Sex steroids and intestinal amino acid transport in rainbow trout, *Salmo gairdneri* Richardson

by

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The effects of some anabolic and naturally-occuring sex steroids on intestinal transport of leucine have been studied in rainbow trout (Salmo gairdneri), in vivo (gut perfusion), and in vitro (everted gut sacs or intestinal strips).

Administration of 17a-methyltestosterone (MT) by injection for a prolonged period of time, enhanced intestinal transport and accumulation of leucine. 11-ketotestosterone (KT) or MT treatment in vitro, by direct addition to incubation media, elicited significant short-term increases in active transport of leucine, without effecting intestinal accumulation. Luminal administration of MT in vivo similarly elicited short-term responses, without effecting leucine accumulation in the intestine or other peripheral tissues. However, neither MT nor KT significantly affected intestinal transport of water in trout. Although long term injection of oestradiol (E2) enhanced intestinal transport and accumulation of leucine, E2 treatment in vitro was without effect. Addition of ouabain or 2,4,dinitrophenol in the presence of MT abolished steroid-stimulated leucine transport, in vitro. No significant differences were observed between immature male or female trout with respect to either transport of leucine and water, or intestinal granular cell density. However, 'apparent' Na+ absorption and percentage fold height were higher in females, while total intestinal thickness and enterocyte heights were greater in males. These sex differences were essentially abolished after gonadectomy.

It is suggested that the short-term effects of the androgenic steroids might be partly mediated through increased activity of Na+,K+,ATPase, and that steroid-induced growth promotion in fish may,to some extent, be a consequence of enhanced efficiency of intestinal function.

Key words: Rainbow trout, sex steroids, intestinal transport, amino acids.

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Abbreviations:

Control+Tween	Control incubation medium contains Tween-80
Control-Tween	Control incubation medium
d	day
e.d.	external diameter
E2	17B-Oestradiol
E2+	E2 added to incubation medium
Gly	Glycine
h	hour
Ileu	L-iso-leucine
I.P.	Intraperitoneal
I.M.	Intramuscular
KT	11-Ketotestosterone
Leu	L-Leucine
Lys	L-Lysine
l	Litre
M	Moles
MT	17a-methyltestosterone
MT+	MT added to incubation medium
Met	L-Methionine
min	minutes
S/M	Serosal/Mucosal ratio
SLI	Serosal leucine increase
TW	Tween-80
Val	L-Valine

Val	L-Valine
u	μ

1. General introduction

Section 1

It is evident from the (mammalian) literature that gonadal hormones elicit a wide range of extragenital responses in the body (Kochakian, 1976), and that minor chemical variations of steroid molecules can alter their physiological properties, thus reducing virilizing effects (Vida, 1969; Camerino and Sciak, 1975). The history of the development and use of steroids in animal production has been reviewed by Kruskemper (1968) and Umberger (1975). Similarly, considerable research has also been undertaken on the potential use of sex steroids as growth-promoting agents in fish culture, and it has been established that gonadal steroids, especially the androgenic steroids, promote weight gain in a number of fish species (Donaldson, et al., 1979; Higgs, et al., 1982; Lam, 1982). Since the discovery of the anabolic properties of sex steroids (Kochakian and Murlin, 1935), a number of studies have demonstrated a correlation between protein-anabolism and nitrogen retention (Kochakian, 1950; Albanese, 1965; Stafford, et al., 1954), and it has repeatedly been shown that a more positive nitrogen balance can be achieved by improved appetite (food intake) and food uptake, or more efficient food utilization (Kapoor, et al., 1975; Cowey and Sargent, 1979). In fish, there is evidence that administration of androgenic steroids enhance food utilization, as is apparent by the improved efficiency in food conversion, and protein efficiency ratios (Simpson, 1976; Matty and Cheema, 1978; Fagerlund, et al., 1978, 1979; Lone and Matty, 1980a; Ince, et al., 1982; Higgs, et al., 1982), and that the growth-promoting actions of androgenic steroids are accompanied by increases in gut proteolytic activity (Lone and Matty, 1981) and hypertrophy of intestinal

granular cells (Yamazaki, 1976).

Recent work carried out on the nutritional requirements of fish in relation to the action of anabolic steroids has shown that the steroid-induced growth response is lower in fish maintained on restricted food rations and, since whole body fat is reduced in steroid-treated fish, it was suggested that utilization of fat as an energy source might be a possible way of increasing availability of amino acids for growth (Fagerlund, et al., 1978; Higgs, et al., 1982). It appears therefore that amino acid requirements for steroid-stimulated protein synthesis (Matty and Cheema, 1978; Lone and Matty, 1980a) might be limiting if insufficient dietary protein is assimilated and absorbed. Thus it follows that increased efficiency of intestinal nutrient absorption might be an important contributing factor in the steroid-induced growth response. However, the question concerning the influence of sex steroids on gastrointestinal function has received little attention. In fish, with the exception of a report by Mugiya and Ichii (1980) on the lack of effect of oestradiol on intestinal transport of calcium, in trout, no other studies hitherto have been performed in this respect. Similarly, in higher vertebrates, only a limited number of reports are available. In this context, morphological changes (Giordano, et al., 1962; Ksiazkiewicz-Szapiro, 1970; Tuohimaa and Niemi, 1968; Wright and Morley, 1971; Wright et al., 1972), and effects on the absorption of water and solutes (Larralde, et al., 1968; Cappelli and Tacconi, 1954; Cappelli and Rossi, 1954; Herreros, et al., 1970; Althausen, 1949; Dunnet and Garnier, 1953; Matty, 1964; Fisher, 1955; Ahmed-Sorour, 1978) have been reported in the alimentary tract

of either castrated or intact rats and mice, and they predominently indicate that sex steroids might exert a stimulatory action on intestinal structure and function.

In view of the reported influence of sex steroids on the physiology of the gastrointestinal system in mammals, and the growth promoting effects in fish, the present studies were carried out to investigate the effects of sex steroids on intestinal transport of amino acids in rainbow trout (Salmo gairdneri).

2. Literature survey

Section 2

2.1. Intestinal structure and amino acid absorption in fish.

Detailed accounts of the functional morphology and adaptational variations in the alimentary canal of fish in relation to phylogeny and feeding habits are given in the reviews by Kapoor, et al (1975), and Fängeand Grove (1979). Morphological variations concerning the relative length of the gut in both gastric and stomachless fish have been considered by Karpevitch (1936), Barrington (1942), Szarski (1956), Pasha (1964), Al-Hussaini (1947), de Groot (1971) and Bryan (1975).

2.1.1. Intestinal structure in teleosts

The absorptive area of the intestinal mucosa in most carnivorous fish, including salmonids, is increased by folds. Intestinal villi and crypt cells, which are commonly found in the intestine of higher vertebrates, are generally absent in fish (Kapoor, et al. 1975). The anterior part of intestine in many teleosts contains auxiliary appendages which are known as pyloric caeca, but they are absent in stomachless, and some gastric species (Rahimullah, 1943, 1945; Fänge and Grove, 1979). The number of pyloric caeca varies in different species (Suyehiro, 1942), and are anatomically similar to other parts of the intestine, but may lack enzyme-secreting cells (Jansson and Olsson, 1960). Various functions have been suggested for the pyloric caeca, including absorption of carbohydrates and fat, resorption of water and ions, or as a reservoir for digestive compartments (Jansson and Olssan, 1960; Kapoor, et al., 1975; Fänge and Grove, 1979), but at present the exact function is uncertain.

The lumen of the intestine is lined with columnar epithelium

containing a brush border or micro villi (apical membrane), although exceptions occur in some species, including the presence of ciliated cells (Al-Hussaini, 1949; Bucke, 1971; Magid, 1975) and the occurrence of mixed types of epithelia (Kapoor, 1957, 1958). It was observed in some species e.g. Salmo irideus, that epithelial cells are separated at the luminal region by tight junctions, and the lateral membranes are connected by fine filaments (Yamamoto, 1966). It was shown by the latter author that in Carassius auratus and S. irideus the epithelial cells contain extensive ribbon-like lamellar structure, situated in the basal cytoplasm, and suggested that they might be involved in the transport of water and nutrients. Goblet cells and pear-shaped cells (see Kapoor, et al., 1975) are commonly found between epithelial cells, and the former occurs in greater number in the posterior region of the intestine (Al-Hussaini, 1949). The epithelial layer in some species e.g. S. irideus, is followed by a filamentous basal membrane which is in contact with connective tissue containing vascular and nervous elements (Yamamoto, 1968). A muscularis mucosa is absent in the intestine of teleosts (Fänge and Grove, 1979) and the submucosa is basally bounded by a stratum compactum which is frequently found in carnivorous fish intestines. In salmonids, it is composed of densely packed collagenous material (Burnstock, 1959), and presumably plays a supportive role, limiting the distension of the gut wall (Kapoor, et al. 1975). Normally associated with the stratum compactum is a layer of granule cells, the stratum granulosum (Al-Hussaini, 1949; Weinreb and Bilstad, 1955; Bullock, 1963; Smith, 1975; Kimura and Kudo, 1975; Ezeasor and Stokoe, 1980; Bergeron and Woodward, 1982). The granule cells are

not found in S. gairdneri until 28 weeks after hatching and after 34 weeks they arrange into a sheet structure associated basally with the stratum compactum (Bergeron and Woodward, 1982). It was suggested by the latter authors that the development of the stratum granulosum might be related to the resistance of the stratum compactum to cellular penetration. Scattered granule cells were localized both in the mucosal region (granulosum internum) and basally to the stratum compactum (granulosum externum) (Ezeasor and Stokoe, 1980). These authors have shown close association of granule cells with certain connective tissue cells described as 'ensheathing' cells, and it was suggested that they might constitute a part of the body defence mechanism (Ezeasor and Stokoe, 1980). However, the function of granule cells remains uncertain and further work is required to elucidate the precise role of these cells in the physiology of the gastrointestinal system. Similar to higher vertebrates, the intestine of teleosts contains a muscularis layer which is composed of circular and longitudinal smooth muscle but striped muscle occurs in the terminal rectal region. However, in some species striped muscle might be present in other parts of the intestine (Rauther, 1940; Ohnesorge and Rauch, 1968; Kilarski and Bigai, 1971).

2.1.2. Intestinal transport

Experiments for the quantitative determination of gastrointestinal function in mammals date back to the end of the

sixteenth century (Sanctorious Sanctorious, 1670), and the details of the methods used for investigation of intestinal absorption since that date have been extensively reviewed by Parsons (1968). The following account is a summary of the current views on the absorption of amino acids in relation to the intestinal transport of electrolytes. Where possible, fish were used as a model to describe intestinal transport but it was frequently neccessary to consider the work carried out on higher vertebrates.

2.1.2.1. Amino acid transport

L-isomers of the naturally-occurring amino acids are transported actively against a concentration gradient (Wiseman, 1968). Most aspects of amino acid transport in higher vertebrates have been reviewed by Wiseman (1974), Schultz and Frizzell (1975), and Munck (1977, 1981). However, there are comparatively few data available on intestinal transport of amino acids in fish, but they nevertheless largely indicate similar mechanisms to those of higher vertebrates (Wilson, 1957; Read et al., 1960; Read, 1967; Musacchia et al., 1961; Huang et al., 1965; Huang and Rout, 1967; Rout, et al., 1965; Mepham and Smith, 1966a & b; Neff and Musacchia, 1967; Smith, 1969; Smith, 1970; Kemp and Smith, 1970; Smith and Kemp, 1971; Smith and Ellory, 1971; Kitchin and Morris, 1971; Ward, 1968; Ingham and Arme, 1977; Boge, et al., 1972, 1977b, 1978, 1979, 1982; Hokazono, et al., 1979; Smith and Sepulveda, 1980). It is now clear that amino acids are transported through a carrier mediated transport system across the brush border membrane and then released into the blood after their exit through the basolateral membrane (Schultz and Curran,

1970; Schultz and Frizzel, 1975; Munck, 1981). In mammals, it has been shown that amino acids enter the brush border membrane via three major transport systems; passive diffusion, Na-dependent carriers, and Na-independent carriers, and it is known that both Na-dependent and Na-independent transport occurs through multiple-carrier mediated systems (Smith and Sepulveda, 1979; Stevens, et al., 1982; Munck, 1981; Paterson, et al., 1979). The aspects of Na-independent carrier system have not satisfactorily been shown in fish, but Na-dependent amino acid transport has been demonstrated in a variety of species (Smith, 1969; Smith, 1970; Ingham and Arme, 1977; Boge, et al., 1977b). Furthermore, there is increasing evidence that amino acid transport in fish may also be mediated through a multiple carrier system (Smith, 1970; Ingham amd Arme, 1977; Smith and Sepulveda, 1980) and the kinetics of amino acid transport in fish seems to conform with the basic kinetics of amino acid transport described in mammals by Curran et al. (1967), Schultz et al. (1967) and Chez et al. (1967) (see Schultz and Curran, 1970; Munck, 1981). Recently, Boge, et al (1982) using intestinal brush border membrane vesicles demonstrated Na-dependent transport of glucose and 2-amino isobutyric acid in Dicentrachus labrax, Mugil cephalus, Anguilla anguilla and Boops salpa. They have demonstrated that the Na-dependent transport was only apparent when there is a Na+ gradient (out > in) across the brush border membrane. The mechanisms of amino acid transport across the basolateral membrane has not been investigated in fish but in higher vertebrates Mircheff et al (1980), using vesicles of rat small intestine basolateral membrane, have suggested that neutral amino

acids are transported mainly by a Na-independent system similar to those of the L-carrier in Ehrlich ascites tumor cells (Oxender and Christensen, 1963; Crane, 1977; Wilson, 1978). However, in addition to Na-independent transport, there is evidence for passive diffusion and low rates of Na-dependent amino acid movement across the basolateral membrane (Schultz and Frizzel, 1975; Munck, 1981). Aspects of mutual inhibition and temperature adaption have been demonstrated in C. auratus and S. gairdneri (Smith, 1970; Ingham and Arme, 1977) but the extensive interactions between amino acids recognized in higher vertebrates (Munck, 1977) have not been studied in fish.

2.1.2.2. Energy sources of active transport

A principal role of the gastrointestinal tract is to convert energy foods into fuels that are transported in the blood. The process of active transport which takes place in the intestine requires metabolic energy to drive the absorption of nutrients against an 'uphill' electrochemical gradient and in this context it has been shown that in different preparations of intestine in vitro, the cellular ATP content is substantially reduced after a short incubation period (Leese and Bronk, 1972; Bronk and Leese, 1973; Faelli, et al., 1976). In fish, indirect evidence supports the involvement of ATP in providing metabolic energy for active transport processes. Inhibition of oxidative phosphorylation by 2,4,dinitrophenol (DNP) allows respiration to proceed in the absence of ATP production (Wilson, 1980). Addition of DNP to in vitro preparationsof intestine in fish reduces active transport of

amino acids (Ingham and Arme, 1977; Boge, et al., 1979). However, there is no evidence that ATP might be directly involved in the transport mechanism at the apical membrane, and it seems more likely that ATP hydrolysis may 'charge up' a store of electrochemical potential energy which affects the rate of transport through the membrane (Parsons, 1975), eg. Na+ gradient. Burrill et al (1976) have found in chicken enterocytes that DNP substantially inhibited the influx of amino acids (leucine, proline, glycine and alanine) both in the presence and in the absence of Na+, although in the latter condition the degree of inhibition was lower, and the reduced absorption was mainly observed for leucine and proline. Thus, they suggested the involvement of other cellular mediators in addition to Na+ (see Parsons, 1975). In trout (S. gairdneri), DNP is without an effect on glycine transport when the intestine is incubated in Na-free media (Boge, et al., 1979). The concept of Na-dependence of solute transport was developed by Crane (1965) and was subsequently proposed as the Na-gradient hypothesis (Christensen, 1970; Schultz and Curran, 1970; Heinz, 1972) and has been critically reviewed by Alvarado (1976), Crane (1977) and Eddy (1977). This hypothesis proposes that at the expense of ATP hydrolysis, the Na "pump" maintains a low cytoplasmic concentration of Na+ which results in an electrochemical gradient across the apical membrane (cytoplasm is electrically negative with respect to the mucosal solution), which drives amino acid-, or sugar-Na co-transport until the electrochemical gradient is abolished. More recent studies, using isolated brush border and basolateral membranes in higher vertebrates, have mainly confirmed the Na-gradient hypothesis, e.g.

Murer and Hopfer (1977) using rat intestinal vesicles have shown that active transport of non-electrolytes occurs in the presence of an electrochemical Na+ gradient across the brush border membrane. Aspects of the Na-gradient hypothesis have not been investigated in fish but indirect evidence from impairment of amino acid transport by ouabain (Boge, et al., 1979) and recent investigations of Boge et al (1982) using brush border membranes of some marine teleosts, suggest the existence of a similar system in fish.

2.1.2.3. Electrolyte transport

In higher vertebrates the intestine and kidneys are the main sites for homeostatic regulation of electrolyte balance but in fish, in addition to these, salt exchange occurs through the epithelial lining of the gills (Maetz, 1971; Maetz and Skadhauge, 1968; Mayer-Gostan and Maetz, 1980). Furthermore, there is evidence for adaptational variations in the mechanism of ionic transport between euryhaline and stenohaline species (Lahlou, 1976). In euryhaline teleosts, intestinal absorption of salt and water increases with an increase in salinity (Hirano and Utida, 1968; Bensahla-Talet, et al., 1974; Maetz and Skadhauge, 1968; Lahlou, et al., 1974), while in stenohaline fish (C. auratus), an increase in salinity results in a decrease in water and Na absorption (Ellory et al., 1972). Unlike higher vertebrates, in vitro preparations of the euryhaline intestine exhibits a serosa-negative potential difference (Hirano and Utida, 1972; Lahlou, et al., 1974; Huang and Chen, 1971; Ando, et al., 1975). It has been shown in winter flounder,

Pseudopleuronectes americanus, that under short-circuit conditions the intestine absorbs almost three times as much Cl- as Na+, and that Cl- transport is dependent upon Na+ in the bathing medium, and is inhibited by ouabain (which suggested that the driving force for Cl- absorption is the Na+ gradient) and that direct coupling between Na+ and Cl- fluxes occurs in the brush border membrane (Field, et al., 1978). Mackay and Lahlou (1980) have also studied the relationship between Na+ and Cl- fluxes in the intestine of the European flounder, Platichthys flesus, and observed that although replacement of Na+ with choline reduced the net Cl- transport by 30%, removal of Cl- totally abolished the net Na+ flux. They suggested a coupled transport system involving Na+ and Cl-, and estimated that the Cl-,Na+ co-transport is responsible for approximately 30% of the net Cl- and total transport of Na+. The suggestion by Mackay and Lahlou (1980) and Field, et al (1978) that Na+ and Cl- might share a common transport system, is consistent with the observation by Frizzell, et al (1979) who demonstrated a coupling between Na+ and Cl- influx across the brush border membrane in flounder intestine. A further study of this subject by Musch, et al (1982) in winter flounder, has shown that luminal presence of K+ stimulates NaCl absorption, and that K+ uptake across the apical membrane depends on the presence of both Na+ and Cl-, and is inhibited by furosemide (inhibitor of coupled Na+ and Cl- uptake). It was subsequently suggested by these authors that flounder intestine contains a Na+,K+,Cl- co-transport system, and that the Na+ gradient maintained by Na+,K+,ATPase activity in basolateral membrane can provide the energy for the transport of Cl-. However,

there is no evidence that other euryhaline species have similar mechanisms for the transport of electrolytes. There is evidence that C. auratus does not contain such a pump (Lahlou, 1976) but salmonids, which are migratory fish and can tolerate sea water, are likely to have mechanisms for control of electrolyte and water absorption to survive in high-salinity conditions. However, the intestinal transport of electrolytes and water has not been extensively studied in migratory salmonids, but there is evidence that, in S. irideus, electrolyte uptake (Na^+, Cl^-, and Ca^{++}) is linear over 3 minutes and the rate is proportional to the external concentration of electrolytes (Lahlou, 1976; Grenesse, 1974). It was suggested by the former author that the high rate of ionic penetration into the enterocytes might, to some extent, be the result of mechanisms other than simple diffusion.

2.2. Growth-promoting effects of sex steroids

The objective of the following section is to underline those aspects most relevent to the present study, since the literature contains an overwhelming body of articles which have been devoted to the actions of sex steroids in fish. Details of gonadal structure in relation to the synthesis of sex steroids have been reviewed by Dodd (1955, 1972, 1975), Lofts (1968), Lofts and Bern (1972), Hoar (1969) and de Vlaming (1974). The occurance and biosynthesis of steroids in fish have been considered in detail by Schmidt and Idler (1962), Idler and Truscott (1972), Ozon (1972a & 1972b), and the effects of

sex steroids in development of primary and secondary sexual characteristics by Dodd (1955), Hoar (1957, 1965) and Chester Jones, et al. (1972).

Sex steroids in vertebrates are classified into three groups with respect to their physiological functions; androgens, oestrogens and progestrogens. All naturally-occurring steroids have in common the basic cyclopentanophenanthrene skeleton but different either in spatial configuration or in the nature of the functional groups located at certain key positions in the molecule (Vida, 1969; Camerino and Sciak, 1975). In recent years there has been considerable progress in understanding the anabolic properties of sex steroids in fish, and in this respect androgenic steroids exert the greatest growth-promoting action. Detailed accounts of the anabolic properties of sex steroids in fish have been reviewed by Donaldson, et al. (1979), and more recently by Higgs, et al. (1982).

2.2.1. Steroid administration and metabolism

It has been emphasized by Higgs, et al. (1982) that the effective anabolic dose of steroids is related to the length of treatment, and it is evident that, below a specific dose which varies with species, there is usually a positive correlation between growth response and dose, at least over short experimental periods (20-56 days). It was suggested by Higgs, et al. (1982) that the probable cause of the reduction in growth response following prolonged administration of a steroid might be the result of

pharmacological effects of the hormone interfering with normal liver and kidney function. There is however no information on the relationship between growth response and route of administration, but half-lives have been determined in certain instances. The half-life of H3-testosterone, for example, is 2.5 hours when administred as a single injection into epaxial muscle in rainbow trout (Schreck, 1973) as compared with 11.2 hours when fed (5 ppm) for 10 days in coho salmon, Oncorhynchus kisutch (Fagerlund and McBride, 1978). In this context, a direct comparison may not be valid because of the differences in isotope equlibration period. However, Fagerlund and Dye (1979) have followed the distribution and disappearance of 3H-methyltestosterone in coho salmon, using oral route of administration, and found a half-life of 13 hours in the plasma. The latter authors suggested that increased retention time of methyltestosterone might be the reason for more potent role of this steroid than testosterone in fish. Since no information is available on the metabolism of synthetic steroids in fish, further studies on the dynamics of steroid metabolism in vivo would be valuable. Similarly, little information is available on the metabolism of sex steroids in various organs or tissues of fish, although androgen metabolites have been largely identified in higher vertebrates (Kochakian, 1975). Fagerlund and McBride (1978) measured H3-testosterone residues in various tissues of coho salmon (O. kisutch), after withdrawal of the steroid, and found relatively longer half-lives of the radionucleide in brain, liver, gall bladder, pyloric caeca and small intestine than those in testis, ovary, blood, muscle and gills. No differences in half-life was

found between male and female fish (McBride and Fagerlund, 1978; Schreck, 1973). In carp (Cyprinus carpio), it was found that after feeding H3-testosterone for 12 days (10 ppm) 97.5% of the total radionucleide recovered was from the gall bladder and alimentary canal (excluding liver), suggesting a hepato-biliary pathway of excretion, 1.37% from liver, kidney and spleen, 0.06% from muscle and the remainder from other tissues (Lone and Matty, 1981a).

The intermediary metabolism of C19 steroids in fish, yields similar by-products to those of mammals both for androgens and oestrogens (Ozon, 1972a & b). However, formation of C11-oxygenated steroids has been shown in the testes of S. salar (Idler and MacNab, 1967) which might be an exception to mammals. A high rate of steroid metabolism has been reported in the liver (Lisboa and Breuer, 1966) and in the skin (Hay, et al., 1976) of rainbow trout (S. gairdneri).

2.2.2. Anabolic activity

It is generally accepted that both naturally-occurring and synthetic steroids stimulate nitrogen retention in mammals (Kochakian, 1950, 1975), and although this has not been fully demonstrated in fish, there is some evidence for similar actions in the latter. Administration of 4-chlorotestosterone acetate in rainbow trout for example resulted in an increased growth rate which was accompanied by increased hematocrit, total serum protein, digestibility and a decrease in total non-protein nitrogen (Hirose and Hibiya, 1968a). Furthermore, administration of methyltestosterone (2.5 ppm) in rainbow trout increased total body weight, food conversion ratio, but reduced visceral fat content,

although carcass lipid composition was slightly increased (Simpson, 1976). In coho salmon (O. kisutch), methyltestosterone increased the total body protein (Fagerlund, et al., 1979), but was without effect on fat content at low concentrations (0.2-1 ppm) although, at a higher concentration (10 ppm), lipid composition was increased (Fagerlund and McBride, 1975). It has been reported by Fagerlund et al. (1978) that both methyltestosterone and testosterone enhance food utilization and food conversion efficiency in O. kisutch, and that methyltestosterone increased protein and moisture content, and decreased the percentage fat composition (both dry and wet weight). Similar, but less pronounced, changes were observed in testosterone-treated fish. Both steroids exerted lower growth-promoting actions when fish fed a restricted food ration, suggesting that improvement of protein utilization in the steroid-treated groups was caused partly by mobilizing body lipid for energy needs thus sparing dietary protein for growth. It is apparent that the main effect of the steroids might be the mobilization of visceral rather than carcass fat in rainbow trout (Simpson, 1976). Fagerlund, et al. (1980) demonstrated increased appetite, food utilization, moisture and decreased fat content following administration of methyltestosterone (0.2-1 ppm) in coho salmon, (O. kisutch). However, Matty and Cheema (1978) found no marked variations in the daily consumption of food in rainbow trout following administration of two synthetic androgens, dimethazine and norethandrolone. Both steroids (2.5-5.0 ppm) significantly increased weight gain and food conversion efficiency. High concentrations of dimethazine (10 & 20 ppm) produced an initial weight increase which diminished after 48

days. These authors also found that administration of both androgens significantly increased protein synthesis and incorporation of 14C-leucine into skeletal muscle protein. Lone (1980) and Lone and Matty (1980, 1980a, b & c) have investigated the effects of androgenic steroids in carp (Cyprinus carpio). Methlytestosterone administration increased growth rate and food conversion efficiency. Steroid treatment for 90 days resulted in percentage weight increases over the controls of 40.4, 39.0, 18.2 and -6.0 for 1.0, 2.5, 5.0 and 10 ppm respectively. After 90 days of steroid feeding, significant increases were observed in total protein, RNA and protein/DNA ratios in liver, kidney, brain and muscle (1980a). Similar anabolic activities were reported for testosterone, 11-ketotestosterone and trenbolone acetate following oral administration of the steroids (Lone, 1980; Lone and Matty, 1980a, b, c). These authors have also reported increases in the proteolytic activity of carp (C. carpio) intestine following administration of testosterone, 11-ketotestosterone and adrenosterone for 60 days (Lone and Matty, 1981). Steroid withdrawal for one month reduced protease activity below control values. In addition to the increasing evidence for the action of steroids on the activity of intestinal proteases, Yamazaki (1976) found increased granulation of acinar cells and hypertrophy of granulocytes in the intestine of horai masu (Oncorhynchus masou) treated orally with methyltestosterone (10 ppm). It has also been shown by Ince, et al. (1982) that oral administration of ethylestrenol increases growth rate in rainbow trout and that the steroid-induced growth response is associated with an increased

food conversion, protein efficiency ratio, and protein digestibility and assimilation. Significant changes in tissue protein, RNA and DNA also occurred in response to ethylestrenol (Lone and Ince, 1983) but with few significant changes in blood chemistry (Lone, et al., 1982).

In general, there is evidence that growth rates in 20 different species of fish may be stimulated by at least 14 androgenic steroids (Donaldson et al., 1979; Higgs et al., 1982). In this context, unlike androgenic compounds and in contrast to mammals (Umberger, 1975; Aschbacher, 1978), oestrogens have little or no anabolic properties in fish, and in some cases growth-retardation has been observed (Ashby, 1957; Ghittino, 1970; Scidmore, 1966; Matty and Cheema, 1978; Bulkley, 1972). However, some positive growth responses have been reported. Cowey et al (1973), for example, found that oral treatment of diethylstilbesterol (0.6 ppm) increased growth rates in plaice, Pleuronectes platessa, and Yu, et al (1979) reported a significant increase (15%) in growth rate of coho salmon, O. kisutch, following oral administration of oestradiol (2.5 ppm). However, it remains to be determined if other varieties of oestrogenic compounds can induce anabolic responses in fish.

The use of natural and synthetic steroids in the production of live stocks and poultry is now widespread (Bird, 1976; Umberger, 1975; McMartin et al., 1978) and may also have important applications in fish culture (Lam, 1982). However, more studies are required on the side effects and fate of growth-promoting steroids before they can be used in commercial fish production although, at the present time, some countries allow growth-promoting agents to be

used on a limited scale, for example, in Singapore, guppy farmers use stilbestrol to stimulate growth rate in males (Lam, 1982). The use of androgens and oestrogens for the production of mono-sex culture may not be subject to the same restrictions, since steroid administration is only necessary during the early stages of development, usually 6 to 12 months before marketing (Schreck, 1974; Yamamoto, 1969). In some cases, the steroid-induced sex- reversed fish are only used as brood stock for production of mono-sex culture. For example, in salmonids, masculinized, genetically female fish (XX♂) are produced by methyltestosterone administration (Johnstone, et al., 1979) and then used to produce all female progenies by a cross with normal female fish (XX) (Yamamoto, 1969; Clements and Inslee, 1968; Jalabert, et al., 1974; Jensen and Shelton, 1979; Jensen and Shelton, 1979; Johnstone, Jensen and Shelton, 1979; Johnstone, et al., 1979). Economic aspects of the commercial production of groupers (Epinephelus salmoides) have been examined by Chua and Teng (1980), who estimated that administration of methyltestosterone (9 ppm) would produce a net weight increase of 79% over the controls which reduces the rearing time for a 500g fish by 33%. In addition Higgs et al (1982) calculated the production cost of pan-size grouper and estimated a saving of $ 1.22 per kilogram of fish in the steroid-treated group. Thus, the current research indicates that application of sex steroids can benefit both farmer and consumer but more studies are needed to establish a standardized practice on a commercial scale.

General materials and methods

Section 3

3.1 Fish maintenance

3.1.1 Animals

Rainbow trout (Salmo gairdneri) were largely obtained from the fish culture unit of the university of Aston and maintained at 11 +/- 3°C, depending on the time of the year, under a constant photoperoid (14h light : 10h dark) in a recirculating system. Two to three weeks before each experiment, fish were transferred to the experimental system which included 250L fibre-glass tanks (unless otherwise stated), plumbed with a recirculating system. Fish were fed a commercial diet (Omega No. 4 or 6; Ewos-Baker Ltd., Sudbury, Suffolk) once a day to satiation, and food witheld 24h prior to experiments. With the exception of the experiments described in section 8, in all experiments male and female fish were used randomly. Experiments carried out on 9-10 month old trout indicated no significant sex differences in intestinal absorption of water and amino acid (section 8). The experimental temperature is given where appropriate.

3.1.2. Recirculating system.

Each recirculating system consisted of 3 fibre-glass circular tanks (250l; 1m i.d.), supplied with water from a header tank (3.5-4 l/min) by gravity flow. Each tank was constructed with a stand pipe at the centre, enclosed by a covering pipe to facilitate self cleaning as shown in Fig. 3.1. Each system included a submerged gravel filter and a faecal trap, and the sediments collected in the faecal trap were removed on alternate days. As a routine procedure nitrates, ammonia, water hardness, oxygen, pH and water temperature

were measured regularly during the experiments [Temperature: 12.8 ± 3.4°C; 02: 9.52 ± 0.97 ppm; PH: 6.84 ± 0.50; NH3: <0.1 ppm; NO3+NO2: 3.15 ± 2.57 ppm; Hardness: 20.6 ± 2.27 ppm (n=149-155, mean ± S.D.)].

3.2. Materials.

Unless otherwise stated, all organic and inorganic chemicals used were of analytical grades (Sigma or BDH). Chemicals used for spectroscopy, or scintillation counting were Analar/spectroscopic, or scintillation grade. Silk sutures were purchased from Sutures Ltd., Clwyd, Wales, and the dental glue (Cicaterine containing antibiotics) was a gift from the Ministry of Agriculture and Fisheries & Food.

