulphonic acid.

Conclusions

The observed tumour inhibitory properties of the hydrazine derivatives investigated, indicate that appreciable activity is not confined to those compounds containing the group, $CH_3NHNHCH_2C_6H_4$-R, being present in a number of structurally diverse mono and di-substituted hydrazines also. There is some evidence to suggest, however, that the introduction of an N-methyl group into the molecule often results in a correspondingly enhanced cytotoxic activity. As the discovery of the anti-tumour properties of hydrazines is still rather less than five years old, it is to be expected that any considerations as to their mode of action will be more tentative than authoritative. It would, however, seem not unreasonable to suggest that the explanation which provides the best rationalisation of the tumour inhibitory data presently available, is that hydrazine itself is the underlying, cytotoxic, common factor.

The Synthesis of Alkyl and Aryl Hydrazines:

Discussion

1. Mono-substituted Hydrazines

(a) General Synthetic Methods:-

Although the methods available for the synthesis of monosubstituted hydrazines are numerous and varied, they can be divided into the following major groups:- (1) the alkylation and arylation of hydrazine (2) the reduction of acid hydrazides (3) the reduction of hydrazones (4) the reduction of nitrosoureas and related compounds (5) the reduction of diazo compounds (6) the addition of Grignard reagents to diazo derivatives and azines (7) the acid hydrolysis of syndones (8) the reaction of chloramine and similar compounds with primary amines and (9) the hydrolysis of diaziridines.

The details and general application of each of these preparative methods will now be discussed.

1. The Alkylation and Arylation of Hydrazine

Monoalkyl hydrazines can, in principle, be prepared by the direct alkylation of hydrazine with the usual reagents such as methyl and ethyl iodides. This method is seldom used however, owing to the difficulty of stopping the reaction at the monosubstituted stage. Thus the alkylation of hydrazine with methyl iodide leads ultimately to the quaternary salt N,N,N-trimethylhydrazinium iodide[105]

$$CH_3I + NH_2.NH_2 \longrightarrow CH_3NHNH_2 \longrightarrow (CH_3)_2NNH_2 \longrightarrow \left((CH_3)_3\overset{+}{N}NH_2\right) I^-$$

Although the use of alkyl halides of longer chain length has been reported by Westphal[106] to give improved yields of the monoalkyl hydrazines, the problem of separating the mono from the more highly substituted derivatives renders the method synthetically unattractive. The best example of direct alkylation seems to be that reported by Brown and Kearley[107] who observed that ethylhydrazine is formed in 32% yield from hydrazine and diethyl sulphate:-

$$Et_2SO_4 + NH_2NH_2 \xrightarrow[EtOH]{KOH} EtNHNH_2$$

The alkylation of hydrazine beyond the monoalkyl stage can however be blocked by using an azine[108]. Thus the alkylation of benzalazine with the alkylating agent will form the hydrazonium salt, which upon acid hydrolysis will yield the corresponding alkyl hydrazine.

$$C_6H_5CH{=}N{-}N{=}CHC_6H_5 \xrightarrow{R_2SO_4} C_6H_5CH{=}\overset{+}{N}(R){-}N{=}CHC_6H_5.RSO_4^- \xrightarrow[H^+]{H_2O} RNHNH_2 + 2C_6H_5CHO$$

Monoaryl hydrazines can however be prepared in quite good yields by the direct arylation of hydrazine with aryl halides, without the use of blocking groups. Thus Biel and his co-workers[109]

have reported the preparation of benzylhydrazine in excellent yield by refluxing hydrazine hydrate (excess) with benzyl chloride.

2. The Reduction of Acyl Hydrazines

Although the reduction of a monoacyl hydrazine would appear to be a useful route to the corresponding alkylhydrazine,

$$RCONHNH_2 \xrightarrow{H_2} RCH_2NHNH_2$$

the difficulty with which such compounds are reduced, makes it a synthetically unsuitable method. This applies particularly in the case of aromatic hydrazides, such as benzhydrazide which remains quite unreduced even after prolonged treatment with lithium aluminium hydride[110]. The possible explanations for this behaviour will be considered in relation to the synthesis of disubstituted hydrazines (page 178).

3. The Reduction of Hydrazones

Monoalkyl hydrazines can be prepared by the catalytic hydrogenation of the corresponding hydrazones in the presence of platinum[111,112].

$$RCH = NNH_2 \xrightarrow[\text{acid}]{H_2/Pt} RCH_2NHNH_2$$

By the direct reduction of an equimolar mixture of acetone, hydrazine hydrate and hydrochloric acid, isopropylhydrazine has been made in one step[17], thus eliminating the need of isolating

the hydrazone.

Semicarbazones can also yield monosubstituted hydrazines. The semicarbazone is hydrogenated in the presence of platinum to the corresponding semicarbazide which is, in turn, hydrolysed to the primary hydrazine. Thus the catalytic reduction of acetone semicarbazone, followed by the acid hydrolysis of the resulting isopropyl semicarbazide, yields isopropylhydrazine[113].

$$(CH_3)_2C{=}NNHC(=O)NH_2 \xrightarrow{H_2/Pt} (CH_3)_2CHNHNHC(=O)NH_2 \xrightarrow{\text{hydrolysis}} (CH_3)_2CHNHNH_2$$

One of the advantages of utilising this route is that these substituted hydrazones are much easier to handle than the corresponding unsubstituted compounds.

When, as is often the case, it is easier to prepare the aldazine or the ketazine than the hydrazone, primary hydrazines can be prepared from the azine.

$$RCHO + NH_2.NH_2 \longrightarrow RCH = NN = CHR$$

aldazine

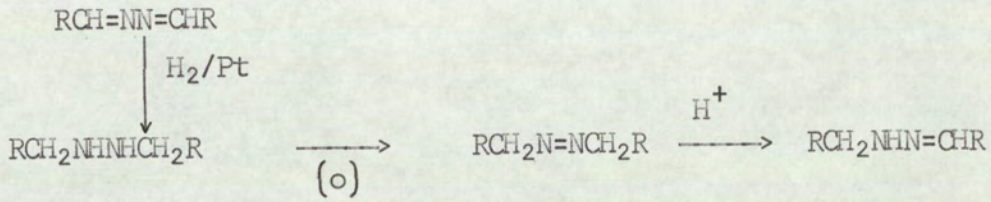
$$R_2C{=}O + NH_2.NH_2 \longrightarrow R_2C = NN = CR_2$$

ketazine

With azines, the amount of reduction must of course be controlled so that only one of the carbon-nitrogen double bonds is reduced. The resulting hydrazone is then hydrolysed in acid solution to the hydrazine.

$$RCH = NN = CHR \xrightarrow{H_2/Pt} RCH_2NHN{=}CHR \xrightarrow{\text{hydrolysis}} RCH_2NHNH_2$$

Some workers prefer to allow the reduction to go all the way to the sym-disubstituted hydrazines, and then to oxidise these to the azo compounds, which are readily isomerised to the same hydrazones that would have been obtained by partial reduction:-



Lithium aluminium hydride is also an effective reducing agent for azines [114]. The oxidation of the dialkyl hydrazine to the azo compound can be almost quantitatively effected with a wide variety of oxidising agents, including cupric chloride [115], potassium dichromate [116], and mercuric oxide [114].

4. Monosubstituted Hydrazines from Grignard Reagents

As discovered by Zerner [117], Grignard reagents can add to the diazo group of such compounds as ethyl diazoacetate and diazomethane to give hydrazines, which can then be hydrolysed to give the hydrazine corresponding to the Grignard reagent. Thus Zerner, by treating diazoacetic ester with methylmagnesium iodide, obtained ethyl glyoxylate methyl-hydrazone, which upon acid hydrolysis gave methylhydrazine. Ethylhydrazine has been made by the same route.

$C_2H_5OCOCHN_2$ $\xrightarrow[(2)\ H_2O]{(1)\ MeMgI}$ $C_2H_5OCOCH{=}NNHMe$

ethyl glyoxalate methyl hydrazone

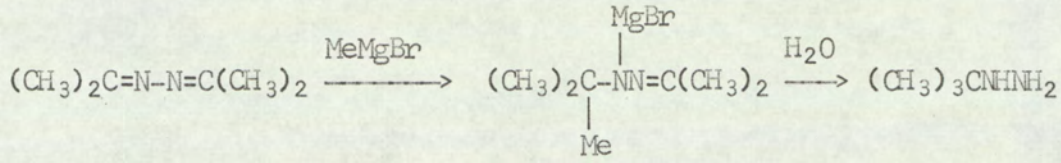
$C_2H_5OCOCH{=}NNHMe$ $\xrightarrow[H_2O]{H^+}$ $MeNHNH_2 + HOOC.CHO + C_2H_5OH$

Because excess Grignard may cause further addition the method is not always successful, although the reaction of t-butylmagnesium chloride with diphenyldiazomethane appears to be one of the better methods for the synthesis of t-butylhydrazine[118].

An analagous reaction to the ones just described have been observed when alkyllithium compounds are treated with nitrous oxide. Thus the reaction of n-butyllithium with nitrous oxide gave butyraldehyde n-butyl hydrazone, which upon hydrolysis with oxalic acid gave n-butylhydrazine oxalate:-[119]

$$n\text{-}C_4H_9Li \xrightarrow{N_2O} n\text{-}C_4H_9NHN{=}CHCH_2CH_2CH_3 \xrightarrow{H_2O/(COOH)_2} n\text{-}C_4H_9NHNH_2(COOH)_2$$

Grignard reagents are also known to add across the carbon nitrogen double bond of azines in much the same way as they do to carbonyl groups. If only one mole of the Grignard reagent is used, the product is a monosubstituted hydrazone. Thus acetone azine is reported to have given 30 - 60% of t-butyl hydrazine when treated with methylmagnesium bromide, followed by acid hydrolysis[120].

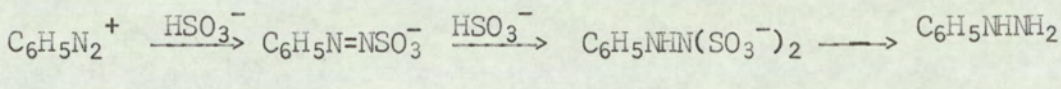


This technique has recently been extended to the use of alkyllithium as the organometallic reagent [121].

5. The Reduction of Diazonium Compounds

Although aliphatic diazo compounds can be reduced to hydrazines, the process cannot be regarded as a useful synthetic procedure for monoalkyl hydrazines as the intermediates required to prepare most diazo compounds can be converted more directly to the hydrazine.

The reduction of aromatic diazonium salts however, is the major source of monoaryl hydrazines [122, 123]. The general method is well illustrated by the synthesis of phenylhydrazine from the benzene diazonium salt, as follows:-



(80% yield)

The reduction can be effected either by sodium bisulphite as illustrated above, or by stannous chloride [124].

6. The Acid Hydrolysis of Syndones

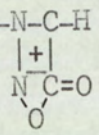
The acid hydrolysis of the mesoionic group of compounds known as syndones, is the basis of a method for the conversion of primary amines to monoalkyl hydrazines.

The synthesis of these oxadiazole derivatives (syndones) is effected in three steps. A primary amine is condensed with chloracetic acid to give an N-substituted glycine which is then nitrosated with nitrous acid. The acetylation of the N-nitroso glycine thus formed, results in ring closure to form the syndone. The overall process, which takes place in good yield, is illustrated below:-

$$RNH_2 + ClCH_2COOH \longrightarrow RNHCH_2COOH$$

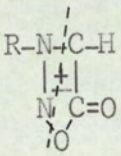
$$RNHCH_2COOH \xrightarrow{HNO_2} RN(NO)CH_2COOH$$

$$RN(NO)CH_2COOH \xrightarrow{Ac_2O}$$



Syndone

The hydrolysis of the syndone with concentrated hydrochloric acid gives the monoalkyl hydrazine (as the hydrochloride).



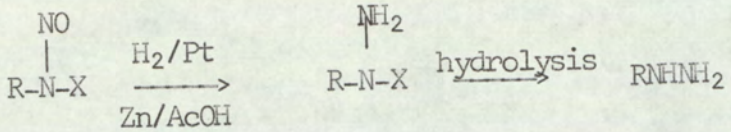
$$\longrightarrow RNHNH_2.HCl$$

Using this technique Fugger and his co-workers[125] have synthesised benzyl, n-butyl and n-hexyl hydrazines.

7. The Reduction of Nitrosoureas and Related Compounds

Monoalkyl hydrazines have also been prepared by the reduction and hydrolysis of substituted N-nitrosoureas, N-nitrosourethanes and N-nitrososulphamic acids by the following

route :-



where X = $-CNH_2$ ---- urea

= $-COC_2H_5$ -- urethane

= $-S(=O)(=O)-OH$ ---- sulphamic acid

As these nitroso compounds are prepared from the corresponding alkyl ureas, urethanes etc., which are in turn obtained from amines, this route constitutes a synthesis of hydrazines from amines. Using this method, ethylhydrazine has been synthesised from N-nitroso-1,2-diethyl urea[105], and methylhydrazine has been obtained from both N-nitroso-N-methyl urethane[126], and N-nitroso-N-methyl sulphamic acid[127].

8. The Reaction of Chloramine and Similar Compounds with Alkylamines

Chloramine (NH_2Cl), produced by the reaction of equimolar quantities of hypochlorite and ammonia in alkaline aqueous solutions, was shown by Raschig[128] to be capable of

reacting with further quantities of ammonia to yield hydrazine. By a modification of the Raschig synthesis, Audrieth and Diamond[129] successfully synthesised a number of monoalkyl hydrazines in 55 - 70% yield by the reaction of chloramine with the appropriate alkylamine (6 -8 fold excess):-

$$NH_2Cl + RNH_2 \longrightarrow RNHNH_2 + HCl$$

A similar reagent, hydroxylamineisomonosulphonic acid (NH_2OSO_3H), often referred to as HOS, also reacts with alkylamines to give primary hydrazines. Thus Gever and Hayes[130] have prepared n-butyl, n-amyl and isopropyl hydrazines in yields of up to 50% using HOS.

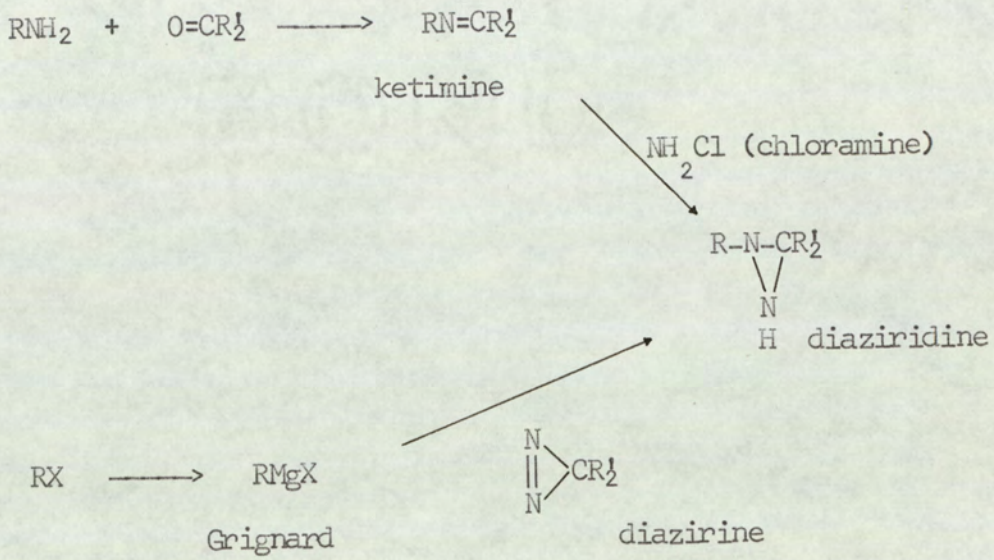
$$RNH_2 + NH_2OSO_3H \longrightarrow RNHNH_2 + H_2SO_4$$

Meusen and Gosl[131] have extended this reaction to include aryl hydrazines such as phenylhydrazine.

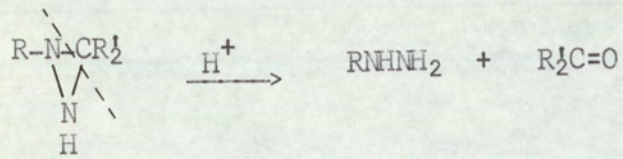
As in the chloramine synthesis, good yields of the hydrazine are dependant upon a considerable excess of the primary amine, although an inorganic base such as potassium hydroxide can be employed in conjunction with a small excess of the amine[130]. The yields in the latter case are however inferior to those obtained using the amine alone. The relative merits of chloramine and HOS in the synthesis of monosubstituted hydrazines, will be later discussed in relation to the synthesis of t-butylhydrazine (page 168).

9. The Hydrolysis of Diaziridines

An interesting method of potential value for the preparation of monosubstituted hydrazines is the hydrolytic cleavage of diaziridines [132, 133]. These three membered ring compounds are obtainable either by the action of chloramine with ketimines or by the addition of Grignard reagents to diazirines, as follows:-



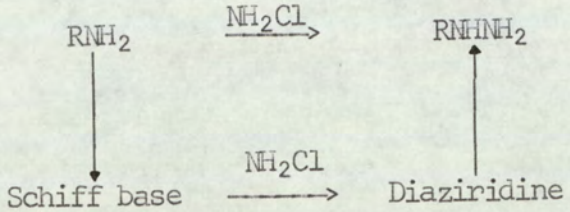
The formation of the diaziridine from the ketimine can also be effected by the use of HOS [132, 134]. The hydrolytic cleavage of the resulting diaziridine ring with the formation of the monosubstituted hydrazine is brought about by aqueous mineral acids:-



The synthesis, for example, of n-butyl, isopropyl and

benzyl hydrazines from such diaziridines in good yields, has been reported by Schmitz [135].

The synthesis of diaziridines and their cleavage to alkyl hydrazines corresponds in effect to the direct reaction of an amine with chloramine as previously described.



The detour via the diaziridine has, however, several significant advantages. For instance, in the direct reaction a considerable excess of the amine is necessary to supress further interaction of the alkyl hydrazine formed, with the aminating agent. Diaziridines are however insensitive to the aminating agent. Furthermore, the range of hydrazines available via the diaziridine route is considerably wider than by direct amination [133].

(b) Experimentally adopted Procedures

The majority of the synthetic routes to monosubstituted alkyl and aryl hydrazines are described above. As some of the methods are more suitable, for instance, for the synthesis of alkyl rather than aryl hydrazines, the particular synthetic procedure adopted will clearly primarily depend on the nature of the hydrazine desired. In addition, the suitability of a particular method to the production of the hydrazine in both the

right form (acid salt or free base, for example), and in the necessary quantities, must be taken into consideration. The methods adopted for the synthesis of methylhydrazine, benzylhydrazine, and t-butylhydrazine are discussed below.

Methylhydrazine sulphate. (Experimental: page 204)

The simplest and most rapid procedure for the synthesis of methylhydrazine sulphate appears, in fact, to be a direct alkylation reaction using dimethyl sulphate, in which the tendency to polyalkylation is blocked by the use of an azine, as previously described (page 154). The azine, benzalazine, was prepared in 90% yield by the reaction of benzaldehyde with hydrazine sulphate in the presence of ammonia:-

$$C_6H_5CHO + N_2H_4.H_2SO_4 \xrightarrow{NH_3} C_6H_5CH{=}NN{=}CHC_6H_5$$

benzal azine

The alkylation of benzalazine with dimethyl sulphate, followed by the hydrolysis of the resulting hydrazonium salt with water, resulted in the formation of the desired methylhydrazine sulphate in 48% yield.

$$C_6H_5CH{=}NN{=}CHC_6H_5 \xrightarrow{(CH_3)_2SO_4} C_6H_5CH{=}\overset{+}{N}(CH_3)N{=}CHC_6H_5.CH_3SO_4^- \xrightarrow{H_2O} CH_3NHNH_2 + C_6H_5CHO$$

Benzylhydrazine (Experimental: page 208) A consideration of

the available methods for the synthesis of benzylhydrazine as the free base, suggested that the direct alkylation of hydrazine with benzyl chloride, as described by Biel and co-workers [109], was the superior procedure.

$$C_6H_5CH_2Cl + NH_2.NH_2.H_2O \longrightarrow C_6H_5CH_2NHNH_2$$

By using a six-fold excess of the hydrazine over the alkylating agent, the benzylhydrazine was obtained in 88% yield, any dibenzylhydrazine present being eliminated by fractional distillation.

t-Butylhydrazine (Experimental: page 213f)

Very few syntheses of this compound are to be found in the literature. The first, based on a direct alkylation of hydrazine with t-butyl chloride was described as having very low yields [106]. As, in addition, it seems doubtful that the described product was, in fact, t-butylhydrazine, the method was therefore considered to be of little value. In support of earlier work [120], Klages and his co-workers [136] reported that synthesis of t-butylhydrazine by a method, which consisted of the addition of methylmagnesium bromide to acetone azine as previously described (page 158). Further investigations into this synthesis by Lakritz [137], who was unable to duplicate this experiment, suggested that there was some question as to whether the product Klages obtained was, in fact, t-butylhydrazine.

In an attempt to establish a synthetic procedure capable of yielding this hydrazine in reasonable quantities, Lakritz made a thorough investigation of many of the general preparative methods for monosubstituted hydrazines. The technique involving the reduction and hydrolysis of a nitrosourethane (page 160) proved unsuccessful, owing to the difficulty experienced in nitrosating t-butylurethane.

$$(CH_3)_3C\text{-}NHCOOC_2H_5 \not\longrightarrow (CH_3)_3CN(NO)COOC_2H_5$$

The hydrolysis of syndones (page 159) also appeared to be a feasible route to t-butylhydrazine, but, although the N-tert-butylglycine required for this reaction was readily obtained from ethyl chloracetate and t-butylamine, the nitrosation of this sterically hindered glycine was also unsuccessful.

$$(CH_3)_3CNH_2 + ClCH_2COOC_2H_5 \longrightarrow (CH_3)_3CNHCH_2COOC_2H_5$$

$$(CH_3)_3CNHCH_2COOC_2H_5 \longrightarrow (CH_3)_3CNHCH_2COOH \longrightarrow (CH_3)_3CN(NO)CH_2COOH$$

The method finally adopted by Lakritz was one involving the reaction of a Grignard reagent with a diazocompound (general method: page 157). This technique, discovered by Zerner[117] and further developed by Coleman[138] consisted of the treatment of t-butylmagnesium chloride with diphenyldiazomethane to give benzophenone t-butyl hydrazone (I). The hydrolysis of this hydrazone with concentrated hydrochloric acid at room temperature gave the desired t-butylhydrazine:-

$$(C_6H_5)_2C{=}N_2 + (CH_3)_3CMgCl \longrightarrow (C_6H_5)_2C{=}NN{-}\overset{\overset{MgCl}{|}}{C}(CH_3)_3$$

$$(C_6H_5)_2C{=}NN{-}\overset{\overset{MgCl}{|}}{C}(CH_3)_3 \xrightarrow{H_2O} (C_6H_5)_2C{=}NNHC(CH_3)_3 \xrightarrow{\text{hydrolysis}} (CH_3)_3CNHNH_2$$

This method, although successful, suffers from the disadvantage of being rather lengthy.

The synthesis of t-butylhydrazine hydrochloride has also been achieved in good yield, but on a small scale, by treating t-butylamine with chloramine[129]; the difficulty of preparing chloramine on a large scale in the laboratory however, severely limits the quantities of the hydrazine which can be so produced.

From the above discussion it is clear that there is still scope for the development of a reasonably rapid synthetic procedure capable of the formation of t-butylhydrazine in useful quantities. Gever and Hayes[130] were able to obtain n-butylhydrazine oxalate in 80% yield bythe reaction of hydroxylamineisomonosulphonic acid (HOS) with n-butylamine. That HOS is relatively easy to synthesise in large quantities suggested that the use of t-butylamine instead of n-butylamine in their method, could afford a useful and efficient route to t-butylhydrazine. This, in fact, proved to be the case (Expt: page 213), the hydrazine being formed as the oxalate, a derivative so far unrecorded, in 34% yield.

$$t\text{-}BuNH_2 + NH_2OSO_3H \longrightarrow t\text{-}BuNHNH_2 \text{ (as oxalate)}$$

The hydrazine was isolated from the excess t-butylamine

employed, by treatment of the reaction mixture with benzaldehyde to give the benzaldehyde t-butylhydrazone. This hydrazone was then hydrolysed by oxalic acid to yield the t-butylhydrazine oxalate:-

$$t\text{-BuNHNH}_2 + C_6H_5CHO \longrightarrow t\text{-BuNHN=CHC}_6H_5 \xrightarrow{(COOH)_2} t\text{-BuNHNH}_2.(COOH)_2$$

The synthesis of the free t-butylhydrazine from the oxalate was achieved (page 214) using the method adopted by Smith and co-workers[118]. The pungent, colourless liquid was finally obtained 100% pure by fractional distillation using a highly efficient spinning band column. It was found that the pure hydrazine reacted rapidly with the air, and it was therefore stored under nitrogen, and at $0^{\circ}C$. Under these conditions it was found that it could be stored for approximately a week with little decomposition, although as was reported by Smith[118], the pure liquid evolved a slow but constant stream of a gas, which was not identified.

Although the synthesis of t-butylhydrazine via its oxalate salt was an acceptable method, it appeared that there was room for substantial improvements in the efficiency of the procedure. Firstly, the recovery of the considerable excess of t-butylamine employed in the reaction, proved impracticable in the existing method. Secondly, the lengthy steam distillation of the acid

reaction liquors to remove the excess benzaldehyde (Experimental: page 213) is not only tedious, but what is more important, it involves the prolonged boiling of an acid solution of t-butyl hydrazine, conditions which are known to favour its decomposition by elimination of the t-butyl group[137] . Thirdly, the conversion of t-butylhydrazine oxalate to the free base is a time consuming procedure. In an attempt to eliminate these experimental drawbacks, and thus to facilitate the rapid synthesis of the free hydrazine, the synthetic procedure detailed on page 214 was adopted. Essentially, the method consisted of reacting the t-butylamine with HOS in the usual manner,and then using fractional distillation to isolate the t-butylhydrazine direct from the reaction mixture. By this means, although considerable quantities of t-butylamine were recovered and the tedious procedures of steam distillation and the basification of the hydrazine oxalate so obtained were eliminated, the resulting yield of t-butylhydrazine was so small as to outweigh any advantage gained in respect of the increased rapidity of the method.

2. 1,2-Disubstituted Hydrazines

(a) General Synthetic Methods:-

The general methods available for the synthesis of 1,2-disubstituted hydrazines are in principle very similar to those already described for the synthesis of monosubstituted hydrazines. The increased chemical complexity of the disubstituted derivatives

however introduces a number of additional preparative difficulties which are not immediately apparent. The principal general methods of synthesis of such hydrazines are:- (1) the alkylation and arylation of monosubstituted hydrazines (2) the reduction of acyl hydrazines (3) the reduction of hydrazones and azines and (4) the addition of Grignards to diazo compounds. The details and general application of each of these preparative methods will now be discussed.

1. Alkylation and Arylation Reactions

Alkylation of monosubstituted hydrazines can be accomplished with facility with such alkylation agents as alkyl halides and sulphates. With small alkyl groups such as methyl, however alkylation takes place on the substituted nitrogen only, and usually continues, as previously described (page 153) until the quarternary ion, $R_3\overset{+}{N}NH_2$, is formed[139]. It might be expected that either considerable steric hindrance or a pronounced reduction in base strength, could result in alkylation, partial or complete, at the unsubstituted nitrogen and thus provide a synthetic route to symmetrical disubstituted hydrazines.

Even t-butylhydrazine is alkylated almost entirely at the substituted nitrogen however,[137] at least by methyl iodide, and it is not until steric hindrance becomes very great, as in the reaction between triphenylmethylhydrazine and trityl chloride that alkylation takes place almost predominantly at the unsubstituted nitrogen.

$$(C_6H_5)_3NHNH_2 + (C_6H_5)_3CCl \longrightarrow (C_6H_5)_3CNHNHC(C_6H_5)_3$$

The pronounced base weakening effect of a phenyl substituent has also been shown[140] to cause alkylation to take place at the unsubstituted nitrogen. Thus the alkylation of phenylhydrazine with ethyl bromide results mainly in 1-phenyl-2-ethylhydrazine

$$C_6H_5NHNH_2 + C_2H_5Br \longrightarrow C_6H_5NHNHC_2H_5$$

As might be expected, the extent of alkylation or arylation at a particular nitrogen atom is to some extent dependant upon which halide is used. Thus reaction at the substituted nitrogen occurs to the largest extent with iodides and least with chlorides[106]. This phenomena may be explained by the probable fact that the C-N bond length in the transition state is greatest with a good leaving group, such as iodide, with the result that the transition state is 'looser' and thus not so sensitive to destabilisation. It should be borne in mind however that any relative increase in the yield of the 1,2-disubstituted hydrazine resulting from the use of an alkyl chloride as opposed to the iodide, may be more than offset by the inferior reactivity of the chloride. Where it is not possible to utilise steric and base weakening effects to cause alkylation to take place predominantly at the unsubstituted nitrogen, it is usual to employ blocking groups to the same end.

One such blocking agent which has been used to this effect

is the nitroso group. In nitrosation, as in other types of electrophilic reactions, attack normally takes place at the more basic nitrogen. Thus methylhydrazine and benzylhydrazine are both nitrosated on the substituted nitrogen.

$$RNHNH_2 + HNO_2 \longrightarrow RN(NO)NH_2$$

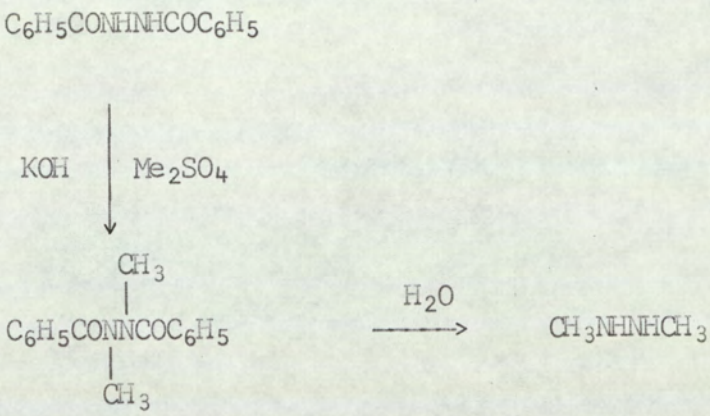
This feature has been utilised by Thiele[141] to prepare a number of 1,2-disubstituted hydrazines. The nitrosation of the monosubstituted hydrazine at the substituted nitrogen forces any alkylation to take place at the unsubstituted atom. The removal of the nitroso group from the 1,2-disubstituted hydrazine is then effected by heating the nitroso derivative in acid solution.

$$RNHNH_2 \xrightarrow{HNO_2} RN(NO)NH_2 \xrightarrow{R'X} RN(NO)NHR' \xrightarrow[H_2O]{H^+} RNHNHR'$$

The attempts to synthesis 1-methyl-2-benzyl hydrazine by this route are later discussed (page 184).