3.3. Scintillation counting

Samples obtained from the media and tissue extracts were counted for 14C activity in glass scintillation vials (Packard Ltd.) using a Tri-Carb 2600 scintillation counter (Packard Ltd.). Each vial contained 10 ml of scintillation fluid (4g 2,5-diphenyloxazole, 500 ml toluene 500 ml 2-ethoxyethanol). In each case, samples were counted with a blank and a standard (14C-L-leucine) for a period of 20 min, using a single channel. The degree of quenching for the solvents used were estimated using a series of standards quenched with different volumes of solvent, as described in the Packard manual. The scintillation counter was subsequently programmed to determine disintegrations per min (DPM) from the counts per min (CPM) for each sample:

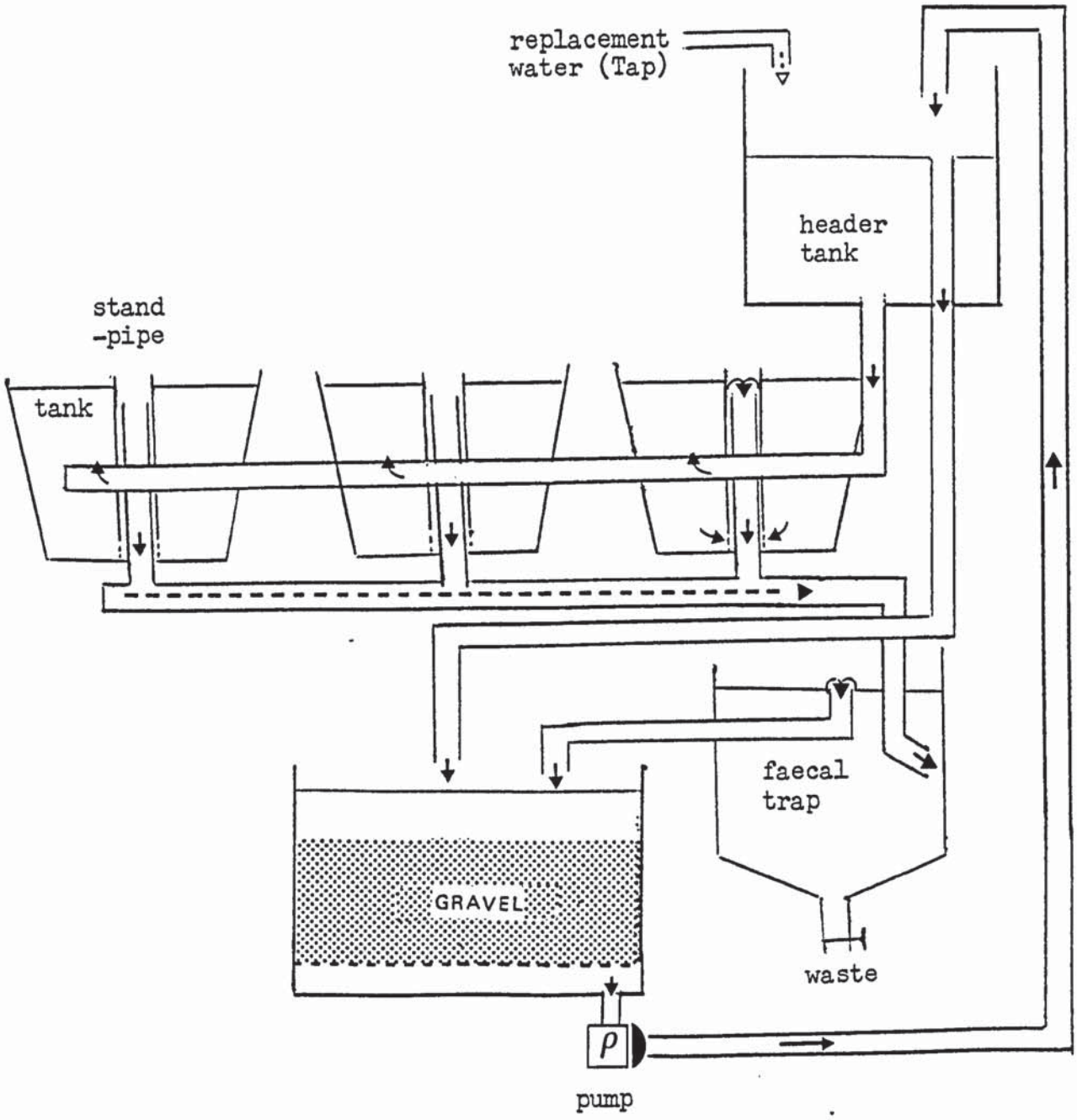
$$DPM=(CPM/\text{percentage counting efficiency})\times 100$$

3.4. Spectroscopy

Concentrations of Na+, K+ and Ca++ were measured using an atomic absorption spectrophotometer (Perkin-Elmer, 373 A.A.S., Nowalk, Connedicut, U.S.A.) operating on an air/acetylene mixture, with a 4 cm burner head. For Na+ and Ca++ measurement the diluted solution was buffered with 2000 ppm of K+ and extinctions were determined at 587.0 (K+) and 422.7 (Ca++) nm, with a slit size of 0.7 mm. K+ samples were buffered with 2000 ppm of Na+, and the extinctions were determined at 766.5 nm (slit size 2.0 mm). In each case a red filter was used to absorb radiation below 650 nm.

For other spectrophotometric determination of compounds, a Cecil (CE272) spectrophotometer was used and all extinctions were determined at room temperature, using glass cuvettes of 1 cm light path. Linear calibration curves were obtained in all cases over the range of substrate concentration, using serial samples of known concentration.

Fig. 3.1. Recirculating system.



NOT TO SCALE

The effects of 17a-methyltestosterone on the intestinal transport of a mixture of amino acids by the intestine of rainbow trout, S. gairdneri , in vitro.

Section 4

4.1. Introduction

Numerous studies, performed primarily in mammals, have shown that a variety of hormones of pituitary, thyroid and adrenal origin, may influence gastrointestinal function, although this site is not their main locus of action (Crane, 1960; Spencer, 1960; Wiseman, 1964; Levin, 1969). In comparison, little information is available concerning the effects of androgenic and oestrogenic steroids on gut physiology, nor have their modes of action been elucidated. However, histological studies of steroid-treated animals suggests, that androgens might enhance protein synthesis in this tissue. For example, Giordano et al. (1962) have demonstrated that testosterone propionate injection in rats resulted in an increased height of the intestinal villi and higher occurrence of goblet cells. Furthermore, these morphological changes were accompanied by a greater number of mitotic divisions and higher cellular RNA content in the intestine. Ksiazkiewicz-Szapiro (1970) has also demonstrated increased height of villi in the testosterone propionate-injected rats, but was not able to observe any variations in the structure of the intestine following castration. Similarly, testosterone propionate administration in mice was shown to reduce the time phase of the enterocyte cell cycle (Tuohimaa and Niemi, 1968) and increased the mitotic index of the crypt cells in the upper jejunum (Wright and Morley, 1971; Wright, et al., 1972). In fish, similar observations have been reported by Yamazaki (1976). Administration of methyltestosterone in horai masu, a variant of rainbow trout (o. masu) resulted in hypertrophy of the intestinal granular cells

and marked increases in granulation and the number of pancreatic acinar cells. Thus, it was suggested by Yamazaki (1976) that, at certain dose-levels, methyltestosterone induces a higher rate of digestion or absorption of the food. In accord with this, Lone and Matty (1981) have demonstrated increased protease activity following oral administration of 11-ketotestosterone, testosterone, or adrenosterone in the intestine of carp (Cyprinus carpio).

In addition to morphological effects, sex steroids have been observed to influence the epithelial transport of water and solutes in a number of mammalian studies. In this context, Riggs et al.(1963), and Riggs and Walker (1963) found that testosterone increased amino acid uptake in rat kidney and reduced amino acid clearance independently of glomerular filtration rate and renal blood flow, and suggested that this steroid might enhance amino acid absorption in kidney tubules. The studies of Cappelli and Tacconi (1954) and Cappelli and Rossi (1954) suggested that testosterone might enhance intestinal water absorption in the female castrated, or adrenalectomized, rat but not in male rats. Goligorsky et al.(1980) have demonstrated that administration of a mixture of sex steroids (oestrogens and androgens) in subtotally nephrectomized rats reduced the intestinal absorption of calcium, a change which is associated with reduced secretion and enhanced absorption of fluid in the intestine (Field, 1981). Larralde et al. (1966) studied the influence of pregnancy on intestinal absorption. A significant increase in the absorption of glucose in the pregnant rat was observed by these workers. Similar results have been reported for intestinal absorption of glycine (Larralde, J., and Gonzalez, M.,

cited in Larralde, et al., 1966). In mammals, there are other reports of an oestrogenic influence on intestinal transport of water and solutes (Herreros et al., 1970; Ahmed-Sorour, 1978). In fish, Mugiya and Ichii (1981) have studied the effects of oestradiol on calcium uptake in rainbow trout. In this species, a single injection of the steroid was without effect on intestinal calcium transport.

In view of the anabolic properties of androgenic steroids in salmonids (Donaldson, et al., 1979), the present study was undertaken to investigate the effects of methyltestosterone on the absorption of a mixture of amino acids by the intestine of rainbow trout, using the everted gut sac technique. These studies suggested that methyltestosterone might exert a stimulatory action on the intestinal transport of amino acids in rainbow trout

4.2. Materials and Methods

4.2.1. Animals

Rainbow trout weighing approximately 170g (range: 110-230g), were maintained at 10°+/- 2 C, under the conditions described in Section 3 in 140 l polythene tanks.

4.2.2. Hormone administration

For injection, 17a-Methyltestosterone (MT) was dissolved in absolute ethanol (15 mg/ml), then further diluted in arachis oil to a final concentration of 1 mg/ml. Fish were injected intraperitonialy under light benzocaine (ethyl-p-aminobenzoate) anaesthesia (1:15000) with MT, at a dose level of 200 ug/100g body weight, depending upon the experimental procedure. Control fish were injected with an equal volume of solvent in each case. For in vitro administration of the steroid, MT was dissolved in a minimum volume of absolute ethanol (40 mg/ml), then diluted in the incubation medium to a concentration of 200 ug/ml, using 5 ug/ml of Tween-80 as surfactant. Control incubation medium contained the same volume of solvent with Tween-80 (section 5). The experimental procedures were as follows;

Experiment 1: No pretreatment; MT was directly added to the incubation medium.

Experiment 2: Fish were injected 4 hours before incubation.

Experiment 3: Fish were injected 8 hours before incubation.

Experiment 4: Fish were injected every two days, for 6 d. (3 injections; days 1, 2 and 3). The last injection was given 24h

before incubation.

4.2.3. Method of intestinal preparation

4.2.3.1. Incubation medium

The composition of the Ringer solution used in the present experiments was as follows (mMoles/l): NaCl 120, KCl 4.8, CaCl2 2, MgSO4 1.2, Na2HPO4 15.1 (pH 7.3) (Boge', et al., 1977b). The incubation medium used contained a mixture (5 mMoles/l each) of Glycine (Gly), L-valine (Val), L-methionine (Met), L-iso-leucine (Ileu), L-leucine (Leu), and L-lysine (Lys).

4.2.3.2. Preparation and incubation of gut sacs

The procedure used in the present experiment was previously described by Smith and Lane (1971). Briefly, fish were sacrificed with a blow on the head, the abdomen opened through the midline, the rectum cut, and the intestine was freed of mesenteries. The anterior end was cut (just posterior to the pyloric caeca), and the intestine (including midgut and hindgut) was transferred to 30 ml of ice-cold Ringer solution, pregassed with 95% O2 : 5% CO2. The intestine was cleaned from the remaining mesenteries and fat, and gently flushed with 10 ml of ice cold, pregassed Ringer solution. The pyloric end of the intestine (including midgut and hindgut) was tied firmly over the groove of a glass rod (1mm d.), with a ligature thread. The intestine was everted by gently forcing the gut over the glass rod, and the everted intestine was cut free with a scalpel. The pyloric end of the everted gut was tied around a glass cannula, whilst the rectal end was closed with a ligature. The sac was filled with 1 ml

incubation medium (serosal solution) using PE 25 polyethylene tubing attached to a G21 hypodermic needle. The everted sac was incubated at 12 +/- 1°C for a total of 60 min in 20 ml of incubation medium (mucosal solution), which was gassed continuously with 95% O2 : 5% CO2. At the end of the incubation period (60 min), the solution inside the sac (serosal) was withdrawn and samples of mucosal and serosal solution were stored at -30° C until analysis. The gut sac was detached from the cannula and the ligature thread removed. The intestine was then blotted and weighed on a Mettler balance.

4.2.4. Analytical procedures

Samples (100 ul) of serosal and mucosal medium were added to 1.9 ml 3% sulphosalicylic acid (20 fold dilution) and the solution was centrifuged at 1500g for 15 min to remove any protein contamination. Aliquots of the supernatant were loaded on the column (18 cm) of an automatic amino acid analyser (Locarte Co., London), and the amino acids were separated by ion-exchange chromatography. The system was operated on the basis of a stepwise increase in the pH of the sodium citrate buffer (3.25/4.25/9.35) for a greater resolution (Moore and Stein, 1954), at 55°C.

4.2.5. Presentation of results

Concentration of the aminoacids were determined (I) and used to calculate the following parameters; Mucosal absorption : u Moles amino acid disappearance from the mucosal solution per g intestinal wet weight per h (II); Serosal concentration per g intestine : u Moles per ml amino acid present in the medium per g intestinal

wet weight per h; Serosal/Mucosal ratio (S/M) : ratio of final serosal concentration (u moles/ml) to final mucosal concentration (u Moles/ml) (III).

$$Ca = Cst.(Aa/Ast) \quad ...(I)$$

$$A = (Ci-Cf).V/W \quad ..(II)$$

$$S/M = Cfs/Cfm \quad .(III)$$

where; Ca= Concentration of amino acid (u Moles/ml); Cst= Concentration of standard (u Moles/ml); Aa= absorbance of amino acid; Ast= absorbance of amino acid standard; A= mucosal absorption (u Moles/g/h); Ci= initial mucosal concentration (u Moles/ml); Cf= final mucosal concentration (u Moles/ml); V= Initial mucosal volume (20 ml); W= Intestinal wet weight; Cfs= final serosal concentration (u Moles/ml); Cfm= final mucosal concentration (u Moles/ml).

Analysis of the serosal solution at the end of these experiments revealed a net decrease (instead of an increase) in the concentration of the amino acids in a number of intestinal preparations. For this reason the serosal amino acid increase per g intestine [(Cf-Ci).V/W] was not included in the results, since in a number of preparations Ci was greater than Cf. The change in serosal amino acid concentration is expressed in terms of the absolute final serosal concentration (u Moles/ml) and serosal concentration per g intestine (u Moles/ml/g). For comparison, absolute final mucosal concentration (u Moles/ml) was also included, and in this case a decrease in final mucosal concentration (Cfm) indicates absorption from the medium.

4.2.6. Statistical analysis

Differences between means were tested using one-way analysis of variance, and multiple comparison of the means (Duncan multiple range test, Duncan, 1955). Differences were considered significant when $P<0.05$.

4.3. Results

The statistical analys s were only carried out for 95% probability and significant variations in the results indicate $P<0.05$.

4.3.1. In vitro effects of MT

At the end of the incubation period, the mucosal concentration of Gly and Met in the MT-treated group were significantly lower than those of the controls. Concentrations of Val, Ileu, Leu, and Lys were also lower in the MT-treated intestine, but the differences were not statistically significant (Table 4.1.a). The results of mucosal absorption per g intestine indicated significantly higher values for Gly, Met, Ileu, and Lys in the MT-treated than those of the control intestine (Table 4.1.b).

Final serosal concentration of three amino acids studied were higher in the MT-treated groups, although significant differences were only observed for Gly. Comparison of the final serosal concentration per g intestine, obtained from the steroid-treated and the controls, revealed no significant differences for any of the amino acids studied (Table 4.1.a & b).

Addition of MT significantly increased the S/M ratios for Gly and Val, but was without effect on the S/M ratios obtained for Met, Ileu, Leu, and Lys (Table 4.1.a).

4.3.2. In vivo effects of MT on intestinal transport of amino acids.

4.3.2.1. MT injection (4 hours)

Injection of MT 4h before incubation did not significantly influence the final mucosal concentration of the amino acids (Table 4.2.a). Comparison of the mucosal absorption per g intestine revealed only a significantly higher Lys absorption in the steroid-treated group (Table 4.2.b).

The final serosal concentrations of Met, Leu, and Lys were significantly higher in the steroid treated than those of the controls. However, the corresponding serosal concentrations per g intestine were not significantly different in the MT-injected when compared to the controls (Table 4.2.a & b).

The S/M ratios of Met and Lys were significantly higher in the steroid-treated group, but the values obtained for Gly, Val, Ileu, and Leu were not significantly different to those of the controls (Table 4.2.a).

4.3.2.2. MT injection (8 hours)

The final mucosal concentration of the amino acids (Gly, Val, Met, Ileu, Leu & Lys) were significantly lower in the MT-injected fish(Table 4.3.a) Determination of the mucosal absorption per g intestine revealed significantly higher values in the steroid-treated fish for all amino acids tested, when compared to the controls (Table 4.3.b)

Although the final serosal concentrations of the amino acids were higher in the MT-injected group, statistical analysis indicated significant differences only for Val and Lys (Table 4.3.a). The corresponding values of the final serosal concentrations per g

intestine were similarly higher in the steroid-injected group, but their respective variations from the controls were not statistically significant (Table 4.3.b). However, the S/M ratios of the amino acids studied were all significantly higher in the MT-injected than those of the controls (Table 4.3.a).

4.3.2.3. MT injections (6 days)

Neither mucosal concentrations nor mucosal absorption per g intestine of the amino acids were significantly different between the steroid-injected and the control fish (Table 4.4.a &b). However, final serosal concentration of the amino acids were significantly higher for Gly, Val, Met, Leu and Lys in the MT-injected group than the controls. The corresponding values of the serosal concentration per g intestine although generally higher in the steroid injected fish, nevertheless were not statistically significant (Table 4.4.a & b).

The S/M ratios were also higher in the steroid-injected fish, but the differences were only significant for Met and Lys (Table 4.4.a).

4.4. Discussion

The aim of the present study was to investigate the effects of an androgenic-anabolic steroid, MT, on the intestinal transport of a mixture of essential amino acids (with possible exception of Gly) (Cowey and Sargent 1979), with different chemical properties.

4.4.1. Experimental technique and amino acid transport.

An S/M ratio of greater than one is generally regarded as an index of transport against a concentration gradient (Wiseman, 1968). The results obtained in the present study indicated S/M ratios of less than one in a number of observations, suggesting perhaps that the amino acids were not transported against their respective concentration gradients in all preparations. By contrast, the active transport of these amino acids (Gly, Val, Met, Ileu, Leu & Lys) have been demonstrated previously in a number of studies dealing with intestinal absorption in fish (Ward, 1968; Smith, 1970; Smith and Lane, 1971; Ingham and Arme, 1977; Boge', et al., 1977b, 1978). In the present study therefore, the results were likely to have been the result of the experimental conditions employed, unaccounted for in the quantitative determination of the transport parameters. The factors which are likely to affect the transmural absorption of the amino acids include; (a) availability of metabolisable energy; (b) interactions between the amino acids; (c) hypo- or hyperosmolarity of the incubation medium; (d) metabolism of the amino acids, and (e) the net movement of water from the mucosal to serosal solution. In the first instance, glucose was not included in the incubation medium due to its reported interference with amino acid transport

(Alvarado, 1966; Chez, etal., 1966; Hokazono, 1979), although the underlying causes remain a subject of debate (Robinson and Alverado, 1971, 1977; Munck, 1981). Glucose-free media have previously been used for the study of amino acid absorption in rainbow trout, using similar everted gut sac preparations (Hokazono, et al., 1979). It is apparent that these authors used lower concentrations of L-lysine (<<5 mM/l) in their study, whilst the present incubation medium contained a mixture of amino acids, each present initially at a concentration of 5 mM/l. Thus, Matthews and Laster (1965) have demonstrated a significant reduction in S/M ratios with increasing concentration of amino acids in the medium (high S/M ratios when amino acid concentration <= 1 mM/l). In view of the concentration and the number of the amino acids used in the present study, there is a possibility that the energy reserve in the enterocytes was not sufficient to sustain the maximum rate of active transport for a period of 60 min. However, since the total absorptive ability of the intestine is not dependent on glycolysis and oxidative phosphorylation (Ingham and Arme, 1977; Present study, section 7), absence of glucose does not necessarily abolish active amino acid transport during 60 min incubation.

Mutual inhibition of amino acids has been demonstrated both in higher vertebrates (Munck, 1977) and in fish (Smith, 1970; Ingham and Arme, 1977). It has been shown that over a substrate range of 0.2-10 mM/l, L-leucine, L-methionine and L-valine are mutually competitive inhibitors of uptake in rainbow trout intestine, and uptake of L-leucine is not inhibited by acidic or basic amino acids (Ingham and Arme, 1977). Furthermore, these authors have suggested

the existence of multiple transport sites for the active absorption of neutral amino acids in the intestine of rainbow trout. In this context however, mutual stimulation of amino acid transport has also been demonstrated in a number of studies in higher vertebrates (Munck, 1977) but this aspect has not been documented in fish. Moreover, significant variations in amino acid absorption from an equimolar mixture of amino acids has been reported for different concentrations of amino acids in the medium. For example, significant variations between absorption of a number of amino acids were observed when the initial concentration of each amino acid was increased from 1-2 to 3 mM/l in rat (Bronck and Leese, 1974) and to 8 mM/l in man (Adibi and Gray 1967). The present results indicated some variations between the absorption of the amino acids. For example higher S/M ratios were obtained for Lys compared to the remaining amino acids in the controls. This observation is in accord with the finding that some neutral amino acids, including leucine and methionine, stimulate the intestinal transport of Lysine (basic amino acid) (Robinson and Felber, 1964; Munck, 1972; Reiser and Christiansen, 1971). The stimulatory mechanism of leucine on lysine absorption has been investigated by Munck and Schultz (1969) in rabbit ileum, and it was suggested that leucine exerts two actions, one of which increases the efflux of lysine across the basolateral membrane, and the second, trans-stimulation of lysine influx through the brush border membrane by intracellularly-located leucine (Munck, 1977). The mechanism for the stimulated efflux across the basolateral membrane is not known, but it has been suggested that the trans-stimulation effect across the brush border membrane might

be due to accelerated exchanges in diffusion (Munck and Schultz, 1969). Alternatively, it was suggested by Munck (1981) that the trans-stimulation effect might be the result of hyperpolarization of the electrical potential difference across the brush-border membrane caused by sodium-neutral amino acid co-transport. A recent study on rabbit indicated that mutual inhibition of amino acids exists for both sodium dependent and sodium independent transport (Stevens, et al., 1982), but this aspect has not been shown in fish.

There is no doubt that the absorption of water and solutes in vitro is influenced by the osmotic pressure and pH of the incubation medium. The media commonly used in investigations of intestinal transport have a basis of inorganic salts, buffered to maintain a constant pH and osmotic pressure. The incubation medium used in the present study was adopted from Boge et al. (1978) which is essentially similar to those of Stokes and Fromm (1964). There is a possibility that the present medium was slightly hyperosmotic with respect to fish plasma, due to the presence of a total of 30 mM/l amino acids. However, since both mucosal and serosal solutions were initially identical, it is unlikely that the net transmural movement of these amino acids were significantly affected.

The present study indicated a poor rate of transport and a net loss of the amino acids from the incubation medium. The total recovery of the amino acids could not be determined, since there is no data on the intestinal accumulation of these compounds. Thus, the possibility of amino acid metabolism cannot be ruled out although there is no clear evidence that essential amino acids undergo

extensive metabolism during their transport across the intestine. Furthermore, the present study (section 7) has shown that L-leucine retains more than 90% of its integrity during transmural absorption, both in control and steroid-treated intestines, in vitro. However, it has been shown in higher vertebrates that glutamic acid and aspartic acid are transaminated and converted mainly to alanine and glutamic acid, and there is evidence that the catabolism of these amino acids in the intestine might be related to the dietary regime of the animals (Wiseman, 1968). In fish, there is large species variation and little correlation between peripheral amino acid catabolism and nutritional status (Cowey, et al., 1979). In any event, although amino acid metabolism might be expected to effect serosal transfer, the effect on mucosal disappearance of solutes is likely to have been minimal under the present conditions.

A net transport of fluid from mucosal to serosal solution would result in an apparent mucosal secretion and an apparent serosal absorption of the amino acids. The present technique, as described by Smith and Lane (1971), was based on the assumption that the final volumes of the serosal and mucosal solutions are not significantly different to the initial volumes. It has been shown that in any in vitro preparation, stimulation of fluid transport is seen only in the presence of glucose (Borry, et al 1961; Newey, et al, 1968). Furthermore, the fluid transport in the present preparation was probably reduced further due to slight hyperosmolarity of the media. But, as shown in the present study (section 7), small fluid absorption occurs even in the absence of glucose. It was suggested by Munk (1981) that the difference in

fluid transport in the presence and absence of glucose might be related to glycolysis of the transported glucose to lactic acid, which results in additional osmotic pressure differences. Thus, it was shown in the present study (section 7) that an inhibitor of glycolysis (NaF) significantly reduced fluid absorption by the intestine. Furthermore, influx of water was higher in the steroid-treated intestine although the differences were not statistically significant. A higher fluid absorption in the MT-treated fish would result in a higher concentration of amino acids in the mucosal solution, and an apparent reduction in amino acid absorption in the steroid-treated compared to the controls.

In general, some or all of these factors may have contributed to the reduced amino acid absorption observed in the present experiments. However, since the experimental conditions were identical for both controls and the steroid-treated fish, a significant difference between the two groups might be taken as a possible indication of the effects of the steroid treatment.

4.4.2. Effects of methyltestosterone on amino acid transport

4.4.2.1. Hormone administration

The intestinal transport of amino acid was investigated in response to doses of MT assumed to be anbolic for this species. However, since no information is available on the effective anabolic doses of these steroids when administraed by injection, it was necessary to derive an approximate concentration on the basis of previous studies in fish using related compounds (Hirose and Hibiya, 1968a & b; Donaldson et al, 1979; Mugiya and Ichii, 1981). The

effectiveness of 17a-methyltestosterone as an androgenic-anabolic compound in fish has been demonstrated by a number of workers (Donaldson, et al., 1979). The metabalism of 17a-methyltestosterone has not been studied in fish but, in mammals, metabolism of MT is similar to testosterone, except that dehydrogenation of the 17B-hydroxyl group does not take place (Kochakian, 1976) and the methylated C-1, markedly protects this compound against aromatization (Vida, 1969). The half lives of labeled testosterone (H3-testosterone) and 17a-methyltestosterone (H3-MT) following oral administration of the steroids in Coho parr were found to be 11.2 hours for testosterone and 13.0 hours for MT (Fagerlund and McBride, 1978; Fagerlund and Dye, 1979). In the present study, MT was dissolved in arachis oil for slow and gradual release to extend the half life of the steroid.

4.4.2.2. Effect of MT on amino acid absorption

It appears from the results that MT enhances the intestinal transport of amino acids, both when added directly to the media, in vitro, and when administered in vivo by injection. Although small differences in absorption were observed between different amino acids, the stimulatory effects of the steroid were apparently not directed towards any particular amino acid. This suggests that the mechanisms involved may not be linked to any amino acid-specific carrier system. Moreover, the results of the in vitro administration of MT indicate that pathways other than protein synthesis might account for MT action since a 60 min incubation period was clearly not sufficient for steroid-stimulated processes leading to protein

synthesis. However, this possibility cannot be ruled out in the studies which involved in vivo administration of MT.

The studies of Kochakian and his coworkers indicated two approaches for the elucidation of the mechanism of anabolic androgenic steroid action. Firstly, the involvement of a wide variety of enzymes including regulatory and RNA-protein synthesis enzymes (Kochakian, 1962; 1964) and secondly, the involvement of steroid metabolites, produced by C-19 steroid oxidoreductases present in the tissue (Clark and Kochakian,1947; Kochakian, 1959; Kochakian, 1976). Moreover, the work carried out in the past decade has revealed that some tissues previously considered to be non-target tissues contain low but significant levels of sex steroid receptors (Katzenellenbogen, 1980). Although the presence of sex steroid reseptors have not been confirmed in the gastrointestinal tract, a number of studies have demonstrated significant influences of androgenic steroids on the activity of the enzymes present in the intestine of the rat and mouse, including oxidases, dehydrogenases, phosphatases, folate-metabolising enzymes, and some glycolytic enzymes (Kochakian, 1976). Thus, it might be possible that some of the 'short-term' effects of MT were mediated by changes in the activity of some intestinal enzymes. Significant effects of MT are apparent in all experimental groups, and the following sequence is seen when comparing the steroid-stimulated amino acid absorption with respect to the time course of steroid administration; 8 hours > 0 hours > 6 days > 4 hours. These results indicate that 8 h steroid treatment is more effective than 4 h. One possible explanation might be the time required for the circulation to deliver an effective

dose of MT to the intestinal tissues, since the concentration of MT achieved in the blood is related to the rate of absorption from the injection site. It will be noted that the steroid was dissolved in arachis oil which only allows slow release of the hormone. The difference observed between the 8 h injection and 6 days injection, suggests that the presence of MT in high concentration might be required to elicit a stimulatory effect on the absorption of the amino acid. In the group receiving MT injection for 6 days, the steroid concentration would be reduced after 24 h due to metabolism and/or excretion. In this context however, there is no information on steroid absorption kinetics, but with an estimated t1/2 for orally administred 3H-MT of 13 h (Fagerlund and Dye 1979), a significant reduction would be expected at the time of transport measurement (24 h after the last injection). Furthermore, there is a possibility of a shorter t1/2 after I.P. injection of MT, since Schreck (1973) has found t1/2=2.5 h for H3-testosterone when injected into epaxial muscle of rainbow trout, compared to t1/2=11.2 h observed by Fagerlund and McBride (1978), following oral administration of H3-testosterone. Thus, it would appear that MT, although exerting an effect when injected for a prolonged period of time, might have a greater effect on absorption when present in larger concentrations in a relatively shorter period of time. This explanation is further supported by the observation that addition of MT into the incubation medium produced a more significant increase in amino acid absorption than injection of the steroid for 6 days, as indicated by the higher score of significant differences observed in the group receiving MT by direct addition to the medium (Fig.

4.1). It is worthy of consideration that the effects observed after in vivo administration of the steroid might be the result of an indirect effect of MT. In this context, there is evidence that testoterone injections in rainbow trout result in an increased plasma thyroxine level (Hunt and Eales, 1979). Thyroid activity has also been shown to influence the morphology and physiology of the gastrointestinal tract in mammals (Levine, 1969). In addition, there is evidence for the involvement of thyroid hormones in smoltification and development of seawater tolerance in salmonids (Nagahama, et al., 1982) and other fish species (Hoar, 1976).

The present results are complicated by the fact that the amino acids were not transported with maximum efficiency, due presumably to some mutual interactions between the amino acids. Thus, although the stimulatory effect of methyltestosterone on the absorption of amino acids by the intestine of rainbow trout was evident, this conclusion is at best tentative. Further investigations using alternative methodologies were therefore conducted, and are discussed in the following sections.

Table 4.1
In vitro effects of methyltestosterone (MT) on intestinal absorption of amino acids.

a MUCOSAL (M) AND SEROSAL (S) CONCENTRATIONS (u Moles/ml) SEROSAL/MUCOSAL RATIOS (S/M)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	4.9113	4.8630	4.8890	4.9671	4.9051	4.7118
+/- S.E.	0.0812	0.0905	0.0765	0.0792	0.0992	0.0405
MT+ (M)	4.6076*	4.6383	4.5563*	4.7226	4.8016	4.5005
+/- S.E.	0.0829	0.0775	0.0789	0.0819	0.0592	0.0906
Cont (S)	4.2020	4.3395	5.1276	4.8223	4.7751	5.1233
+/- S.E.	0.0477	0.2562	0.1070	0.1004	0.0381	0.09305
MT+ (S)	4.7140*	4.7886	4.7576	5.0468	4.7426	4.8726
+/- S.E.	0.1775	0.3006	0.14307	0.1307	0.15105	0.1812
Cont (S/M)	0.8565	0.8917	1.0487	0.9711	0.9753	1.0871
+/- S.E.	0.0148	0.0466	0.0130	0.0163	0.0195	0.0153
MT+ (S/M)	1.0253*	1.0336*	1.0453	1.0700	0.9872	1.0853
+/- S.E.	0.0459	0.0664	0.0333	0.0313	0.0254	0.0473

b MUCOSAL ABSORPTION PER GRAM (u Moles/g) (M) SEROSAL CONCENTRATION PER GRAM (u Moles/ml/g) (S)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	0.9880	1.4299	0.5486	-0.1531	1.8726	2.4755
+/- S.E.	0.5990	0.9227	0.6264	0.7905	1.4316	0.1978
MT+(M)	3.7207*	3.1120	3.8739*	2.3323*	1.1307	4.7190*
+/- S.E.	0.7719	0.4741	0.4128	0.5288	0.5219	2.3165
Cont (S)	10.1230	10.3991	12.3989	11.6613	11.5090	12.4173
+/- S.E.	1.6968	1.8136	2.1274	2.0072	1.9313	2.1478
MT+ (S)	11.3227	11.4588	11.3712	12.0875	11.3965	11.6734
+/- S.E.	1.4032	1.4416	1.3216	1.4543	1.4199	1.4220

The values are mean +/- S.E. of 6 observations. (*) indicates significant difference to the respective control ($P<0.05$). MT was added directly to the incubation medium (200 u g/ml).

Table 4.2
In vivo effects of methyltestosterone (MT) on intestinal absorption of amino acids (4 h after MT injection).

a MUCOSAL (M) AND SEROSAL (S) CONCENTRATIONS (u Moles/ml)
SEROSAL/MUCOSAL RATIOS (S/M)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	5.0255	5.0395	4.9907	4.7038	4.7497	4.7762
+/- S.E.	0.0520	0.0577	0.2755	0.0398	0.0459	0.0460
MT (M)	5.1317	5.3060	4.8447	4.7073	4.7702	4.3415
+/- S.E.	0.0173	0.1238	0.0735	0.0508	0.0705	0.0428
Cont (S)	3.8720	4.1367	3.8025	3.7773	3.6380	4.0367
+/- S.E.	0.0912	0.0558	0.0702	0.0385	0.1039	0.0874
MT (S)	4.0057	4.0155	4.1705*	3.6504	3.9717*	4.6010*
+/-S.E.	0.0880	0.0735	0.0959	0.0673	0.0380	0.1223
Cont (S/M)	0.7708	0.8214	0.7686	0.8032	0.7660	0.8459
+/- S.E. .0208		0.0193	0.0431	0.0111	0.0223	0.0266
MT (S/M)	0.7804	0.7590	0.8611*	0.7759	0.8329	1.0600*
+/- S.E.	0.0144	0.0321	0.0199	0.0189	0.0096	0.0301

b MUCOSAL ABSORPTION PER GRAM (u Moles/g) (M)
SEROSAL CONCENTRATION PER GRAM (u Moles/ml/g) (S)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	-0.1929	-0.27207	0.2952	2.6831	2.3487	2.0303
+/- S.E.	0.4739	0.5767	2.3439	0.3564	0.5520	0.3958
MT (M)	-1.21306	-2.7283	1.5331	2.7374	2.0189	5.9405*
+/- S.E.	0.1971	1.0225	0.8050	0.6330	0.55194	0.2394
Cont (S)	8.8849	9.4805	8.7381	8.6629	8.3540	9.2229
+/- S.E.	0.8206	0.8018	0.8396	0.7477	0.8080	0.6691
MT (S)	9.1484	9.1615	9.4965	8.3331	9.0600	10.4760
+/- S.E.	0.7321	0.6795	0.6189	0.63845	0.6545	0.6880

The values are mean +/- S.E. of 4 observations. (*) indicates significant difference to the respective control ($P<0.05$). MT was injected (I.P.) 4 hours befor the incubation (200 ug/100g).