Another device commonly employed to block undesired alkylations is the use of such compounds as sym-diformyl and dibenzoyl hydrazines. Such compounds can be alkylated once on each nitrogen atom by most unhindered alkylating agents, giving dialkyl hydrazides which can then be easily hydrolysed to symmetrical dialkyl hydrazines

by boiling with mineral acids[142]. The overall process is illustrated below:-



In contrast to the previously described procedure of using nitroso groups as blocking agents, this method suffers from the disadvantage that it is only useful for the synthesis of hydrazines with identical groups on each nitrogen.

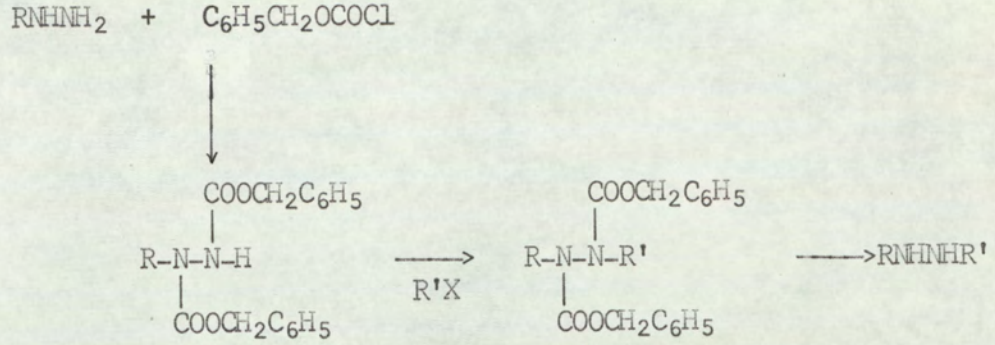
A further blocking agent which has been employed in the synthesis of 1,2-disubstituted hydrazines is benzyl chloroformate[143]. This reagent is readily prepared by the action of phosgene on benzyl alcohol in toluene:-

$$C_6H_5CH_2OH + COCl_2 \longrightarrow C_6H_5CH_2OCOCl$$

benzyl chloroformate

Benzyl chloroformate reacts with both nitrogen atoms of the primary hydrazine, and the resulting compound is then alkylated with the appropriate agent. The protecting groups are readily

removed by hydrogen bromide in glacial acetic acid to yield the disubstituted hydrazine:-



This synthetic procedure is useful for the synthesis of 1,2-disubstituted hydrazines with different substituents on each nitrogen atom, and has been thus used by Zeller [10] and his co-workers to prepare a number of such hydrazines of pharmacological interest.

2. The formation and reduction of acyl hydrazines

The basis of this route to 1,2-disubstituted hydrazines consists essentially of the formation of the symmetrical acyl derivatives of primary hydrazines and their subsequent reduction to the corresponding dialkyl compounds, as follows:-

$$RNHNH_2 \xrightarrow{R'COCl} RNHNHCOR' \xrightarrow{H_2} RNHNHCH_2R'$$

One of the difficulties sometimes encountered in this synthetic procedure lies however in the initial preparation of the appropriate acyl hydrazine.

The acetylation of hydrazines somewhat resembles alkylation in that the alkylated nitrogen is often preferred to the substituted one. Thus, the benzoylation of methylhydrazine with benzoyl chloride, or benzoic anhydride first yields N-methyl benzhydrazide, which then undergoes further benzoylation to give 1,2-dibenzoyl methylhydrazine

$$CH_3NHNH_2 + C_6H_5COCl \longrightarrow \underset{\displaystyle COC_6H_5}{\overset{\displaystyle |}{CH_3N\text{-}NH_2}} \longrightarrow \underset{\displaystyle COC_6H_5}{\overset{\displaystyle |}{CH_3\text{-}N\text{-}NHCOC_6H_5}}$$

On the other hand, the acylation of primary hydrazines seems to be much more sensitive to steric crowding than does alkylation. Thus, t-butylhydrazine is <u>first</u> acylated by benzoyl chloride on the <u>un</u>substituted nitrogen to give 1-t-butyl-2-benzoyl hydrazine, which then further reacts to yield 1,2-dibenzoyl-t-butyl hydrazine[137]. The net result of these two factors, i.e. base strength and steric hindrance, on the acylation pattern of monosubstituted hydrazines is that in many cases a mixture of acyl derivatives of a particular hydrazine is obtained, which are often difficult to separate.

A recent study by Hinman and Fulton[143] has however revealed interesting differences in the acylation of alkylhydrazines with anhydrides and esters. They found that methylhydrazine was predominantly substituted at the unalkylated nitrogen by esters, whereas anhydrides caused almost exclusive acylation at the

substituted nitrogen.

$$RNHNH_2 + R'COOEt \longrightarrow RNHNHCOR'$$

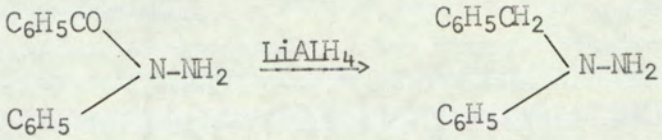
$$RNHNH_2 + (R'CO)_2O \longrightarrow RN(COR')NH_2$$

The fact that these authors also found that the percentage of the 1-alkyl-1-acyl hydrazine formed in the acylation dropped sharply with increasing size of the ester, suggests that the reason why esters acylate mainly at the unsubstituted nirogen is steric in origin. The acylation of alkyl hydrazines with esters thus affords a useful route to those 1-alkyl-2-acyl hydrazines which are difficult to prepare by the use of more common acylating agents. The employment of this technique to synthesise 1-methyl-2-benzoyl hydrazine is discussed on page 187 .

The second step in the preparation of 1,2-dialkyl hydrazines is the reduction of the corresponding 1-alkyl (or aryl)-2-acyl hydrazine. The reagent of choice for this reduction is lithium aluminium hydride, a reagent which appears to be effective for a wide variety of such hydrazides. Thus, the reduction of 1,1-dimethylformhydrazide yields 1,1-dimethyl-2-methyl hydrazine [144],

$$HCONHN(CH_3)_2 \longrightarrow MeNHNMe_2$$

and 1-benzoyl-1-phenyl hydrazine is converted to 1-benzyl-1-phenyl hydrazine [110].



Although the reduction of acetyl, propionyl and butyryl hydrazides has been successfully achieved [145,146], it is clear that the reduction of secondary hydrazides, which contain the group -CONH-, is considerably slower than that of teriary hydrazides, which contain a -CONR- group. For example, whereas 1-benzoyl-1-methyl hydrazine is reduced to the corresponding hydrazine in reasonable yield within three hours, benzoylhydrazine is completely unaffected under the same conditions [110]. It is of interest in this respect that tertiary amides ($RCONR_2$) are also reduced much more rapidly than secondary amides (RCONHR) [147].

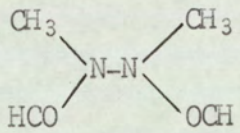
Two principal suggestions have been put forward to explain why secondary hydrazides are so resistant to reduction. Firstly, it is well known that the replacement of an active hydrogen with lithium aluminium hydride is accompanied by the evolution of hydrogen gas and the formation of a complex between the substrate and some form of the reducing agent. It has been suggested that the reduction of the functional group (the carbonyl in this case) in the substrate may be hindered or entirely prevented by the precipitation of this complex [148]. The insolubility of some acyl hydrazines themselves in the organic solvents customarily used in such reductions, such as tetrahydrofuran and ether, may also contribute to lengthy reaction times. The formation of a hetero-

geneous reaction mixture because of either of these factors would seem at first sight to explain the lack of reactivity observed in acyl hydrazines containing acidic hydrogens, as these compounds are not only less soluble in tetrahydrofuran and ether, but are also more likely to form complexes.

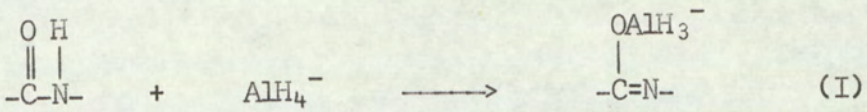
That other factors are also involved is suggested by the observations that a compound such as 1-benzoyl-2,2-dimethyl hydrazine

$$C_6H_5CONHN(CH_3)_2$$

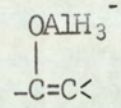
is not reduced in a homogeneous solution[149], whereas such compounds as 1,2-diformyl-1,2-dimethylhydrazine,



are rapidly reduced even in heterogeneous media[150]. Following experimental work on a series of variously substituted benzoyl hydrazines, Hinman[110] has concluded that the principal cause of the slow reduction of compounds containing the group -CONH- is due to the inactivation of the functional group by a mechanism which is peculiar to compounds containing this particular group. Assuming that replacement of the active hydrogen is the first reaction, the author suggests that inactivation may occur by the formation of the following complex:-



This complex (I) so formed resembles that of the enolate ion,



which is reported to resist attack by lithium aluminium hydride [148,151]. Thus if the complex (I) also resists reduction, then the addition of water at the end of the reaction would merely regenerate the original functional group.

Summarising, it would seem, owing to the difficulties often encountered in the synthesis and reduction of 1-alkyl-2-acyl hydrazines, that this particular route to 1,2-dialkyl hydrazines is far less attractive than it first appears. The attempts to prepare 1-methyl-2-benzyl hydrazine and 1-t-butyl-2-benzyl hydrazine by the reduction of the appropriate acyl hydrazines with lithium aluminium hydride, are later discussed (pages 188 and 192, respectively).

3. The Reduction of Azines and Hydrazones

The synthesis of 1,2-disubstituted hydrazines by the reduction of azines and hydrazones is closely analagous to the previously described (page 155) formation of monosubstituted compounds.

The preparation of aromatic and aliphatic azines is generally simply effected by the reaction of hydrazine with the appropriate aldehyde or ketone:-

$$RCHO + NH_2.NH_2 \longrightarrow RCH{=}NN{=}CHR$$

aldazine

$$R_2C{=}O + NH_2.NH_2 \longrightarrow R_2C{=}N{-}N{=}CR_2$$

ketazine

In contrast to the reduction of hydrazides, the reduction of azines presents relatively few experimental difficulties and has therefore been extensively used to synthesise symmetrical hydrazines [114, 152]

$$\text{e.g.} \quad R_2C = NN = CR_2 \xrightarrow{2H_2} R_2CHNHNHCHR_2$$

Thus, Renaud and Leitch [114] have reported excellent yields of 1,2-diethyl, 1,2-diisopropyl and 1,2-di-n-propyl hydrazines from acetaldazine, dimethylketazine and propionaldazine respectively. Although a number of different reducing agents have been employed to effect this reduction, hydrogenation over platinum in the presence of acid appears to be one of the most general methods [152, 153]. One of the disadvantages of this reagent however, is that the reduction sometimes tends to cause the cleavage of the N-N bond to give amines. Lithium aluminium hydride has also been found to effectively reduce azines to

disubstituted hydrazines [114], and in contrast to the catalytic method, it gives a pure product free of the amine.

The reduction of hydrazones is another similar route to 1,2-disubstituted hydrazines:-

$$RNHNH_2 + R'CHO \longrightarrow RNHN{=}CHR' \xrightarrow{H_2} RNHNHCH_2R'$$

Hydrazones are nearly always prepared from ketones or aldehydes and the appropriate monosubstituted hydrazine, it being usually sufficient to mix the carbonyl compound and the hydrazine in aqueous or partly alcoholic solution with the addition of a trace of acid as a catalyst. The formation of aryl hydrazones is a reaction which has a very favourable equilibrium [154], and it thus is unnecessary to remove the water formed in the reaction, or to avoid using water as a solvent. In the case of alkyl hydrazones however it is often necessary to remove the water formed in the course of the reaction to obtain a reasonable yield [155], although the preparation of methyl hydrazones of benzaldehyde seems to be less sensitive in this respect [156].

Although sodium amalgam will reduce hydrazones to hydrazines [157], the two most commonly used reagents for this reduction are hydrogen over platinum or palladium at room temperature [153,157], and lithium aluminium hydride [145,150]. The attempts to form 1-t-butyl-2-benzyl hydrazine by the reduction of benzaldehyde t-butyl hydrazone by both catalytic hydrogenation and lithium aluminium hydride is discussed later in the text (page 194).

4. The Addition of Grignard Reagents to Diazo Compounds

As has earlier been discussed with regard to the synthesis of monosubstituted hydrazines, Grignard reagents are capable of reacting with diazo compounds to form hydrazones. It has also been observed by Coleman and his co-workers[138] that the addition of a large excess of a Grignard reagent to the diazo compound results in the reduction of the azo-methine linkage as well as addition to the diazo group. Thus the reaction of phenylmagnesium bromide with diazomethane has been reported[138] as yielding 1-benzyl-2-phenyl hydrazine by a reaction which may be represented as follows:-

$$CH_2{=}N_2 + C_6H_5MgBr \longrightarrow CH_2{=}N{-}N\begin{matrix}C_6H_5\\MgBr\end{matrix} \xrightarrow{C_6H_5MgBr}$$

$$C_6H_5CH_2{-}N(MgBr){-}N(MgBr){-}C_6H_5 \longrightarrow C_6H_5CH_2NHNHC_6H_5$$

However, when benzylmagnesium chloride was substituted for phenylmagnesium bromide, the product was reported[138] as being 1-methyl-2-benzyl hydrazine, rather than the expected 1-phenethyl-2-benzyl hydrazine.

This method would thus appear to be suitable for the preparation of a considerable variety of symmetrical disubstituted hydrazines containing different substituents on each nitrogen. The attempts made to synthesise 1-methyl-2-benzyl hydrazine by the procedure outlined above, are discussed on page 188.

(b) Experimentally Adopted Procedures

As in the case of monoalkyl hydrazines, of the general methods available, the actual method of synthesis adopted for any one particular 1,2-disubstituted hydrazine will primarily depend on the nature of the desired compound. The methods adopted and the experimental difficulties experienced in the attempted synthesis of 1-methyl-2-benzyl hydrazine and 1-t-butyl-2-benzyl hydrazine are described below.

1-Methyl-2-benzyl hydrazine

The synthesis of this particular hydrazine was attempted by the following routes:- (1) The methylation of benzylhydrazine (Expt: page 209f). (2) The synthesis and reduction of 1-methyl-2-benzoyl hydrazine (Expt: page 206), and (3) the reaction of benzylmagnesium chloride with diazomethane.

(1) As is emphasised in the general discussion on the alkylation of monosubstituted hydrazines, the alkylation of a compound such as benzylhydrazine with an alkylating agent such as methyl sulphate which suffers little from steric hindrance, is likely to result in the predominant formation of 1-benzyl-1-methyl hydrazine unless the α nitrogen of the benzylhydrazine is blocked. The synthesis of 1-methyl-2-benzyl hydrazine reported by Thiele[141], involves the initial nitrosation of benzylhydrazine with nitrous acid, the resulting α-nitroso group acting as the blocking agent.

$$C_2H_5CH_2NHNH_2 + HNO_2 \longrightarrow C_6H_5CH_2\underset{\displaystyle NO}{\underset{|}{N}}NH_2$$

The presence of the nitroso group on the substituted nitrogen then forces the subsequent alkylation to occur at the unsubstituted atom. The nitroso group is then removed from the 1-methyl 2-nitrosobenzyl hydrazine by acid hydrolysis to give the disubstituted hydrazine.

$$C_6H_5CH_2\underset{\underset{NO}{|}}{N}NH_2 + Me_2SO_4 \longrightarrow C_6H_5CH_2\underset{\underset{NO}{|}}{N}\text{-}NHMe \xrightarrow[H_2O]{H^+} C_6H_5CH_2NHNHMe$$

Despite the rigorous adherence to the experimental procedure detailed by Thiele, considerable difficulty was experienced in repeating the synthesis. The first attempt (page 209) to nitrosate benzylhydrazine with nitrous acid gave a good yield of the expected N-nitrosobenzylhydrazine, the identity of which was confirmed by spectral and analytical analysis in the usual way. However, all attempts to repeat this synthesis failed to yield any N-nitroso-benzylhydrazine. Instead, the addition of the benzylhydrazine (as the sulphate) to the sodium nitrite solution caused the vigorous release of a brown gas, and the formation of a yellow oil, which rapidly turned brown on warming. The oil smelt strongly of benzaldehyde. In an attempt to determine the correct experimental conditions to effect the synthesis of the nitrosohydrazine, the reaction was repeated using a variety of experimental conditions (page 210). In every case the reaction between the benzylhydrazine and nitrous acid resulted in a vigorous effervescence and the

formation of further quantities of the water insoluble oil. Further investigations into this reaction by Blair and Taylor[181] have shown that the formation of the oil is favoured by acidic conditions. The major components of the oil so formed were found to be benzyl alcohol and benzaldehyde when the reaction was carried out at 0°, but at much higher temperatures (90°) benzyl alcohol was the predominant constituent. These authors[181] have suggested that the formation of benzyl alcohol is due to the reaction of benzyl carbonium ions, probably formed under the acidic reaction conditions, with water,

$$C_6H_5CH_2^{+} + H_2O \longrightarrow C_6H_5CH_2OH$$

and that benzaldehyde may result from the oxidation of the benzyl alcohol. The formation of the N-nitrosobenzylhydrazine was found to be favoured by neutral or alkaline conditions.

Despite several attempts (Expt: page 210), the methylation of N-nitrosobenzylhydrazine with methyl sulphate by the experimental procedure of Thiele, failed to yield any of the desired 1-methyl-2-nitrosobenzylhydrazine.

Owing to the difficulties experienced in the synthesis and methylation of nitrosobenzylhydrazine, this particular route to 1-methyl-2-benzyl hydrazine was not further pursued.

(2) The synthesis of 1-methyl-2-benzyl hydrazine was next attempted by a route which involved the preparation and reduction

of 1-methyl-2-benzoyl hydrazine.

$$CH_3NHNH_2 + C_6H_5COCl \longrightarrow CH_3NHNHCOC_6H_5 \longrightarrow CH_3NHNHCH_2C_6H_5$$

As previously indicated in the general discussion of the preparation of acyl hydrazines (page 176), the benzoylation of methylhydrazine with benzoyl chloride results in initial acylation at the substituted atom to give 1-methyl-1-benzoyl hydrazine, a reaction which is followed by further acylation to give 1,2-dibenzoyl methylhydrazine. The synthesis of the latter compound is detailed in the experimental section (page 212). However, as already discussed, the use of methyl benzoate as the acylating agent results in predominant substitution at the unsubstituted nitrogen. Thus, 1-methyl-2-benzoyl hydrazine was prepared (as the hydrochloride) using the experimental procedure of Hinman and Fulton [143], by the reaction between methylhydrazine and methyl benzoate

$$CH_3NHNH_2 + C_6H_5COOCH_3 \longrightarrow CH_3NHNHCOC_6H_5 + CH_3OH$$

By increasing the reaction time of two hours used by Hinman and Fulton to twenty-four hours, the yield of the hydrazide was raised from 8 to 20%. It is likely that this yield could be further elevated by also increasing the mole ratio of ester to hydrazine. The 1-methyl-2-benzoyl hydrazine hydrochloride

prepared as described above was converted to the free hydrazide by neutralisation with sodium hydroxide (Expt: page 206).

The reduction of the resulting 1-methyl-2-benzoyl hydrazine was attempted using a two-fold excess of lithium aluminium hydride (Expt: page 206). No reduction however occurred, the only reaction products isolated being starting material and a small quantity of yellow oil which, as indicated by its infra red and NMR spectra, possessed no aromatic character. The failure of lithium aluminium hydride to effect the reduction of 1-methyl-2-benzoyl hydrazine, which was not altogether unexpected in view of the difficulty experienced by previous workers in reducing such benzoyl groups[110], necessitated the adoption of another route to the desire 1-methyl-2-benzyl hydrazine.

(3) The addition of a Grignard reagent to a diazo compound is, as previously discussed (page 183), capable of yielding a 1,2-disubstituted hydrazine. Coleman and his co-workers using this route have reported the isolation of 1-methyl-2-benzyl hydrazine (as the hydrochloride) as a result of the reaction between an excess of benzylmagnesium chloride and diazomethane.

$$CH_2{=}N_2 + C_6H_5CH_2MgCl \longrightarrow CH_3NHNHCH_2C_6H_5$$

Using the identical experimental procedure to that detailed by Coleman, the above reaction was repeated (Expt: page 207). The product obtained from the reaction was a white solid of indefinite

melting point (112° - 116°d) which rapidly turned yellow on exposure to the air. This compound was not 1-methyl-2-benzyl hydrazine as indicated by the absence of an absorbtion corresponding to a methyl group in the NMR spectrum. No further investigation into the identity of the product was made. The reaction was repeated using an identical experimental procedure, but again none of the desired 1-methyl-2-benzyl hydrazine was obtained.

1-t-Butyl-2-benzyl hydrazine

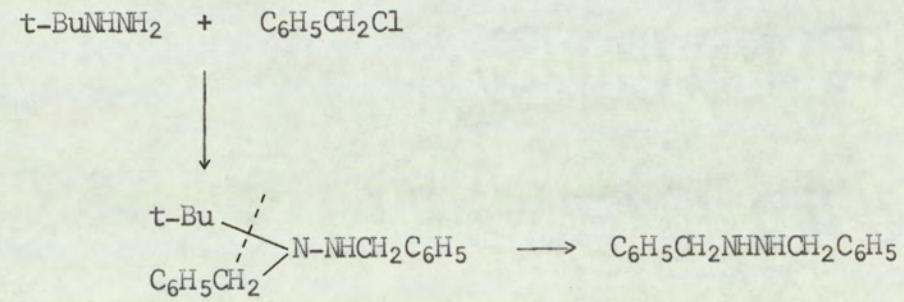
The synthesis of this hydrazine was attempted by the following procedures:- (1) the direct alkylation of t-butylhydrazine with t-butyl halides (2) the formation and reduction of 1-t-butyl-2-benzoyl hydrazine (3) the synthesis and reduction of benzaldehyde t-butyl hydrazone.

1. As is considered in the general discussion on the alkylation of monosubstituted hydrazines (page 171) it is to be expected that, where steric factors are sufficiently pronounced, alkylation or arylation will occur on the substituted nitrogen. Thus, although t-butylhydrazine is alkylated on the unsubstituted nitrogen by methylating agents, it is not unreasonable to expect that the larger alkylating agents such as benzyl halides would cause alkylation, at least to some extent, on the unsubstituted nitrogen. Furthermore, as also previously mentioned, such substitution is more likely to occur with benzyl chlorides and

bromides than iodides. The synthesis of 1-t-butyl-2-benzyl chloride was therefore attempted by the direct arylation of t-butyl-hydrazine with either benzyl chloride or benzyl bromide. Although a considerable variety of experimental conditions were employed (Expt: page 220f) to effect this arylation, no 1-t-butyl-2-benzyl hydrazine could be isolated.

The stirring of equimolar quantities of benzyl chloride and t-butylhydrazine in ethanol at room temperature for 72 hours, failed to cause any arylation as evidenced by the recovery of t-butylhydrazine (as its hydrochloride) in good yield at the end of the reaction. An analagous experiment was carried out by refluxing the same reagents for 8 hours under an atmosphere of nitrogen (Expt: page 221). Once again only the original t-butyl-hydrazine was recovered from the reaction liquors. The repetition of this experiment using the same conditions, but using a higher boiling solvent (propanol) and benzyl bromide as the arylating agent, failed to yield any significant products. That arylation of t-butylhydrazine does however take place is suggested by the results of the experiment detailed on page 222. In this experiment a mixture of t-butylhydrazine and benzyl chloride was refluxed in ethanolic solution for 16 hours. By removing the solvent by blowing dry nitrogen over the surface of the still heated reaction mixture, the temperature of the liquors was gradually increased

as evaporation of the solvent took place. The colourless oil which finally resulted was then worked up as described on page 222. The first compound isolated from the reaction mixture was identified as 1,2-dibenzylhydrazine. The presence of this compound strongly suggests that not only did arylation of the t-butylhydrazine take place, but that it occurred on both nitrogen atoms to give 1,2-dibenzyl t-butylhydrazine. It can reasonably be assumed that the isolated 1,2-dibenzylhydrazine originates from the latter compound by the elimination of a t-butyl group, as follows:-



That such eliminations can readily occur under the hot, acid conditions as used in the reaction work up procedure, is a well established feature of the chemistry of tertiary alkyl hydrazines[101,158]

Work up of the remaining reaction mixture by fractional crystallisation, yielded four main fractions (page 223).

Examination of these indicated that the identity of the first (E) was t-butylhydrazine (as the hydrochloride), and the second (F) was probably a mixture of benzyl hydrazines. Despite

repeated recrystallisation of the remaining two fractions, G and K, it proved difficult to elucidate their exact composition. From instrumental data and elementary analysis it seems likely however, that both fractions were mixtures of t-butyl and benzyl hydrazines. Thus, summarising, it is clear from these experiments that the arylation of t-butylhydrazine does not readily take place, and even when it does occur it is likely to result in a variety of products. It would thus appear to be unsuitable as a synthetic route to 1-t-butyl-2-benzyl hydrazine.

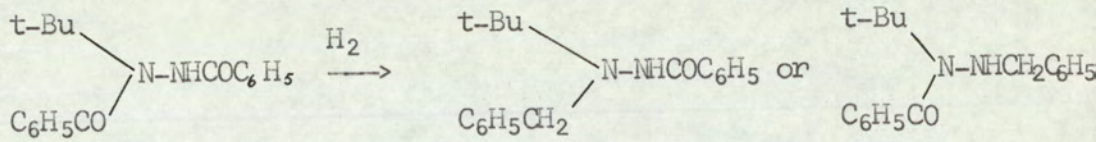
2. The synthesis of 1-t-butyl-2-benzyl hydrazine was also attempted using the general method which involves the preparation and reduction of an acyl hydrazine, in this case, 1-t-butyl-2-benzoyl hydrazine.

This hydrazide was prepared (as the hydrochloride) by the reaction of equimolar quantities of benzoyl chloride and t-butylhydrazine in benzene solution (Expt: page 217). The neutralisation of the 1-t-butyl-2-benzoyl hydrochloride gave the free 1-t-butyl-2-benzoyl hydrazine (page 218).

As previously discussed (page 178) it has been suggested that the difficulty almost invariably experienced in the reduction of benzoyl hydrazides such as 1-t-butyl-2-benzoyl hydrazine, may be partly due to the precipitation from the reaction medium, of the complex formed between the reducing agent (lithium aluminium hydride) and the hydrazide. According to Bernatek[159],

this heterogeneity can be minimised by the use of methylal (formaldehyde dimethyl acetal) as the reaction medium, as this compound is claimed to possess solvent characteristics for these metal complexes which are superior to those of either ether or tetrahydrofuran. However, even using methylal as a solvent and a reaction time of 24 hours, the lithium aluminium hydride reduction of 1-t-butyl- 2-benzoyl hydrazine was unsuccessful, the original hydrazide being recovered in 80% yield.

In the context of the reduction of benzoyl derivatives of t-butylhydrazine, it is of interest that in contrast to 1-t-butyl-2-benzoyl hydrazine, the diacylated derivative, 1,2-dibenzoyl t-butylhydrazine was found to undergo partial reduction with lithium aluminium hydride, in as little as thirty minutes. (Expt: page 216). Elemental analysis and spectral data of the reduction product indicated that it was either 1-t-butyl-1-benzyl-2-benzoyl hydrazine (I) or 1-t-butyl-1-benzoyl-2-benzyl hydrazine (II).



(I) (II)

The fact that 1-t-butyl-2-benzoyl hydrazine resisted reduction

and that, as previously discussed (page 178), tertiary amide groups are more susceptible to reduction than the corresponding secondary amide groups, suggested that the reduction product of 1,2-dibenzoyl t-butylhydrazine was in fact compound (I). This was confirmed by the unambiguous synthesis of (I) by benzoylation of 1-t-butyl-1-benzyl hydrazine (Expt: page 228). The two products were found to be identical in all respects.

3. The third synthetic procedure adopted for the preparation of 1-t-butyl-2-benzyl hydrazine involved the formation and attempted reduction of the hydrazone, benzaldehyde t-butylhydrazone, as illustrated below:-

$$t\text{-}BuNHNH_2 + C_6H_5CHO \longrightarrow t\text{-}BuNHN{=}CHC_6H_5 \xrightarrow{H_2} t\text{-}BuNHNHCH_2C_6H_5$$

The preparation of benzaldehyde t-butylhydrazone (Expt: page 230) was effected by the condensation of t-butylhydrazine, obtained from t-butylamine and hydroxylamineisomonosulphonic acid in the usual way, with benzaldehyde in a weakly acid solution. The hydrazone was finally purified by fractional distillation under reduced pressure. The hydrochloride of the hydrazone was made by the passage of anhydrous hydrogen chloride into a rigorously dried solution of the hydrazone in ether.

The reduction of benzaldehyde t-butylhydrazone was first

attempted using lithium aluminium hydride (Expt: page 232). In order to eliminate the oxidation of any 1-t-buty-2-benzyl hydrazine formed both the reaction and the work up of the reaction mixture was conducted under an atmosphere of nitrogen. The reduction proved unsuccessful, the only product which was obtained from the reaction being the original hydrazone (as the hydrochloride). The repetition of the experiment using a 24 hour reaction time again yielded the starting material. The reduction of the hydrazone was next attempted using catalytic hydrogenation over a 20% palladium-on-charcoal catalyst (Expt: page 233). Although the uptake of hydrogen virtually ceased after an exactly equimolar equivalent of the gas had been absorbed within 45 minutes, no 1-t-buty-2-benzyl hydrazine could be isolated. An examination of the infra red and NMR spectra of the reduction product indicated that by far the major component was starting material. No evidence of a benzyl group was detectable in the NMR spectrum.

It is rather difficult to understand why 1-t-butyl-2-benzyl hydrazine cannot be obtained by the reduction of the corresponding hydrazine. It is known however that such symmetrical hydrazines are very easily oxidised to the corresponding azo compounds, which can in turn rearrange to the hydrazone as follows:-

$$RNHNHCH_2R' \xrightarrow{(o)} RN{=}NCH_2R' \longrightarrow RNHN{=}CHR'$$

It is thus possible that reduction to the hydrazine does in fact

take place, as is suggested by the appropriate uptake of hydrogen in the catalytic hydrogen experiment, but that the hydrazine, being very susceptible to oxidation, rapidly reverts to the original hydrazone.