Table 4.3
In vivo effects of methyltestosterone (MT) on intestinal absorption of amino acids (8 h after MT injection).

a MUCOSAL (M) AND SEROSAL (S) CONCENTRATIONS (u Moles/ml) SEROSAL/MUCOSAL RATIOS (S/M)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	5.2190	5.3605	5.2060	5.4438	5.3585	4.9766
+/- S.E.	0.0284	0.0399	0.0454	0.0784	0.0556	0.0484
MT (M)	4.7250*	4.5456*	4.4913*	4.7455*	4.6493*	4.4015*
+/- S.E.	0.0284	0.0840	0.0293	0.0470	0.0558	0.0284
Cont (S)	4.1010	4.2195	4.0290	4.3660	4.3911	4.1186
+/- S.E.	0.0895	0.0574	0.1282	0.1167	0.0436	0.1015
MT (S)	4.1625	4.5566*	4.3063	4.5080	4.6631	4.5101*
+/- S.E.	0.0967	0.1116	0.1110	0.1198	0.0807	0.1043
Cont (S/M)	0.7861	0.7873	0.7737	0.8038	0.8198	0.8282
+/- S.E.	0.0194	0.0125	0.0227	0.0308	0.0110	0.0236
MT (S/M)	0.8812*	1.0041*	0.9589*	0.95008*	1.0030*	1/0253*
+/- S.E.	0.0226	0.0312	0.0247	0.0246	0.0151	0.0287

b MUCOSAL ABSORPTION PER GRAM (u Moles/g) (M) SEROSAL CONCENTRATION PER GRAM (u Moles/ml/g) (S)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	-2.6414	-4.3621	-2.6152	-5.7101	-4.2646	0.3067
+/- S.E.	0.3575	0.4931	0.6785	1.3564	0.5435	0.5233
MT (M)	3.4346*	5.7316*	6.4100*	3.2510*	4.3471*	7.6966*
+/- S.E.	0.3162	1.1555	0.3726	0.6292	0.5821	0.7570
Cont (S)	12.7026	13.1201	12.5748	13.5655	13.6062	12.8593
+/- S.E.	1.1180	1.2763	1.3969	1.3163	1.1840	1.3449
MT (S)	13.3057	14.5704	13.7426	14.4265	14.9049	14.3852
+/- S.E.	0.9621	1.0633	0.9505	1.0861	1.0476	0.9958

The values are mean +/- S.E. of 6 observations. (*) indicates significant difference to the respective control ($P<0.05$). MT was injected (I.P.) 8 hours before the incubation (200 ug/100g).

Table 4.4
In vivo effects of methyltestosterone (MT) on intestinal absorption of amino acids (MT injected for 6 d).

a MUCOSAL (M) AND SEROSAL (S) CONCENTRATIONS (u Moles/ml)
SEROSAL/MUCOSAL RATIO (S/M)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	4.4396	4.6773	4.9206	4.6002	4.3583	4.3343
+/- S.E.	0.0277	0.1250	0.0243	0.0626	0.0312	0.1285
MT (M)	4.5340	4.7786	4.7893	4.6623	4.4846	4.3120
+/- S.E.	0.0169	0.1301	0.2781	0.1168	0.0699	0.0792
Cont (S)	3.5773	3.7010	3.8986	3.5627	3.3010	4.1096
+/- S.E.	0.0447	0.2145	0.0444	0.0581	0.0473	0.1223
MT (S)	3.8976*	4.1390*	4.4513*	3.9551	3.5793*	4.5950*
+/- S.E.	0.0373	0.0276	0.0950	0.0435	0.0191	0.1262
Cont (S/M)	0.8057	0.7947	0.7922	0.7746	0.7576	0.9512
+/- S.E.	0.0051	0.0657	0.0058	0.0115	0.0161	0.0535
MT (S/M)	0.8596	0.8677	0.9365*	0.8457	0.7985	1.0670*
+/- S.E.	0.5909	0.0297	0.0631	0.0225	0.0150	0.0437

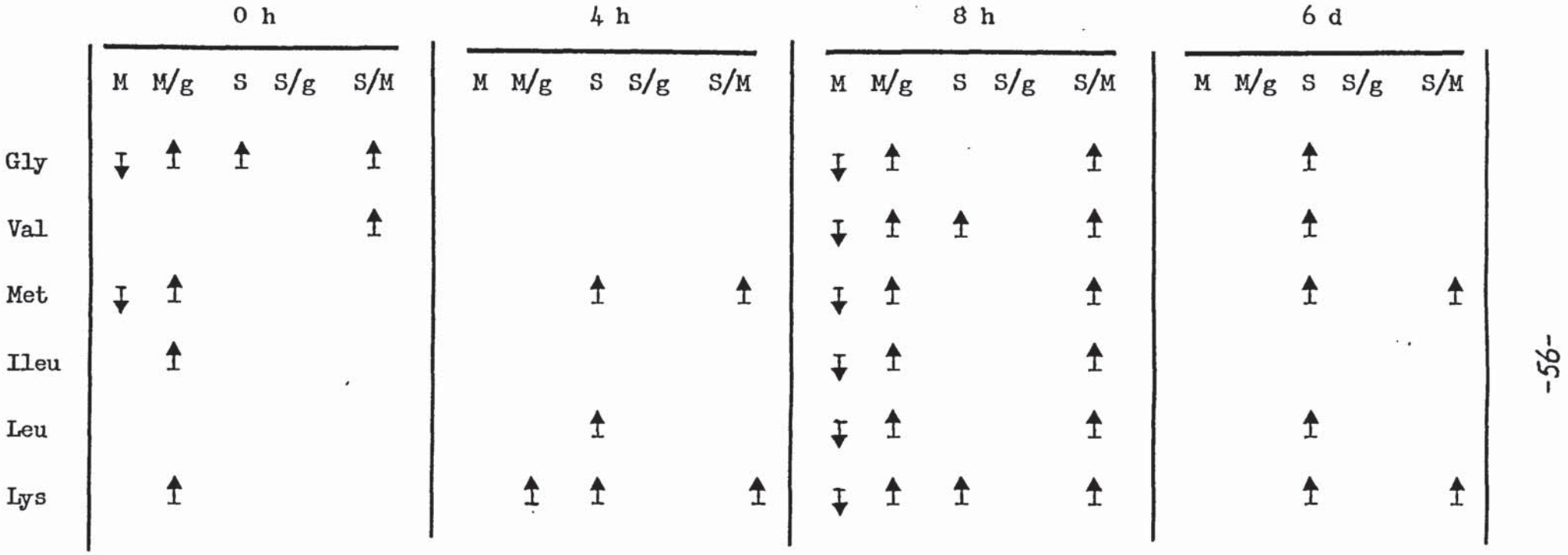
b MUCOSAL ABSORPTION PER GRAM (u Moles/g) (M)
SEROSAL CONCENTRATION PER GRAM (u Moles/ml/g) (S)

TREATMENT	Gly	Val	Meth	Ileu	Leu	Lys
Cont (M)	3.3453	5.1530	1.3012	4.3343	3.8107	3.7005
+/- S.E.	0.6608	1.8859	0.2341	0.70051	0.6675	0.3333
MT(M)	3.1885	4.1762	3.1545	4.9315	3.5145	4.6117
+/- S.E.	0.31126	2.2762	5.6876	0.5103	0.4962	0.1703
Cont (S)	15.7363	16.1229	17.2693	8.7260	14.6036	18.0156
+/- S.E.	2.1124	1.6759	2.7316	1.2284	2.2310	2.1917
MT (S)	20.1273	21.3237	22.8554	11.30087	18.4640	23.5520
+/- S.E.	2.4838	2.3961	2.2099	1.1754	2.1856	2.0814

The values are mean +/- S.E. of 3 observations. (*) indicates significant difference to the respective control ($P<0.05$). MT was injected (I.P.) every 2 days for 6 d (200 ug/100g).

Fig 4,1

Summary of the results showing the significant changes observed following the administration of 17α-methyltestosterone.



↑ indicates significant ($P<0.05$) increases in the parameters shown following the administration of the steroid.
↓ indicates significant ($P<0.05$) decrease in the final mucosal concentration (increase in solute disappearance from the medium). M: final mucosal conc. ; M/g: mucosal absorption per g intestine; S: final serosal concentration; S/g: final serosal concentration per g intestine; S/M: serosal/mucosal ratio; 0 h: MT was added to the incubation medium (200 µg/ml); 4 h: MT injection given 4 h before incubation; 8 h: MT injection given 8 h before incubation; 6 d: MT injection for 6 days, last injection 24 h before incubation. All injections were given at 200 µg/100 g body weight.

Effects of 17a-methyltestosterone and 17ß-oestradiol on intestinal transport and absorption of L-(14C)-leucine in vitro in rainbow trout (Salmo gairdneri).

SECTION 5

5.1. Introduction

The experiments described in Section 4 indicated that MT might exert a stimulatory effect on the transport of a mixture of amino acids by the intestine of rainbow trout, both when administered in vitro by direct addition into the incubation medium, and when administred in vivo, by injection. The present study was aimed to investigate this possibility, using a single amino acid (L-leucine) to circumvent the problems discussed previously. Furthermore, the technique of transport measurement was improved for greater sensitivity by using a 14C-labelled leucine to distinguish between the endogenous leucine and the absorbed amino acid from the medium. In addition, the influence of oestradiol on intestinal transport of leucine was studied for comparative purposes, since, there are few data on oestrogenic influences on gastrointestinal function. Alterations in fluid and solute absorption have been observed in a number of studies, and it appears that under certain conditions, oestrogens might influence the absorptive ability of the intestine. However, there is little consistency to be found in the literature regarding the oestrogenic influence on intestinal absorption. For example, Dunnet and Garnier (1953) have demonstrated increased fluid absorption following oestradiol and progesterone administration. In accord with this finding, Althausen (1949) reported reduced glucose absorption in ovariectomized rats. However, Ahmed-Sorour (1978) studied the intestinal transport in ovariectomized mice and found that ovariectomy increases intestinal transport of fluid and glucose, and that oestrogen-replacement therapy reverses this

condition. Furthermore, while Harralde, et al. (1966) reported significant increases in the intestinal absorption of glucose and glycine in pregnant rats, Harreros, et al. (1970) using the everted sac technique, found that diethylstilbestrol inhibited glucose accumulation in the serosal fluid, without effecting the mucosal entry of the sugar. In fish (rainbow trout), oestrogenization is without effect on the intestinal transport of calcium (Mugiya and Ichii, 1980).

The present results indicate that MT significantly enhances the active transport of leucine, both when added directly to the media, and when administered by injection, while E2 stimulates the amino acid transport only when injected for a prolonged period of time.

5.2. Materials and Method

5.2.1. Animals

Rainbow trout (Salmo gairdneri), of approximate weight 200g (range: 180-230 g) were maintained in 250 l fibre-glass tanks at 14 +1°C, as described in Section 3.

5.2.2. Hormone administration

17a-Methyltestosterone (MT) or 17B-oestradiol (E2) were administered in vivo by injection (200 ug/100g body weight) or in vitro by direct addition to the incubation medium (200 ug/ml), as described in Section 4. For in vivo administration, two groups of 12 trout were injected (intramuscular) with MT or E2, every two days for 10 days. A third group of trout received the same volume of solvent (control injected). The effects of Tween-80 were investigated in control experiments, and the control incubation media contained the same volume of solvent with Tween-80 (control+Tween) and without Tween-80 (control-Tween).

5.2.3. Incubation medium

The incubation medium used in the present study was based on the trout Ringer, modified from Stokes and Fromm (1964) for higher osmolarity, closer to trout serum (Fig. 5.1). The Ringer solution was composed of (mMoles/l.): NaCl 125.8, KCl 4.3, CaCl2 1.0, MgSO4 1.2, KH2PO4 3.2, and Na2HPO4 14.5 (PH 7.3; 279 mOsm/l.). To the

Ringer solution was added 1 mM ATP, 5 mM L-leucine , and 0.05 uCi/ml L-(U-14C)-leucine (specific activity 300 mCi/mMol; Radiochemical Centre, Amersham.). The addition of ATP has been shown to increase active transport of L-lysine by trout intestines (Hokazono et al., 1979) and was thus employed in the present study to ensure availability of sufficient energy for leucine transport, since glucose was excluded from the medium due to its reported interference with amino acid absorption (Section 4). The concentration of leucine was chosen to lie within the range commonly employed for amino acid transport studies in vitro (Ingham and Arme, 1977). Serosal and mucosal solutions were identical at the begining of the incubation period, and in those experimentswhere the steroids were added directly to the medium (MT+; E2+), each was present in both solutions.

5.2.4. Preparation and incubation of gut sacs

The intestine was removed between the midgut (just posterior to the pyloric caeca) and rectum, and everted as described in Section 4. The sac was filled with 0.8 ml of incubation medium (serosal solution), and the everted sac was incubated at 14 $\pm$ 1 ^{o}C for a total of 60 min. in 10 ml of incubation medium (mucosal solution), which was gassed continuously with 95% O2 : 5% CO2. An additional experiment was conducted in which non-everted sacs were used to determine the direction of leucine transport and extent of leakage. In this case, 14C-leucine was added only to the serosal solution.

5.2.5. Media and tissue analysis

A sample of the mucosal solution (200 ul) was taken at 0, 20, 40 and 60 min, and a sample of serosal solution, after 60 min. The volumes of serosal and mucosal solutions were determined at the termination of the incubation, and each sac was then blotted dry. The ligature thread was removed and a 1 cm section of mid and hind gut were extracted for 24 h in 1 ml 80% ethanol. Each sample was dried for 24 h at 90°C and the ethanol-extracted dry weight obtained (modified from Bamford and James, 1972). To determine the ethanol-extracted dry weight of the whole intestine, the remaining tissue was treated in similar fashion. For radioactive counting, duplicate 100 ul aliquots of the ethanol extracts, and serosal and mucosal media, were added to 10 ml of scintillation fluid (4g 2,5-diphenyloxazole, 500 ml toluene, 500 ml ethoxyethanol). The samples were counted for 20 min in a Packard Tricarb 2660 scintillation counter, and the results corrected for quenching. The following formulae (modified from Smith and Lane, 1971) were used to calculate the mucosal absorption (I), and SLI (II).

$$A = (C1.V1 - C2.V2)/ W \quad \text{.....(I)}$$

$$B = (C2.V2 - C1.V1)/ W \quad \text{..... (II)}$$

$$C = (\text{DPM sample} / \text{DPM standard}) \times \text{concentration standard} \quad \text{..(III)}$$

A: Mucosal absorption (u moles/g ethanol dry wt.)

B: Serosal leucine increase (u moles/g ethanol dry wt.)

C1: initial concentration (u moles/ml), (III)

C2: concentration after t min incubation (III)

V1: initial volume (ml)

V2: volume after t min incubation (ml)

W: ethanol-extracted dry weight

DPM: decomposition per min

5.2.6. Presentation of results

Sample count rates (DPM) were used to calculate the following parameters:

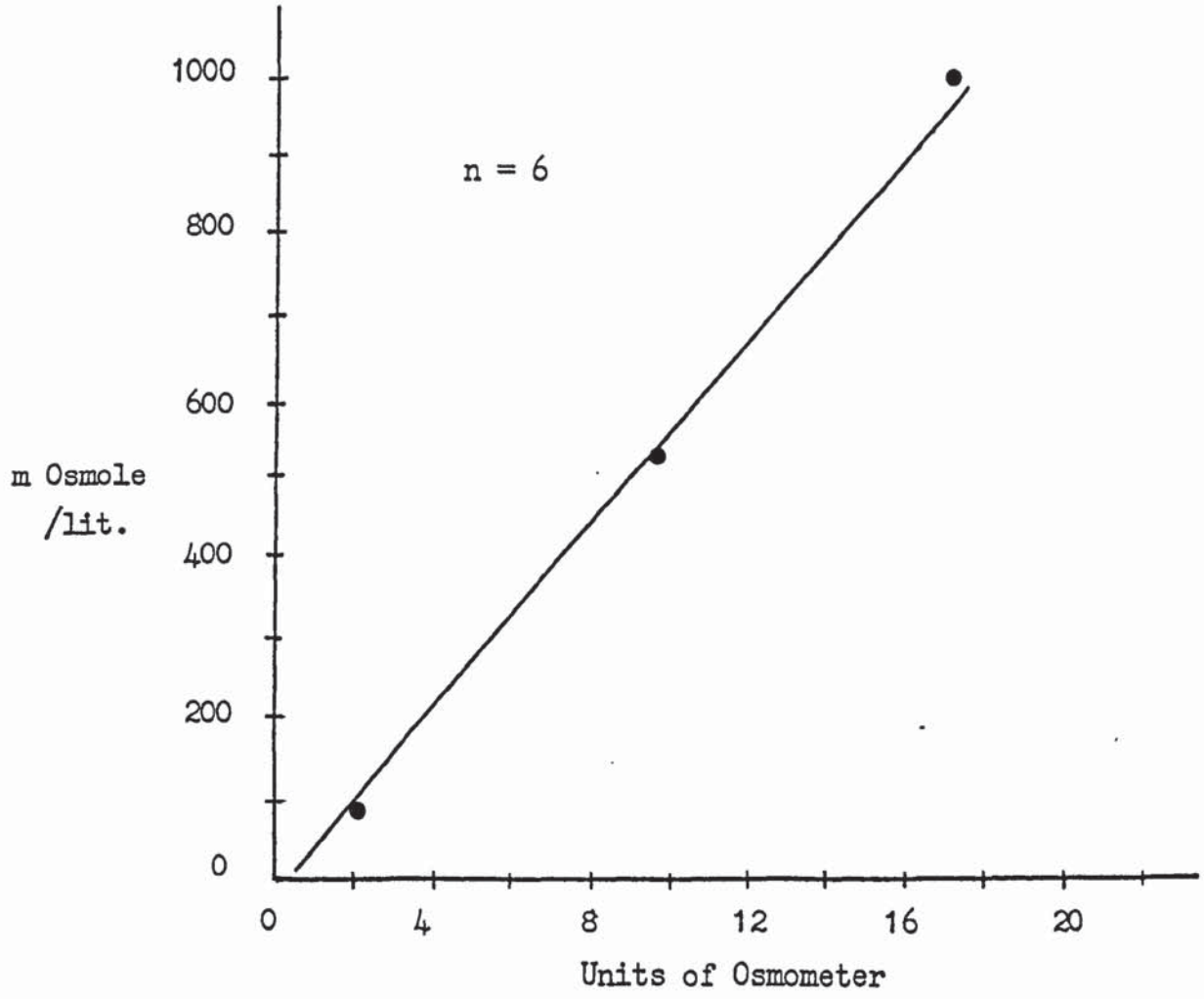
mucosal absorption = u moles leucine absorbed from the mucosal solution per g of ethanol-extracted dry weight of whole intestine during 20, 40 and 60 min of incubation; serosal leucine increase (SLI) = u moles leucine appearance in the serosal solution per g of ethanol-extracted dry weight of whole intestine after 60 min of incubation; serosal/mucosal (S/M) ratio = final serosal concentration/final mucosal concentration; intestinal accumulation = u moles leucine accumulated in the intestinal tissue (mid gut or hind gut) per g of ethanol-extracted dry weight after 60 min of incubation.

5.2.7. Statistical analysis

Statistical analysis of the results for mucosal absorption was performed using two-way analysis of variance in conjunction with split-plot design, F-test and T-test (Ridgman, 1975), and regression analysis with T-test (Bailey, 1981). Results of intestinal accumulation, SLI and S/M ratios were compared using student's T-test and F-test. Differences were considered significant when $P<0.05$

Fig. 5.1

Standard curve for determination of osmolarity.



Medium	Osmolarity (m Osm./l)
Rainbow trout serum	285 ± 5.7
Boge et al. (1977)	263 ± 6.4
Stokes & Fromm (1964)	271 ± 5.9
Habibi et al. (1983)[1]	279 ± 4.8

[1] Ringer solution used in the present study.
Values are mean ± S.E. of 6 observations

5.3. Results

5.3.1. Control and validation experiments

Experiments were conducted initially to test the validity of the everted gut sac technique for the study of L-(14C)-leucine transport. For this purpose, everted sacs were obtained either from untreated fish, or from control-injected fish. The results indicated that radioleucine was transported against its concentration gradient, giving S/M ratios greater than 1.0 in every instance. The possibility of leakage from the ligature sites of the gut sacs was tested in a further series of experiments using non- everted sacs prepared from untreated fish. The values obtained (u moles /g /60 minutes) were: 9.88 ±1.40 for serosal absorption; 0.17 ±0.01 for mucosal leucine increase, and 2.53 ±0.32 for intestinal accumulation (mean ±S.D. of 3 observations). Since the mean values were less than the calculated S.D. of the corresponding values of the everted sacs, under the present conditions, no significant quantity of leucine was being transported from the serosal to the mucosal solution, nor was there any evidence of leakage from the ligature sites. The effect of Tween 80 was also investigated in a further experiment and was found to increase significantly ($P<0.05$) mucosal absorption of radioleucine after 40 minutes and SLI (Table 5.1), but was without effect on intestinal accumulation when compared with control sacs incubated in the absence of Tween 80 (control-Tween). For this reason the effects of MT+ and E2+ were analysed with reference to

control sacs incubated in media containing Tween 80.

5.3.2. Effects of MT and E2 on mucosal absorption of radioleucine, SLI and S/M ratio

The results presented in Table 5.1 show the effects of MT and E2 when added into the incubation medium containing everted sacs taken from untreated fish (control-Tween, control+Tween, MT+ and E2+). Addition of MT to the incubation media (MT+) significantly ($P<0.05$) increased mucosal absorption after 20 min., when compared with the controls, but was without effect after 40 and 60 min. Direct MT addition also significantly ($P<0.001$) increased SLI and S/M ratios. Addition of E2, however, was without effect on any of the parameters studied. Injection of either MT or E2 whilst exerting no effect on mucosal absorption of radioleucine, nevertheless significantly ($p<0.001$) increased SLI and S/M ratios when compared with those of control injected fish (Table 5.2)

5.3.3. Effects of MT and E2 on intestinal accumulation of radioleucine

Addition of MT and E2 to the incubation media was without effect on overall intestinal accumulation of radioleucine when compared with the control fish (Fig. 5.2.). Analysis of mid and hind gut radioleucine content however, revealed a generally higher level in the mid gut of control, MT+ and E2+ groups, although the difference was only significant ($P<0.05$) in the MT+ group. By contrast, MT

injection elicited significant increases in overall intestinal accumulation of radioleucine, with a significantly higher ($P<0.05$) level in the hind gut region. No regional variations were however observed in the E2 injected group, but a significantly ($P<0.05$) higher radioleucine accumulation was detected in the mid gut region when compared to those of control injected fish (Fig. 5.3).

5.4. Discussion

The viability of the everted gut sac preparation used under the present conditions was indicated by S/M ratios greater than 1.0 in all experiments. Thus, in agreement with previous observations (Ward, 1968; Ingham and Arme, 1977), L-leucine was transported against its concentration gradient.

5.4.1. Effects of Tween-80

The effect of Tween 80 (Polysorbate 80), was also investigated in control experiments. It is seen in the results that Tween 80 significantly enhanced mucosal absorption and SLI, without effecting the efficiency of transport, as indicated by S/M ratios not being significantly different in the control-Tween and control+Tween groups. This suggests that Tween 80 may exert its action by increasing passive diffusion of the amino acid without significantly affecting the active transport mechanism. Furthermore, regression analysis of the mucosal absorption curves obtained from MT+, E2+ and control+Tween groups indicated a similar interaction of absorption with time in the three groups. Thus, a significant complexing interaction between Tween 80 and steroids is unlikely. These observations are supported by those of Levy and Anello (1968), who reported similar enhancing effects of Polysorbate 80 on secobarbital absorption in goldfish. The present data is also in accord with earlier mammalian studies on the effects of Tweens on fat and vitamin absorption (Wiseman, 1964).

5.4.2. Effects of methyltestosterone

The results of steroid treatment indicated that MT stimulates the intestinal transport of L-leucine both when added directly to the medium (12.3 % increase), and when injected for 10 d (13.8 % increase) as apparent from the S/M ratios. However, comparison of the absorption parameters revealed that MT enhances the amino acid transport, either by increasing mucosal absorption and SLI when added in vitro or by increasing intestinal accumulation and SLI when administered in vivo by injection (Table 5.3). Results of in vitro treatment suggest a rapid action of MT on the mucosal epithelial cells. However, it is not certain whether the steroid action is directed on the brushborder membrane or on the basolateral membrane. Both in vitro and in vivo treatment of MT resulted in an increased serosal leucine transfer against the concentration gradient, suggesting perhaps that the steroid might influence the energized transport of the amino acid. Furthermore, the results obtained from the MT+ indicates that MT might stimulate amino acid absorption through mechanisms other than those associated with the classical RNA-protein axis. However, it appears from the results that MT might have different mechanisms of action for its 'long-term' and 'short-term' effects, since differences were observed between the transport parameters measured in the MT+ and MT-injected groups. In this context, the results of MT injection could be taken to indicate an in vivo action of MT on the intestine, including a possible effect on smooth muscle proteogenesis, and hence, increased intestinal accumulation. However, it will be noted that the method used to extract 14C-leucine from the gut tissue removed only solute leucine and not the incorporated amino acid. It

would nevertheless be of relevance to investigate this possibility further since, in trout, MT has been shown to stimulate amino acid incorporation into skeletal muscle protein (Matty and Cheema, 1978). Furthermore, the possibility that, in vivo, secondary factors may have added to, or potentiated, the steroid- induced effects is worthy of consideration.

5.4.3. Effects of oestradiol

Administration of E2 in vitro was without a significant effect on leucine transport and, although the effects of E2 injection were similar to MT on SLI, a significantly smaller stimulation of L-leucine accumulation occurred. Moreover, the mucosal absorption in the E2-injected was lower than those observed in the MT-injected group, although neither were significantly different to the controls. The stimulatory effects of oestradiol observed in the present study is in accord with the report that oestradiol injection increases both the potential difference and short- circuit current across the intestine of the male mouse intestine (Matty, 1964). Of particular note is the discrepancy between mucosal absorption and SLI in the E2-injected group. In this group, whilst little variation is apparent in mucosal absorption, there is a significant increase in SLI when compared to the controls. One possible explanation for this might be the large variations obtained. Thus, no significant differences were obtained between the mucosal absorption of the E2-injected and MT-injected fish. In addition, some discrepancies are evident concerning the recovery of 14C-L-leucine between mucosal absorption and serosal appearance. This apparent lack of correlation

has also been reported by other workers (Stokes and Fromm, 1964; Smith, 1969; Smith and Lane, 1971). It is known however, that L-leucine, an essential amino acid for fish as well as mammals, retains 90% or more of its integrity during transport across the trout intestine (Ingham and Arme, 1977; present study, Section 7). Therefore, it seems unlikely that the discrepancies were due to metabolism of the amino acid. Since, the quantity of 14C-L-leucine not recovered by ethanol extraction, represented the 'apparent' incorporated amino acid in tissue protein. One further factor which may be of significance is the extent of uptake and metabolism of the steroids by gut tissue. Evidence that intestinal tissue is capable of substantial steroid uptake in fish has been provided in previous studies (Lone and Matty, 1981a), although details of steroid metabolism per se by gut tissue in vitro are lacking.

5.4.4. Regional variations of intestinal accumulation

The tendency for a higher leucine accumulation in mid gut than hind gut in the untreated controls, although not statistically significant, nevertheless is in accord with previous observations on the transport of this and other amino acids by fish intestines (Neff and Musacchia, 1967; Ingham and Arme, 1977; Boge et al., 1979). Regional patterns of leucine accumulation have also been found in the rat small intestine (Larsen et al., 1967). Moreover, the studies of Boge et al.(1979) on glycine transport in trout in vivo showed that regional accumulation was a function of amino acid concentration; a higher uptake in hind gut at low concentrations (0.5-2.0 mM) and a preferential accumulation in mid gut at higher

concentrations (2.0-10.0 mM). Since net water fluxes were found to be higher in mid gut, irrespective of glycine concentration, and no regional distribution variations in oxygen consumption or quantitative differences in mucosa were apparent (Boge, 1972), it was concluded that these factors were unlikely to be responsible for the pattern of accumulation observed. In the present study, the tendency for a higher mid gut leucine accumulation was also apparent in the MT+ and E2+ group whereas in the injected fish, this pattern was reversed in the case of the control and MT injected groups. Overall, the greatest impact on regional and total accumulation, whether directly or indirectly, was clearly exerted in the MT injected groups. In view of the results of Boge et al. (1979) outlined earlier, it would be of value to investigate further steroid effects on absorption and accumulation over a wide range of amino acid concentrations.

In general, the present results are in accord with the observations reported in Section 4, and they suggest a greater effeciency of radioleucine transport in the MT+, MT and E2 injected groups. These results, when viewed in the context of the known growth-promoting effects of anabolic androgenic steroids in fish, are of particular interest and are in accord with those of Lone and Matty (1981) and Ince, et al. (1982) who demonstrated increased proteolytic activity in carp intestine, and enhanced protein digestibility and assimilation in rainbow trout following administration of anabolic-androgenic steroids.

Summary:

(1) MT significantly enhances L-leucine transport, either by increasing mucosal absorption and SLI when added directly to the media, or by increasing intestinal accumulation and SLI when administered in vivo by injection.

(2) It is suggested that the in vivo effects of MT on intestinal accumulation might be partly the result of increased amino acid accumulation by the smooth muscle layer, while the in vitro effects of MT resulted from a direct effect of the steroid on intestinal epithelial cells.

(3) Whereas injection of E2 elicited significantly higher intestinal accumulation and SLI, addition of the steroid to the media was without effect on any of the parameters studied.

(4) Of additional interest was the observation that Tween-80 increases the intestinal transport of leucine. It was suggested that Tween-80 might increase the passive diffusion of the amino acid.

Table 5.1.

In vitro effects of methyltestosterone and oestradiol on the mucosal absorption of L-leucine, serosal leucine increase (SLI) and serosal/mucosal (S/M) ratio.

		u Moles of leucine absorbed/g ethanol dry weight				
		SAMPLING TIME (MIN)				
TREATMENT	(n)	20	40	60	SLI	S/M
Con-TW	(4)	64.810^{a}	74.458^{bg}	78.200^{cg}	9.431^{h}	1.372^{p}
+/- S.D.		16.777	16.429	12.953	3.957	0.043
Con+TW	(10)	90.041^{a}	112.209^{be}	132.662^{d}	14.879^{j}	1.449^{p}
+/- S.D.		22.905	27.574	32.841	2.740	0.087
MT+	(10)	111.532^{f}	134.497^{e}	149.689^{d}	19.372^{k}	1.628^{r}
+/- S.D.		21.200	29.287	26.868	2.967	0.110
E2+	(10)	87.960^{a}	108.812^{be}	126.493^{d}	14.154^{j}	1.413^{p}
+/- S.D.		22.770	25.957	27.814	2.094	0.077

Superscripts containing dissimilar symbols are significantly different. Differences were considered significant when $P<0.05$. Con-TW: Control medium without Tween-80; Con+TW: Control medium contain Tween-80; n: sample size (number of fish).

Table 5.2.

Effects of intramuscular injection of methyltestosterone and oestradiol on mucosal absorption of L-leucine, serosal leucine increase (SLI), and serosal/mucosal (S/M) ratio.

		u Moles of leucine absorbed/g ethanol dry weight				
		SAMPLING TIME (MIN)				
TREATMENT	(n)	20	40	60	SLI	S/M
Con inj	(10)	65.882^{a}	88.094^{b}	99.783^{c}	18.279^{d}	1.343^{f}
+/- S.D.		22.564	24.188	22.730	3.313	0.061
MT inj	(10)	86.902^{a}	101.419^{b}	113.232^{c}	25.186^{e}	1.529^{g}
+/- S.D.		25.672	28.267	28.911	5.109	0.092
E2 inj	(10)	69.939^{a}	86.178^{b}	97.996^{c}	26.501^{e}	1.520^{g}
+/- S.D.		27.900	26.711	32.920	5.853	0.113

Superscripts containing dissimilar symbols are significantly different. Differences were considered significant when $P<0.05$. Con inj: control injected; MT inj: MT injected (200 ug/100); E2 inj: E2 injected (200 ug/100g).