(c) Suggested Alternative Syntheses for 1-methyl-2-benzyl and 1-t-butyl-2-benzyl hydrazines

1. The recent isolation of sodium methyl hydrazine[160] formed by the reaction of sodamide and methylhydrazine,

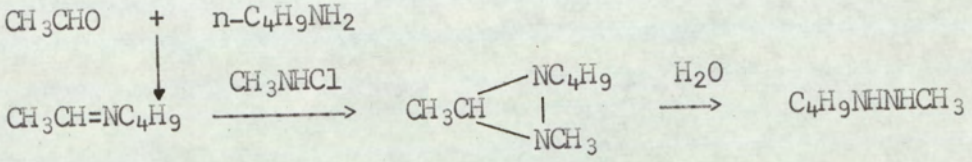
$$CH_3NHNH_2 \xrightarrow[ether]{NaNH_2} CH_3NHNHNa$$

suggests that this, and similar compounds, could be useful intermediates in the synthesis of symmetrical disubstituted hydrazines, as it would reasonably be expected that aryl and alkyl halides would react preferentially with the nitrogen bearing the sodium atom. The synthesis of, for example, 1-methyl-2-benzyl hydrazine should be possible by the arylation of sodium methyl hydrazine with benzyl chloride.

$$CH_3NHNHNa \xrightarrow{C_6H_5CH_2Cl} CH_3NHNHCH_2C_6H_5$$

2. The use of diaziridines in the synthesis of monosubstituted hydrazines has been previously discussed. The synthetic applications of such compounds have now been extended to include the synthesis of 1,2-disubstituted hydrazines[133,161,162]. Essentially this preparative method consists of the reaction of an N-chloroalkylamine (instead of chloramine itself as used in the synthesis of monosubstituted hydrazines) with a Schiff base to give the diaziridine. The latter can

then be hydrolysed to the symmetrical hydrazine by treatment with aqueous 2N hydrochloric acid. The synthesis of 1-n-butyl-2-methyl hydrazine using this technique is illustrative of the general method:-



Schiff base Diaziridine

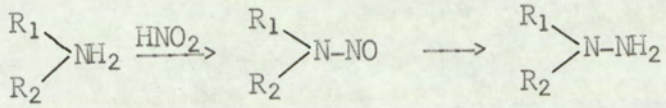
It would thus seem reasonable to suggest that by the reaction between the appropriate Schiff base and alkylchloramine, both 1-methyl-2-benzyl hydrazine and 1-t-butyl-2-benzyl hydrazine could also be prepared by this route.

3. 1,1-Disubstituted Hydrazines

(a) General Synthetic Methods

The principal routes to 1,1-disubstituted hydrazines are as follows:- (1) the reduction of nitrosamines (2) the direct amination of secondary amines and (3) the partial reduction of 1,2-dicarbethoxy arylhydrazines.

1. For unsymmetrical disubstituted hydrazines the almost invariably chosen route is the reduction of a nitrosamine prepared from a secondary amine.



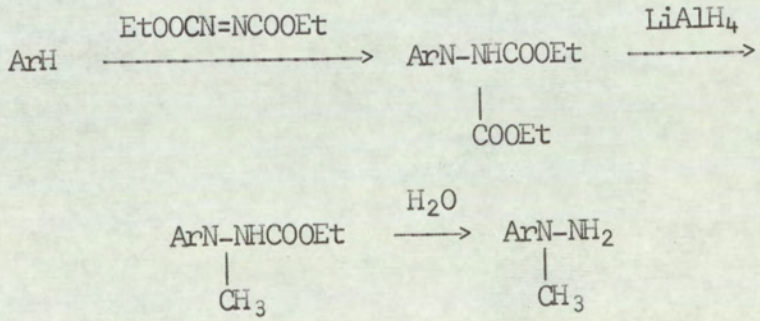
The appropriate nitrosamines can readily be prepared from dialkyl, alkyl aryl, and diarylamines. The reduction has been effected by a wide variety of reducing agents, among them being lithium aluminium hydride[163], zinc and acetic acid[164], aluminium amalgam[165] and sodium in alcohol[165]. None of these reagents can really be considered as general however, as the success of any one particular reductive method appears to be very dependant upon the specific nitrosamine. Thus the reduction of t-butylbenzylnitrosamine by means of sodium and ethanol gave 1-t-butyl-1-benzyl hydrazine in 31% yield, whereas the use of zinc and acetic acid resulted only in the regeneration of the secondary amine; lithium aluminium hydride gave a mixture of unidentified products[165]. The most reliable reagents seem to be aluminium amalgam and sodium in ethanol.

2. The direct amination of primary amines to give monosubstituted hydrazines, has been extended to the synthesis of 1,1 disubstituted hydrazines[166]. Thus Meusen and Gösl[131] have reported the synthesis of 1,1-di-n-butylhydrazine in 34% yield from the reaction between di-n-butylamine and hydroxylamineisomonosulphonic acid (HOS):-

$$(C_4H_9)_2NH + NH_2OSO_3H \longrightarrow (C_4H_9)_2NNH_2$$

The use of chloramine as the aminating agent in analagous syntheses has also been reported.

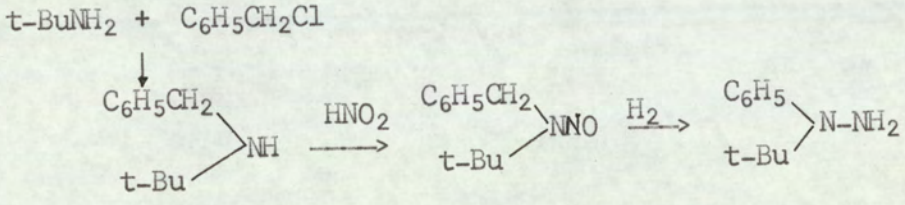
3. A rather unusual route to N-methyl arylhydrazines is that due to Huisgen and his co-workers.[167] Their method consists of the reaction of azodicarboxylic ester with an aromatic hydrocarbon to yield the arylhydrazine-1,2-dicarboxylic acid-diethyl ester. Treatment of the latter with lithium aluminium hydride (which preferentially reduces the ester group on the substituted nitrogen, as has been previously observed, page 193), followed by hydrolysis yields the N-methyl arylhydrazine. The overall reaction is represented below:-



(b) Experimentally Adopted Procedures

1-t-Butyl-1-benzyl hydrazine (Expt: page 226f)

The synthesis of this hydrazine was carried out by the following route:-



The secondary amine, N-t-butylbenzylamine, which was obtained in 90% yield by the direct alkylation of t-butylamine with benzyl chloride, was then nitrosated with nitrous acid to give the t-butylbenzylnitrosamine. The slow recrystallisation of the latter from petroleum ether gave a product, the melting point of which (45°-46°) corresponded closely to the literature value.[165] Rapid recrystallisation of the nitroso compound from petroleum ether yielded a higher melting point form (platelets, 55°-56°) which has not previously been reported. By the slow recrystallisation of this higher melting form, the lower melting form was again obtained. The infra spectra (in carbon tetrachloride) of both forms were found to be absolutely identical.

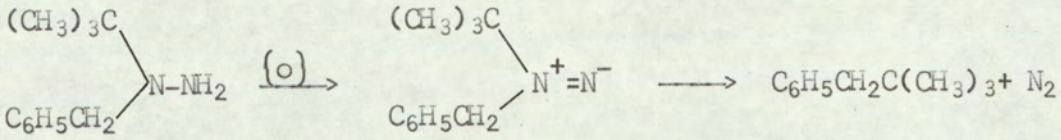
The reduction of the t-butylbenzylnitrosamine was first attempted using sodium and ethanol as the reducing agent (Expt: page 226). The components of the final reaction mixture were separated by fractional distillation under reduced pressure. Although 1-t-butyl-1-benzyl hydrazine was obtained, the yield was very poor. That a relatively high yield of the secondary amine, t-butylbenzylamine, was also obtained, indicated that considerable cleavage of the N-N bond had taken place, a fact which could account for the low yield of the hydrazine. The reduction of the nitrosamine was repeated using aluminium amalgam as the reducing agent, instead of sodium in ethanol. The relative yields of 1-t-butyl-1-benzylhydrazine and the corresponding secondary

amine were 6.7 and 27% respectively, thus indicating that the cleavage of the N-N bond was still the dominant feature of the reaction. The reduction using aluminium amalgam was repeated under identical conditions except that the fractional distillation was carried out under nitrogen. The yield of the hydrazine obtained was raised to 20% by this means.

The fractional distillation of the reaction products of both the sodium/ethanol and the aluminium amalgam reductions, gave a fraction which boiled at 54-55^{o} at 5.0 mm Hg, a value considerably lower than either the amine or the hydrazine. This material possessed the physical and chemical properties of a hydrocarbon and was, in fact, identified (page 227) as 1-phenyl-2,2-dimethylpropane ($C_6H_5CH_2C(CH_3)_3$). The isolation of this compound confirms the work of Carpino and his co-workers[165], who observed that the same compound is formed during the oxidation of 1-t-butyl-1-benzyl hydrazine with mercuric oxide. The formation of such hydrocarbons from 1,1-disubstituted hydrazines is thought to involve the intermediate formation of azamines, by the removal of two hydrogen atoms from the hydrazine[168].

$$R_2NNH_2 \xrightarrow{-H_2} R_2N^{+}=N^{-}$$

These charge separated diimides, as they are otherwise known, are easily fragmented, probably with the formation of free radicals, which can, in turn, dimerise to give the corresponding hydrocarbon[169]. The formation of 1-phenyl-2,2-dimethyl propane by this mechanism is illustrated below:-



That a considerable drop (14% to 4%) in the yield of this hydrocarbon occurred when the fractional distillation was carried out under an atmosphere of nitrogen, further emphasised that its mode of formation was oxidative in nature.

4. Identification of the Synthesised Hydrazines

The establishment and confirmation of the identity of the hydrazines synthesised was carried out using the normal spectral and analytical techniques. Although hydrazine derivatives have sometimes been reported to give inconsistent analytical results[170], little difficulty was experienced in obtaining satisfactory values for the particular hydrazines whose synthesis is reported in this thesis. The use of mass spectrometry proved to be a useful tool to confirm the identity of the hydrazines. Most compounds gave prominent parent peaks, whilst hydrazines in the form of their acid salts gave, as one might expect, the parent peak of the free base. The infra-red, ultra-violet and NMR spectra of the compounds were recorded where appropriate, and used for structural elucidation in the usual way.

EXPERIMENTAL

Part 2: The Synthesis of Mono and Disubstituted Hydrazines

Apparatus and Chemicals:- Melting points and boiling points are uncorrected. Physical constants quoted as "lit." without qualification are from the "Dictionary of Organic Cpds"[171] and the "Handbook of Chemistry".[172]

NMR and IR spectra were determined on Perkin Elmer R10 (60 Mc) and Infracord - 237 instruments, and UV spectra on the Perkin - Elmer 137 - UV. Mass spectra were carried out on the AEI MS9, and GLC on either the Beckmann GC - 2 or the Pye 'series 104' dual flame ionisation chromatograph. Hydrogenation was performed using the Gallenkamp General Purpose Hydrogenation Apparatus HR - 160.

IR assignments are based on the texts of Bellamy,[173] Flett,[174] and Nakanishi,[175] and for NMR, Jackman.[176] The IR spectra of all new compounds are deposited in the Chemistry Department collection.

Samples of 1 - Methyl - 2 - benzylhydrazine hydrochloride and N -isopropyl - α - (2-methylhydrazino) - p - toluamide hydrochloride (Natulan) were supplied by F. Hoffman - La Roche and Co. Ltd., Basel, Switzerland.

Microanalyses were by Drs. G. Weiler and F. B. Strauss, Oxford, Dr. A. Bernhardt, Mulheim, W. Germany, and Mrs. B. Taylor, Chemistry Department, University of Aston.

Hydrazine dihydrochloride, Hydroxylamine sulphate, Phenylhydrazine hydrochloride, and semi-carbazide hydrocloride

These compounds were purified by recrystallisation of the appropriate standard grade laboratory reagents. Hydrazine dihydrochloride (H_2O), m.p. 197^0 - 198^0 (lit., 198^0); Hydroxylamine sulphate (H_2O), m.p. 170^0 - 172^0d (lit., 172^0d); Phenylhydrazine hydrochloride (H_2O), m.p. 247^0 - 249^0 (lit., 249^0d); Semicarbazide hydrochloride (aqueous ethanol), mp. 172^0 - 174^0d. (lit., 173^0).

Benzalazine

Benzalazine was synthesised in 90% yield by treating hydrazine sulphate with benzaldehyde, according to the procedure given in "Organic Syntheses".[108] Recrystallisation of the product from aqueous ethanol gave yellow needles, m.p. 91^0 - 92^0 (lit., 91-92^0); IR, in CCl_4, maxima (cm^{-1}) at 1665 (C = N), 710, 690 (phenyl nucleus); NMR, in CCl_4, singlet at 1.22τ (benzal protons), and multiplets centred at 2.05τ and 2.50τ (phenyl protons), with respective intensities of two and ten; UV, in EtOH, maxima in mμ(log ε): 300 (4.55), 308 (4.52), lit.,[177] 300 (4.56) 308 (4.54). Found : C,80.54; H,5.76; N,13.25. Calcd. for $C_{14}H_{12}N_2$: C, 80.70; H, 5.81; N, 13.47%.

Methylhydrazine sulphate

Methylhydrazine sulphate was prepared from benzalazine and dimethyl sulphate according to the method described in "Organic Syntheses".[108]

A yield of 48% was obtained, of product m.p. 142^0 - 144^0, which upon a further recrystallisation from 80% aqueous ethanol, furnished an analytical sample of the same melting point. IR, KBr disc, maxima (cm^{-1}) at 3200 (NH), 2480 ("ammonium band"), 1600, 890 (NH_2) NMR, in TFA, singlets at 6.6τ and 6.7τ; in TFA/D_2O (1:1), one singlet at 6.7τ. (Found : C,8.33; H,5.54; N,19.51. Calcd. for $CH_8N_2O_4S$: C,8.32; H,5.59; N,19.45%).

1 - methyl - 2 benzoylhydrazine hydrochloride

The reaction between equimolar quantities of freshly distilled methyl benzoate and methylhydrazine under the experimental conditions detailed by Hinman and Fulton,[143] produced 1 - methyl - 2 - benzoylhydrazine hydrochloride in 20% yield. An increase in the reflux time from 2hr. to 24hr, as suggested by these authors, raised the yield of the hydrazine from 8% to the above figure. Two recrystallisations from absolute ethanol gave the product as white needles, m.p.209^0 - 211^0 (lit.,[143] 209^0 - 210^0); IR, KBr disc, maxima (cm - ') at 3345 w, 3160s (secondary amide, NH), 1685s (secondary amide, C = O), 710, 690 (phenyl nucleus); NMR, in TFA, multiplet centred at 2.30τ (five aryl protons), singlet at 6.70τ (three methyl protons). UV, in EtOH, maximum in mμ(log ε): 229 (4.10). (Found: C, 51.46; H, 5.94; N, 14.96; Cl, 19.21. Calcd. for $C_8H_{11}ClN_2O$: C, 51.50; H, 5.94; N, 15.01; Cl,19.00%).

1 - methyl - 2 - benzoylhydrazine

A solution of 1 - methyl - 2 - benzoylhydrazine hydrochloride (10g) in water (10ml.), was made basic with 35% sodium hydroxide (100ml.) and the resulting solution extracted with chloroform (4 x 30ml.). The chloroform extract was concentrated to approximately one-tenth of the original volume, and n - hexane was added to the boiling solution until it became turbid. Cooling this solution in an ice bath caused the separation of the 1 - methyl - 2 - benzoyl hydrazine as white needles (2.2g., 27%), m.p. 81-83^0 (lit.,[143] 83^0 - 85^0).

Attempted reduction of 1 - methyl - 2 - benzoylhydrazine

To a vigorously stirred slurry of lithium aluminium hydride (1g., 0.025 mole) in dry tetrahydrofuran (30 ml.) at 0^o, was added, during 30 min, a solution of 1 - methyl - 2 benzoylhydrazine (2g., 0.013 mole) in tetrahydrofuran (40ml.) and the resulting mixture heated to reflux for 30 min. The reaction was carried out under an atmosphere of nitrogen throughout. The reaction liquors were allowed to cool, and the excess lithium aluminium hydride was decomposed and dissolved by the cautious dropwise addition of water, followed by 30% sodium hydroxide (50ml.). The organic layer was separated off and dried (K_2CO_3). The passage of anhydrous hydrogen chloride into this solution precipitated a white solid (0.3g) having an infra red spectrum identical with that of 1 - methyl - 2 - benzoylhydrazine hydrochloride.

The acid mother liquors were reduced to a small volume, and after being saturated with solid sodium hydroxide, were extracted with

chloroform (4 x 20 ml.). The passage of dry hydrogen chloride into the dried ($MgSO_4$) chloroform extract, gave no precipitate. Evaporation of this chloroform solution, gave a yellow oil, which showed no evidence (IR and NMR) of aromatic character, and was, therefore not further investigated.

Attempted synthesis of 1 - methyl - 2 benzylhydrazine hydrochloride

Following the general procedure of Coleman and Gilman,[138] a solution of diazomethane[178] (0.06 mole) in dry ether was added dropwise to a stirred solution of benzylmagnesium chloride[179] (0.20) mole in ether. The reaction mixture was stirred for three hours under nitrogen at room temperature, and was then hydrolysed with 50% ammonium chloride (50 ml.). The resulting ethereal layer was filtered off and dried ($MgSO_4$). The passage of anhydrous hydrogen chloride caused the precipitation of a white solid (4.4g), mp. 112° - 116° d., which was found to turn yellow rapidly when in contact with the air, and was therefore collected and stored under nitrogen. IR, KBr disc, maxima (cm - ') at 3130, 2995, 2400 (very broad), 800, 755, 700 and 645; NMR, in D_2O, showed singlets at 2.30τ and 5.6τ but showed no signal corresponding to a CH_3 - N - group, and it was therefore concluded that the product was not the desired 1 - methyl - 2 - benzylhydrazine hydrochloride.

The reaction was repeated, using the same experimental procedure. A white solid (3.1g) again resulted, m.p. 110° d (approx), the NMR spectrum of which was also characterised by the absence of a signal corresponding to a CH_3 - N group. No further investigation into its identity was made.

Benzylhydrazine

Adopting the general experimental procedure as used by Biel and his co-workers,[109] a solution of benzyl chloride (63g., 0.5 mole) in ethanol (300 ml.) was added dropwise over a period of one hour to a refluxing solution of 98% hydrazine hydrate (144g., 2.9 mole) in ethanol (1000 ml.), after which the mixture was refluxed for a further six hours. The ethanol was then removed by distillation at atmospheric pressure and the residue extracted with ether. Fractionation of the dried (K_2CO_3) extract through a 30 cm. Vigreux column yielded 54g.(88%) of benzylhydrazine, b.p. 98^0/4mm.IR, neat liquid, maxima (cm - ') at 3300, 3170 (NH), 745, 710 (phenyl nucleus); NMR, in CCl_4, singlets at 2.75τ(five aryl protons), 5.9τ(two methylene protons), 6.2τ(three NH protons).

Benzylhydrazine Sulphate

A solution of benzylhydrazine (12g., 0.1 mole) in absolute ethanol (100 ml.) was cooled to 0^0, and acidified with concentrated sulphuric acid to pH 3. Evaporation of the resulting solution gave a white solid (19.8g.) which upon recrystallisation from absolute ethanol yielded benzylhydrazine sulphate as lustrous plates (18.3g), m.p. 160^0 - 162^0 (lit., 161^0 - 162^0). A further recrystallisation from the same solvent gave an analytical sample, m.p. 160^0 - 162^0; IR, KBr disc, maxima (cm - ') at 3280, 3160 (primary amine), 2600 ("ammonium band"), 750, 700 (phenyl nucleus); NMR, in a 1:1 mixture of TFA/D_2O, singlets at 2.35τ (aryl protons), and 5.4τ (methylene protons) with

respective intensities of five and two. (Found : C, 38.39; H, 5.48; N, 13.18. $C_7H_{12}N_2O_4S$ requires : C, 38.19; H, 5.48; N, 12.72%).

N - nitroso benzylhydrazine

To a 50% solution of sodium nitrite (40ml.) at room temperature, (7g., 0.06 mole) of benzylhydrazine sulphate was added in one portion, and the resulting mixture warmed on a boiling water bath for five minutes. The yellow oil which rapidly formed on the surface, solidified on cooling the mixture to 0^0 in an ice bath, and was filtered off. Recrystallisation of the crude material from water (200 ml.) yielded N - nitroso benzylhydrazine (3.4g., 71%) as white needles, m.p. 69.0^0-70.5^0.

A further recrystallisation from water gave an analytical sample, m.p. 69.5^0 - 70^0 (lit.,[180] 71^0); IR, KBr disc, maxima (cm - ') at 3305, 3200 (NH), 710, 690 (phenyl nucleus); NMR, in $CDCl_3$, singlets at 2.44τ(five aryl protons), 4.15τ(two NH protons) and 4.60τ(two methylene protons.) UV, in EtOH, maximum in mμ(log ε) 252 (3.85) (Found: C, 55.61; H, 6.04; N, 27.86. Calcd. for $C_7H_8N_3O$: C, 55.60; H, 6.00; N, 27.81%).

Attempts to repeat this synthesis using identical experimental conditions failed to produce the desired N - nitroso benzylhydrazine. The addition of the benzyl hydrazine sulphate to the sodium nitrite solution caused vigorous effervescence, and the formation of a yellow oil, which rapidly turned brown upon warming. The oil, which was heavier than water, had an odour distinctly reminiscent of benzaldehyde.

In an attempt to establish the correct conditions for the consistent formation of the nitroso hydrazine, the following variations in the experimental procedure were made :

(a) The benzylhydrazine sulphate (10% solution in distilled water), was added dropwise with stirring to a 50% solution of sodium nitrite at room temperature (21^0).

(b) The above conditions were repeated but using a 5% aqueous solution of the benzylhydrazine sulphate.

(c) An aqueous solution of benzylhydrazine sulphate (5%) was cooled to 0^0, and added dropwise with stirring to an ice-cold solution of sodium nitrite (50%).

(d) To a 5% aqueous solution of the hydrazine sulphate an excess of sodium nitrite was added in small portions.

All the above variations in reaction conditions resulted in vigorous effervescence, and the formation of the yellow oil. No N - nitroso benzylhydrazine was obtained. Further investigation into this reaction by Blair and Taylor[181] has demonstrated that the reaction is extremely dependent upon pH, and that the yellow oil contains both benzaldehyde and benzyl alcohol.

Attempted synthesis of 1 - N - nitrosobenzyl - 2 methyl hydrazine

Following the general procedure of Thiele,[141] N - Nitroso benzylhydrazine (1.1g., 0.07 mole) was dissolved in hot water (40ml), and 25 ml. of 8% sodium hydroxide added. To this stirred mixture at 30^0 - 35^0, methyl sulphate (5 ml.) was added over a period of fifteen

minutes, the stirring being continued for a further ten minutes after addition. The solution was then cooled to 0^0 for thirty minutes and the yellow oily layer which formed was extracted into ether (2 x 20 ml.)

The addition of petroleum ether (40^0 - 60^0) to the dried (K_2CO_3) extract failed to yield any of the desired 1 - N - nitrosobenzyl - 2 - methyl hydrazine.

The aqueous reaction liquors were made strongly alkaline by the addition of sodium hydroxide pellets, and again extracted with ether (2 x 20 ml.). The extract was washed with water (2 x 10 ml.) and dried as before. Addition of petroleum ether to the extract again failed to produce the expected hydrazine.

The reaction was repeated, but as before, no 1 - N - nitroso benzylhydrazine was obtained.

Attempted nitrosation of methylhydrazine

In spite of rigorous adherence to the nitrosation procedure as given by Thiele,[141] no 1 - N - nitroso - 1 - methyl hydrazine was obtained.

1, 2 - Dibenzoyl hydrazine

1, 2 - dibenzoyl hydrazine was prepared according to the directions given in "Organic syntheses".[142] The crude hydrazine, m.p. 229^0 - 239^0, was purified by recrystallisation from absolute ethanol to give (in 65% yield) the pure material, m.p. 244.5^0 - 245.5^0 (lit. 241^0); IR, KBr disc, maxima (cm^{-1}) at 3210s (NH), 1640s (secondary amide, C = O); U.V., in EtOH, maximum in $m\mu$(log ε) at 230 (4.04).

Gagnon[182] gives 232 (4.2); NMR, in TFA, multiplet centred at 2.3τ(aryl protons.) (Found : C, 69.60; H, 5.03; N, 11.70. Calcd for $C_{14}H_{12}N_2O_2$: C, 69.97; H, 5.03; N, 11.67%).

Hydrogenolysis of 1, 2 - dibenzoylhydrazine[183]

A stirred mixture of 1, 2 - dibenzoylhydrazine (2g, 0.8 mM) and Raney Nickel (6g.) in 95% ethanol (100 ml.), was heated to reflux for 3 hr, and then allowed to cool. After removal of the nickel catalyst by filtration, the filtrate was evaporated to dryness in vacuo. Extraction of the resulting solid residue with hot water (3 x 10 ml.) gave benzamide (1.1g., 54%) m.p. 122.5^0 - 123.5^0, undepressed by admixture with a known sample, leaving a water - insoluble material (0.7g, 35%) m.p. 244^0 - 245^0, which was identified by mixed melting point with a known sample, as unchanged 1, 2 - dibenzoylhydrazine.

1, 2 - dibenzoyl - 1 - methyl hydrazine

Benzoyl chloride (15.5g., 0.11 mole) was added with stirring during 10 min., to a solution of methylhydrazine, (5g., 0.11 mole) in 10% sodium hydroxide (100 ml.). After vigorous agitation for 15 min., the solution was cooled to 0^0, and the white solid present was filtered off. Recrystallisation from aqueous ethanol gave 1, 2 dibenzoyl - 1 - methyl hydrazine (8.30g., 30%) as white needles, m.p. 143.5^0 - 145^0 (lit., m.p. 141^0,[184] m.p. 144^0 - 145^0[167]); IR, KBr disc, maxima (cm-') at 3200s (secondary amide, NH), 1675s (secondary amide, C = O); U.V, in EtOH, maximum in mμ(log ε) 226 (4.16); NMR, in $CDCl_3$, multiplet centred at 2.5τ(10 aryl protons) and a singlet

at 6.6τ(3 methyl protons). (Found : C, 70.81; H, 5.61; N, 11.16. Calcd. for $C_{15}H_{14}N_2O_2$: C, 70.80; H, 5.52; N, 11.03%).

t - Butylhyrazine oxalate

A procedure similar to the one adopted by Gever and Hayes[130] for the synthesis of N - butylhydrazine oxalate, was employed.

Care was taken to rigorously exclude contact with metal ions during the preparation of the precursors of, and their conversion to, t - butylhydrazine oxalate, in order to minimise catalytic decomposition of the hydrazine, and any compounds subsequently derived from it.

Freshly distilled t - butylamine (1480g, 20.4 moles) was mixed with water (600ml.), and heated to reflux. The heating was turned off, and a solution of freshly prepared hydroxylamineisomonosulphonic acid[185] (200g., 1.7 moles) in water (600 ml.) was added, with stirring, during 1 hr. On completion of the addition the solution was cooled, the white inorganic precipitate filtered off and discarded, and the filtrate acidified with glacial acetic acid (1200 ml.), and then warmed to 50^0 with acid-free benzaldehyde (360g.) for 10 min. The resulting emulsion was cooled, and extracted with ether (4 x 200 ml.). The ether extracts were combined and added to oxalic acid dihydrate (280g.) in water (1000ml.) and the mixture steam distilled until no more benzaldehyde came over. (12 hr.) The residue was concentrated to dryness in vacuo and the resulting solid was recrystallised from aqueous ethanol (90%) to give t - butylhydrazine oxalate (108g., 33%) as robust white needles, m.p. 187^0d; IR, KBr disc, maxima (cm-') at

3450m, 3420m, 3320s, 3210m, 3190m (free and bonded NH), 2490s (ammonium band, NH^+ or NH_2^+); NMR in TFA, singlet at 8.20τ. (Found : C, 40.77; H, 7.75; N, 15.59. $C_6H_{14}N_2O_4$ requires: C, 40.45; H, 7.86; N, 15.73%).

t - Butylhydrazine (Method 1)

By adopting the procedure of Smith et.al.,[118] t - butyl hydrazine was obtained from t - butylhydrazine oxalate in 45% yield. The final purification of the free hydrazine was accomplished by fractional distillation at atmospheric pressure, using a Buchi*spinning band column. The colourless, pungent t - butylhydrazine so obtained, was shown by GLC (5 ft. column of silicon SE - 30 (20%) on Chromosorb at 40°) to be pure, and had b.p. 116°/756.5 mm (lit.[118] 109 /749 mm). Despite storage at 0° under nitrogen, it evolved gas slowly when in contact with impurities. IR, neat liquid, maxima (cm-') at 3340m, 3240m (NH_2), 1390 - 1370s (doublet, gem dimethyl), 1218s, 1233s, (skeletal, t - butyl); NMR, neat liquid, singlet at 7.78τ; Mass spectrum, predominant ions (m/e) at 88 (parent), 73 (parent minus one methyl) and 57 (t - butyl).

t - Butylhydrazine (Method 2)

To a boiling solution of freshly distilled t - butylamine (592g., 7.6 moles) in water (240 ml.), a solution of hydroxylamine isomonosulphonic acid (100g., 0.85 mole) in water (240 ml.) was added with stirring during 30 min. The solution was allowed to cool, the

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white inorganic material which precipitated, filtered off, and the resulting filtrate fractionated under nitrogen through a 25 cm. Vigreux column. The first fraction (360g.) was essentially pure t - butylamine, which was put to one side for re-use. The final fraction (35g.) was treated with successive potions of solid sodium hydroxide until a water layer no longer appeared, and then allowed to stand over barium oxide for 4 hr. As GLC analysis of the product indicated a very small yield (0.4g.) of t - butylhydrazine the final distillation was not attempted.

1, 2 - Dibenzoyl - 1 - t - butylhydrazine

Finely powdered t - butyl hydrazine oxalate (1g., 5.3 mM) was dissolved in 10% sodium hydroxide solution (20ml.) at room temperature, and the sodium oxalate which precipitated was filtered off and discarded. To the stirred filtrate, benzoyl chloride (1.6g., 10mM) was added, and the stirring continued until all the excess benzoyl chloride had been hydrolysed. The resulting white solid was filtered off and recrystallised from aqueous ethanol to give 1, 2 - dibenzoyl - 1 - t - butylhydrazine (2.5g., 77%) as colourless, fine needles, m.p. 171.5^0 - 174^0. Further recrystallisation from the same solvent furnished an analytical sample, m.p. 173^0 - 174^0 (lit.,[186] 174^0 - 175^0); IR, maxima (cm-') at 3305s (secondary amide, NH), 1695s (secondary amide, C = O), 1220, 1205 (skeletal, t - butyl) UV, in EtOH, maximum in $m\mu$(log ε) at 221 (4.12); NMR, in $CDCl_3$, multiplet centred at 2.60τ(aryl protons, 10), singlet at 8.42τ(t - butyl protons, 9); (Found: C, 73.10; H, 6.90; N, 9.70. Calcd. for $C_{18}H_{20}N_2O_2$: C, 72.92; H, 6.81; N, 9.45%).