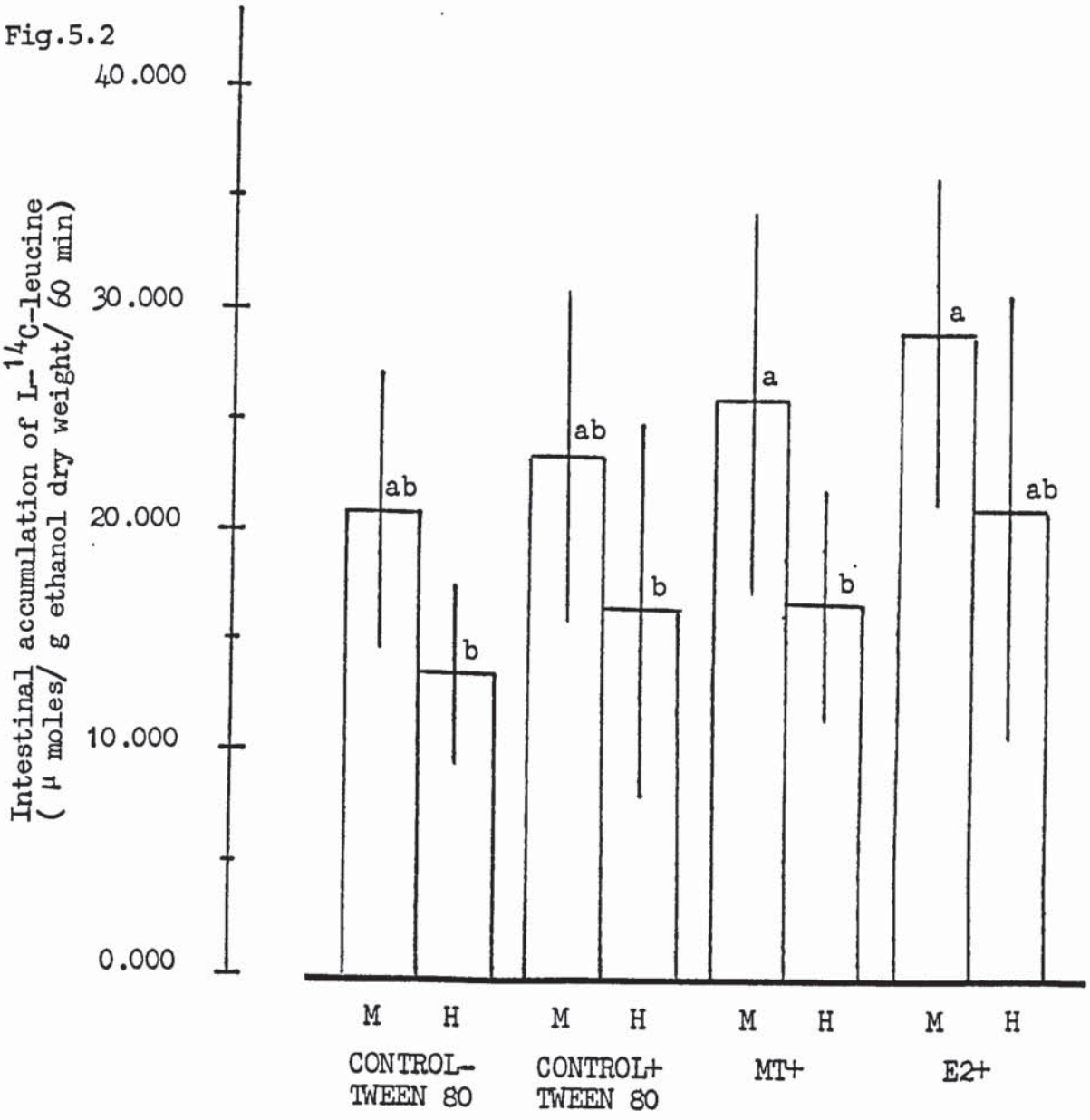
Table 5.3

Percentage difference in transport parameters following steroid administration.

	Percentage change						
	mucosal absorption incubation time (min)					intestinal accumulation	
	20	40	60	SLI	S/M	Midgut	Hindgut
MT+	+23.86^{s}	+19.86	+12.83	+30.19^{s}	+12.35^{s}	+9.66	+1.88
MT injected	+31.90	+15.12	+13.47	+37.78^{s}	+13.84^{s}	+37.78^{s}	+139.07^{s}
E2+	-2.31	-3.02	-4.65	-4.87	-2.48	+23.33	+28.40
E2 injected	+6.15	-2.17	-1.79	+44.98^{s}	+13.17^{s}	+48.30^{s}	+19.79

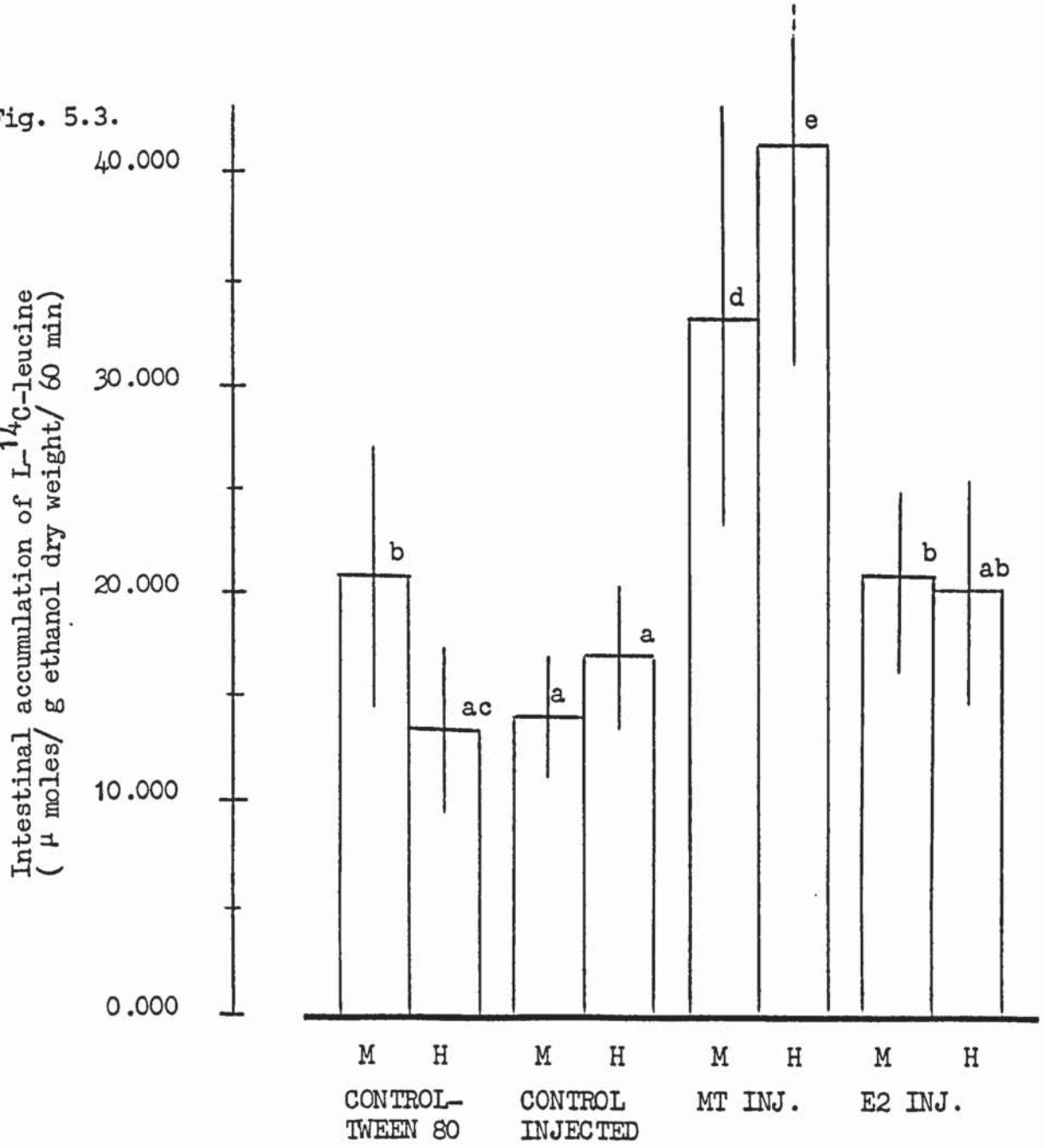
Superscript (s) indicates significant difference with respect to control. Values are percentage increase (+), or decrease (-) in mucosal absorption, SLI, S/M and intestinal accum ulation with respect to control values [% change = (Mean$_t$ / Mean$_c$).100-100]; where; Mean$_t$: mean value of the steroid treated ; Mean$_c$ = mean value of the corresponding control.

Fig.5.2



In vitro effects of MT and E2 on L-leucine accumulation by everted gut sacs of rainbow trout (Salmo gairdneri). Values are mean +/- S.D. of 10 observations for Control+Tween, MT+ and E2+ and 4 observations for Control-Tween. Superscripts containing dissimilar symbols are significantly ($P<0.05$) different. M: mid gut; H: hind gut; MT+: MT added to the incubation medium (200 ug/ml); E2+: E2 added to the incubation medium (200 ug/ml).

Fig. 5.3.



Effects of intramuscular injection of MT and E2 on L-leucine accumulation by everted gut sacs of rainbow trout (Salmo gairdneri). Values are mean +/- S.D. of 10 observations for Control injected, MT injected and E2 injected and 4 observations for control-Tween. Fish were injected with MT or E2 (200 ug/100g), every two days for 10 d. Control injected fish received the same volume of solvent. MT inj.: MT injected; E2 inj.: E2 injected.

Effects of steroids and sex reversal on intestinal absorption of L-14C-leucine in vivo, in rainbow trout, S. gairdneri .

Section 6

6.1. Introduction

The present study has shown that methyltestosterone and, to a lesser extent, oestradiol enhance the transport of amino acids by the intestine of rainbow trout (section 5). Stimulation of leucine transport after in vitro administration of methyltestosterone was associated with increases in mucosal absorption and serosal transfer. The increase in mucosal absorption was apparent 20 min after exposure of the mucosal and serosal surface to the steroid, and it was suggested that MT might exert a direct action on the mucosal epithelial cells of the intestine. The experiments presented in this Section were undertaken to further investigate this possibility using a different experimental approach in which the total period of steroid exposure was extended to 120 min, by way of an in vivo gut perfusion technique. The dose of steroid was reduced to 50 ug/ml to circumvent the use of surfactant, and to examine the effects of the steroids at a lower dose level. A further advantage of this technique was that the intestinal absorption of the amino acid could be studied on intact, unanaesthetised fish after exposing the mucosal surface to the steroid, a condition which simulates oral administration of the steroid for inducing anabolic response in fish (Donaldson, et al., 1979). Furthermore, in view of the increasing importance attached to the production of all-female trout by using a cross between masculinized genetically female (XX^{δ}) and normal female trout (Johnstone et al., 1979), additional studies were conducted on intestinal absorption of L-leucine in sex-reversed fish to determine any variations from normal fish.

6.2. Materials and Methods

6.2.1. Experimental animals

For the steroid treatment studies, rainbow trout (Salmo gairdneri) of approximate weight 220g (range: 190-25) were used. The masculinized, genetically female rainbow trout (XX♂), of the same size and range, were generously provided by Avon Fisheries, Pewsey, UK. The sex-reversed fish were produced by oral administration of MT (3 ppm) during the first 90 days of feeding (Johnston, et. al., 1978). Fish were maintained as described in Section 3 on a commercial diet, but food witheld 24 h prior to catheterization.

6.2.2. Gut perfusion technique

Gut catheterization

The perfusion procedure used in the present experiment was based on that described by Boge et al (1977a) but with certain modifications. Briefly, a section of intestine, which included the mid- and hindgut (between 0.5 cm posterior to pyloric caeca and 0.5 cm anterior to anus) was catheterized using two translucent vinyl catheters (int. dia. 3.0; ext. dia. 4.25 mm; Portex Ltd., Kent, Uk.), and to each of which was attached a 1 cm grooved glass cannula (int. dia. 3.5; ext. dia. 4.0 mm). Insertion of the catheters was performed with trout under light benzocaine anaesthesia, through a 1 cm lateral incision for the anterior

attachment, and a 1 cm ventral incision for the posterior attachment (the intestinal artery and hepatic portal vein remained intact and unobstructed). The incision wounds were closed with individual silk sutures (Fig. 6.1, 6.2 & 6.3), and the fish placed in a rigid, plastic mesh chamber (5x20x35 cm) immersed in a 8 l rectangular plastic tank. The tank was supplied with a temperature-controlled (14 +/-1°), recirculating system, and vigorous aeration.

6.2.3. Perfusion protocol

Fish were allowed to recover for 24h following gut catheterization (i.e. a total of 48h food deprivation), after which the catheters were connected to an open-circuit (non-recycling) perfusion system, which included a multichannel peristaltic pump (Watson Marlow), as shown diagramatically in Fig. 6.4. The intestine of the unanaesthetised trout was then perfused five times at 30 min intervals with 1 ml of perfusion medium (perfusion period 1 to 5), during a total experimental period of 130 min, at a rate of 0.1 ml/min (10 min for each perfusion), at 14 ±1 °c.

6.2.4. Perfusion medium

The composition of the perfusion medium used was as follows (mMoles/l): NaCl 125.8; KCl 4.3; CaCl2 1.0; MgSO4 1.2; KH2PO4 3.2; Na2HPO4 14.5 (PH 7.3; 279 mOsm/l). To this was added 5 mM L-leucine, and 0.05 uCi/ml L-(U-14C)-leucine For steroid treatment either 17a-methyltestosterone (MT) or 17B-oestradiol (E2) was dissolved in absolute ethanol (10 mg/ml) ; then diluted further in perfusion medium to give a final concentration of 50 ug/ml. At this

concentration, the steroids could be suspended completely in the aqueous medium without resort to the use of surfactants (cf. Section 4). These concentrations are within the effective oral dose range of related steroids, known to produce anabolic responses in salmonids (Donaldson, et. al., 1979). Perfusion of the XX♂ fish was carried out using a steroid-free medium, but containing the same volume of solvent as for the other groups (MT-treated, E2-treated, control).

6.2.5. Medium and Tissue analysis

During each perfusion period (1 to 5), the total perfusate was collected, and the volume (V) determined. A sample (100 ul) of the perfusate was then counted for 14C activity. At the termination of the final perfusion period (period 5; 130 min), fish were sacrificed by immersion in benzocaine (1 : 4000) and 1 ml of blood withdrawn from the ductus Cuvieri with a heparinised syringe. Plasma was separated by centrifugation and a sample (100 ul) counted for 14C activity. The section of intestine between the two catheters was removed, blotted dry, and divided into a mid- and hindgut section. Samples of dorsal skeletal muscle, liver and spleen tissue were also removed, and each gut and tissue sample (approximately 800 mg) was extracted in 1 ml 80% ethanol for 24h, and the extracts counted for 14C activity. Each sample was then dried for 24h at 90°, and the ethanol-extracted dry weight determined.

6.2.6. Calculations

Sample count rates, in terms of decomposition per minute (DPM), were used in the following formulae to calculate leucine absorption.

$$Cs = (DPMs \cdot Cst) / DPMst \quad .....(I)$$

$$A = (Cs1 \cdot V1 - Cs2 \cdot V2) / W \cdot T \quad .....(II)$$

where,

Cs : Concentration of sample (umoles/ml); Cst: Concentration of standard (umoles/ml); DPMs: DPM sample; DPMst: DPM standard; A: Intestinal absorption (II); Cs1: Initial concentration of perfusion medium (I); Cs2: Final concentration of perfusate (I); V1: Initial volume of perfusion medium (1 ml); V2: Final volume of perfusion medium (ml); W: Ethanol-extracted dry weight of perfused intestinal section (mid- and hingut); T: Total time of perfusion (10 min at a rate of 0.1 ml/min);

6.2.7. Presentation of results

Intestinal absorption : uMoles L-leucine disappearance from perfusate per g ethanol-extracted dry weight of intestine per minute (II); Intestinal accumulation : uMoles L-leucine solute extracted per g ethanol-extracted dry weight per 120 minutes; Muscle 14C accumulation : uMoles 14C solutes accumulated per g ethanol-extracted dry weight per 120 minutes. Similar terms were used to express 14C accumulation in liver and spleen tissue; Plasma 14C concentration: uMoles 14C solutes per ml plasma. It is important to emphasise that the results of muscle, liver and spleen accumulation,

and plasma 14C content have been expressed in terms of 14C solutes only. Intestinal accumulation, however, was expressed in terms of 14C-leucine accumulation, since there is good evidence that this amino acid is not metabolised to any significant degree in the intestinal epithelium of trout (Section 7).

6.2.8. Statistical analysis

A two-way analysis of variance (ANOVA) in conjunction with split-plot design, F-test and T-test (Ridgman, 1975) were used for statistical analysis of the absorption results. Differences between intestinal accumulation and tissue accumulations of the solutes were analysed using a one-way analysis of variance(Duncan multiple range test; Duncan, 1955). Differences were considered significant when $P<0.05$.

6.3. Results

6.3.1. Intestinal absorption of L-14C-leucine

Perfusion experiments were performed in four groups of trout; controls, MT-treated, E2-treated and sex-reversed ($XX\delta$) fish. Figure 6.5 shows the intestinal leucine absorption data for each group, at the five separate perfusion periods of 10 min (period 1 to 5) over 120 min. Analysis of the combined results (ANOVA) indicated that over 120 min, radioleucine absorption was significantly greater in the MT-treated trout than the controls ($P<0.005$). Further statistical analysis however revealed that, whereas in periods 1 and 2, leucine absorption (uMoles/g /min) was significantly higher in MT-treated fish than the controls (period 1: 3.37 ± 0.37 vs. 1.60 ± 0.16 uMoles/g /min, $P<0.005$; period 2: 2.66 ± 0.162 vs. 1.18 ± 0.41 uMoles/g/min, $P<0.01$), in periods 3 to 5, the values although higher were not statistically different from the controls. A comparison of the control absorption data with those of the E2-treated and sex-reversed fish, indicated that neither treatment significantly affected leucine absorption at each of the perfusion periods. A further observation of importance was that in all groups, including the controls, leucine absorption showed a gradual decline with time. This effect is likely to have been more the result of some degree of tissue damage during luminal perfusion, or stress, rather than a possible result of perfusate pH change, since in the present preparation, the perfusion medium was not recycled .

6.3.2. Intestinal accumulation

Figure 6.6 shows the intestinal (mid- and hindgut) accumulation of radioleucine in the various groups at the termination of the perfusion experiments (period 5). It can be seen that neither MT nor E2 treatment exerted any significant effect on either total or regional accumulation of the amino acid when compared with the controls. In the sex-reversed group, however, total intestinal accumulation was significantly higher than in the controls ($P<0.05$), together with a marked regional accumulation in midgut ($P<0.05$). Such regional differences in radioleucine accumulation were also observed previously using everted gut sacs of trout, although in these in vitro studies, the extent of accumulation was considerably higher than in the present experiments (cf: Section 5).

6.3.3. 14C solutes in skeletal muscle, liver, spleen, and plasma

Figures6.7 and 6.8 show the 14C solute accumulation in dorsal skeletal muscle, liver and spleen, and the 14C content in plasma after 120 min of perfusion (period 5). Although the accumulation of 14C solutes in muscle was generally higher in the MT and E2 groups than the controls, the differences were not statistically significant. However, a significantly higher accumulation of label was found in the skeletal muscle of the sex-reversed group ($P<0.05$). Comparison of the results obtained from the steroid treated, sex-reversed and controls revealed no significant variations for 14C accumulation in liver and spleen. Plasma 14C content after 120 min is shown partly to demonstrate that the surgical interventions required for gut catheterization did not effect the normal routes of transfer of leucine from intestinal lumen to blood.

6.4. Discussion

6.4.1. Effects of steroids on leucine absorption

The present results have shown that MT significantly increases L-leucine absorption by the intestine of rainbow trout, in vivo. In addition, it is apparent that MT exerts a similar effect on intestinal transport when present at a smaller concentration (50 ug/ml) than that observed previously, using everted gut sacs of trout, in which direct addition of MT (200 ug/ml) to the incubation medium enhanced mucosal absorption of the amino acid after a 20 min exposure period (Section 5). The in vivo technique, however, more closely simulated the situation when trout are fed a MT-supplemented diet, and thus provided data of comparative value. Furthermore, the present work established that exposure of the apical membrane (brush border) to the steroid is sufficient to evoke MT-stimulated leucine transport. However, it should be noted that absorption of MT after a sort time-lag would expose other parts of the enterocytes (basolateral membrane and cytosolic organelles) to the steroid. Taken together, these results suggest that MT exerts a relatively rapid action at the mucosal epithelial level, whilst bearing in mind that MT absorbed from lumen to blood may also have contributed to some degree. An alteration in membrane fluidity by a non-specific action of the steroid is considered unlikely to account for these effects, since a similar action of E2 was not detected either in vitro or in vivo. In this context however, a more specific action of MT on the apical membrane is a possibility, and/or an increase in

the activity of enzymes associated with intestinal transport, since there is evidence that anabolic-androgenic compounds can modify the activity of some gastrointestinal enzymes (Kochakian, 1976). In addition, the results obtained in Section 5 demonstrated an increase in the active transport of leucine following the administration of MT in vitro. Thus, the results obtained in the present experiment, and those reported in Section 5 suggest a possible effect of MT on the energized transport of the amino acid, presumably by effecting enzymes or other cellular mediators. A further possibility which should be considered in any in vivo preparation is an effect on intestinal blood flow which would favour enhanced nutrient absorption. The latter however, does not explain MT action in vitro. The mode of action of MT, in the context of enhanced amino acid absorption, is examined in more detail in Section 7.

6.4.2. Intestinal and tissue accumulation

Also in accord with the previous in vitro findings was the lack of effect of MT or E2 on intestinal accumulation of L-leucine. However, unlike the in vitro preparations, in the present experiment the circulation was intact, and movement of the 14C solutes into blood would generate a concentration gradient across the basolateral membrane. Thus, in the present in vivo condition, the lack of difference in amino acid accumulation could also be the result of a higher rate of leucine entry into the vascular system in the MT-treated group, possibily due to a greater concentration gradient produced by MT-stimulated increase in luminal absorption. It is

however not certain to what degree the concentration gradient across the basolateral membrane effects the movement of leucine through this membrane. In this context, both active transport and passive diffusion of amino acids across the basolateral membrane have been demonstrated in higher vertebrates (see Munck, 1981). The present values obtained for leucine accumulation were approximately nine-fold lower than those found previously in vitro (cf: Section 5). This apparent discrepancy is, nevertheless, readily explicable on the basis of a fundamental difference between the two experimental conditions. In the present in vivo studies, intestinal blood circulation remained intact (as evidenced by appearance of 14C solutes in plasma), thus leucine could be freely taken into blood after transepithelial transport. By contrast, in non-stripped in vitro preparations (e.g. everted gut sacs), absorbed solutes must necessarily pass through submucosa, smooth muscle and serosa, before appearing in the serosal compartment. Inspite of this difference, it is apparent nevertheless, that the same pattern of regional accumulation of leucine (higher in mid- than in hindgut), is found both in vivo and in vitro, thus conforming to that which has been observed by other workers (Ingham and Arme, 1977; Boge et al., 1979). The observed lack of variation in plasma 14C concentration despite a significantly higher 14C-leucine absorption in the MT-treated group could be the result of increased uptake of the amino acid in peripheral tissues, as reflected in the higher concentration of 14C-solute detected in skeletal muscle and liver extracts of this group when compared to controls, although the differences were not statistically significant.

6.4.3. Sex-reversed

In the sex-reversed trout, overall intestinal accumulation of leucine, and muscle 14C solutes, were significantly increased, although amino acid absorption per se was unaffected. At present, there appear to be no data on sex steroid levels during the early stages of development of the sex-reversed rainbow trout, which could have accounted for the present results. Measurements have however, been performed in larger trout over a one year period (initial mean wt. 422g ; final wt. 1824g) and, although seasonal peaks were noted, no significant differences in plasma androgen levels were found in sex-reversed and control male trout (Scott et al., 1980). In the present study, using 220g trout, there were no precocious spawners, and since the initial MT treatment used to produce the sex-reversed condition was only carried out during the first 90 days of feeding (Johnstone et al., 1979), the effects observed presumably were related to some change in physiological status, rather than to MT residues. Further studies will be required to explain the variations observed in intestinal transport of leucine in sex-reversed from those of normal male and female fish at a similar stage of development.

6.5. Summary:

1) A technique (modified from Boge, et al., 1977a) is described for the measurement of amino acid transport on unanaesthetized rainbow trout, in vivo.

2) The present results provide further evidence for the direct

stimulatory action of MT on intestinal absorption of leucine, in vivo, in a short period of time (within 10 min), which is not compatible with the time-course of steroid-stimulated protein synthesis.

3) Administration of E2 in vitro was without effect on intestinal transport of L-leucine.

4) Sex reversal, whilst having no effect on leucine absorption, significantly increased intestinal accumulation of leucine, and accumulation of 14C solutes in skeletal muscle. The reasons remain uncertain, but are not likely to be related to MT-residues.

Fig. 6.1

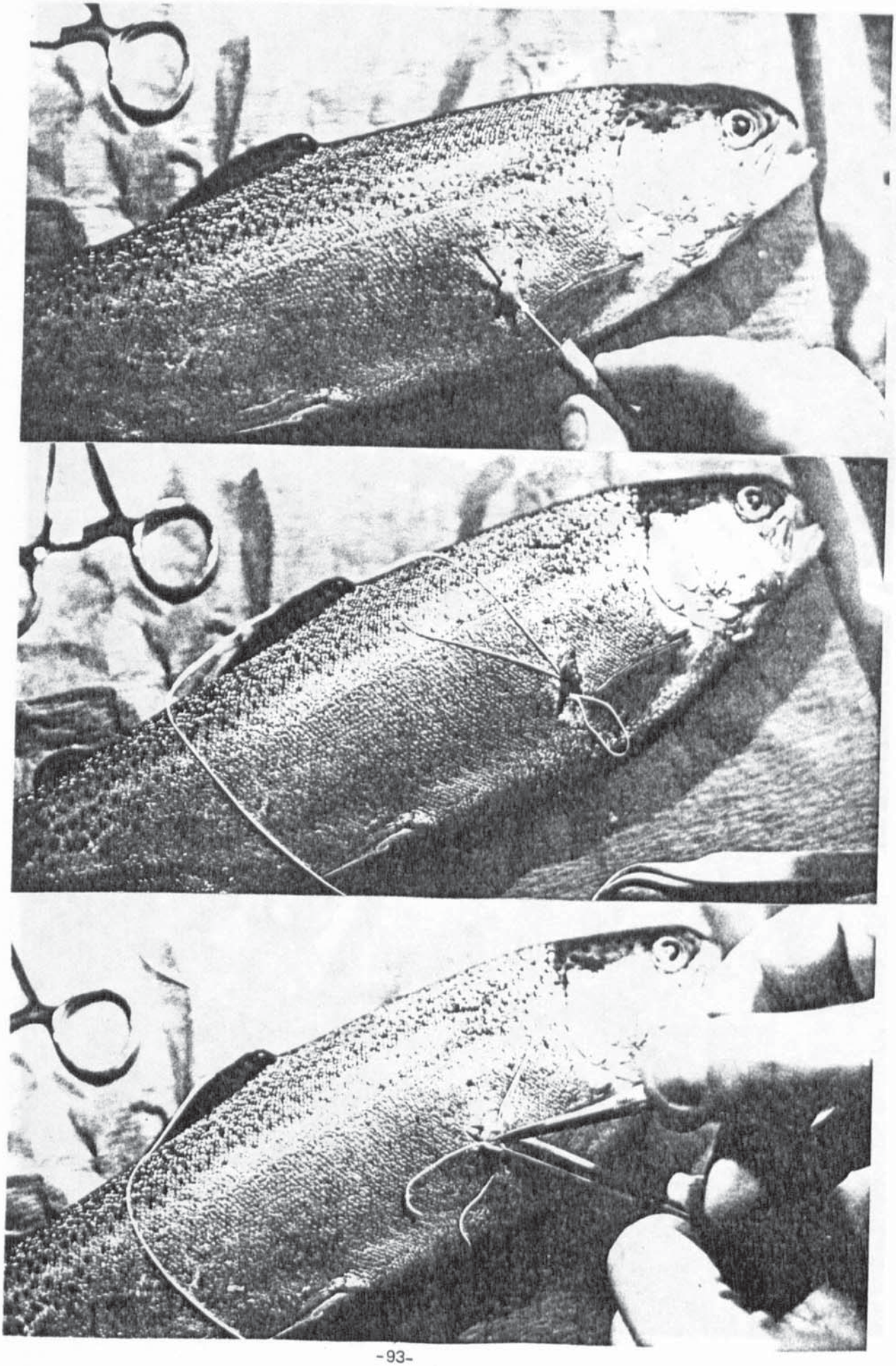


Fig. 6.2

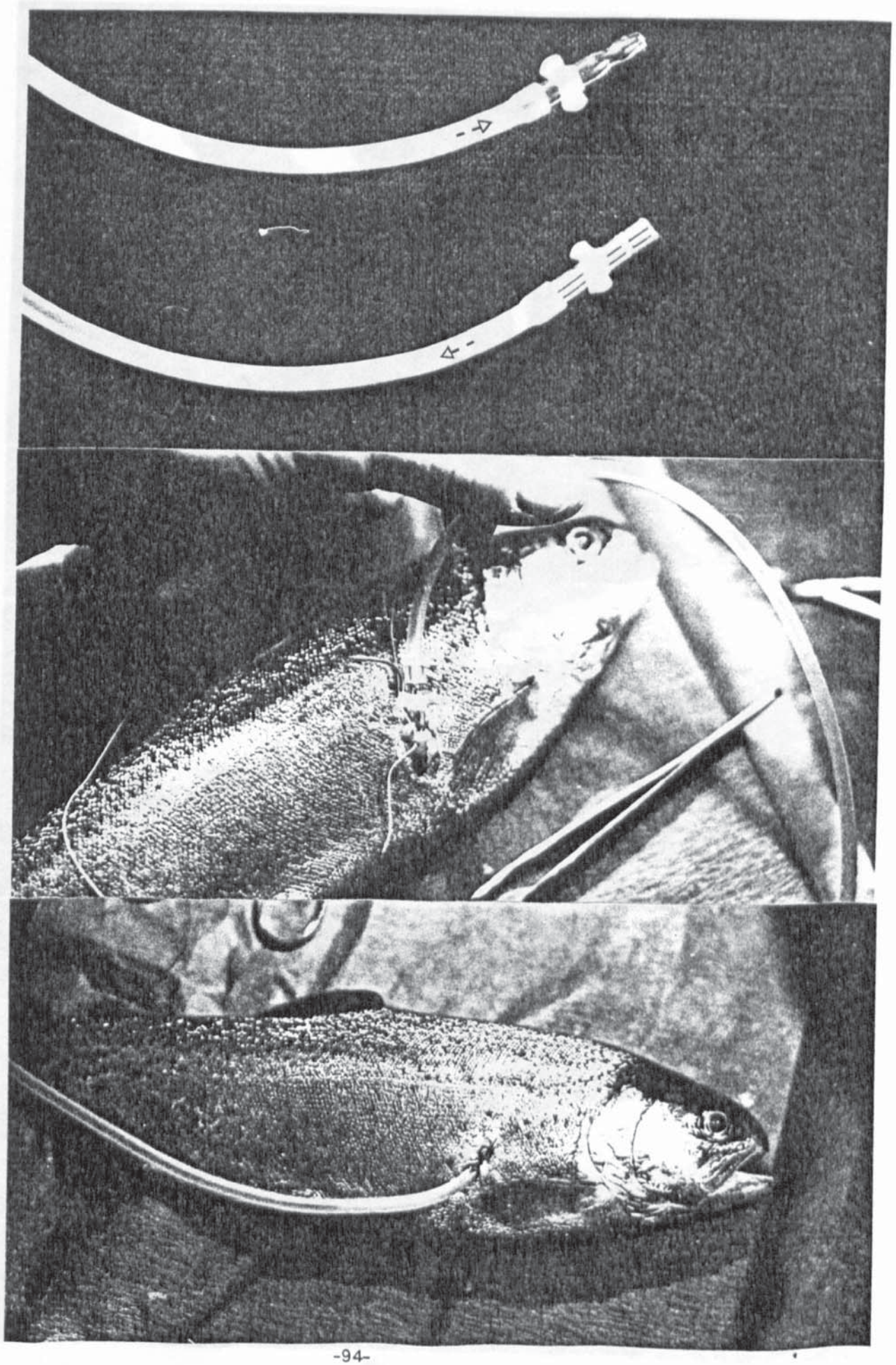


Fig.6.3

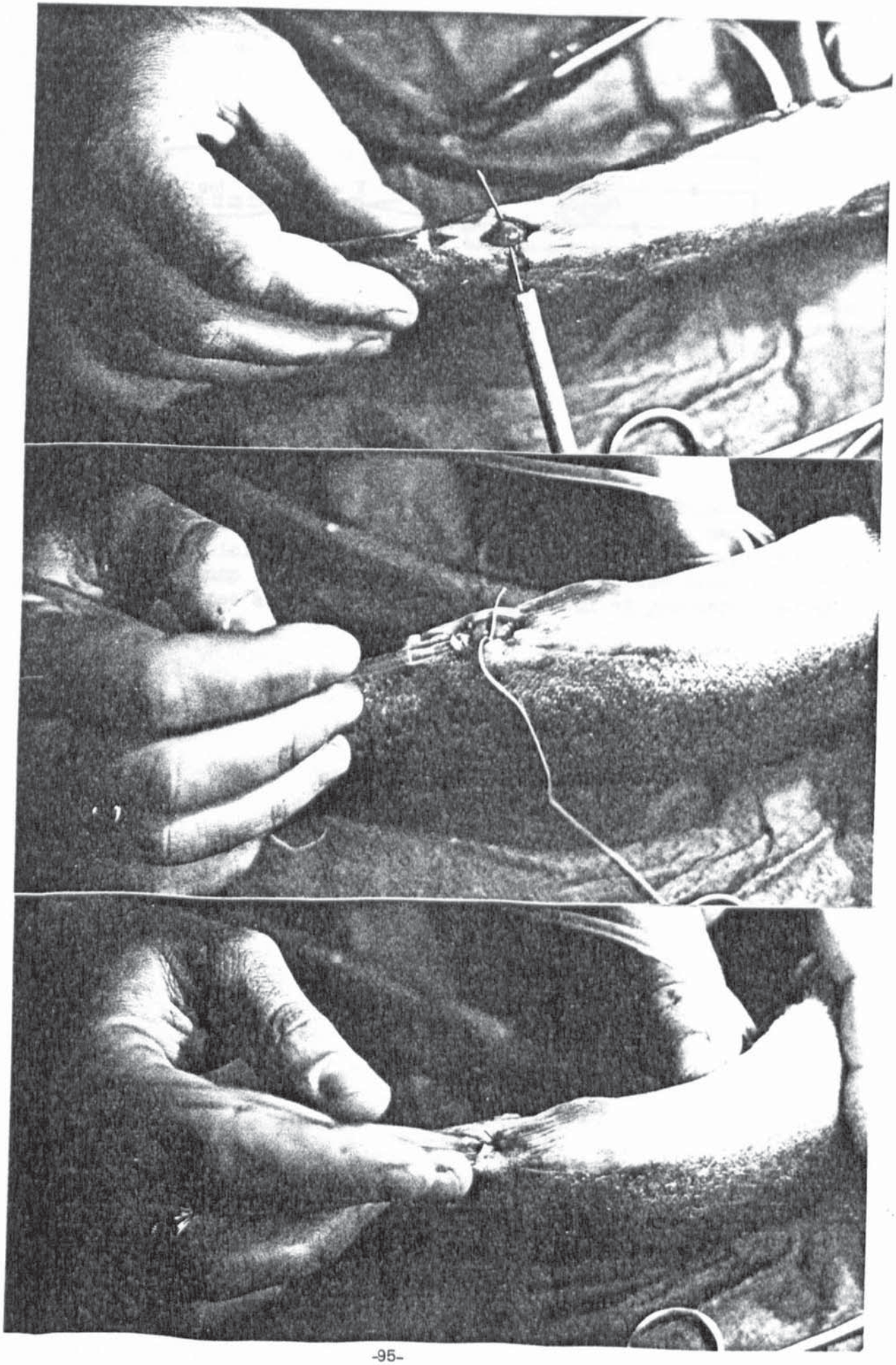
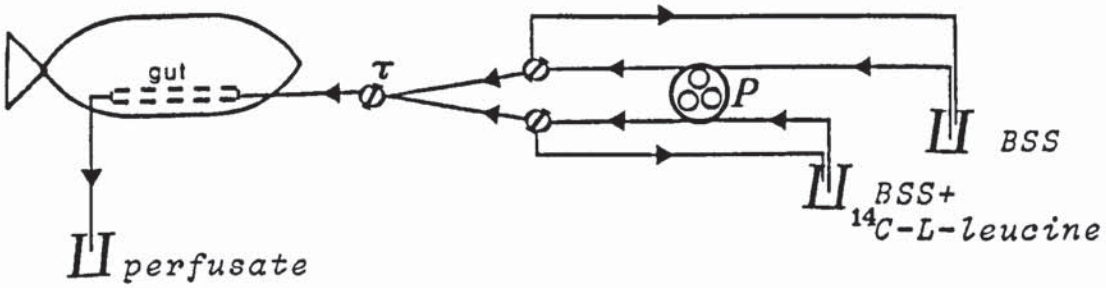


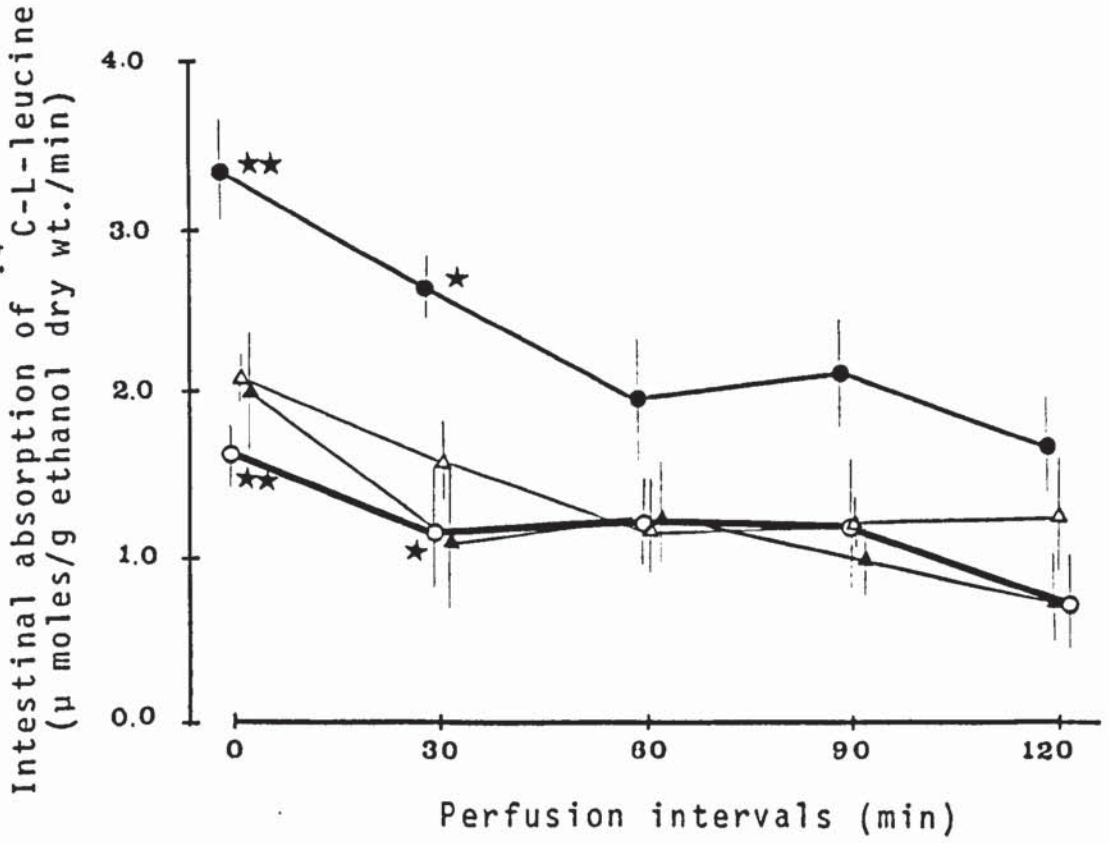
Fig.6.4

Perfusion circuit



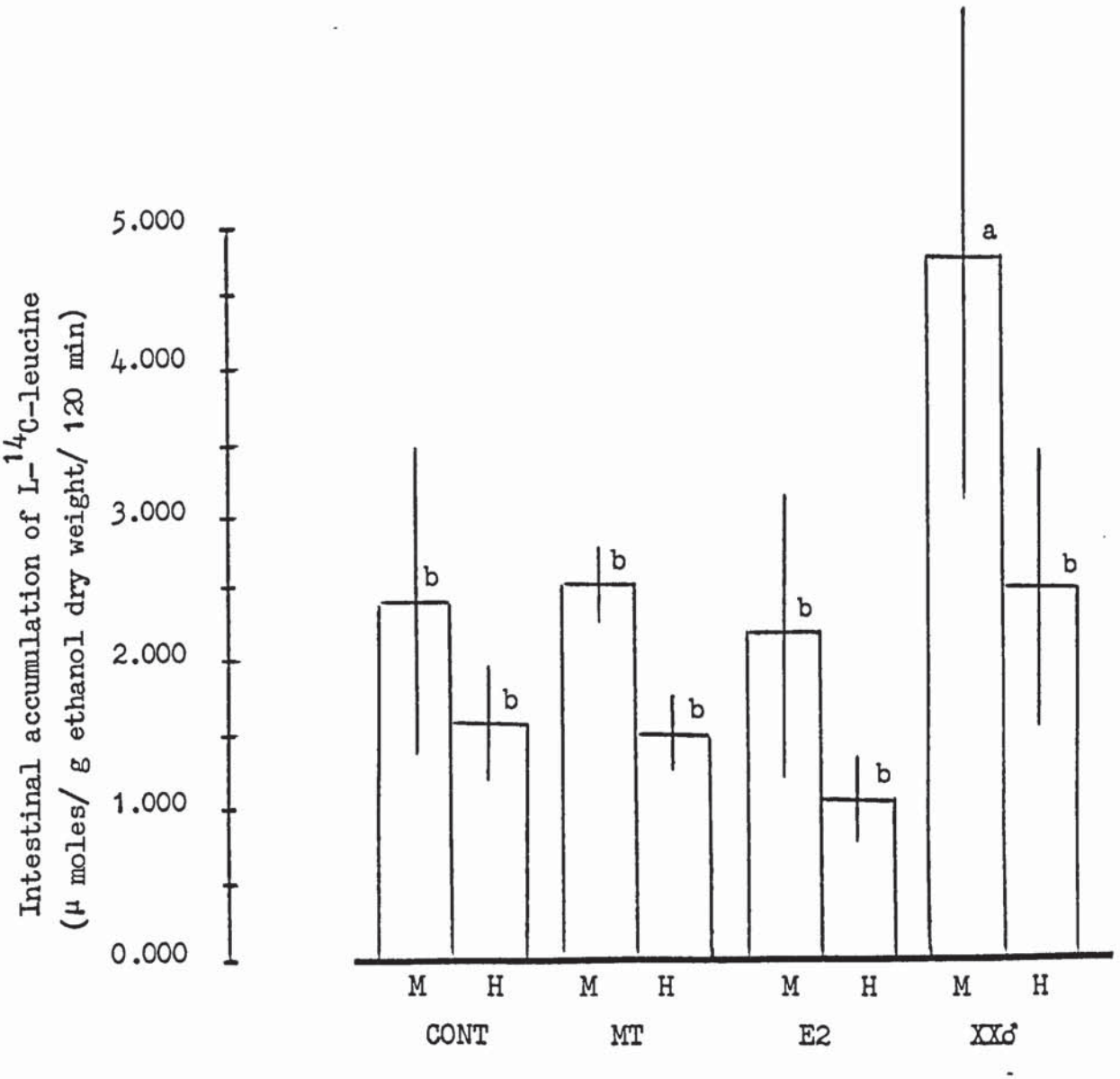
Open circuit (non-recycling) perfusion system. BSS: Balanced salt solution (trout Ringer); BSS+ 14C-L-leucine: perfusion medium containing radioleucine (5 mM/l); τ: three-way adaptor tap; P: psristaltic pump (0.1 ml/min). The intestine of each fish was perfused five times at 30 min intervals with 1 ml of perfusion medium.

Fig. 6.5



Intestinal absorption of L-^{14}C-leucine in four groups of rainbow trout. Control, ○——○; MT-treated, ●——●; E2-treated, △——△; and sex-reversed, ▲——▲. The intestine of each fish was perfused 5 times at 30 min inter-vals. Each value is mean +/- S.E. of 4 observations. Total comparison of data indicated significant differences between control and MT-treated ($P<0.005$). Values marked with (★) are significantly different (★, $P<0.01$; ★★, $P<0.005$).

Fig 6.6.



Intestinal accumulation of solute leucine in the mid- (M) and hindgut (H) region of control (CONT), MT-treated (MT), E2-treated (E2) and the sex-reversed (XXδ). Values are mean +/_ S.E. 4 observations, and those containing dissimilar symbols are significantly different ($P<0.05$).

Fig. 6.7

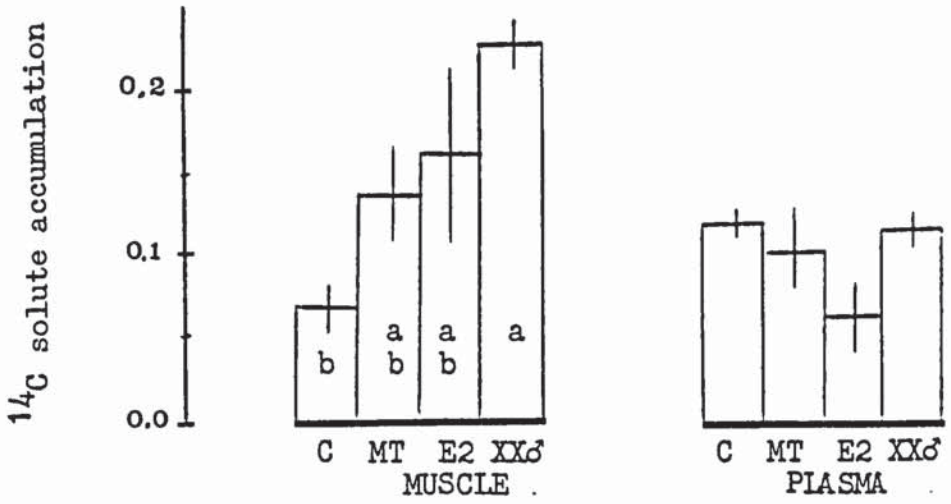
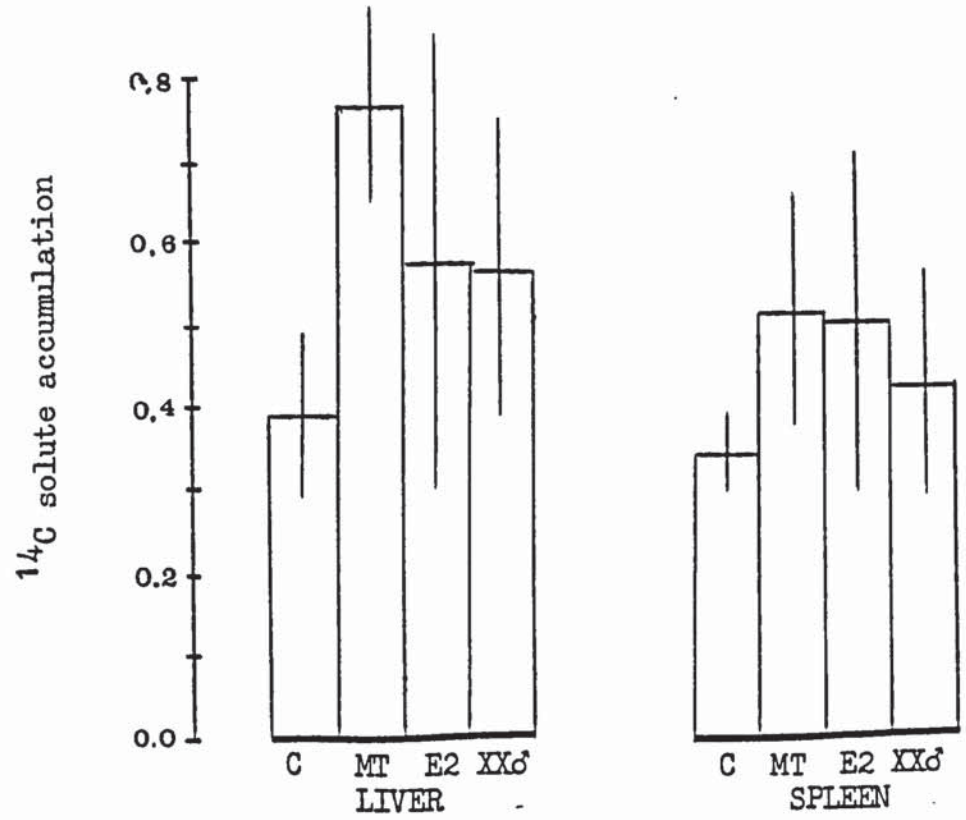


Fig. 6.8



Tissue accumulation of ^{14}C solute (μ moles/ g ethanol dry weight/ 120 min). Values are mean +/- S.E. of 4 observations. Those containing dissimilar symbols are significantly different ($P<0.05$). Differences in plasma, liver and spleen are not significant.

A study of the steroid-stimulated L-leucine transport in relation to the action of inhibitors in the intestine of rainbow trout, S. gairdneri, in vitro.

Section 7

7.1. Introduction

In previous studies, it was shown that methyltestosterone stimulates the intestinal transport of L-leucine both when injected in vivo, and when added directly to the isolated intestine, in vitro (Sections 4 and 5; Habibi, et.al., 1983). The possibility of a direct effect of MT was further investigated by adding the steroid into the medium bathing the mucosal surface, using a perfusion technique in vivo (Section 6; Habibi and Ince, 1983). These studies demonstrated an increased rate of leucine absorption in the intestine of rainbow trout, 10-20 min after in vitro administration of the steroid. However, little is known about the short-term effects of the steroids in general but, in fish and higher vertebrates, evidence is accumulating that adrenal and gonadal steroids influence the cellular transport of ionic and non-ionic solutes by mechanisms distinct from their classical effect on the mRNA-protein axis. For example, oestradiol enhances the rate of cellular calcium exchange in rat uterine cells 2.5 min after addition of the hormone in vitro (Pietras and Szego, 1975). Homo and Simon (1981) found a rapid and transient increase in calcium uptake 5 min after addition of dexamethasone (a synthetic glucocorticoid compound) to isolated mouse thymocytes in vitro. The early biochemical events leading to increased transport of solutes, induced by steroids, have not been investigated . However, a study of the effects of oestradiol on water and electrolyte uptake in the rat uterus, after a 6 hours time-lag, suggested that the 'early' response to the steroid did not depend on the initial

steroid uptake and its binding to the cytosol receptor, but might have been due to an effect on K+ movement across the membrane, possibly by mediation of a microtubule-assisted membrane mechanism (Fujimoto and Morrill, 1978; Kalimi and Fujimoto, 1978). In vivo, administration of testosterone propionate was found to increase amino acid uptake in rat kidney (Riggs, et.al.,1963), and skeletal muscle (Riggs and Walker, 1963). In this context, a recent study has shown that some steroids, including testosterone and cortisol, increase the activity of Na+,K+,ATPase in rat synaptosomal membrane (Alivisatos, et.al., 1981).

Glucocorticoids, in particular cortisol, were previously shown to increase the intestinal transport of ionic and non-ionic solutes both in higher vertebrates (Field, 1981) and in fish (Hirano and Utida, 1968; Ellory, et al., 1972; Mayer, et al., 1967; Porthe'-Nibelle and Lahlou, 1975). It was suggested that the effects of glucocorticoids might be mediated by the increased activity of Na+,K+,APTase (Charney, et al.,1975; Epstein, et al., 1971; Pickford, et al., 1970; Lahlou, 1976; Bentley, 1981). It is therefore conceivable that effects of androgens on amino acid transport in the intestine are also a consequence of an interaction with the Na "pump" and intracellular Na+ activity, and thus Na-dependent amino acid transport processes. In trout, only the Na-dependent mechanism has been satisfactorily demonstrated (Ingham and Arme, 1977) and inhibition of Na+,K+,ATPase by ouabain has been shown to impair the intestinal transport of certain amino acids (Boge, et al., 1979).

The work carried out in the previous sections indicated that

steroids might increase the active transport of leucine. Thus, the present study was intended to investigate the short-term effects of methyltestosterone and 11-ketotestosterone on the transport of L-leucine and water by the intestine of rainbow trout, in relation to the influence of metabolic inhibitors (NaF & DNP) and ouabain, a specific inhibitor of Na+,K+,ATPase. In this way it was hoped that some insight into the mechanisms of steroid action on amino acid transport would be gained. Experiments were subsequently carried out using strips of trout intestine in vitro, a technique which allows simultaneous comparison of the effects of different agents, with each fish acting as its own control. The results suggest that MT-stimulated leucine transport might be partly the result of increased Na+,K+,ATPase activity.

7.2. Materials and Method

7.2.1. Animals

Rainbow trout weighing approximately 250g (range: 230-280g), were maintained in 2501 fiber-glass tanks as described in Section 3.

7.2.2. Administration of hormone and inhibitors

17a-Methyltestosterone (MT), 11-ketotestosterone (KT), ouabain, 2,4,dinitrophenol (DNP), and sodium fluoride (NaF) were obtained from Sigma (London). Steroids were dissolved in absolute ethanol (MT, 12.5 mg/ml; KT, 2.5 mg/ml), then further diluted in the incubation medium to a concentration of 50 ug/ml (MT) and 10 ug/ml (11-keto). Ouabain (0.1 mM/1), DNP (0.01 mM/1), and NaF (5 Mm/1) were added to incubation media either alone or in combination with each steroid. The incubation medium of all experimental groups (including control) contained the same volume of solvent, 5 mM L-leucine (Sigma), and 0.05 uCi/ml L-(U-14C)-leucine (specific activity 30 mCi/mM; Radiochemical center, Amersham, UK.). The Ringer solution used contained (mM/1): NaCl, 125.8; KCl, 4.3; CaCl2, 1.0; MgSO4, 1.2; KH2PO4, 3.2; Na2HPO4 14.5 (PH 7.3; 279 mOsm/1).

7.2.3. Preparation and incubation procedure

The absorption and accumulation of L-leucine and water were measured using strips of rainbow trout intestine, in vitro. The technique used in the present study was based on those described by Agar, et al. (1954), and Schachter, et al. (1960) with certain modifications. Briefly, a section of intestine, which included the

mid- and hindgut, was removed and washed in 10 ml of Ringer solution. The mid- and hindgut sections were separated, and each cut into 6 'rings' of approximately 4 mm length, which in turn, were cut transversely to expose their mucosal surface . The intestinal strips were then randomly divided into 6 groups, each of which contained a portion of mid- and hindgut, and transferred to incubation vessels containing 4 ml of medium (2 strips per vessel). Designation of the groups was as follows: 1, control; 2, inhibitor; 3, MT; 4, MT+inhibitor; 5, KT; 6, KT+inhibitor. Incubation vessels were gassed continously with 95% O2: 5% CO2, and shaken at 100 Os/min in a constant-temperature water bath (14°C), over a period of 60 min.

7.2.4. Medium and tissue analysis

7.2.4.1. L-leucine transport

Samples (100 ul) of the medium were withdrawn after 5, 10, 20, 30, 40, and 60 min and at the termination of the incubation period (60 min), mid- and hindgut slices were removed and blotted dry, and each extracted for 24 h in 1 ml 80% ethanol. The extracted tissues were then dried for 24 h at 90°C, and the ethanol-extracted dry weights obtained. Samples of medium and ethanol extracts (100 ul) were counted for 14C activity in a Packard Tricarb 2660 scintillation counter, and the results corrected for quenching.

7.2.4.1.1. Chromatography of leucine

In order to determine the percentage recovery of 14C-leucine, as an index of leucine metabolism, 5 ul samples of medium (obtained after 60 min) and tissue extracts were chromatographed (thin-layer

silica gel '60' plates of 5 x 20 cm; Sigma) in butanol/acetic acid/water (12/3/5, v/v) for 4 h. After drying, the same volume of sample (5 ul) was applied at the origin, and the solvent front marked (14C marker) for determination of Rf values. Each TLC plate was scanned for 14C activity using a Tracerlab 4π chromatogram scanning system (SC-525 model, with a TLC scanning attachment; Tracerlab, Massachusetts). The 14C activity in the original sample and in the separated leucine band were used to calculate the percentage recovery of L-leucine present as 14C-labelled compound with respect to the 14C-L-leucine standard. It was found that the leucine band represented the only detectable region of 14C activity, and it was not necessary to calculate the Rf value (0.5039 +/- 0.0092; mean of 12 +/- S.D.) for each sample. Sample chromatograms of the 14C-scan obtained from the medium of control and MT-treated group are shown in the Appendix (Fig. 7.b). The area under each curve was determined by cutting and weighing each section.

7.2.4.2. Measurement of tissue fluid uptake and water flux.

The tissue fluid uptake was measured by weight differences of the strips of mid- and hindgut before and after incubation. To obtain a consistent result, the strips of intestine were placed between several thickness of soft tissue paper and then blotted under the pressure of a perspex ruler (24.7 g) for 15 seconds. The strips were then weighed immediately on a Mettler balance.

Tissue water flux was measured using phenol red (phenosulphonphthalein), a non absorbable extracellular space marker (Soergel and Hogan, 1967). The media were prepared by dissolving

phenol red in the Ringer solution (5 ug/ml) containing 5 mM/l L-leucine. Steroid and inhibitors were then added to the solution as described above. Samples (100 ul) of the medium were withdrawn after 2, 4, 5, 10, 20, 30, 40, and 60 min and added to 2ml 3N NaOH (Hart and Schanker, 1966; Diamond, 1964). Phenol red concentration was measured colorimetrically at 560 nm (Cecil CE272 spectrophotometer). The mean phenol red concentration after 2 and 4 min of incubation was determined and regarded as the initial phenol red concentration (Ci), and those measured after 5 min of incubation as the final phenol red concentration (after t min of incubation; Cf). It were assumed that the changes in the volume during the initial 4 min was much smaller than those during the next 56 min of incubation. The Ci/Cf ratios (Appendix, Table 7.a) were then used to determine the volume of the medium at each time interval (Schedl, 1966). It was necessary to conduct separate experiments for the determination of water flux during the 60 min incubation period to avoid colour interference, due to phenol red, with 14C-illuminesance measurement (scintillation counting). Values of the intestinal water flux obtained by this technique were then used to correct for the changes in the volume of the incubation medium used to measure leucine absorption during the 60 min period. The viability of this volume correction procedure was apparent from the small and statistically non-significant differences obtained between the Ci/Cf ratios of the intestinal strips of different weights (Table 7.1). This was due to the fact that both Ci and Cf are dependent, and in direct relationship with the weight of the intestine, within the narrow range of the weights of the strips used in the experiments.

Thus, a significant correlation ($P<0.01$) was obtained between tissue dry weight and tissue fluid content after 60 min of incubation (Fig. 7.1). an indication that Ci/Cf ratios might be used to determine volume changes in similar preparations.

7.2.5. Presentation of the results

Sample count rates (DPM) were used to determine the concentration of the amino acid (Section 5). Leucine absorption: uMoles leucine disappearance from the incubation medium per g ethanol extracted dry weight of intestine per sampling time (I); Intestinal accumulation of solute leucine (IAL.sol): uMoles leucine solute extracted per g ethanol-extracted dry weight per 60 min; Apparent intestinal accumulation of incorporated leucine (AIAL.inc): uMoles of tissue-incorporated leucine not extractable by ethanol per g ethanol extracted dry weight of intestine (mid- and hindgut) per 60 min (II); Tissue water uptake: g water taken up by the intestine per g of ethanol dry weight per 60 min.

The following formulae were used to calculate the parameters shown:

$$A = [Cl.Vl - Ct.Vt] / W \qquad ..(I)$$

A = Leucine absorption (uMoles/g ethanol dry wt./sampling time 't').

Vl = Initial volume (4 ml).

Vt = Ci/Cf X 4 (ml)

Ci = Initial phenol red concentration.

Cf = Phenol red concentration after time 't'.

Cl = Initial leucine concentration (uMoles/ml).

Ct = Leucine concentration after time 't'.

t = Incubation intervals (5,10,20,30,40,60 min).

$$AIAL.inc = A60 - IAL.sol \quad ..(II)$$

AIAL.inc was calculated on the basis that ethanol extracts only contained the solutes amino acid. Assuming total recovery of the labelled amino acid, the difference between leucine absorption obtained at the end of the incubation (A60) and the IAL.sol would be AIAL.inc which is not extracted by ethanol.

7.2.6. Statistical analysis

A two-way analysis of variance (ANOVA) in conjunction with split-plot design, F-test, and T-test (Ridgman, 1975) were used for statistical analysis of the leucine absorption results. The analysis of variance for 2 factorial (Bailey, 1981) was used to determine the significance of interactions between steroids and inhibitors with respect to 'control' leucine absorption (results were arranged for 2^n factorial design i.e. n=2, F values for blocks and treatment were all significant at the 5% level). Differences in intestinal accumulation were analysed by one-way analysis of variance (Duncan multiple range test; Duncan, 1955). All analyses were carried out on the HP 2000 and Harris 500 computer facilities of the University of Aston.

7.3. Results

7.3.1. Percentage recovery of L-leucine

The results of L-leucine recovery in the medium and ethanol extracts are shown in Table 7.2.a & b. Using thin-layer chromatography, an almost complete recovery of L-leucine was obtained in control medium (96.8 + 2.5%), and in medium containing MT(102.4 + 5.5%) and KT (95.5 + 8.5%). The values given represent the mean ± S.D. of 3 observations made from pooled samples of 4 media. The average percentage recovery of L-leucine in incubation media containing either inhibitors alone, or in combination with steroids, amounted to 95.8 ± 7.2% (range: 85.7-106.9%). The corresponding recovery was also determined in ethanol extracts for both mid- and hindgut in all groups, and gave an average value of 92.8 + 6.7% (range: 82.1-104.3%). These results are consistent with the view that during intestinal absorption, essential amino acids undergo minimal metabolism, at least in rainbow trout (Ingham and Arme, 1977).

7.3.2. Effects of hormones on intestinal absorption of L-leucine

The effects of MT and KT on the absorption of leucine are shown in Table 7.3. MT significantly increased ($P<0.02$) the overall absorption of leucine (total absorption during 60 min). Analysis of individual absorption values revealed that leucine absorption in the MT group was significantly higher after 10 ($P<0.02$), 20 ($P<0.02$), 30 ($P<0.001$), 40 ($P<0.01$), and 60 ($P<0.01$) min of incubation. Similarly, KT administration resulted in a significantly

higher overall leucine absorption ($P<0.02$), and the individual absorption values in the KT-treated group were significantly higher after 20 ($P<0.02$), 30(0.02), 40 ($P<0.001$), and 60 ($P<0.01$) min of incubation. Comparison of these results with the values not corrected for volume change revealed a very small, statistically non-significant, difference between the two groups.

The results show a general decline of the rate of leucine absorption with time in all groups (Fig. 7.2), presumably due to degeneration of some epithelial cells, and the re-entry of the absorbed amino acid into the medium. The viability of similar preparations however, has been demonstrated in rainbow trout, using rings of everted intestine (Ingham and Arme, 1977), and intestinal strips (Boge, et al., 1979). It is seen from the present results that the stimulating effect of MT is significantly higher during the first 20 min of incubation, followed by a more steady increase in the rate of leucine absorption during the latter 40 min of incubation. This initial increase in the rate of amino acid absorption however, is not apparent in the KT-treated group, and the results indicate a more steady increase in this group which becomes significant in the periods after 20 min of incubation.

7.3.3. Effects of inhibitors on intestinal absorption of leucine

Ouabain, an inhibitor of Na+,K+,ATPase, significantly reduced the control leucine absorption after 60 min of incubation. Moreover, administration of ouabain resulted in significant inhibition of both MT- and KT-stimulated absorption of the amino acid (Table 7.4.1). MT-stimulated leucine absorption was significantly reduced after 10

($P<0.05$); 40 ($P<0.05$); and 60 ($P<0.001$) min of incubation, while KT-stimulated transport was reduced after 40, and 60 min of incubation ($P<0.05$).

DNP, an uncoupler of oxidative phosphorylation, significantly reduced control leucine absorption after 30 ($P<0.05$), and 60 ($P<0.02$) min of incubation, and significantly attenuated steroid-induced amino acid absorption after 20-60 min of incubation (Table 7.4.2).

NaF a glycolytic inhibitor, which blocks the formation of phosphoenol-pyruvate from 2-phospho glyceric acid, significantly inhibited the absorption of control leucine after 20-60 of incubation (20, $P<0.05$; 30, $P<0.05$; 40, $P<0.02$; 60, $P<0.01$). NaF, also reduced the MT- and KT-stimulated amino acid absorption as shown in Table 7.4.3.

The significance of these results for studying the mode of action of MT and KT on the absorption of leucine is more readily explicable when they are presented in a format which compares the action of the inhibitors alone, and when in conjunction with steroids. These results were thus analysed for further statistical comparison of the differences obtained between control and inhibitor-treated, and those obtained between steroid- and steroid+inhibitor-treated groups. The results were subsequently tested to estimate if the inhibitors significantly modified the action of steroids, by calculating the interactions between steroid and inhibitor. The analysis were carried out on the basis of a 'one steroid : one inhibitor' relationship, and the interactions were estimated using 2 factorial design (i.e. each factor at 2 levels of

treatment; factor 1: control, control+inhibitor; factor 2: steroid, steroid+inhibitor). The degree of interaction between steroid and inhibitor would thus indicate the extent by which the two compounds influenced absorption by acting on the same cellular mediator.

7.3.3.1. Effects of ouabain

Analysis of variance by factorial design revealed a significant interaction between ouabain and control leucine absorption ($P<0.01$), reducing total amino acid absorption from the medium, and a significant interaction between the inhibitor and MT, attenuating the effect of MT on leucine absorption ($P<0.01$; Fig. 7.3.a). No significant interactions were obtained between KT and ouabain.

7.3.3.2. Effects of DNP

Statistical analysis indicated a significant interaction of DNP with control leucine absorption, reducing total amino acid absorption ($P<0.01$), and a significant interaction between DNP and MT ($P<0.05$), depressing the MT-induced leucine absorption (Fig. 7.3.b). No significant interactions were found between KT and DNP.

7.3.3.3. Effects of NaF

Factorial analysis indicated a significant interaction between the inhibitor and control amino acid absorption ($P<0.01$). No significant interactions however, were obtained between the inhibitors and any of the steroids (Fig. 7.3.c).

7.3.4. Effects of steroids and inhibitors on intestinal accumulation of leucine.

7.3.4.1. Intestinal accumulation of leucine

Effects of steroids and inhibitors on intestinal accumulation of solute leucine (IAL.sol) are shown in Fig. 7.4.a. IAL.sol values represent the compartment of leucine which is not incorporated in the tissue (protein), but present as free, extractable solute. The results of steroid treatment on IAL.sol. indicated that, neither MT nor KT affected overall or regional accumulation of solute leucine, when compared with the controls. Ouabain administration however, significantly reduced the control IAL.sol both in mid- and hindgut ($P<0.05$). In the presence of ouabain and MT, IAL.sol was similar to those of ouabain alone, and significantly lower than the solute leucine accumulation in the MT-treated group ($P<0.05$). Although the solute leucine accumulation in the ouabain-treated was significantly lower ($P<0.05$) than those in the KT-treated group, combination of KT and ouabain resulted in no significant changes in IAL.sol when compared to KT-treated intestine.

Administration of DNP significantly reduced the control IAL.sol ($P<0.05$). Combination of DNP and MT, or DNP and KT, resulted in IAL.sol values similar to those of DNP alone, but each were significantly lower ($P<0.05$) than the MT- and KT-treated groups.

NaF administration resulted in a significantly lower solute leucine accumulation when compared to the controls. Although the IAL.sol in NaF-treated was significantly lower than those of MT-treated group, the accumulation of solute leucine in NaF+MT treated was not significantly different from those of the MT-treated group. Moreover, comparison of IAL.sol in NaF-, KT-, and KT+NaF-treated groups revealed that the solute leucine absorption was generally

lower in NaF-, and KT+NaF- than those of KT-treated intestine (differences only significant for hingut accumulation; $P<0.05$).

The results shown in Fig. 7.4.b represent the 'apparent' intestinal accumulation of the incorporated leucine (AIAL.inc) which is not freely extractable. Both MT and KT significantly increased the control AIAL.inc ($P<0.05$), while ouabain administration significantly reduced the control AIAL.inc. Combination of ouabain and MT, or ouabain and KT treatment resulted in a significantly lower AIAL.incthan those of MT- or KT-treated intestine ($P<0.05$). Furthermore, no significant differences were obtained between the AIAL.inc of ouabain and those of ouabain+MT, or ouabain+KT. Although lower AIAL.inc values were obtained in the DNP-treated group than the controls, the differences were not statistically significant due to large variations (32.6006 +/- 13.849 vs 53.6153 +/- 6.1624, mean +/- S.E.). The AIAL.inc in MT+DNP-, or KT+DNP-treated groups however, were significantly lower than those of MT-, or KT-treated groups ($P<0.05$). The AIAL.inc obtained in the DNP-treated intestine was not significantly different to those of MT+DNP, or KT+DNP.

NaF, either alone, or in combination with steroids, significantly supressed the AIAL.inc when compared to control, MT-, or KT-treated group ($P<0.05$). No significant differences were obtained between NaF and NaF+MT, or NaF and NaF+KT.