Attempted hydrogenolysis of 1, 2 - dibenzoyl - t - butylhydrazine

A stirred mixture of the hydrazine (0.9g., 3mM), and Raney nickel (10g.) in 95% ethanol (80 ml.) was heated to reflux for 5 hr., and allowed to cool. Removal of the Raney nickel by filtration, followed by evaporation of the filtrate to dryness in vacuo, produced a white solid (0.65g.), m.p. 174^0 - 175^0 which was identified (IR spectrum and mixed m.p.) as the starting material.

1 - t - Butyl - 1 - benzyl - 2 - benzoyl - hydrazine

The reduction was carried out under an atmosphere of nitrogen. To a cooled (0°) slurry of lithium aluminium hydride (0.8g., 20mM) in anhydrous tetrahydrofuran (20ml.), a solution of 1, 2 - dibenzoyl - t - butyl hydrazine (2.4g., 8 mM) in dry tetrahydrofuran (10 ml.) was added with stirring during 30 min. The mixture was then refluxed for a further 30 min., cooled, and the excess lithium aluminium hydride was destroyed by the cautious addition of water, followed by 10% sodium hydroxide (50 ml.). The tetrahydrofuran layer was separated, washed with water (2 x 10 ml.), dried ($MgSO_4$) and evaporated. Recrystallisation of the resulting white solid from aqueous ethanol gave 1 - t - butyl - 1 - benzyl - 2 - benzoyl - hydrazine (1.85g., 81%) as delicate, colourless needles, m.p. 162^0 - 163^0, undepressed by admixture with a known sample, prepared by the benzoylation of 1 - t - butyl - 1 - benzylhydrazine (see page 228). The IR, NMR and mass spectra were identical to those of the known material.

1 - t - Butyl - 2 - benzoylhydrazine hydrochloride

To a stirred mixture of t - butylhydrazine (1.76g., 0.02 mole) benzene (20ml.) and 10% sodium hydroxide (20ml.), a solution of benzoyl chloride (2.80g., 0.02 mole) in benzene (20ml.) was added, dropwise, during 6 min. On completion of the addition, the mixture, which turned yellow after approximately 20 min., was stirred for a further 4 hr. The aqueous layer was rejected, and the organic phase washed with 10% sodium hydroxide (2 x 5 ml.), followed by water (10ml.), and finally, extracted with 10% hydrochloric acid (5 x 15 ml.). The organic layer was discarded, and the combined acid extracts were made strongly basic with 20% sodium hydroxide (40ml.), the white solid which precipitated, being extracted into ether (2 x 20ml.). The ether layer was washed with water (2 x 5 ml.) and dried ($MgSO_4$) overnight. The passage of dry hydrogen chloride into the dry solution caused the precipitation of a white solid, which was collected, and recrystallised from ethanol/ether to give 1 - t - butyl - 2 - benzoylhydrazine hydrochloride, (1.4g., 31%) as colourless needles, m.p. 228^{0}d. The m.p. was found to be unusually dependent upon the heating rate. IR, KBr disc, maxima (cm-') at 3345w, 3140s (secondary amide, NH), 1680s (secondary amide, C = O), 1380s (doublet, gem dimethyl), 1255s, 1200s (skeletal, t - butyl); NMR, in TFA, multiplet centred at 2.15τ(five aryl protons), singlet at 8.35τ(nine t - butyl protons); (Found : C, 58.15; H, 7.62; N, 12.22. $C_{11}H_{17}ClN_2O$ requires: C, 57.65; H, 7.48; N, 12.25%).

1 - t - Butyl - 2 - benzoylhydrazine

25% sodium hydroxide was slowly added, with stirring, to a cooled (0°) solution of 1 - t - butyl - 2 - benzoylhydrazine hydrochloride (4g.) in 1% hydrochloric acid (100ml.) until precipitation was complete (pH ≃ 10).

The precipitate was extracted into ether (2 x 50 ml.), and the combined extracts washed with water (2 x 5 ml.), dried ($CaSO_4$) and evaporated to dryness.

Recrystallisation of the residue, from aqueous ethanol yielded a colourless crystalline solid (2.5g.), m.p. 84^0 - 90^0. The mother liquors, from the foregoing recrystallisation, deposited, on standing, a further 100mg. of crystalline material, m.p. 93^0 - 93.5^0, whose IR spectrum was identical to that of the main product. The fact that the main reaction product, the melting point of which remained unchanged and ill - defined despite successive recrystallisations from aqueous ethanol, analysed correctly as the desired 1 - t - butyl - 2 - benzoyl hydrazine (lit.,[186] m.p. 93.5^0), was assumed to indicate that the product was a mixture of two crystalline forms; IR, maxima (cm^{-1}) at 3360w, 3320m, 3280, 3260 (secondary amide, NH), 1630 (secondary amide, C = O) 1255, 1210 (t - butyl skeletal). (Found: C, 69.18, H, 8.47; N, 14.55. Calcd. for $C_{11}H_{16}N_2O$: C, 68.72; H, 8.39; N, 14.55%).

Attempted reduction of 1 - t - butyl - 2 - benzoylhydrazine

Methylal (dimethoxymethane), was purified according to the method of Kratzl,[145] and was stored under nitrogen.

The reaction itself, and the work up procedures, were carried out under an atmosphere of nitrogen. Into a cooled (0°) flask, equipped with a magnetic stirrer and a nitrogen inlet, oxygen - scavenged methylal (15ml.) was introduced, followed by lithium aluminium hydride powder (0.8g., 20mM). A solution of 1 - t - butyl - 2 - benzoylhydrazine (0.8g., 4mM) in methylal (15ml.) was added dropwise during 30 min., to the vigorously stirred slurry thus formed, and the mixture heated to reflux for $1\frac{1}{4}$ hr. The solution was then re-cooled (0°), and, after cautious destruction of the excess lithium aluminium hydride by the addition of wet ether (10ml.), 2N sodium hydroxide (5ml.) and water (5ml.), the remaining inorganic salts were removed by filtration. The organic layer was separated off, evaporated to dryness under a stream of nitrogen, and the residue taken up in sodium - dried ether. Passage of anhydrous hydrogen chloride into the dried ($MgSO_4$) solution, yielded a pure sample (0.6g) of 1 - t - butyl - 2 - benzoylhydrazine hydrochloride, m.p. 220^{0} - 222^{0}d., as identified by its IR spectrum and mixed m.p. with the known material.

In a repeat experiment, in which the reflux time was extended to 24 hr., the unchanged starting material (as the hydrochloride) was again the only product recovered.

Arylation of t - butylhydrazine (a)

t - butyl hydrazine oxalate (2g., 11.2 mM) was dissolved in a stirred mixture of n - butanol (30 ml.) and ethanol (3 ml.), and a sufficient quantity of 60% sodium hydroxide (2 ml.) added to give a neutral solution of the free hydrazine. Benzyl chloride (1.26g., 10 mM) was then added during 10 min., and the resulting reaction mixture heated to reflux for 72 hr. The inorganic solids which precipitated upon allowing the solution to cool, were filtered off, and the acid filtrate (pH 3) evaporated to dryness at room temperature under a stream of nitrogen.

Addition of ether (20 ml.) to the yellow oil which resulted, caused the separation of the organic and aqueous phases. Extraction of the latter with further 10 ml. portions of ether which were subsequently combined, dried ($MgSO_4$) and then evaporated, yielded 55 mg. of an unidentified white crystalline solid, m.p. 77^0 - 79^0.

To the aqueous phase benzoyl chloride (0.5g) was added and the mixture agitated at room temperature for 1 hr. The white solid (0.2g.) which precipitated, recrystallised from aqueous ethanol as white needles, m.p. 168^0 - 171^0, and was identified as essentially pure 1, 2 - dibenzoyl - t - butylhydrazine by its IR spectrum.

Arylation of t - butylhydrazine (b)

Benzyl chloride (3.42g., 27 mM) in anhydrous ethanol (30 ml.) was added during 4 hr., to a stirred solution of t - butylhydrazine (2.56g., 28 mM) in dry ethanol (10 ml.), and the stirring continued

for 72 hr. at room temperature. Removal of the solvent from the acid (pH 3) reaction mixture left a yellow residue, which after repeated washing with dry ether, was recrystallised from chloroform/ carbon tetrachloride to give a white solid (2.3g) m.p. 170^0 - 176^0. The NMR spectrum of the material showed one peak only, at 8.2τ (corresponding to a t - butyl group), thus indicating that the desired alkylation had not taken place.

Arylation of t - butylhydrazine (c)

Freshly distilled benzyl chloride (1.95g., 15 mM) in absolute ethanol (5 ml.) was added to a vigorously stirred solution of t - butylhydrazine (1.33g, 15 mM) in ethanol (35 ml.) and the mixture refluxed for 8 hr. under an atmosphere of nitrogen. The reaction mixture was allowed to cool, the solvent removed by vacuum evaporation, and the resulting concentrate taken up in dry chloroform (10 ml.). Passage of dry hydrogen chloride into the dried ($MgSO_4$) solution precipitated a white solid, m.p. 188^0 - 189^0; NMR in D_2O, singlet at 8.3τ(t - butyl). Recrystallisation from aqueous ethanol furnished pure t - butylhydrazine hydrochloride (1.1g) m.p. 189^0 - 190^0; undepressed by admixture with the known material. No other products were obtained from the reaction.

The experiment was repeated under identical conditions using benzyl bromide as the alkylating agent, and n - propanol as the solvent. The only product obtained was a small amount (50 mg) of an unidentified white solid, m.p. $>240^0$; NMR, in D_2O, singlet at 8.6τ(t - butyl); IR, maxima (cm-') at 3320s, 3270w, 3190w (NH), 1380s (doublet, gem dimethyl), 1255w, 1200s, (t - butyl skeletal).

Arylation of t - butylhydrazine (d)

The reaction was carried out under an atmosphere of dry nitrogen.

To a stirred solution of t - butylhydrazine (2.8g., 0.03 mole) in absolute ethanol (10ml.), a solution of benzyl chloride (4g., 0.03 mole) in ethanol (30ml.) was added during 15 min., and the mixture gently refluxed for 16 hr. The solvent was then removed by the passage of a stream of nitrogen over the surface of the heated reaction liquors. Extraction of the colourless oil which resulted with chloroform (20ml.),left behind a white solid, which after two recrystallisations from aqueous ethanol gave a pure sample of 1, 2-dibenzyl hydrazine hydrochloride (0.7g), m.p. 220.5^0 - 222^0 (lit., m.p. 222^0 - 225^0) IR, KBr disc, maxima (cm^{-1}) at 3200s (NH), 755s, 700s (mono - substituted benzene nucleus); NMR, in TFA, singlets at 2.60τ(ten aryl protons) and 5.55τ(four methylene protons); mass spectrum predomenant ions (m/e) at 212 (parent minus hydrogen chloride), 106 ($C_6H_5CH_2NH^+$), 91 ($C_6H_5CH_2^+$), 77 ($C_6H_5^+$). (Found: C, 67.88; H, 6.94; N, 11.34. Calcd. for $C_{14}H_{17}ClN_2$: C, 67.60; H, 6.88; N, 11.28%).

The chloroform extract was dried ($MgSO_4$) for 48 hr, during which time the colour of the solution changed from a pale yellow to a deep red. Passage of dry hydrogen chloride into the cooled (0^o) extract precipitated 2.1g of a pink solid (A), m.p. 120^0 - 130^0, which upon recrystallisation from ethanol/ether gave a pink crystalline product (B) of indeterminate melting point, yield 1.8g.

Fractional recrystallisation of a portion of B (0.4g.) from chloroform yielded three distinct substances:- F, (50mg.) m.p.

122° - 128° (insoluble in chloroform), E, (170 mg) m.p. 188^0 - 189^0 (difficultly soluble) and G (100 mg) m.p. 96 - 98^0 (very soluble). Recrystallisation of the remainder of B (1.4g) from the same solvent gave a further material, K, (1.04g) m.p. 122^0 - 130^0, which was also very soluble in chloroform.

Examination of substances E, F, G, K:-

E: m.p. 188^0 - 189^0; NMR, in D_2O, singlet at 8.8τ; mixed m.p. with a known sample of t - butylhydrazine hydrochloride was undepressed. Substance E was therefore identified as t - butylhydrazine hydrochloride.

F: m.p. 122^0 - 128^0; NMR, in D_2O, singlets at 2.65τ(five aryl protons) and 5.8τ(two methylene protons). It is therefore, probable that substance F was a mixture of benzylhydrazine hydrochlorides.

G: m.p. 96^0 - 108^0; soluble in water and chloroform; IR, KBr disc, maxima (cm-') at 3320s, 3270s, 3190w, 3160s (NH), 2480 ("ammonium band"), 1380s (doublet, gem dimethyl), 1255, 1210 (t - butyl skeletal) 755, 700 (phenyl nucleus); NMR, in D_2O, singlets at 2.55τ, 5.77τ and 8.73τ with respective intensities of five, two and nine; (Found: C, 45.51; H,8.96; N,19.85.)

From the above data it seems probable that fraction G is a mixture comprised of almost exactly equal quantities of t - butyl and benzylhydrazine hydrochlorides. The elemental analysis for such a mixture would be :- C, 48.2; H, 9.0; N, 20.5%.

K: m.p. 122^0 - 130^0 (approx.); soluble in chloroform and water; NMR, in D_2O, singlets at 2.6τ(aryl), 5.6τ(methylene) and 8.6τ(t - butyl) with respective intensities of five, two and a half, and nine respectively, IR, KBr disc, maxima (cm-') at 3320s, 3210s, (NH), 2500 ("ammonium band"), 1380 (doublet, gem dimethyl), 1255w 1210s (t - butyl, skeletal), 750s, 732m, 700s (phenyl nucleus).

The melting point of the material obtained from successive recrystallisations (EtOH/ether) of K remained indefinite, but the NMR and IR spectra were materially altered. The singlets in the NMR spectrum at 2.6τ and 5.6τ were markedly reduced, as was the infra red absorption at 3210 cm-'. From the above information it appears likely that the unidentified reaction product was a mixture, once again, of t - butyl and benzyl hydrazines.

Arylation of t - butylhydrazine (e)

To a stirred solution of t - butylhydrazine oxalate (2g., 11.2mM) in isopropanol (35 ml.), a solution of 35% sodium hydroxide (3 ml.) was added. Freshly distilled benzyl bromide (2g.) was then added dropwise (10 min.) to the neutral solution, and the reactants refluxed for 48 hr. The inorganic material which precipitated on cooling was filtered off, and the filtrate evaporated on a water bath to give a yellow oil, which was, in turn basified with 4N sodium hydroxide (10 ml.) and extracted into ether (5 x 10 ml.) The passage of anhydrous

hydrogen chloride into the combined and dried ($MgSO_4$) extract precipitated a yellow solid (160 mg) m.p. 172^0 - 180^0, which recrystallised from aqueous ethanol to give a pure sample of t - butylhydrazine hydrochloride, m.p. 189 - 190^0(lit.,[118] 189^0) undepressed by admixture with the known material.

The mother liquors, resulting from the passage of the hydrogen chloride into the ether extracts, were evaporated to dryness, and the tacky yellow solid which resulted was washed with anhydrous ethanol. Recrystallisation from the same solvent gave a white crystalline material (86 mg) m.p. 195^0 - 200^0. The NMR and IR spectra both indicated the absence of a t - butyl group, however, and thus, no further attempt to elucidate its structure, was made.

The above experiment was repeated with the following modifications:

(a) Isopropanol was replaced by ethanol as the solvent.

(b) To remove any hydrogen bromide liberated during the reaction, calcium carbonate (1g) was added to the reaction mixture.