7.3.5. Effects of steroids on intestinal fluid absorption and tissue water uptake

Intestinal fluid absorption was measured during 60 min incubation in the control and the steroid-treated groups and the

volume changes obtained shown in Fig. 7.5. The results of fluid absorption in other experimental groups are presented in Appendix 7.a, in terms of Ci/Cf ratio. The results of fluid absorption show similar trends to those of the amino acid, with a tendency for higher rates of fluid absorption in the steroid-treated groups. The fluctuations observed in the absorption of fluid are likely to be due to the limited sensitivity of the marker used (phenol red) and the fact that the contraction of the muscles in the short strips to some degree restricts the subepithelial space (Wilson, 1962), thus effecting homogeneous exchange of water molecules, and solutes. Intestinal water fluxes in the groups receiving inhibitor and steroid+inhibitor treatment are summarised in Table 7.a (Appendix) in terms of Ci/Cf ratios. These results generally indicate a reduced water uptake in the groups receiving inhibitor treatment, of which NaF was found to be the strongest.

The intestinal water uptake measured at the end of the incubation period (60 min) was significantly higher ($p<0.05$) in the midgut than in hindgut in all experimental groups (Table 7.5; Fig. 7.6.a & b). Neither MT nor KT, significantly affected the overall or regional uptake of water by the intestine when compared with the controls. Results of overall water absorption indicates that the metabolic inhibitors NaF and DNP were more effective in reducing water uptake than ouabain, suggesting that the mechanism involved is more dependent on metabolic energy than the activity of the Na+,K+,ATPase at the basolateral membrane. Furthermore, marked regional differences were observed in all groups receiving inhibitor treatment, especially those receiving ouabaine treatment. In the

latter group, the hindgut was significantly effected by ouabain ($p<0.05$), while the corresponding inhibition in midgut was slight and not significantly different to the groups incubated in inhibitor-free media. The intestinal water uptake was reduced both in mid and hindgut of the groups treated with DNP and NaF. Similarly, hindgut was more affected in these groups.

7.4. Discussion

7.4.1. Effects of steroids on leucine transport

The results of the present study indicate that MT, and to a lesser extent KT stimulate L-leucine absorption by strips of trout intestine, in a relatively short time which can more readily be explained in terms of a direct effect on the plasma membrane and the membrane associated enzymes, or the cytoplasmic mediators involved in the control of cellular transport, since the generally- accepted mechanism of action of sex steroids (through their effect on the nucleus and protein synthesis), cannot account for the responses observed during 60 min of incubation. In this context, the rapid effects of steroids on cellular ion transport (Pietras and Szego, 1975; Homo and Simon, 1981), intestinal ion transport (Frizzell and Schultz, 1978) and kidney amino acid transport (Riggs, et al., 1963) have been demonstrated in mammals. It is also apparent from the results that these steroids do not effect the intestinal accumulation of solute leucine (IAL.sol.) when added directly to the medium. This is consistent with the results obtained in the previous experiments in vitro and in vivo (Section 5 & 6; Habibi, et al., 1983; Habibi and Ince, 1983). Both MT and KT, however, significantly increased the 'apparent' intestinal accumulation of the incorporated leucine. This finding is in accord with the report that anabolic steroids increase the incorporation of leucine into skeletal muscle protein of trout (Matty and Cheema, 1978).

7.4.2. Effects of inhibitors

The results obtained from intestinal strips incubated with

ouabain, DNP, and NaF indicate that these inhibitors significantly reduce intestinal absorption and accumulation of leucine. The present results on the effects of ouabain and DNP are in agreement with those of Ingham and Arme (1977), who used rings of everted trout intestine, and of Boge, et al. (1979), using strips of trout intestine. The intestinal transport of leucine in the presence of NaF in control experiments, however, was lower than that reported by Boge and coworkers for glycine. This apparent difference might be the result of the difference in the amino acids used. Also in accord with the present results on the reduced accumulation of the solute and 'apparent' incorporated leucine following ouabain administration, Kypson and Hait (1971) reported a significant decrease in the accumulation of solute leucine, and incorporation of the amino acid into tissue protein, 90 min after addition of ouabain (0.1 mM) to slices of rabbit atrial tissue, in vitro. In the present experiments, administration of DNP, NaF and ouabain, resulted in a significant reduction of amino acid transport, yet none of these agents completely abolished the influx of leucine. This suggests the presence of a transport-system which is partly dependant on metabolic energy produced through glycolysis and oxidative phosphorylation, and in part energised through the electro-chemical gradient maintained by the Na+ "pump".

7.4.2.1. Steroid-inhibitor interactions

The interactions of ouabain and DNP with MT-stimulated leucine absorption indicate, albeit indirectly, that the action of MT on amino acid absorption involves cellular mediators which might also

be affected by ouabain, and by DNP. The results shown in Fig. 7.2 indicate that MT has an initial stimulatory action on the absorption of leucine during the first 20 min, followed by a smaller, but more steady increase during the latter 40 min of incubation. KT however, produced no significant interactions with the inhibitors, and resulted in no initial increase in leucine absorption. Moreover, the KT-stimulated amino acid absorption became significant during the latter 40 min of the incubation which is not significantly different to the absorption values obtained in the MT-treated group, at similar time intervals (20–40 min). It is suggested therefore, that the significant interactions obtained between MT and ouabain, or MT and DNP were partly the result of MT-stimulated leucine absorption occurring during the first 20 min of incubation. Furthermore, it would appear that both MT and KT exert similar actions during 20–60 min of incubation, although, comparison of total absorption during 60 min indicated that the KT-stimulated leucine absorption was not completely abolished by the inhibitors.

7.4.2.1.1. Effects of DNP

The observed interaction between MT and DNP suggests that the initial MT-stimulated leucine transport is possibily related to metabolic energy, although a nonspecific action of DNP, unrelated to its role as a metabolic inhibitor cannot be ruled out (Chez, et al., 1967). Moreover, it is not certain if MT directly influenced mitochondrial metabolism and ATP production within the short time of incubation, although there is evidence for a longer-term action of androgens on mitochondrial metabolism in mammalian systems

(Williams-Ashman and Reddi, 1971). There is however, one possible explanation for an indirect effect of the steroids on mitochondrial metabolism, since changes in intracellular (cytosolic) Na+ and K+, effect the Ca++ accumulation activity of mitochondria. There is evidence that inhibition of Na+,K+-ATPase could lead to the release of Ca++ into the cytosol (Noak and Greeff, 1975).

7.4.2.1.2. Effects of ouabain

The cardiotonic steroid ouabain is known for its specific inhibition of Na+,K+,ATPase activity in the basolateral membrane of the enterocytes. At this concentration (0.1 mM/l), ouabain could cause other intracellular changes, which in mammals may be related to cellular K+ depletion (Kaplan, 1978). The metabolic changes secondary to intracellular K+ depletion however, can not account for the initial inhibition of the MT-stimulated leucine transport. Moreover, in fish, there is evidence that the intestine is slightly more permeable to K+ than to Na+ (Moreno and Diamond, 1974), therefore, K+ depletion may not be as severe as that reported for mammals. A more likely mechanism for the short-term effects of ouabain on amino acid transport is through its effect on the Na+ gradient maintained by Na+,K+,ATPase activity (Schultz, et al., 1967). The time course of the effects observed in the present study is consistent with the reported time measured for ouabain to reach half maximal concentration (t1/2 : 6.5 min), and produce half maximal effects (t1/2 : 5.8 min) in mammalian atrial tissue, superfused with blood containing ouabain (Dutta, 1981). Moreover, there is evidence for a very rapid onset of ouabain effect (t< 1

min) in rat kidney proximal tubules (Heidenrieich and Osswald, 1981), and chicken enterocytes (Kimmich, 1970a & b), when applied at the cellular level, in vitro.

7.4.3. Steroid-induced transport and Na-gradient

The present findings raise a number of fundamental questions; Does MT effect leucine absorption by affecting the Na+ gradient across the luminal membrane? If so, is the effect on the Na+ gradient linked directly with the stimulation of Na+,K+,ATPase, or is it related to changes in membrane permeability? The interaction between ouabain and MT suggested that both agents effect a common cellular factor which is also related to leucine transport. Sodium is the most likely mediator involved in this process. The hypothesis that MT may act by increasing the Na+ gradient across the luminal membrane, presumably by increasing the activity of Na "pump" at the basolateral membrane and thus, effecting Na-amino acid co-transport, tends to be indirectly supported by some recent findings. Thus, oral administration of MT in salmonids subjected to a seawater challenge resulted in an increased plasma Na+ concentration which declined between 1-2 weeks after termination of steroid treatment (Higgs, et al., 1982). In fish, there is no direct evidence to indicate that androgens can effect the activity of Na+,K+,ATPase. In mammals however, recent studies on the effects of steroids on the functional activity of ouabain-sensitive Na+,K+,ATPase in dog synaptosomal plasma membrane suggested a low-capacity binding of testosterone to the membrane, resulting in an increased activity of this enzyme (Alivisatos, et al., 1981). In addition, the kinetic data in these

studies indicated that the changes observed in ATPase activity were related to the number of specific binding sites and the ability of the activator to cross the membrane. Furthermore, Fransworth (1968) reported that addition of testosterone increased the Mg++, Na+,K+,ATPase activity in rat ventral prostate, which is consistent with androgen-regulated secretory processes in this tissue, and there are other reports of both direct effects, and synergistic actions of testosterone, directed on Na+,K+,ATPase activity in mammals (Coffey, et al., 1968; Mitropoulos, et al., 1982).

7.4.3.1. Non-specific actions of steroids on the Na-gradient

In view of the high concentration of the steroids used in the present study, there is a possibility that some of the responses observed were the result of non-specific actions through binding with other steroid receptors, such as the glucocorticoid receptor, since there is evidence that the specificity of steroid action decreases when present in pharmacological doses. This "spillover" of specificity has been exemplified in a number of studies which have shown the oestrogen- and progestrogen-like responses produced by pharmacological concentrations of androgens (Rochefort, et. al., 1972; Schmidt and Katzenellenbogen, 1979; Schmidt, et. al., 1976), and in reports of the occupancy of glucocorticoid receptors by mineralocorticoids, and vice versa (Katzenellenbogen, 1980). Furthermore, Field (1978) has suggested that the short-term effects of both classes of corticoids on intestinal transport are caused by stimulation of mineralocorticoid receptors, wheareas their long-term effects are related to stimulation of glucocorticoid receptors. In

fish, the presence of the principal mineralocorticoid, aldosterone, is debatable (Idler and Truscott, 1972). However, cortisol which is present in most teleosts has been shown to have both glucocorticoid and mineralocorticoid activities. The effects of cortisol on Na+,K+,ATPase in fish is well documented, and has been investigated in relation to the role of corticosteroids in osmoregulation and seawater adaptation of euryhaline species (Bentley, 1981). Cortisol administration in freshwater-adapted eels increases the intestinal transport of Na+ (Hirano and Utida, 1968). Furthermore, there is evidence that, in eels, plasma cortisol levels increase during seawater adaptation, and remain high for only a short period of 2-4 h (Hirano and Utida, 1971). Cortisol was also shown to increase the activity of Na+,K+,ATPase in the intestinal mucosa of freshwater eels (Epstein, et al., 1971) and seawater Fundulus heteroclitus (Pickford, et al., 1970). In this context, there is evidence that steroids, including adrenal, gonadal and cardiotonic steroids effect Na+,K+,ATPase activity by their binding to receptor sites associated with this enzyme (Alivisatos, et al., 1981; Erdmann, 1981). However, it is unlikely that these agents affect the same receptor sites on Na+,K+,ATPase, since the steroid hormones produce stimulatory effects, while ouabain results in inhibition of the enzyme activity. It has been shown that the stimulation of Na+,K+,ATPase is related to the interaction of the activator with high-affinity binding sites (S sites), while the inhibition of this enzyme is due to interaction with low-affinity sites (I sites) (Godfraind, 1981). In this context, although there is evidence that the binding sites for the inhibition of Na+,K+,ATPase by glycosides

might be located on the outside of the basolateral membrane of the enterocytes (Armstrong, 1975), the exact position of the stimulatory sites are not certain.

7.4.4. Relationship between Na+,K+,ATPase activity and amino acid transport.

It would seem possible that MT might have an effect on the activity of Na+,K+,ATPase. However, it is uncertain whether a stimulating action of MT on Na+,K+,ATPase could explain the steroid-induced leucine absorption. For example, there is evidence that increased Na+ and water absorption following administration of glucocorticoids do not coincide (in time) with increased Na+,K+,ATPase activity in mammalian intestine (Binder, 1978). Furthermore, Frizzell and Schultz (1978) have shown a significant increase in active Na+ absorption after a time lag of 30- 60 min, following in vitro administration of aldosterone to rabbit colon and suggested that the aldosterone-induced Na+ absorption was the result of increased permeability of the luminal membrane to Na+, and not due to increased saturation of the Na "pump". Charney et al. (1975) however, reported that administration of adrenal steroids enhance Na+, glucose, and water transport, and Na+,K+,ATPase activity concomitantly in rat colon. These authors have suggested that the transport of solutes and water were associated with increased enzyme activity. Ellory, et al. (1972) have studied the effects of cortisol on goldfish intestine; they concluded that apical uptake was not influenced by the steroid and suggested that cortisol might exert its action by affecting Na+,K+,ATPase activity

(Lahlou, 1976). The initial pattern of the MT-stimulated leucine transport (0-20 min) obtained in the present study is not consistent with the hypothesis based on increased membrane permeability to Na+ alone. Thus, increased permeability to Na+ might be expected to reduce amino acid absorption, because of the reduced Na+ gradient across the luminal membrane. An increase in leucine transport was observed following administration of MT, observations which are in accord with those of Charney et al. (1975) who suggested a direct link between steroid-induced transport and Na+,K+,ATPase activity. An increased permeability of the luminal membrane to Na+ after 30 min of incubation however, would be consistent with the reduced rate of the MT-stimulated leucine transport observed in the present study.

7.4.5. Timecourse of steroid action

Alivisatos et al. (1981) have shown a sigmoidal increase in ATPase activity in response to testosterone administration in dog synaptosomal membrane, in vitro, while cortisol evoked a smaller and approximately linear response. The time course for testosterone action on ATPase activity reported by these authors, suggests a 13%, and 88% increase in the activity of this enzyme after a 30- and 60 min time-lag, respectively. In view of the data reported by Alivisatos and coworkers, it is unlikely that increased ATPase activity was responsible for the early (10 min) MT-induced amino acid absorption, although an increase in the activity of this enzyme during the latter 40 min of incubation seems a likely explanation for the increased amino acid transport observed. The early effects

of sex steroids on cellular absorption of water, cations, and non-ionic solutes have been reported in a number of studies carried out on mammals both in vitro and in vivo (Noall and Allen, 1961; Riggs, et al., 1963; Roskoski and Steiner, 1967; Kalimi and Fujimoto, 1978; Pietras and Szego, 1975; Wasserman, et al., 1980). The study of Noall and Allen (1961) for example, clearly showed that oestradiol administration significantly increases the absorption of a-aminoisobutyric acid in rabbit uterus after 30 min (135% increase) exposure to the hormone, in vivo. Moreover, it would appear from their results that a shorter time exposure to oestradiol would have increased the amino acid absorption. In this context, it was suggested that the limiting factor was the time required for the circulation to deliver the steroid to the uterine cells. These authors however were unable to show a similar effect of oestradiol when administered, in vitro. Furthermore, Riggs and coworkers (1963) have shown that administration of testosterone propionate significantly increased the uptake of amino acids in rat kidney, 30 min after hormone administration, in vivo. A more rapid effect of steroids was reported by Pietras and Szego (1975) who showedthat oestradiol administration can influence rates of cellular Ca++ exchange in rat endometrial cells as early as 2.5 min after addition of hormone, in vitro. Neither of these studies however, suggested a mechanism for the early action of the sex steroids, but a recent investigation on the rapid effects of glucocorticoids on mouse thymocyte Ca++ uptake (Homo and Simon, 1981) suggested a possible role of Ca++ in the regulation of early steroid responses (Kaiser and Edelman, 1978). It has been shown that functionally, the

intermediary role of cyclic nucleotides is affected by Ca++ concentration (Cheung and Storm, 1982) and it is clear that both cyclic nucleotides and Ca++ effect intestinal transport (Munck, 1981; Field, et al., 1980; Field, 1981), but the mechanisms are not fully understood.

7.4.6. Effects of steroids on water transport

The results of MT and KT administration on fluid transport indicated that steroid treatment was without a significant effect on intestinal water flux and tissue water uptake. However, the tendency for higher fluid absorption in the steroid-treated groups during the latter 30 min of incubation are in accord with the observations on the steroid-stimulated leucine absorption during similar time intervals. It is widely accepted that an increase in amino acid influx is associated with an increase in Na+ influx through a carrier-mediated Na-amino acid cotransport system (Schultz and Frizzell, 1975), and a simultaneous increase in ionic extrusion through the basolateral membrane would generate a region of hypertonicity within the lateral intercellular space which draws water in response to the osmotic difference (Diamond and Bossert, 1967).

The present experiments suggested that the observed action of steroids on leucine absorption might be related to the transport of Na+. In higher vertebrates, the principal ionic mediators involved in the process of fluid transport are Na+ and Cl-. However, the exact mechanism of isotonic transport remains in doubt, mainly because there is uncertainty about the routes of fluid movement

(Diamond, 1979). The current model for isotonic fluid absorption suggests that solutes enter the intercellular space both through the enterocytes and through the 'leaky' junctions; the resulting osmotic pressure difference draws water through the same pathways, and results in isotonic absorption (Schultz, 1981).

A great degree of uncertainty exists in the information available on the mechanism of fluid transport in fish, and the ionic compounds involved in this process (Na^+, Cl^-, K^+, H^+, HCO_3^-, NH_4^+). Moreover, adaptive changes observed in the transport of salt and fluid in the intestine of euryhaline, and stenohaline, fish suggest a dual control of ionic entry in the apical and basolateral membrane of the enterocytes (Lahlou 1976). However, evidence is accumulating that Cl^- plays an important role in the control of fluid absorption and osmoregulation in vertebrates, especially in teleost fish (Zandunaisky and Degnan, 1981). Unlike higher vertebrates, euryhaline fish, such as flounder and eel, transport Cl^- in excess of Na^+ in their intestine, thus resulting in a serosa-negative potential difference (Huang and Chen, 1971; Ando, et al., 1975). However, in stenohaline freshwater species (eg. goldfish) the transepithelial potential is serosa-positive, indicating transport of cations in excess of anions (Ellory, et al., 1973). Trout (S. irideus), however, can only tolerate a stepwise increase of external osmolarity and retain serosa-positive potential in all salinities (Lahlou, 1976). Recently Musch et al. (1982) have provided evidence that the intestinal brush border membrane of winter flounder contains a Na^+, K^+, Cl^- co-transport system which is inhibited by furosemide, a strong inhibitor of Cl^- transport, but it is not known

if the same is true of other euryhaline species. Furthermore, Musch and coworkers have shown that in flounder intestine, the Na+,K+,Cl- system is electrogenic and transports more cations than anions, although the exact stoichiometry remains to be determined. Adaptation and transfer of goldfish to hyperosmotic medium is associated with a reduction in net transmural and mucosal uptake of Na+ (Ellory, et al., 1972). By contrast, the net transepithelial transport of Na is increased in the salt water-adapted trout, while there is a significant reduction in the mucosal entry of Na+, Cl- and Ca++ (Bensahla-Talet, et al., 1974; Lahlou, 1976). It was suggested by Lahlou (1976) that apical entry and active serosal transport are modified separately in the fish intestine during salt water adaptation, and the reduced mucosal entry of ions in trout with a poor rate of gill ion extrusion is vital for survival in sea water. In other euryhaline species of fish such as flounder and eel with an efficient gill ion extrusion system, the intestinal control of ionic entry may be of less importance, although it is not certain to what degree Na+ and Cl-, or other ionic compounds contribute to the control of fluid transport in trout. However, the present study indicated a small dependence of water absorption on the activity of the Na+ "pump" at the basolateral membrane of the enterocytes in the midgut, as evidenced by a small influence of ouabain on water absorption in this region. Boge', et al. (1979) also reported a stronger action of ouabain on water absorption in the hindgut region of rainbow trout intestine. The generally stronger action of ouabain observed by these authors might be the result of the higher incubation temperature (20°C), used in their studies. Thus, there is

evidence that the inotropic action of ouabain changes with temperature (Greeff and Hofner, 1981). Boge et al. (1979) have also reported a reduced rate of water uptake in the presence of DNP, and a strong inhibitory action of NaF, consistent with the present findings. Finally, the tendency for a higher water absorption in the steroid treated group, may support the hypothesis that the action of MT and KT is effected through the activation of the Na+ "pump" during the latter 40 min of incubation. However, the mechanism of the rapid action of MT on leucine absorption observed during the initial 20 min of the experiment remains less certain.

It would be of value to further investigate the possible mechanisms of steroid action on amino acid absorption at the cellular level, since at present the suggestions put forward are based on techniques which permit bidirectional transmural fluxes. The methodology required to determine the exact mechanisms should account for the movement of solutes across the mucosal brushborder membrane, solute exchange across the basolateral membrane, and transepithelial fluxes by diffusional movement through extracellular shunt pathways.

Summary:

(1) The intestinal transport of leucine and fluid was studied in response to in vitro administration of MT and KT.

(2) Both MT and KT increased the intestinal absorption of the amino acid without significantly affecting fluid transport.

(3) The results indicated that none of the inhibitors (NaF, DNP or

ouabain) individually abolish the intestinal absorption of the amino acid, although each significantly reduce it.

(4) It would appear that ouabain and DNP significantly modify the action of MT, although their effect on KT is uncertain.

(5) It is suggested that the steroid-induced leucine transport during the latter 40 min of incubation might be partly the result of an increased activity of Na+,K+,ATPase.

(6) In view of the large concentration of the steroids used, the possibility of a non-specific action is discussed in relation to the experimental data available on adrenal steroids.

Table 7.1
Statistical comparison of the Ci/Cf ratios obtained from intestine of different weights.

WET WEIGHT (g)	DRY WEIGHT	MEAN Ci/Cf	S.D.	D.F.	T VALUE	P
0.1868	0.0254	1.0000	0.0059			
CONTROL				12	0.1937	>> 0.05
0.2452	0.0362	0.9992	0.0091			
0.2732	0.0369	0.9888	0.0105			
MT				12	0.7938	>> 0.05
0.1597	0.0236	0.9957	0.0207			
0.2229	0.0309	1.0039	0.0087			
KT				12	1.5435	>> 0.05
0.1438	0.0205	0.9962	0.0099			

The "MEAN" values shown are mean of 7 Ci/Cf ratios obtained during 60 min incubation of each intestinal tissue. The means were compared by student's T-test. T value for $P \leq 0.05$ with 12 degree of freedom (D.F.) is 2.10.

Fig. 7.1
Correlation of intestinal dry weight and tissue water content after 60 min incubation.

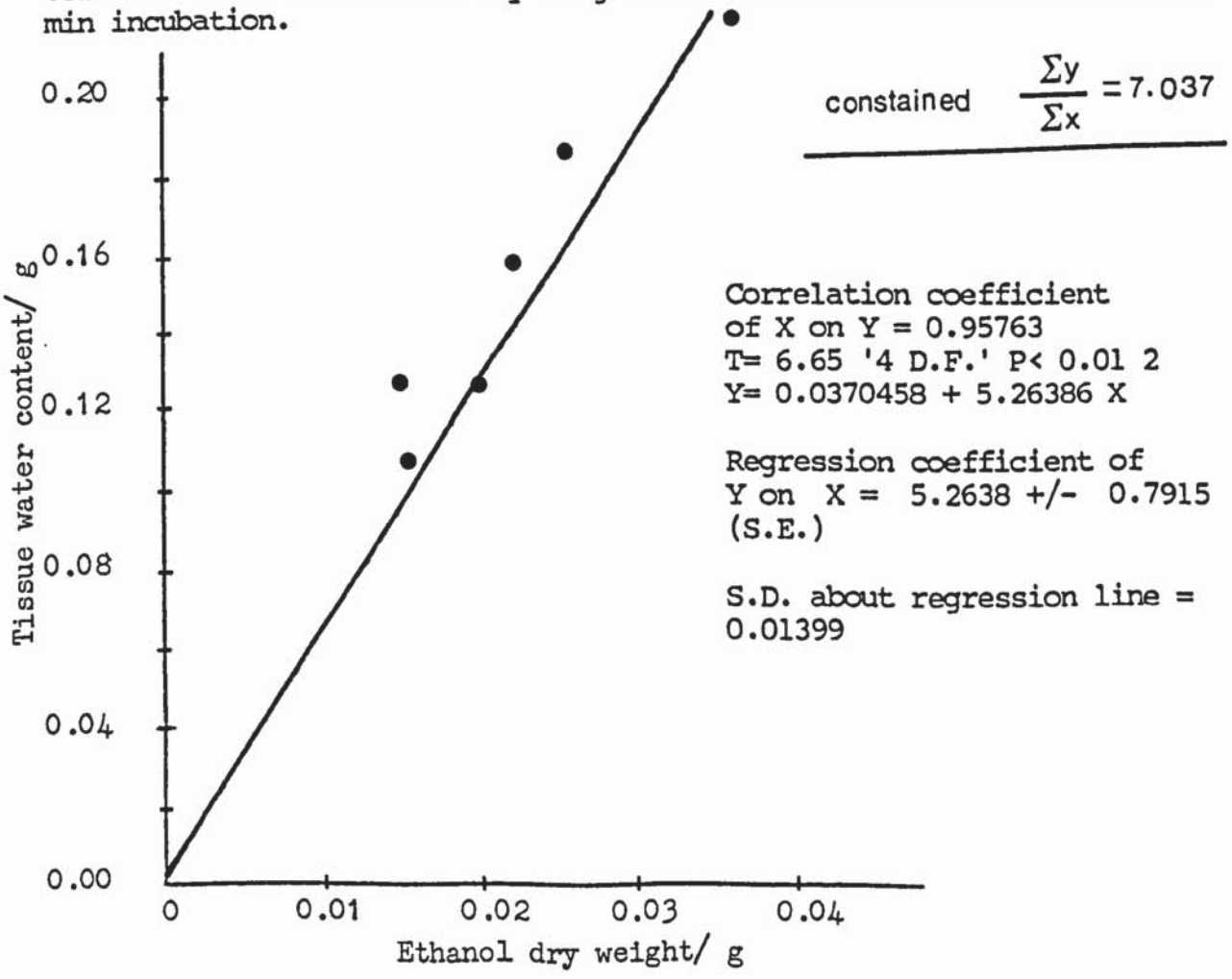


Table 7.2

Percentage recovery of L-leucine from the incubation media and ethanol extracts in different experimental groups.

	Percentage recovery of L-leucine					
	TREATMENTS					
INHIBITOR	CONT	MT	MT+INH	INH	KT	KT+INH
a-Incubation media						
OUA	96.40	99.80	105.86	91.50	102.86	106.98
DNP	99.63	98.68	95.16	100.74	97.61	93.18
NaF	94.63	108.77	89.50	85.70	86.11	93.71
b-Ethanol extracts						
OUA (M)	98.52	98.62	96.38	82.73	100.17	94.36
OUA (H)	88.35	82.39	82.16	96.18	104.36	96.55
NaF (M)	98.52	98.62	96.33	83.86	100.17	89.18
NaF (H)	88.35	82.39	90.50	97.38	104.36	94.15
DNP	NOT AVAILABLE					

Values shown represent the observations made from pooled samples of 4 media. M: midgut; H: hindgut; OUA: ouabain; DNP: 2,4,dinitrophenol; Naf: sodium fluoride; MT: methyltestosterone; KT: 11-ketotestosterone; INH: inhibitor

Table 7.3

Effects of 17a-methyltestosterone (MT) and 11-ketotestosterone (KT) on the absorption of 14C-L-leucine by the strips of intestine.

	u Moles of leucine absorbed/g ethanol dry weight					
	SAMPLING TIME (MIN)					
TREATMENT	5	10	20	30	40	60
CONTROL	27.7198	38.5640	52.7649	64.2126	68.3931	112.2310
+/- S.E.	3.2511	7.1164	5.7294	7.4662	9.4675	7.6850
		**	**	****	***	***
MT	34.4817	66.3798	82.4804	110.2850	108.6720	150.7250
+/- S.E.	7.0420	7.1187	7.1589	9.8587	8.3067	11.1252
			**	**	****	***
KT	23.5183	45.9230	81.8248	95.2046	111.1160	146.2050
+/- S.	3.3031	5.9445	8.3736	10.9565	9.1742	9.8187

Values are mean +/- S.E. of 12 observations. Combined (ANOVA) indicated significant differences between control and MT ($P<0.001$), and between control and KT ($P<0.02$). Superscripts (*) indicate significant variations between individual means compared to those of controls (** $P<0.02$; *** $P<0.01$; **** $p<0.001$).

Table 7.4.1

Effects of 17a-methyltestosterone (MT), 11-ketotestosterone (KT), and ouabain (OUA) on the absorption of 14C-L-leucine by the strips of intestine.

u Moles of leucine absorbed/g ethanol dry weight

TREATMENT	SAMPLING TIME (MIN) 5	10	20	30	40	60
CONTROL	25.7814	37.6043	44.6667	57.2539	88.8811	109.6450
+/- S.E.	6.7821	16.2600	10.1735	5.1342	16.3860	12.3884
		a			a	* aa
MT+	30.7022	54.9047	70.8212	84.8188	94.1763	136.9500
+/- S.E.	8.6682	13.8906	12.2968	11.5864	15.2351	18.6691
		a			a	aa
MT+OUA	16.7847	21.0471	39.4125	58.6698	60.9011	75.2679
+/- S.E.	2.1996	5.7765	10.9858	11.3296	13.1259	14.5162
						*
OUA+	8.2967	36.6799	45.1682	64.7895	71.6238	69.3063
+/- S.E.	2.6308	14.8291	7.5149	4.3438	7.7071	2.3843
					b	b
KT+	22.3584	50.1665	66.5703	77.2396	109.228	137.315
+/- S.E.	5.5405	9.8163	6.7503	11.3514	15.3632	18.1372
					b	b
KT+OUA	20.7166	30.1970	47.8409	68.5070	70.6142	100.8480
+/- S.E.	7.0980	8.1287	10.0144	9.9008	10.0778	10.0352

Values are mean +/- S.E. of 4 observations. Combined (ANOVA) indicated significant differences between MT and MT+OUA ($P<0.02$). Superscripts (*) indicate significant variations between individual means compared to those of controls (* $P<0.05$), and the values sharing similar symbols are significantly different (a $P<0.05$; aa $P<0.001$; b $P<0.05$).

Table 7.4.2
Effects of 17a-methyltestosterone (MT), 11-ketotestosterone (KT), and 2,4,dinitrophenol (DNP) on the absorption of 14C-L-leucine by the strips of intestine.

	u Moles of leucine absorbed/g ethanol dry weight					
	SAMPLING TIME (MIN)					
TREATMENT	5	10	20	30	40	60
CONTROL	29.4103	44.9597	56.3163	57.9758	46.4369	109.8790
+/- S.E	5.8941	14.1513	13.5436	20.3592	11.0215	16.5442
		a	*	aaaa ****	aaa ****	aaaa ****
MT+	57.0723	78.8561	97.4094	124.0880	114.3490	183.7810
+/- S.E.	13.7640	12.7302	12.0703	10.0788	15.0289	18.0258
		a		aaaa	aaa	aaaa
MT+DNP	60.3346	38.3130	77.7566	44.4820	54.1641	77.4885
+/- S.E.	14.9255	10.4089	8.2392	8.3166	13.6057	12.4505
				*		**
DNP+	15.5213	78.6724	64.7691	33.1629	49.2833	66.4688
+/- S.E.	2.5221	8.1903	6.5308	11.0692	7.2331	15.4741
			bbb	* bbb	**	bbb
KT+	31.8214	35.4740	71.7288	99.4581	91.5426	137.5790
+/- S.E.	2.5843	11.9267	10.0490	21.5042	7.3011	7.9904
			bbb	bbb		bbb
KT+DNP	14.2134	43.1202	17.1686	48.9883	89.3965	76.1905
+/- S.E.	7.7888	7.3135	6.6499	5.9156	5.4986	1.3364

Values are mean +/- S.E. of 4 observations. Combined (ANOVA) indicated significant differences between control and MT ($P<0.001$), MT and MT+DNP ($P<0.001$), KT and KT+DNP ($P<0.02$). Superscripts (*) indicate significant variations between individual means compared to those of controls (* $P<0.05$; ** $P<0.02$; **** $P<0.001$). The values sharing similar symbols are significantly different (a $P<0.05$; aaa $P<0.01$; aaaa $P<0.001$; bbb $P<0.01$).

Table 7.4.3
Effects of 17a-methyltestosterone (MT), 11-ketotestosterone (KT), and sodium fluoride (NaF) on the absorption of 14C-L-leucine by the strips of intestine.

	u Moles of leucine absorbed/g ethanol dry weight					
	SAMPLING TIME (MIN)					
TREATMENT	5	10	20	30	40	60
CONTROL	27.9678	33.1281	57.3116	77.4081	69.8613	117.1690
+/- S E	5.7712	8.2848	6.4195	9.2295	17.1663	14.5011
MT+	45.6705	65.3787	79.2107	* aaa 121.9490	* aa 117.4900	aa 131.4440
+/- S E	12.8179	10.3117	11.9867	22.5451	13.9403	11.6884
MT+NaF	33.6806	29.2481	36.9874	aaa 46.8903	aa 58.7416	aa 60.4713
+/- S E	9.7438	10.3799	9.6503	7.8800	19.4937	10.0823
NaF+	17.8374	14.3882	14.0195	34.6170	** 29.1170	*** 41.9754
+/- S E	3.6472	2.8759	11.1899	13.4518	9.7384	14.6011
KT+	16.3751	52.1285	107.1750	108.9160	*** b 132.5770	* bbb 163.7220
+/- S E	2.8751	9.7402	17.2910	23.5147	18.8170	22.6771
KT+NaF	21.1125	23.3815	30.3671	85.1394	b 84.1699	bbb 44.3677
+/- S E	2.3957	16.7901	13.0217	7.1479	14.2325	16.4567

Values are mean +/- S E of 4 observations. Combined (ANOVA) indicated significant differences between control and MT ($P<0.05$), MT and MT+NaF ($P<0.02$), control and KT ($P<0.02$), KT and KT+NaF ($P<0.02$). Superscripts (*) indicate significant variations between individual means compared to those of controls (* $P<0.05$; ** $P<0.02$; *** $P<0.01$; **** $P<0.001$). The values sharing similar symbols are significantly different (a $P<0.05$; aaa $P<0.01$; bbb $P<0.01$; bbbb $P<0.001$).

Table 7.5
Effects of 17a-methyltestosterone (MT), 11-ketotestosterone (KT), and inhibitors (INH) on tissue water uptake by the strips of trout intestine.

g of water /g ethanol dry weight

	TREATMENTS					
INHIBITOR	CONT	MT	MT+INH	INH	KT	KT+INH
OUA (M)	1.0766	1.6059	1.1522	1.2652	0.9189	0.8340
+/- S.E.	0.3572	0.5351	0.0369	0.6031	0.2096	0.1750
DNP (M)	1.3392	1.1118	0.8280	0.3290	1.0850	0.1330
+/- S.E.	0.1653	0.3166	0.2104	0.0305	0.1517	0.1652
NaF (M)	1.2407	1.7648	0.4077	0.3010	1.3478	0.3852
+/- S.E.	0.1931	0.0551	0.5153	0.1510	0.7099	0.5576
OUA (H)	0.3577	0.2542	0.1298	0.1624	0.5223	0.3056
+/- S.E.	0.2232	0.0754	0.2556	0.5832	0.0112	0.2718
DNP (H)	0.6117	0.1447	0.0804	-0.237	0.6133	-0.056
+/- S.E.	0.4635	0.0173	0.3355	0.3412	0.3193	0.2311
NaF (H)	1.2346	1.0883	-0.200	-0.763	0.8896	0.4369
+/- S.E.	0.4528	0.1872	0.0021	0.6581	0.2304	0.4480

Values are mean +/- S.E. of 2 observations. Observations were tested by one-way analsis of variance and Duncan multiple range test (Duncan, 1955), and the values containing dissimilar superscripts are significantly different ($P<0.05$). M: midgut; H: hindgut; OUA: ouabain; DNP: 2,4,dinitrophenol; Naf: sodium fluoride.

Fig. 7.2

Effects of methyltestosterone and 11-ketotestosterone on the rate of leucine absorption by the strips of rainbow trout intestine.

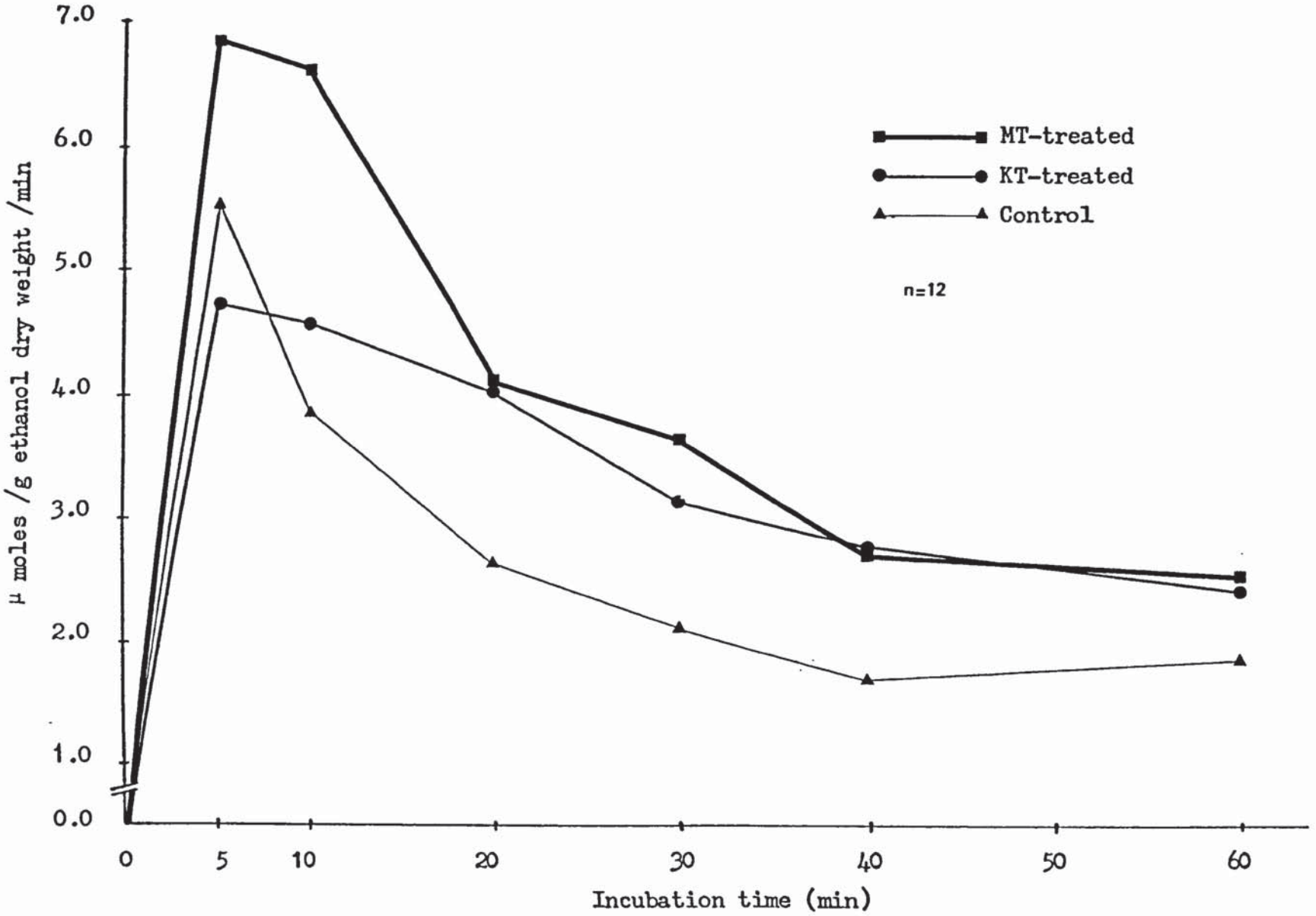
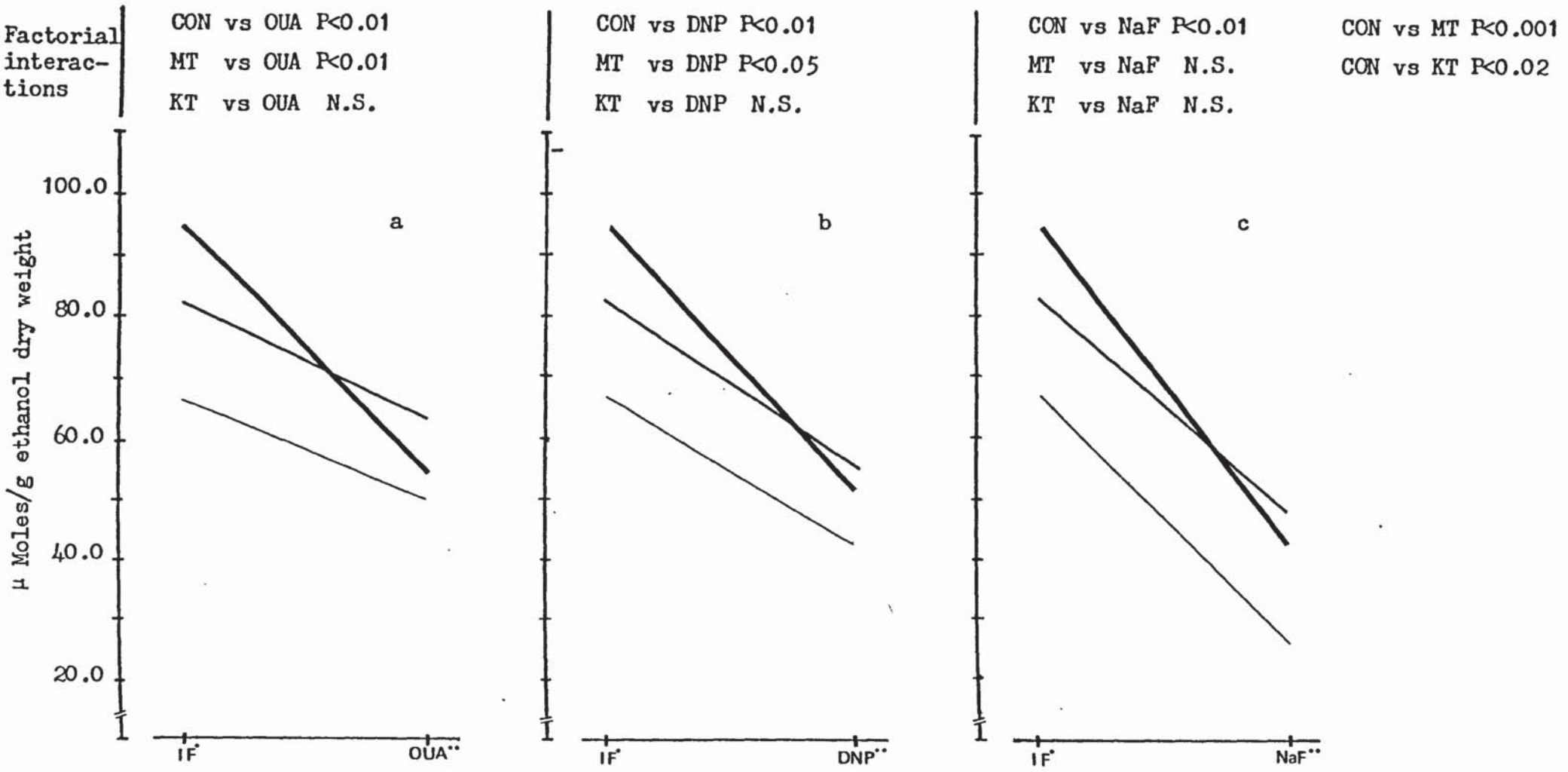


Fig. 7.3

Factorial analysis (z^2) of the absorption of L-leucine in presence of steroids and inhibitors during 60 min incubation.



IF : Inhibitor free; OUA : ouabain treated; DNP : DNP treated; NaF : NaF treated; CON : control; ——, MT-treated; ——, KT-treated; ——, steroid free. Values are mean of 12 (*) and 4 (**) observations, each with 6 replicates. Interactions are apparent from the relative slopes of the lines

Fig. 7.4.b Effects of steroids and inhibitors on the apparent intestinal accumulation of the incorporated leucine (AIAL.inc).

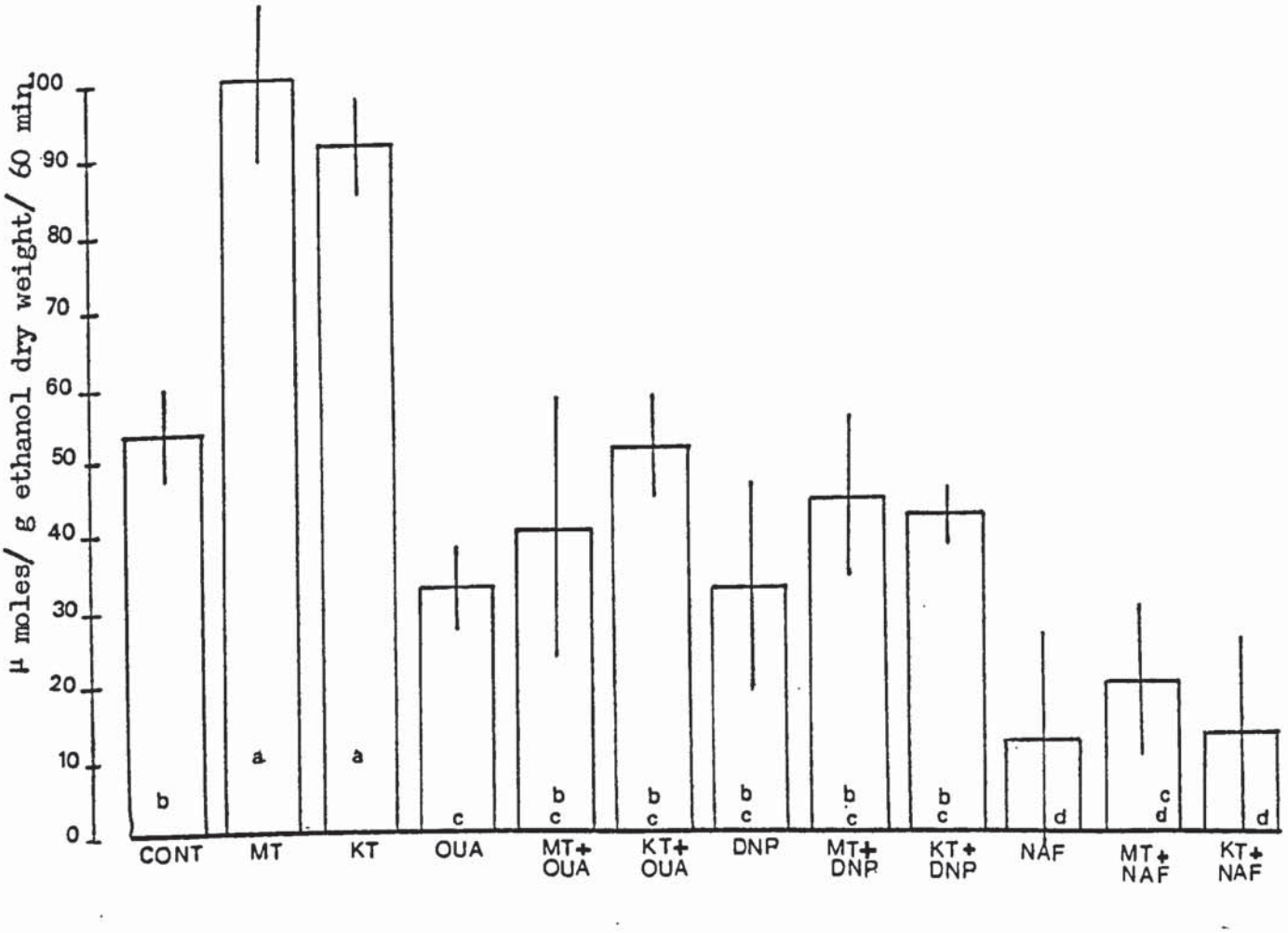
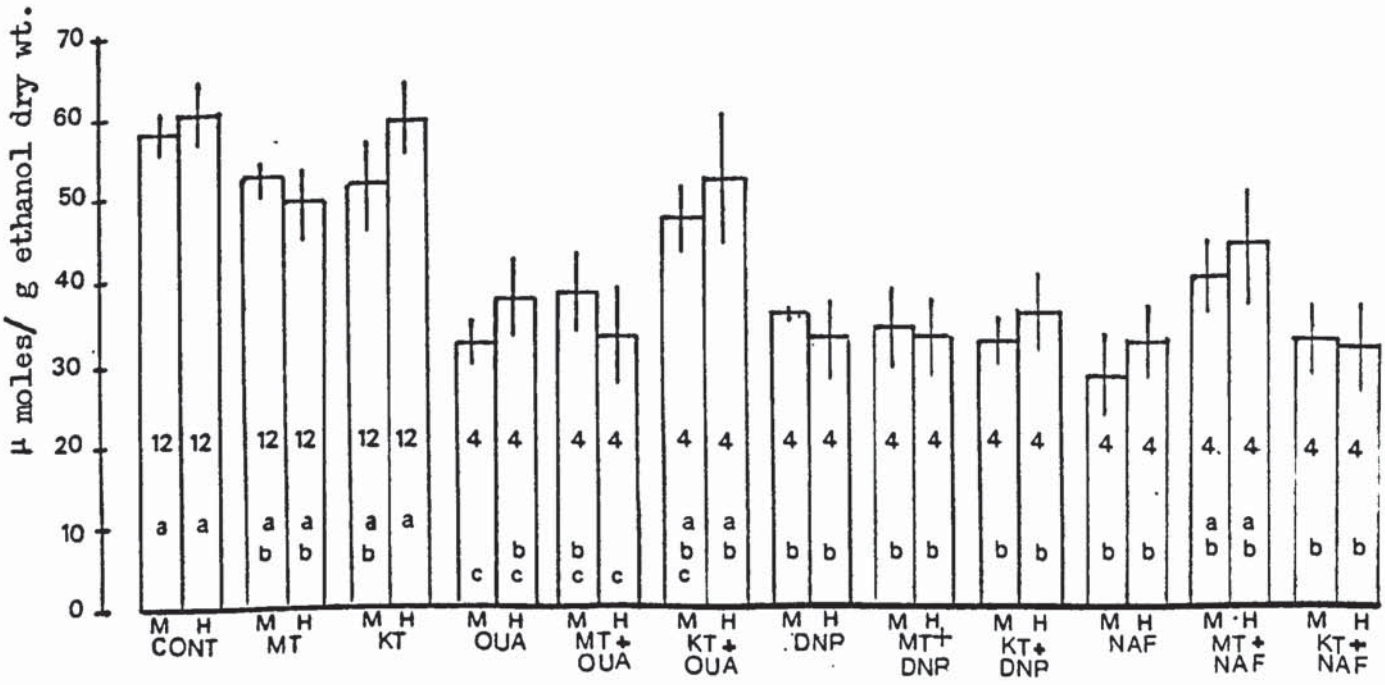
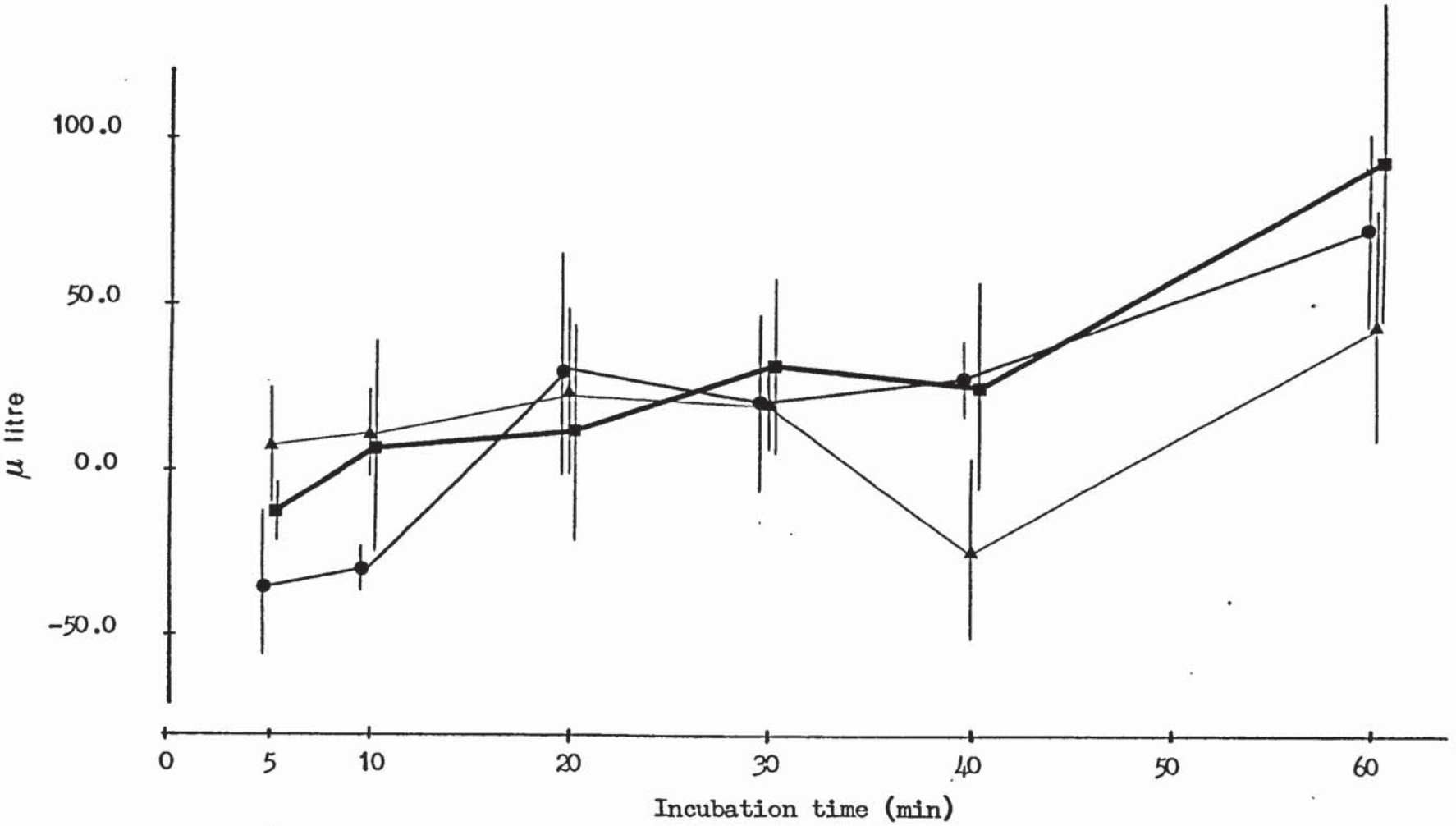


Fig. 7.4.a Effects of steroids and inhibitors on the intestinal accumulation of solute leucine (IAL.sol).



Values are mean +/- S.E. of 12 or 4 observations (as shown). Columns containing dissimilar symbols are significantly different ($P<0.05$). M: midgut; H: hindgut

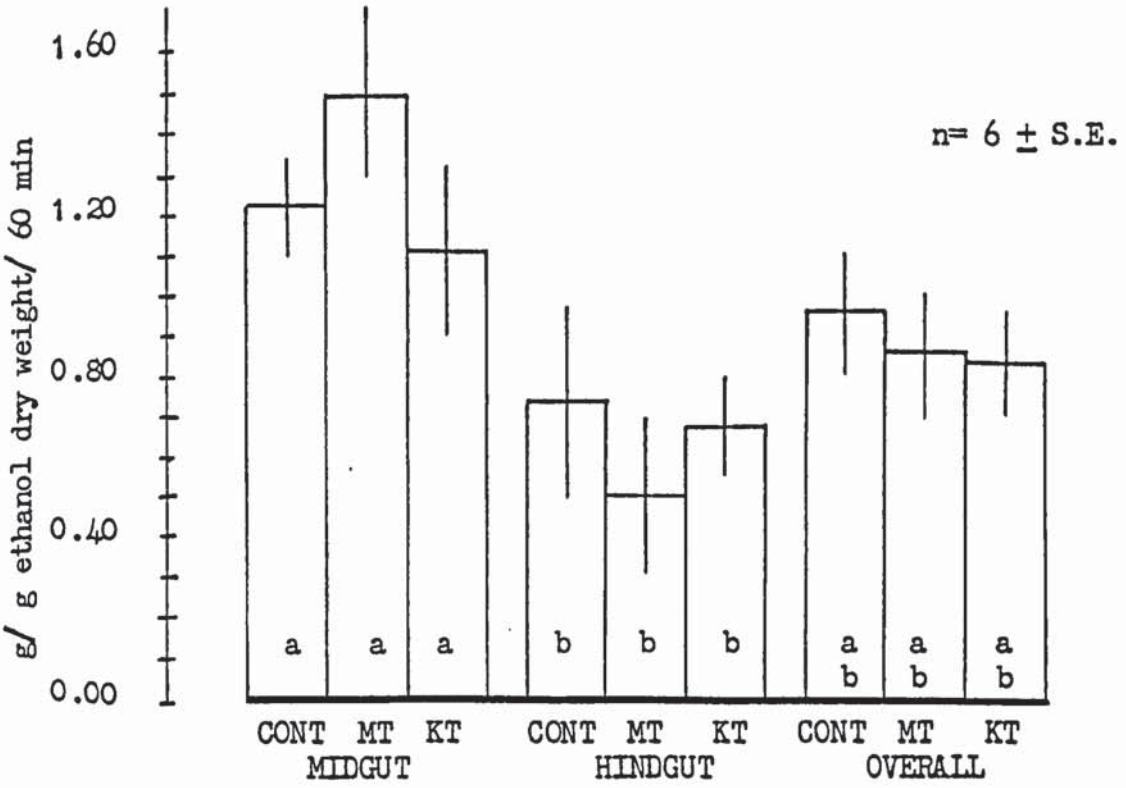
Fig. 7.5 Effects of methyltestosterone and 11-ketotestosterone on intestinal fluid absorption during 60 min incubation period using phenol red



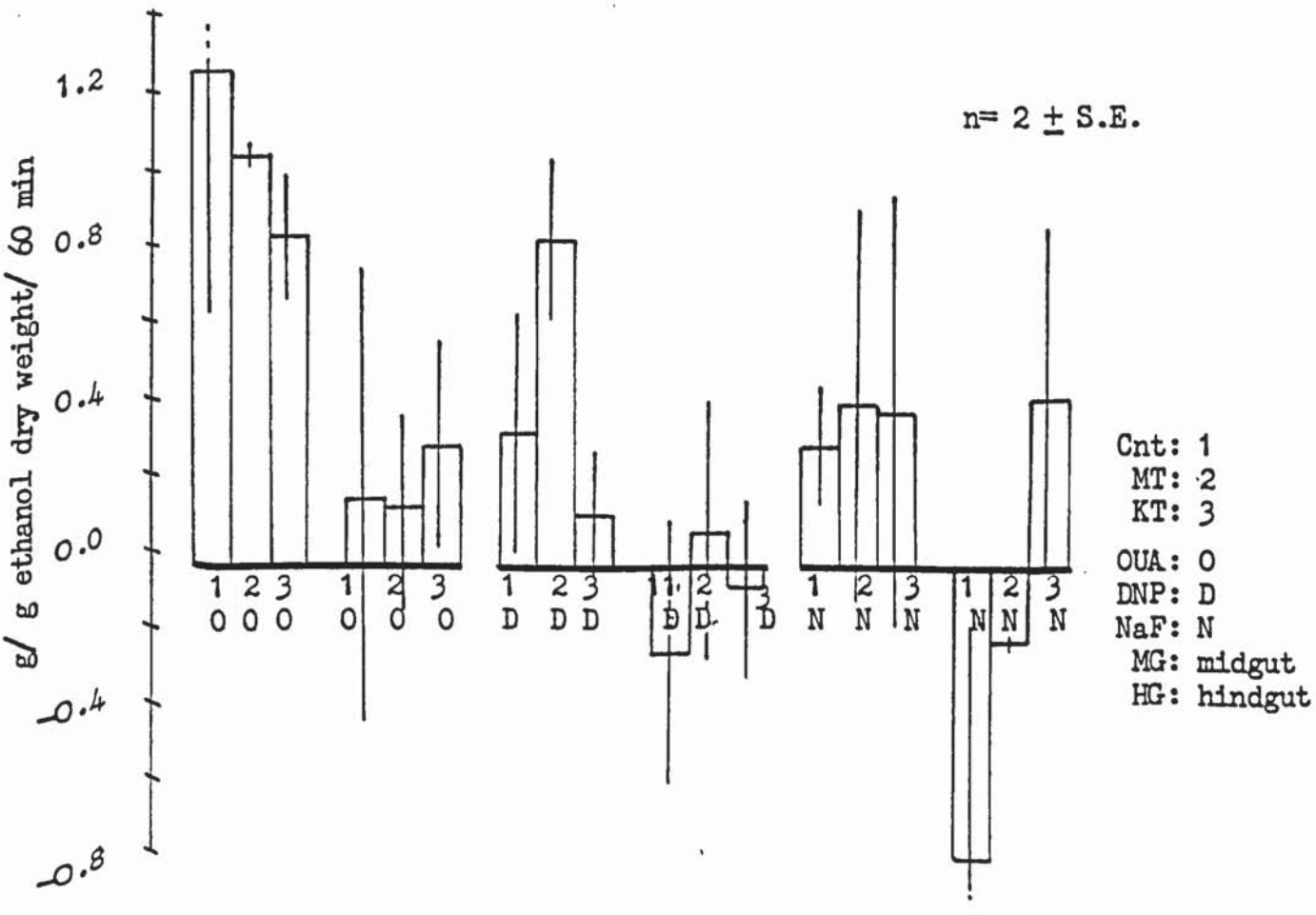
Values are mean +/- S.E. of 6 observations. Differences are not statistically significant. ■——■ MT-treated; ●——● KT-treated; ▲——▲ control.

Fig. 7.6 Effects of methyltestosterone (MT), 11-ketotestosterone (KT), ouabain (OUA), DNP & NaF on the uptake of water by strips of intestine.

a. Effects of steroids



b. Effects of inhibitors and inhibitors+steroids



Some observations on structural and functional differences in the intestine of immature male and female rainbow trout (S. gairdneri).

Section 8

8.1. Introduction

The influence of sex steroids have been investigated on the absorption of water and amino acids by the intestine of rainbow trout, as part of the present study. It was shown that pharmacological doses of 17a-methyltestosterone and 11-ketotestosterone, and to a lesser extent 17B-oestradiol, stimulate the intestinal absorption of amino acids (Sections 4, 5, 6 & 7; Habibi et al., 1983; Habibi and Ince, 1983), but there is no information on the physiological role of gonadal hormones on the control of gastrointestinal function in fish. In mammals however, there is evidence that the ratesof absorption of water and glucose are higher in female than in male rats (Fisher, 1955), and ovariectomy impairs the intestinal absorption of glucose (Althausen, 1949). In addition, Matty (1964) demonstrated that the short-circuit current across the intestine was higher in female than in male mice, and that ovariectomy was without effect on the potential difference or short-circuit current across the intestine. Other investigators studying mainly the morphology and rate of cell division in the mammalian gastrointestinal tract have observed that, although sex steroid administration resulted in hyperthrophy of intestinal tissue, gonadectomy was without significant effect (Curriere, 1966; Ksiazkiewicz-Szapiro, 1970; Tuohimaa and Niemi, 1968).

The aims of the present experiments were to investigate sex differences in intestinal absorption of fluid and solutes, and to examine the morphological variations in the intestine of immature male and female rainbow trout subjected to castration and

ovariectomy. In this section, a technique is described for castration and ovariectomy, using lateral a route to avoid any interference with the alimentary tract during surgical operation. The results indicated no sex differences in the intestinal transport of leucine and water, although it would appear that sodium transport might be higher in the intestine of female fish. These findings are discussed in relation to the histological differences observed in the intestine of male and female trout.

8.2. Materials and methods

8.2.1. Animals

Rainbow trout (S. gairdneri) aged from 8-9 months (approximate length 12 cm) were reared at Aston. Fish were maintained at 14 +/- 1° C, as described in Section 3.

8.2.2. Gonadectomy

The technique described below was developed to avoid any interference with the gastrointestinal tract. By contrast, the surgical method described by Robertson (1958) utilizes the midventrad route for access to the gonads, and involves retracting the alimentary canal and liver for visualization of the cephalic attachment of the gonads.

Operative Procedure

Fish were anaesthetized (6-7 min) in a weak solution of benzocain (approximately 1:17000 w/v), and a 5-6mm, superficial, lateral incision made at the position and angle shown in Fig. 8.1. Using a blunt pair of forceps and a blunt seeker, the tissue was teased away and an incision was made between the lateral bones through the lateral body wall (lateral bones remained uncut). The gonad could be visualized easily by focussing a beam of light into the body cavity, and then was exteriorized, using a blunt glass seeker (Fig. 8.2.a,b,c & d). The ovary or testis was pulled slightly, a fine pair of cissors (spring bow) was inserted into

the cavity and the cephalic attachment was cut. This method is specially suitable for complete removal of the gonads at the anterior end, since the position of the incision is about 5mm posterior to the cephalic attachment. The posterior part of the gonad was detached by gently pulling the gonad forward. It was found that the posterior detachment occurs at the duct region, approximately 5-6mm anterior to the genital opening. For sham operations, the gonads were examined but maintained intact. The incision wound was closed with individual silk sutures. For bilateral gonadectomy, the same procedure was repeated on the other side of the fish. The closed wounds were covered with dental glue containing antibiotics (Fig. 8.3.e,f & g), and the fish was returned to the tanks after superfusing the gills with oxygenated water. Mortalities were approximately 2.8%, and the total operation time was 10-11 min. Generally, food was then witheld for 24h, although in most cases, fish could resume feeding after 4-5h.

8.2.3. Preparation and incubation procedure

The absorption and accumulation of water and solutes were measured using strips of rainbow trout intestine, in vitro. The technique was similar to that described in Section 7 but with the following modifications. Two strips of midgut and two strips of hindgut were obtained from each fish. The strips were then divided into two groups, each containing a portion of mid- and hindgut, and transferred to incubation vessels (1 & 2), containing 5 ml of the medium. The incubation media of both groups consisted of trout Ringer solution (section 7) and 5 mM/l L-leucine. Group 1 was used

for the measurement of amino acid transport, and to the incubation media was added 0.05 uCi/ml L-14C-leucine. Group 2 was used for the determination of Na+, K+, and Ca++ (tracer-free medium). Determination of tissue water content and intestinal water flux was similar to that described in Section 7, using weight differences and the non-absorbable marker, phenol red. The incubation vessels were gassed continously with 95% 02 : 5% C02, and shaken at 100 Os/min in a constant-temperature water bath (14°C), over a period of 40 min.

8.2.4. Media and tissue analyses

8.2.4.1. L-leucine transport

Samples (100 ul) of media were withdrawn after 2,4,6,8,10,15,20,25,30, and 40 min. At the end of the incubation period (40 min), the mid- and hindgut were extracted in 80% ethanol, and samples of the media and the ethanol extracts were assayed for 14C-activity, and ethanol-dry weights determined. Samples of media and extracts were also chromatographed to determine the percentage recovery of 14C-L-leucine, as described in Section 7.

8.2.4.2. Electrolyte transport

Samples (100 ul) of media were withdrawn after 2,4,6,8,10,20,30, and 40 min, and at the end of the incubation period mid- and hindgut were removed and extracted. Determination of electrolyte transport was carried out only for sham-operated male and female, castrated and ovariectomized. The total number of fish used in each group was 4, but the samples obtained from 2 fish were pooled, resulting in

the replicate number of 2 for any group. Na+,K+, and Ca++ concentration were determined by atomic absorption flame photometry after appropriate dilutions (Section 3).