Again, none of the desired 1 - t - butyl - 2 - benzylhydrazine was isolated.

N - t - butylbenzylamine

N - t - butylbenzylamine b.p. 91^0/ 12 mm. was synthesised in 89% yield by adopting the procedure of Bortnick;[187] IR, neat liquid, maxima (cm-') at 3320w (secondary amine NH) 1370s (gem dimethyl); NMR, neat liquid, multiplet centred at 2.8τ(five aryl protons)

singlets at 6.46τ(two methylene protons), 9.0τ(t - butyl protons) and 9.6τ(one proton, NH).

N - t - Butylbenzylnitrosamine

The nitrosation of N - t - butylbenzylamine with sodium nitrite in glacial acetic acid according to the directions of Carpino,[165] gave a good yield (80%) of N - t - butylbenzylnitrosamine, which recrystallised from dry petroleum ether (b.p. 40^0 - 60^0) as very pale yellow needles, m.p. 45^0 - 46^0 (lit.,[165] 45^0 - 46^0) IR, in CCl_4, maxima (cm-') at 1465s, 1445s (N-NO), 690s (monosubstituted benzene nucleous); NMR, in $CDCl_3$, multiplet centred at 2.90τ(five aryl protons), singlets at 5.17τ(two methylene protons) and 8.50τ(t - butyl protons). (Found: C, 68.56; H, 8.27; N, 14.54. Calcd. for $C_{11}H_{16}N_2O$: C, 68.67; H, 8.39; N, 14.58%).

Rapid recrystallisation from petroleum ether (b.p. 60^0 - 80^0) yielded a higher melting form of the nitrosamine, m.p. 54^0 - 55^0 (platelets), the NMR and IR spectra of which were identical in every respect to that of the lower melting form. By slow recrystallisation of the higher melting form from petroleum ether (b.p. 60^0 - 80^0), pale yellow needles of the lower melting form were again obtained.

Reduction of N - t - butylbenzylnitrosamine (Na in EtOH)

Following the general procedure for the reduction of nitrosamines to hydrazines as given by Zimmer,[163] N - t - butylbenzylnitrosamine (40g., 0.21 mole) was dissolved in absolute ethanol, the solution cooled to 0^o, and freshly cut sodium (23g., 1 mole) added with

stirring during 8 hr. The addition of fresh ethanol (50 ml.) and further stirring for 30 hr. at room temperature was necessary to effect the complete solution of the added sodium. Phase separation of the reaction mixture was achieved by the addition of water (200 ml.) and petroleum ether, b.p. 40^0 - 60^0(200 ml.); the resulting aqueous layer was repeatedly extracted with fresh portions of petroleum ether (10 x 150 ml.). Removal of the solvent from the combined and dried ($MgSO_4$) extract, left a yellow oil which was vacuum distilled (5 mm.) through a short Vigreux column, leaving unreduced N - t - butylbenzylnitrosamine (14g., 35%) in the distillation flask. GLC (5 ft. column of silicon SE - 30 (20%) on Chromosorb at 150°) analysis of the distillate showed three well separated components with peak areas (a) 24, (b) 70, (c) 6, in order of emergence.

Fractional distillation through a 20 cm. helices-packed column, gave: (a) 54 - 56^0/5.0mm, 2.9g; (b) 80 - 82^0/5.0mm, 12.1g; (c) 92 - 94^0/5.0mm, 1.0g.

Fraction (a) exhibited the chemical and physical properties of a hydrocarbon, and was identified as 1 - phenyl - 2,2 - dimethylpropane (lit., b.p. 86 - 88/20mm[165] and 185.5 - 186/757.6mm.[188] IR, in CCl_4, maxima (cm^{-1}) at 3080, 3060, 3020 (aryl CH), 2950, 2900, 2800 (aliphatic CH), 2000 - 1700 (four peaks, monosubstituted benzene nucleus); NMR, neat liquid, singlets at 2.70τ(aryl protons), 7.45τ(methylene protons) and 9.1τ(t - butyl protons); mass spectrum, predominant peaks (m/e): 148 (parent). 91 ($C_6H_5CH_2^+$), 77 ($C_6H_5^+$), 71 (t - $C_4H_9CH_2^+$) and 57 (t - $C_4H_9^+$).

Fraction (b) was found to be essentially pure N - t - butylbenzylamine, by comparison with a known sample.

Fraction (c) was identified as the desired 1 - t - butyl - 1 - benzylhydrazine (lit., b.p. 96^0 - 99^0/3 mm[165] and 102^0 - 103^0/5mm.[189] IR, neat liquid, maxima (cm-') at 3340, 3210 (NH_2), 760 725, 700 (benzene nucleus); The free 1 - t - butyl - 1 - benzylhydrazine was stored under nitrogen.

Benzoyl derivative:- m.p. 163 - 164^0(aqueous ethanol); IR, KBr disc, maxima (cm-') at 3265s (secondary amide, NH), 1655s (secondary amide, C = O), 1370s (gem dimethyl), 1255w, 1210s (t - butyl, skeletal); NMR, in $CDCl_3$, multiplet at 2.3 - 2.8 with singlet showing through at 2.45τ, singlets at 3.3τ(NH), 5.88τ(methylene protons) and 8.67τ(t - butyl protons) with respective intensities of ten, one, two and nine; mass spectrum, predominant ions (m/e) at : 282 (parent), 281, 267 (parent minus methyl), 225 (parent minus t - butyl), 177 (parent minus benzoyl), 105 ($C_6H_5CO^+$), 91 ($C_6H_5CH_2^+$), 77 ($C_6H_5^+$) and 57 ($t\text{-}C_4H_9^+$). (Found: C, 76.05; H, 7.90; N, 10.10. $C_{18}H_{22}N_2O$ requires: C, 76.00; H, 7.81; N, 9.85%).

The overall yields of the four products isolated from the reaction, based on the nitrosamine, were (1) unreduced nitrosamine, 35% (2) 1 - phenyl - 2, 2 - dimethylpropane, 9.5% (3) N - t - butylbenzylamine 36% (4) 1 - t - butyl - 1 - benzyl hydrazine, 3%.

Reduction of N - t - butylbenzylnitrosamine (Aluminium amalgam)

Aluminium foil (0.005") was amalgamated by the method of Vogel.[190]

The reduction of N - t - butylbenzylnitrosamine (40g.) according to the procedure of Carpino[165] gave the following products (yields calculated on the nitrosamine):

(a) 1 - phenyl - 2, 2 - dimethylpropane, b.p. 56^0/5.0mm (yield 4.2g., 14%)

(b) N - t - butylbenzylamine, b.p. 80^0 - 82^0/5.0mm (yield 9.1g., 27%)

(c) 1 - t - butyl - 1 - benzylhydrazine, b.p. 93^0 - 94^0/5.0mm (yield 2.5g., 6.7%).

The reduction was repeated under identical conditions, except that all fractional distillations were carried out under nitrogen, and the following yields of the same products were obtained: (a) 4.5% (b) 42% (c) 20%.

1 - t - Butyl - 1 - benzylhydrazine hydrochloride

Passage of dry hydrogen chloride into a solution of 1 - t - butyl - 1 - benzylhydrazine (4.1g.) in dry ether (50ml.), precipitated the hydrochloride as a white solid, which recrystallised from benzene (sodium dried) as clubs, m.p. 199^0d (sealed tube, 196^0d); IR, KBr disc, maxima (cm^{-1}) at 3320s, 3230s, 3170s (NH_2), 2700 - 2200 (intense broad adsorption, "ammonium band"), 1370, (gem dimethyl); NMR, in TFA, singlets at 2.47τ (aryl), 5.52τ(methylene) and 8.33τ(t - butyl) with relative intensities of five, two and nine; Mass spectrum, predominant ions (m/e) at: 215, 213 (parent), 178 (parent minus (HCl), 163, 148, 91 ($C_6H_5CH_2^+$), 77 ($C_6H_5^+$), 57 (t - $C_4H_9^+$), 37, 35 (chlorine isotopes).

Found: C, 61.57; H, 8.94; N, 12.92. $C_{11}H_{19}ClN_2$ requires: C, 61.49; H, 8.92; N, 13.05%).

Benzaldehyde 1 - t - butyl - 1 - benzylhydrazone

Equimolar quantities of acid - free benzaldehyde and 1 - t - butyl - 1 - benzylhydrazine reacted spontaneously and exothermically at room temperature to give a viscous yellow liquid, which, after heating on a boiling water bath for 10 min., solidified on cooling. Recrystallisation from ethanol gave benzaldehyde 1 - t - butyl - 1 - benzylhydrazone as white clubs, m.p. 84^0 - 86^0 (lit.,[165] 84^0 - 86^0); IR, KBr disc, maxima (cm^{-1}) at 1605m (-C = N-), 1370 - 1360 (doublet, gem dimethyl), 760, 730, 700 (benzene nucleus); NMR, in $CDCl_3$, multiplet at 2.4 - 2.9τ(C_6H_5C=) with a singlet showing through at 2.74 (C_6H_5 - C<), singlets at 3.01 (benzal CH), 5.60 (methylene), 8.60 (t - butyl) with intensities of ten, one, two and nine respectively; Mass spectrum, predominant ions (m/e) at : 266 (parent), 251 (parent minus methyl), 91 ($C_6H_5CH_2^+$), 90 ($C_6H_5CH=^+$), 77 ($C_6H_5^+$), 57 (t - $C_4H_9^+$); (Found: C, 81.18; H, 8.38; N, 10.44. Calcd. for $C_{18}H_{22}N_2$: C, 81.16; H, 8.33; N, 10.52%).

Benzaldehyde t - butylhydrazone

t - Butylamine (370g., 5.1 mole) was mixed with water (150ml.) and heated to reflux. With stirring, a solution of hydroxylamine-isomonosulphonic acid (50g., 0.42 mole) in water (150 ml.) was added dropwise during 30 min. After completion of the addition the solution was cooled, acidified with glacial acetic acid (300 ml.) and warmed to 50^o with acid-free benzaldehyde for 10 min. The resulting emulsion was cooled and the yellow hydrazone layer extracted

into ether (2 x 250 ml.). Removal of the solvent from the dried ($MgSO_4$) extract, gave a yellow liquid (55g) which was distilled, under nitrogen, through a 20cm. helices packed column to give the following fractions:-

(a) 34^0 - 36^0/2.0mm, 31.2g. (b) 86^0 - 88^0/2.0mm, 22.3g.

Fraction (a) was benzaldehyde, as identified by comparison with a known sample.

Fraction (b) was found to be the desired benzaldehyde t - butyl hydrazone (yield 29%, based on the hydroxylamineisomonosulphonic acid); IR, neat liquid, maxima (cm-') at 3240, broad (NH), 1365, 1360 (doublet, gem dimethyl), 760s, 720s, 700s (benzene nucleus); NMR, neat liquid, multiplet at 2.3 - 2.9τ(aryl and benzal protons), singlets at 4.02τ(NH) and 8.82τ(t - butyl). UV, in EtOH, maxima in mμ(log ε) 224 (3.76) 290 (4.05); lit.,[146] for benzaldehyde n - butylhydrazone 222(3.85) and 289(4.05). Mass spectrum, predominant ions (m/e) at 176 (parent), 161, 105, 77 ($C_6H_5^+$), 57 (t - $C_4H_9^+$).

Benzaldehyde t - butyl hydrazone hydrochloride

The passage of dry hydrogen chloride into a rigorously dried ($MgSO_4$, followed by $CaSO_4$) solution of benzaldehyde t - butyl hydrazone in ether, precipitated an amorphous white solid, m.p. 185^0 - 187^0, which crystallised from dry benzene to give benzaldehyde t - butylhydrazone hydrochloride, m.p. 189^0 - 190^0; IR, KBr disc, maxima (cm-') at 3140, weak and broad (NH), 2670, 2580 ("ammonium band"), 2430, 2010, 1685 (C = $\overset{+}{N}$-H), 1380, 1370 (doublet, gem dimethyl),

770, 700 (benzene nucleus); NMR, in TFA, singlets at 1.18τ(CH, benzal) 8.38τ(t - butyl) and a multiplet at 2.0 - 2.6τ(aryl) with respective intensities of one, nine and five. (Found: C, 63.56; H, 8.22; N, 13.21. $C_{11}H_{17}ClN_2$ requires C, 62.50; H, 8.04; N, 13.25%).

On gentle warming, a dilute aqueous solution of the hydrazone hydrochloride, a strong odour of benzaldehyde was immediately noticeable.

Attempted reduction of benzaldehyde t - butyl hydrazone ($LiAlH_4$)

Pure, dry tetrahydrofuran was prepared as follows: Ordinary grade THF was fractionated through a 30 cm. packed column, and the fraction boiling at 66° refluxed with freshly cut sodium for two 3 hr. periods. Treatment of the product with, and distillation under nitrogen from, lithium aluminium hydride gave tetrahydrofuran of the required purity.

The reaction was carried out under an atmosphere of nitrogen throughout. To 20ml. of tetrahydrofuran cooled to 0°, and scavenged of oxygen by the passage of nitrogen for 10 min., lithium aluminium hydride (0.9g., 24mM) was added with stirring. On completion of the dropwise addition (30 min.) of a solution of benzaldehyde t - butyl hydrazone (1.0g., 5.8mM) in THF (10ml.), the cooled (0°) reaction mixture was stirred for a further 30 min., and then refluxed for the same period. The solution was allowed to cool, and the excess lithium aluminium hydride destroyed and dissolved by the successive addition of wet ether (20ml.), 2N NaOH (5ml.), and water (4ml.). The

resulting organic layer was evaporated to dryness with a stream of nitrogen, and the residue taken up in dry ether (20ml.). The passage of anhydrous hydrogen chloride into the dried (K_2CO_3) ether solution caused it to turn yellow and to precipitate a white solid, (0.59g) m.p. 179^0 - 182^0, which recrystallised from benzene to give a product, m.p. 185 - 187^0 identified by its IR spectrum as essentially pure benzaldehyde t - butyl hydrazone hydrochloride. The NMR spectrum of the reduction product shows an additional singlet at 7.6τ, which is not present in the corresponding spectrum of the known material.

The reaction was repeated using a reflux time of 24 hr. and a product (0.27 g) m.p. 179^0 - 182^0(unrecrystallised) was again obtained, the IR spectrum of which was identical to that of the material obtained in the first attempt. NMR, in TFA, singlets at 1.18, 7.8 and 9.2τ, and a multiplet centred at 2.3τ.

Attempted reduction of benzaldehyde t - butylhydrazone (Pd/C)

The hydrogenation at atmospheric pressure of benzaldehyde t - butylhydrazine over 20% palladium - on charcoal catalyst, in ethanol, resulted in the uptake of 1.04 molar equivalents of hydrogen. The catalyst was filtered off, the resulting filtrate evaporated to dryness, with a stream of nitrogen, and the residue taken up in sodium-dried ether. The passage of anhydrous hydrogen chloride into the dried ($CaSO_4$) solution yielded a white solid (0.35g), m.p. indefinite. IR, KBr disc, maxima (cm-') at 3210 (NH), 2660, 2580 ("ammonium band"), 2430 (-C=N-H), 1380 (doublet, gem dimethyl), 1255, 1210 (skeletal,

t - butyl), 765, 695 (benzene nucleus); NMR, in TFA, singlets at 1.18τ (benzal CH), 5.65τ(methylene), 8.35, 8.50τ(t - butyl) and a multiplet centred at 2.3τ(aryl). Comparison of the above IR and NMR spectra with those of known benzaldehyde t - butylhydrazone hydrochloride indicate that this compound is the major component of the reaction product; the minor components were not identified.

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