8.2.5. Histological preparations

Intestinal tissues (5mm) were obtained from the anterior part of the midgut (immediately posterior to the pyloric caecum). Each intestinal section included a pyloric caecum for identification of the exact position in the serial sections. A freshly-dissected specimen from each fish was fixed at room temperature for 24h in Bouin's solution. The tissues were embedded in wax, sectioned at 6-8 um thickness (several sections from each specimen) and then stained with hemotoxylin and eosin (Luna, 1968). The epithelial cell height, the distance between the tip of the fold to serosa and the distance from the tip to the base of each fold were measured with a micrometer eyepiece.

8.2.6. Presentation of the results

The terms used to describe leucine and fluid transport are similar to those described in Section 7. The present results also include the absolute concentration of 14C-L-leucine (u moles/l) during 40min incubation, since it appears from the results that gonadectomy might have affected the distance between the tip of the fold to serosa, and the unit weight of the intestine. The values obtained for Na+,K+ and Ca++ are also expressed in terms of absolute concentrations, since it was not possible to distinguish between absorption by the intestine and endogenous secretion.

8.2.7. Statistical analysis

The results obtained for intestinal absorption and leucine concentrations were analysed with a two-way analysis of variance (Split-plot), F-test and T-test. Differences between intestinal accumulation of water and leucine were tested by a one-way analysis of variance (Duncan multiple range test; Duncan, 1955). Differences were considered significant when $P<0.05$.

8.3. Results

8.3.1. percentage recovery of L-leucine.

L-leucine recovery was determined both in the incubation medium and in the ethanol extracts. Samples obtained from male and sham-operated male, or female and sham-operated female were pooled together (n=8 for each; 4 male and 4 sham-operated male, or 4 female and 4 sham-operated female), and samples from castrated or ovariectomized fish were also pooled (n=4 for each) and results are shown in Table 8.1. The percentage recovery of L-leucine obtained from the medium ranged between 89.30 - 111.09% (98.93 +/- 4.57; mean +/- S.E., n=4), and those obtained from the ethanol extracts ranged between 90.69 - 112.88% (103.97 +/- 2.49, n=8). The results indicated no variations between the experimental groups, and little or no metabolism of 14C-L-leucine during transmural transport.

8.3.2. L-leucine absorption.

The intestinal absorption of L-leucine was measured in 6 groups of rainbow trout; male, female, sham-operated male, sham-operated female, castrated and ovariectomized. The results are shown in Tables 8.2 & 8.3. Table 8.2 shows the absorption values expressed per g of ethanol-extracted dry weight of intestine, and Table 8.3 gives the absolute concentration of L-leucine during the 40 min incubation period. Comparison of the results obtained from intact males and sham-operated males, or between intact females and sham-operated females, indicated no significant differences in amino acid absorption which could be attributed to surgical intervention.

8.3.2.1. Sex differences.

Comparison of the results, either expressed per g of dry intestine or absolute concentrations between male and female, or between sham-operated male and female, indicated no significant sex differences in the ability of the intestine to transport L-leucine. The results obtained from the intact, or sham-operated fish were compared to the gonadectomized group, and it can be seen that neither castration nor ovariectomy resulted in any significant variations on either intestinal absorption per unit weight or the absolute leucine concentration during the 40 min incubation. However, a significant difference was found after 40 min incubation between the sham-operated female and ovariectomized fish ($P < 0.05$).

8.3.2.2. Intestinal accumulation of leucine.

The intestinal accumulation of solute leucine (IAL.sol) and apparent intestinal accumulation of the incorporated leucine (AIAL.inc) were determined as described in Section 7. The results are shown in Fig. 8.4.a & b. Comparison of the IAL.sol values obtained from male, female, sham-operated and gonadectomized fish indicated no significant variations between midgut, hindgut, or total intestinal accumulation of the amino acid, in any of the experimental groups. Similarly, no significant differences were found between the AIAL.inc of the intact, sham-operated and the gonadectomized fish.

8.3.3. Electrolyte absorption.

Concentrations of Na^+, K^+ and Ca^{++} were determined in the

incubation medium, and in the ethanol extracts of the intestine taken from sham-operated and gonadectomized fish. The transport parameters are expressed in terms of absolute concentration, since the endogenous secretion of electrolytes from the serosal side could not be distinguished from those present initially in the medium. Thus, the results only show the absolute changes observed in the concentration of Na+, K+ and Ca++ during 40 min incubation period. Furthermore, the intestinal accumulation of the electrolytes are not included due to high variability. One possible reason for the observed variation is the fact that ethanol extracts were diluted to the same extent as the samples obtained from the medium, and because of large differences in concentration, they were probably outside the calibration range.

8.3.3.1. Na+ Concentration.

Comparison of Na+ concentration indicated no significant differences between the sham-operated female and the gonadectomized fish. However, Na+ concentration was significantly lower in sham-operated females when compared to sham-operated males after 8 and 20 min of incubation ($P<0.05$) (Table 8.4). Since the mechanism of amino acid transport is directly linked to Na+ influx, for comparison, variations in Na+ concentration from the initial value (133.05 u moles/ml) were also determined and the results are shown in Fig. 8.5. It is apparent that Na+ was absorbed linearly during the initial 6 min of incubation, followed by an equilbrium phase (8-20 min), and then a decrease in absorption (20-40 min). However, lower values were obtained for Na+ absorption in the sham-operated males,

during the latter 30 min of incubation (10-40 min).

8.3.3.2 K+ and Ca++ Concentrations.

The results obtained for K+ were highly variable and inconsistent p sumably due to problems related to the atomic absorption spectrophotometry, since the same diluted solutions were used for estimation of Ca++ concentration which showed little variations. Furthermore, it is unlikely that the variations observed were due to K+ flux. For this reason mean K+ concentrations are not included, and the results shown in Table 8.5 represent individual determination of K+ concentration in the media. Measurement of Ca++ in the media revealed no significant variations between the sham-operated male and female, and the gonadectomized groups. No consistent pattern of absorption is apparent for Ca++, and the results indicate a fairly steady equilibrium throughout the incubation period, in all experimental groups (Table 8.6).

8.3.4. Intestinal fluid absorption and tissue water uptake.

The intestinal fluid absorption was measured using phenol red as a non-absorbable extracellular space marker (Section 7), in male, female, sham-operated and gonadectomized fish. The results are expressed in terms of Ci/Cf ratios (c.f. Section 7) (Table 8.7), and in ul of fluid flux per sampling time (Fig. 8.6). Statistical comparison of the results indicated no significant variations between male, female, sham-operated and the gonadectomized groups. Similarly, comparison of the results on intestinal water uptake revealed no significant variations between the experimental groups. However, in accord with results shown in Section (7), water uptake

is generally higher in the midgut than hindgut (Fig. 8.7).

8.3.5. Histological study.

8.3.5.1. Granulation.

The intestine of fish used in the present study were characterized by containing a broad and dense stratum compactum and aggregates of granule cells. Both stratum granulosum internum and stratum granulosum externum were apparent in all sections examined. A section of the intestine obtained from 30 months old rainbow trout, is also included for comparison (Fig. 8.8). The granular cell density seems higher in the older fish, both in the granulosum internum and externum. However, neither in the experimental fish (9-10 months old), nor in the 30 months trout, were granular cells arranged into a distinct band, as reported by Bergeron and Woodward (1982). As was apparent by visual comparison, no marked variations are seen between the normal male, female, sham-operated, or gonadectomized fish (Fig. 8.9, 8.10, 8.11 & 8.12).

8.3.5.2. Epithelial and fold heights.

The number of observations for intestinal thickness were limited by the availability of complete folds in the sections (90 transverse sections through the centre of the intestine). Distinctions between the folds were made purely by observation, and no instruments were used for determination of angles. The specimens prepared from the ovariectomized fish yielded unsuitable sections for determination of intestinal thickness (an example is shown in Fig. 8.12), thus no data on distance between tip of the fold to serosa is available for this group. It can be seen in Fig. 8.13a that the intestinal

thickness is higher in male fish when compared to those of female, castrated fish. Comparison of intestinal thickness in castrated male and sham operated female, revealed no marked variations between the two groups. The distance between the tip to the base of the fold was also measured in each case, and the percentage fold height in the whole intestine was determined. It would appear from the results that percentage fold height is higher in female than in male intestines, with no marked variations between intact and castrated males.

The epithelial heights (enterocyte length) were measured in all groups and the results are shown in Fig. 8.13b. It can be seen that epithelial heights were higher in the intestine of male fish than those in female. Similarly, castration resulted in reduced epithelial heights when compared to intact male fish, and no marked differences with those of female fish. No marked variations in epithelial heights were observed between intact and ovariectomized females, although the values are slightly lower in the latter group.

8.4. Discussion.

Before discussing the present results, it is relevant to consider the likely effects of gonadectomy, and hormonal differences in male and female rainbow trout. Although gonadal steroids were not measured, there is evidence for the presence of androgens in the plasma of immature (19 months) trout (12.9 +/- 2.6 and 8.9 +/- 2.8 ng/ml of testosterone in male and female fish respectively) (Schreck, et al., 1972). Also shown by the latter authors was a reduction of plasma testosterone concentration in castrated males from 27.4 to 6.8 ng/ml, 21 days after castration, and a reduction from 13.2 to 4.8 ng/ml, 42 days following gonadectomy in the plasma of ovariectomized fish. However, there are apparently no data on the concentrations of sex steroids in rainbow trout aged less than 19 months. The presence of other androgens and oestrogens have also been demonstrated in rainbow trout (Simpson and Wright, 1977; Scott, et al., 1980; Whitehead, et al., 1979; Lambert, et al., 1978), but there is no information on the plasma concentration of these steroids in immature fish. The fish used in the present study ranged between 9-10 months in age, and histological evidence suggests that the testicular tissue at this stage consists of small nests of spermatogonia, grouped into lobules by septa composed of fibrous tissue and blood vessels (Robertson, 1958). However, it is not clear to what extend the lobular boundary cells and interstitial tissues in the gonad of immature trout contain the steroid-secreting cells. In the present study, measurement of intestinal aborption was

carried out 8 weeks after gonadectomy, and there was no visible sign of gonadal regeneration. In this context, examination of the castrated and ovariectomized fish after 6 months also indicated no visible evidence of gonadal regeneration. Since the gonads in fish are the main site of sex steroid production (Christensen, 1975; Fowcell, 1975), any morphological or physiological changes observed in the intestine of trout after gonadectomy could be related to the effects of gonadal hormones. However, the possibility of an indirect effect, secondary to gonadal steroids or gonadectomy cannot be ruled out, since in the latter, a reduction in plasma sex steroid concentration would effect neuroendocrine secretion in the pituitary (Donaldson, 1973).

8.4.1. Sex differences in intestinal absorption.

Eight weeks following gonadectomy, there was no significant change in leucine transport by the intestine, nor were there differences in intestinal leucine absorption between male and female fish. Similarly, the results indicated no significant sex differences in fluid absorption by the intestines of immature trout. These observations are in contrast to those reported in mammals which suggest that the rate of absorption of water and glucose are markedly greater in female than in male rats (Fisher, 1955; Fisher and Parsons, 1955), and that ovariectomy might reduce glucose absorption (Althausan, 1949). In this context, the present results are also different to reports that ovariectomy in mice increases intestinal transport of fluid and glucose (Ahmed-Sorour, 1978). The observed lack of variation in male and female fish however, does not

necessarily indicate the absence of sex steroids in immature rainbow trout, since significant differences were observed between 'apparent' Na+ absorption and histological parameters measured in male and female fish. The present results suggest that Na+ absorption might be higher in the intestine of intact female than in male fish, whereas no significant differences were observed following castration and ovariectomy. It is clear that the active transport of amino acids across the brushborder membrane is coupled with the entry of Na+, through a Na-amino acid co-transport mechanism (Smith, 1970; Schultz and Frizzell, 1975; Munck, 1977,1981). However, unlike the consistent increase in amino acid absorption during the 40 min incubation period, Na+ absorption was observed only during the initial 8-10 min of incubation, followed by an equilibrium phase, and a decrease in the female and gonadectomized group. In male fish, Na+ absorption was only apparent during the initial 2-8 min, followed by a decrease which became steady during the latter 20 min of incubation. The apparent lack of Na+ absorption in the female and gonadectomized group during 20-40 min of incubation is explicable in terms of the entry of endogenous Na+, mainly from the the serosal side into the media (Schultz, 1981). However, the lower values observed in the male could have either been the result of a decreased absorption, or an increased secretion of Na+. The higher rate of Na+ absorption in female trout is in accord with the previous finding that in rats, the short-circuit current across the intestine is higher in female than in male rats (Matty, 1964). The fact that no significant variations were obtained between the female and the gonadectomized

group, favours the increased secretion of Na+ in males, or an inhibitory action of male sex hormone on Na+ absorption, rather than a stimulatory effect of female sex steroids. This however, is in complete contrast to the observed pharmacological effects of androgenic compounds on intestinal absorption of amino acids (Sections 4,5,6, & 7).

8.4.2. Morphological differences.

Histological studies indicated no apparent sex differences in the granulation of the intestine (granule cell density). However, the significance of this observation is uncertain since little is known of the function of the stratum granulosum in fish (Kapoor, et al., 1975). It has previously been shown that the ontogeny of the stratum granulosum in the trout intestine is related to the age of fish (Bergeron and Woodward, 1982). This finding is consistent with the present observation that intestinal granule cell density is higher in the 30 month-old trout than in 10-11 month old fish. Ezeasor and Stoke (1980) have studied the distribution, morphology and cytochemistry of granule cells in the intestine of rainbow trout, and suggested that granule cells might constitute a part of the body defence mechanism, both mechanical and humoral, which develops in response to environmental demands. Measurement of intestinal thickness and epithelial heights revealed that whereas intestinal thickness is higher in males than in females, the percentage fold height in the intestine is greater in the latter group. However, it is not certain if a higher percentage of fold

height in the intestine would reflect a greater absorptive area in females, since the relative area of mucosal surface depends on the internal diameter of the intestine, the number of folds per unit area, and the average width of the intestinal folds at the base region, as well as the average height of the folds. Determination of these parameters were not possible due to distortion of intestinal tissue in most sections. In this context, Fisher (1955) has found that the mean length of the whole small intestine in rat is approximately 10% longer in the female than in the male, but it was suggested that the higher rate of glucose and water absorption in female rats were not due to differences in intestinal dimensions. One further difference observed in the present study was the greater length of enterocytes in male trout, a difference which was abolished 8 weeks following gonadectomy. Previous studies in mammals however, have indicated no significant morphological changes following castration in the rat intestine (Carrier, 1966 ; Ksiazkiewicz-Szapiro, 1970), although androgen therapy increased the height of the intestinal villi and the population of crypts cells (Giordano, et al., 1962).

8.5. Summary:

(1) A technique is described for gonadectomy in rainbow trout, using the lateral route to avoid interference with the gastrointestinal tract.

(2) No significant differences in water and leucine transport were found in the intestine of intact male and female immature rainbow trout.

(3) There was a tendency for higher Na+ absorption in the female intestine

(4) No sex differences in intestinal granular cell density were apparent.

(5) It would appear that while epithelial height and total intestinal thickness are higher in males, percentage fold height is greater in female fish.

Table 8.1.

Percentage recovery of ^{14}C-L-leucine from the medium and ethanol extracts.

	Sh. Op. ♂ + ♂	Sh. Op. ♀ + ♀	Castrated	Ovariectomized
Medium	99.57	95.76	111.09	89.30
Extracts midgut	112.88	110.73	90.69	103.36
Extracts hindgut	105.00	100.00	100.68	108.42

Values represent a single determination of the solutions made up of pooled samples of 8, or 4 observations. Sh. Op. ♂ + ♂ : pooled samples of sham operated male and intact male ; Sh. Op. ♀ + ♀ : pooled samples of sham operated female and intact female.

Table 8.2

Effects of gonadectomy on the absorption of ^{14}C-L-leucine by the intestine of rainbow trout.

	μ moles /g ethanol dry weight /sampling time									
	INCUBATION TIME (min)									
	2	4	6	8	10	15	20	25	30	40
Male	17.2356	24.7949	34.9419	42.6796	51.8410	55.1877	63.3360	70.7844	76.2154	79.6181
+/- S.E.	4.4537	4.7323	6.1425	13.0121	10.3791	7.9071	7.2109	5.9068	6.198	6.5861
Female	18.2446	21.9978	30.8205	40.2179	47.3955	53.0540	67.3798	69.9486	75.9027	75.4688
+/- S.E.	2.7846	5.3071	4.2204	2.9162	5.6743	8.9622	5.1634	6.3085	6.042	8.2566
Sh. Op. ♂	22.5674	24.6166	29.4751	40.7310	36.2541	48.6178	66.1989	64.9973	79.9420	78.1405
+/- S.E.	8.7816	6.2145	4.2227	9.2908	3.3971	12.0149	13.9339	13.9667	18.8379	16.6263
Sh. Op. ♀	19.6903	23.3370	36.0842	33.6052	37.0508	59.8874	66.6592	79.6399	75.9268	80.0911
+/- S.E.	5.5238	3.6001	5.6494	7.5446	9.8662	10.0598	11.0469	15.9685	8.0437	8.6148
Castrated	22.4520	27.3334	34.8024	46.5094	55.2879	58.7925	68.0010	71.6776	76.8914	83.7652
+/- S.E.	6.1071	5.5845	3.8355	10.6228	10.1008	7.6814	16.7722	7.1738	6.1949	6.2793
Ovari. X	18.0379	25.2831	38.2793	46.7653	48.3013	57.6969	64.7071	72.8851	75.9130	82.9202
+/- S.E.	2.8988	3.9481	10.5557	23.5074	19.0734	9.201	15.4876	10.314	9.2443	6.2288

The values are mean +/- S.E. of 4 observations. Intestinal absorption were measured 8 weeks after gonadectomy, or sham-operation. Sh. Op. ♂: sham-operated male; Sh. Op. ♀: sham-operated female; Ovari. X: ovariectomized. Paired differences between the groups are not statistically significant.

Table 8.3.

Variations in the absolute concentration of ^{14}C-L-leucine in the intact, sham operated and gonadectomized fish.

	L-leucine concentration (μ moles/ml)									
	INCUBATIO TIME (min)									
	2	4	6	8	10	15	20	25	30	40
Male	4.9351	4.9152	4.9174	4.8667	4.8507	4.8182	4.8085	4.7581	4.7292	4.7396
+/- S.E.	0.0220	0.0150	0.0284	0.0601	0.0538	0.0442	0.0421	0.0356	0.0358	0.0405
Female	4.9469	4.9339	4.9359	4.9119	4.90773	4.8875	4.8555	4.8239	4.8184	4.7960
+/- S.E.	0.0156	0.0255	0.0251	0.0356	0.0356	0.0493	0.0415	0.0452	0.0461	0.0533
Sh. Op. ♂	4.9488	4.9345	4.9257	4.9084	4.8899	4.8804	4.8548	4.8319	4.7887	4.7855
+/- S.E.	0.0148	0.0080	0.0287	0.0137	0.0459	0.0352	0.0389	0.0313	0.0289	0.0228
Sh. Op. ♀	4.9492	4.9395	4.9253	4.9237	4.9074	4.8570	4.8511	4.8027	4.8347	4.8087
+/- S.E.	0.0156	0.0117	0.0161	0.0214	0.0274	0.0207	0.0227	0.0345	0.0168	0.0191
Castrated	4.9254	4.9115	4.9021	4.8664	4.8483	4.8306	4.8066	4.7934	4.7823	4.7388
+/- S.E.	0.0230	0.0195	0.0144	0.0345	0.0312	0.0228	0.0464	0.0113	0.0088	0.0125
Ovari. X	4.9326	4.8996	4.8657	4.8277	4.8320	4.7929	4.7663	4.7436	4.7367	4.6826
+/- S.E.	0.0086	0.0214	0.0400	0.0899	0.0777	0.0478	0.0696	0.0320	0.0308	0.0385

The values are mean +/- S.E. of 4 observations. Intestinal absorption were measured 8 weeks after gonadectomy, or sham operation. Differences are not statistically significant. Abbreviations as in Table 8.2.

Table 4

Effects of gonadectomy on the concentration of Na^+ (absolute quantity).

	Concentration of Na^+ in the incubation medium (μ moles/ml)							
	INCUBATION TIME (min)							
	2	4	6	8	10	20	30	40
Sh. Op. ♂	131.58	130.38	130.52	134.49	132.77	134.00	133.92	133.63
+/- S.E.	0.97	1.04	2.08	0.57	1.30	0.38	0.31	0.40
Sh. Op. ♀	132.32	129.83	130.62	130.28	130.03	130.03	133.51	134.62
+/- S.E.	0.05	0.23	0.51	0.40	0.24	0.12	0.38	0.09
Castrated	131.16	130.28	129.94	131.17	130.46	131.38	132.96	134.11
+/- S.E.	0.55	0.48	0.44	0.85	0.66	0.44	0.52	0.36
Ovari. X	132.02	131.19	130.43	130.90	130.27	130.67	133.44	132.06
+/- S.E.	0.66	0.68	0.30	0.21	0.49	0.59	0.78	0.64

Values are mean +/- S.E. of 2 observations, each made up of pooled samples of 2 experiments. Sh. Op. ♂: sham operated male; Sh. Op. ♀: sham operated female; Ovari.X: ovariectomized.

Table 8.5.

Effects of gonadectomy on the concentration of K^+ (Absolute quantity).

	Concentration of K^+ in the incubation medium (μ moles/ml)							
	INCUBATION TIME (min)							
	2	4	6	8	10	20	30	40
Sh. Op. ♂	8.5720	10.1079	7.3600	9.7419	8.7655	5.1900	7.8667	10.9930
Sh. Op. ♂	6.6050	7.8226	8.7493	3.8226	7.8226	6.8471	11.4159	8.7440
Sh. Op. ♀	8.2400	9.0303	3.8760	10.4230	8.1197	7.8920	7.0076	9.8920
Sh. Op. ♀	7.2115	6.9181	8.2848	5.7982	9.4071	4.8890	7.7240	7.6026
Castrated	9.7403	9.0769	8.1703	12.2462	2.0741	4.6893	7.7150	8.0690
Castrated	6.4705	7.5677	0.9960	7.3826	8.2200	7.5116	2.3740	6.5921
Ovari. X	5.0950	8.0433	11.0944	8.8709	7.9225	6.6774	8.3379	5.9560
Ovari. X	8.9942	2.5240	7.5530	6.9595	3.8380	7.9853	7.6670	8.0782

Values represent single determination of K^+ concentration in the solutions made up of pooled samples obtained from two incubation media. Mean values are not shown due to high variability. Abreviations as in Table 8.4.

Table 8.6.

Effects of gonadectomy on the concentration of Ca^{++} (absolute quantity).

	Concentration of Ca^{++} in the incubation medium (μ moles/ml)							
	Incubation time (min)							
	2	4	6	8	10	20	30	40
Sh. Op. ♂	0.9363	0.9679	0.9727	0.9819	1.0563	1.0345	1.0649	1.0521
+/- S.E.	0.0531	0.0362	0.0396	0.0212	0.0373	0.0398	0.0377	0.0400
Sh. Op. ♀	0.9963	0.9928	1.0083	1.0225	1.0001	1.0006	1.0033	0.9944
+/- S.E.	0.0230	0.0305	0.0254	0.0210	0.0138	0.0161	0.0142	0.0053
Castrated	1.0069	1.0030	1.0079	1.0048	1.0414	1.0041	1.0067	1.0041
+/- S.E.	0.0075	0.0430	0.0121	0.0011	0.0224	0.0104	0.0133	0.0162
Ovari. X	1.0156	0.9666	1.0020	0.9996	1.0818	1.0302	0.9951	0.9941
+/- S. E.	0.0127	0.0608	0.0095	0.0071	0.0666	0.0235	0.0024	0.0063

Details as in Table 8.4. Differences are not statistically significant.

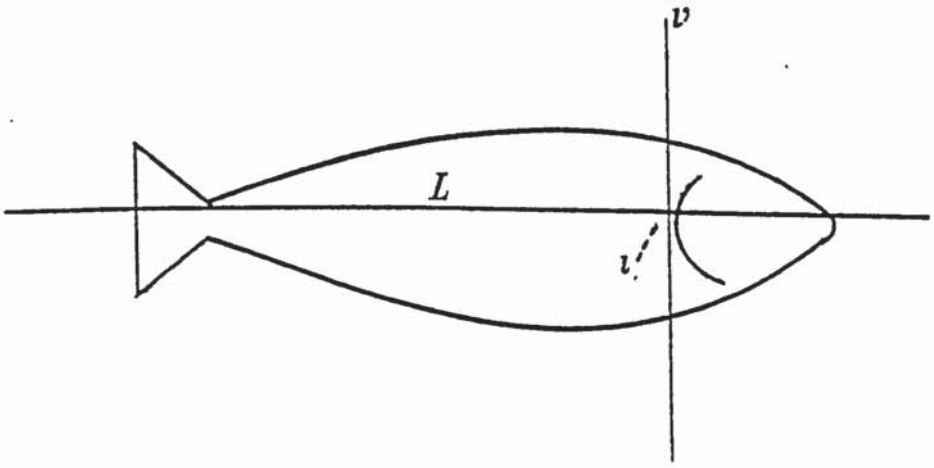
Table 8.7

Ci/Cf ratios for determination of tissue water flux.

	INCUBATION TIME (min)							
	6	8	10	15	20	25	30	40
Male	0.9915	0.9950	0.9906	0.9970	0.9936	0.9991	1.0013	0.9964
+/- S.E.	0.0115	0.0036	0.0014	0.0016	0.0019	0.0027	0.0013	0.0118
Female	0.9948	0.9949	0.9910	0.9911	0.9903	0.9951	0.9928	0.9972
+/- S.E.	0.0050	0.0040	0.0033	0.0057	0.0069	0.0050	0.0060	0.0026
Sh. Op. ♂	0.9956	0.9968	0.9972	0.9960	0.9915	0.9973	0.9995	1.0007
+/- S.E.	0.0122	0.7071	0.0054	0.0039	0.0171	0.0035	0.0004	0.0289
Sh. Op. ♀	0.9963	0.9979	0.9994	0.9985	0.9962	0.9995	0.9944	0.9975
+/- S.E.	0.0036	0.0040	0.0082	0.0077	0.0166	0.0093	0.7031	0.0024
Gonad.X	0.9971	0.9965	0.9949	0.9963	0.9961	0.9960	0.9947	0.9991
+/- S.E.	0.0040	0.0044	0.0059	0.0047	0.0038	0.0039	0.0049	0.0014

Values are mean +/- S.E. of 2 observations. Abbreviations as in Table

Fig. 8.1



L Lateral line

ι Incision

v Vertical axis

Diagram showing the angle and position of incision for gonadectomy. For bilateral gonadectomy, a similar incision was made on the other side.

Fig.8.2

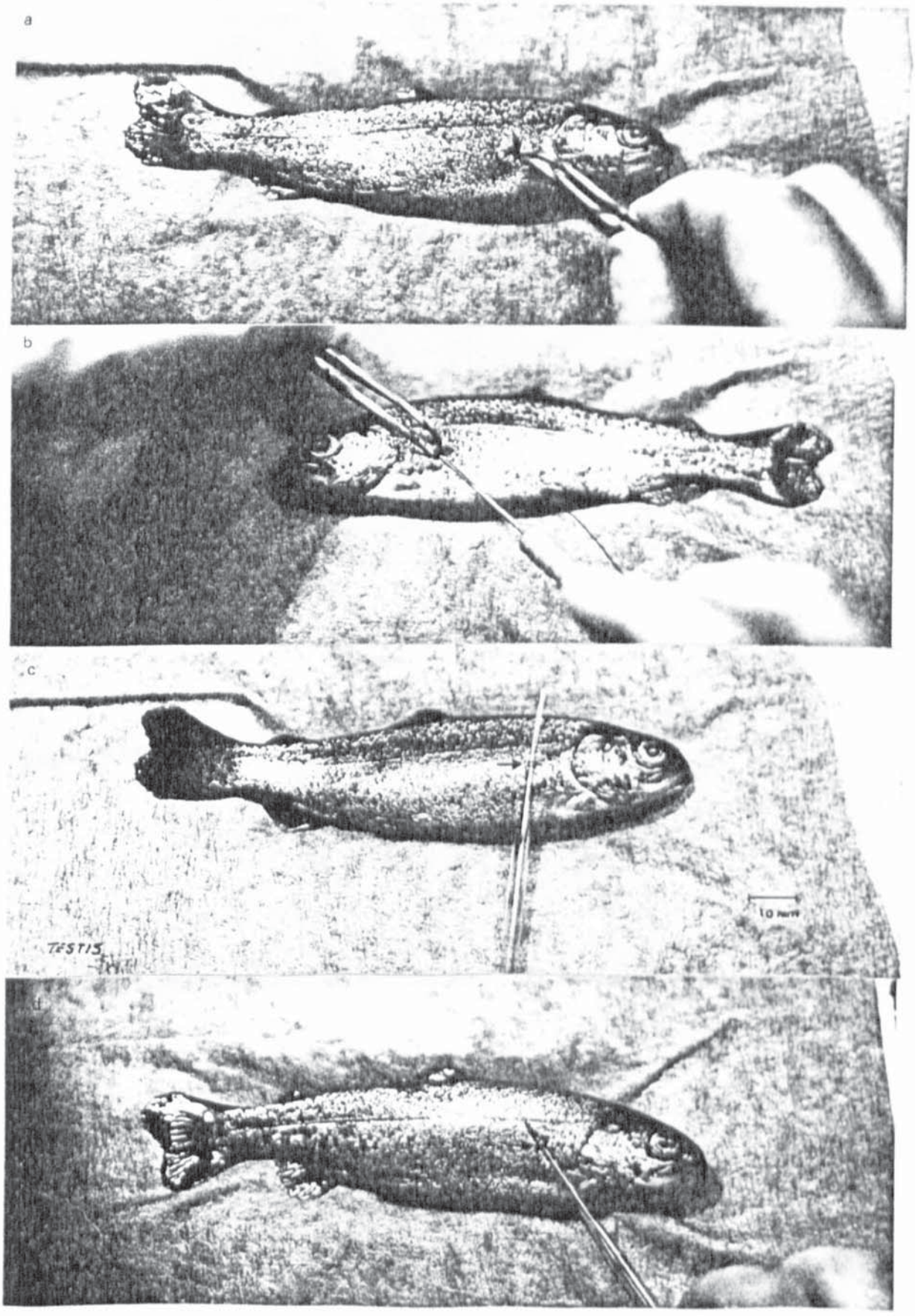
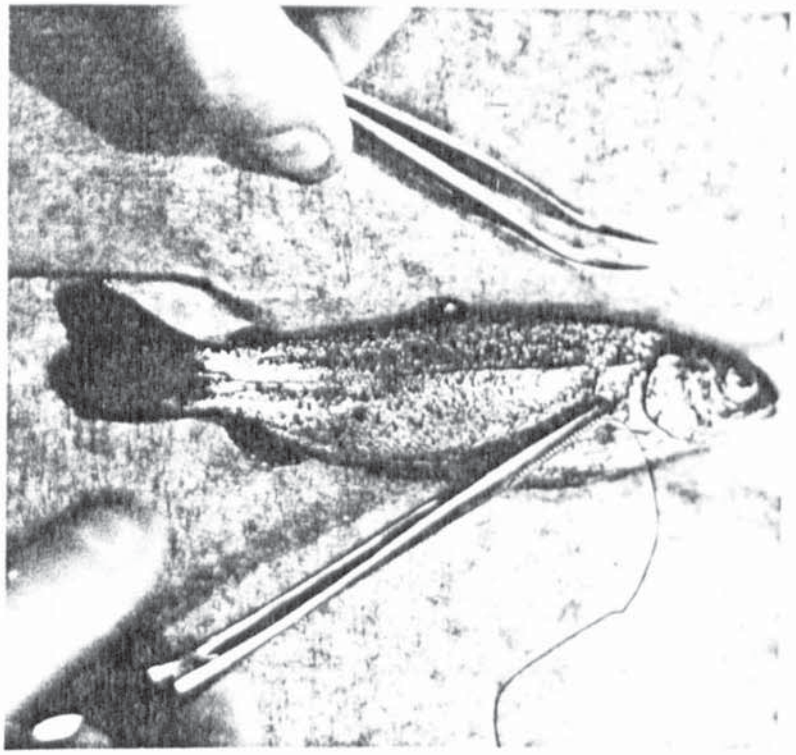


Fig. 8.3



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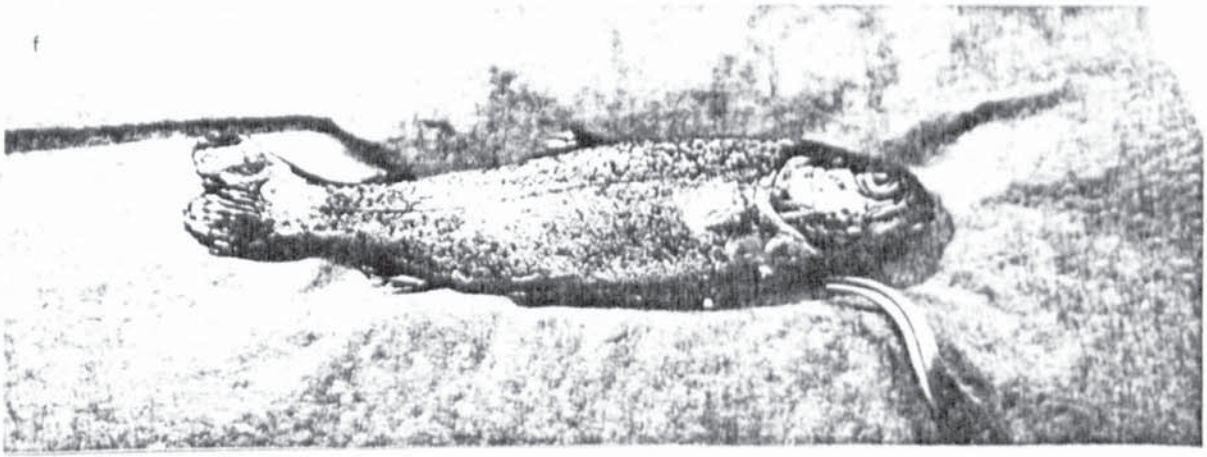
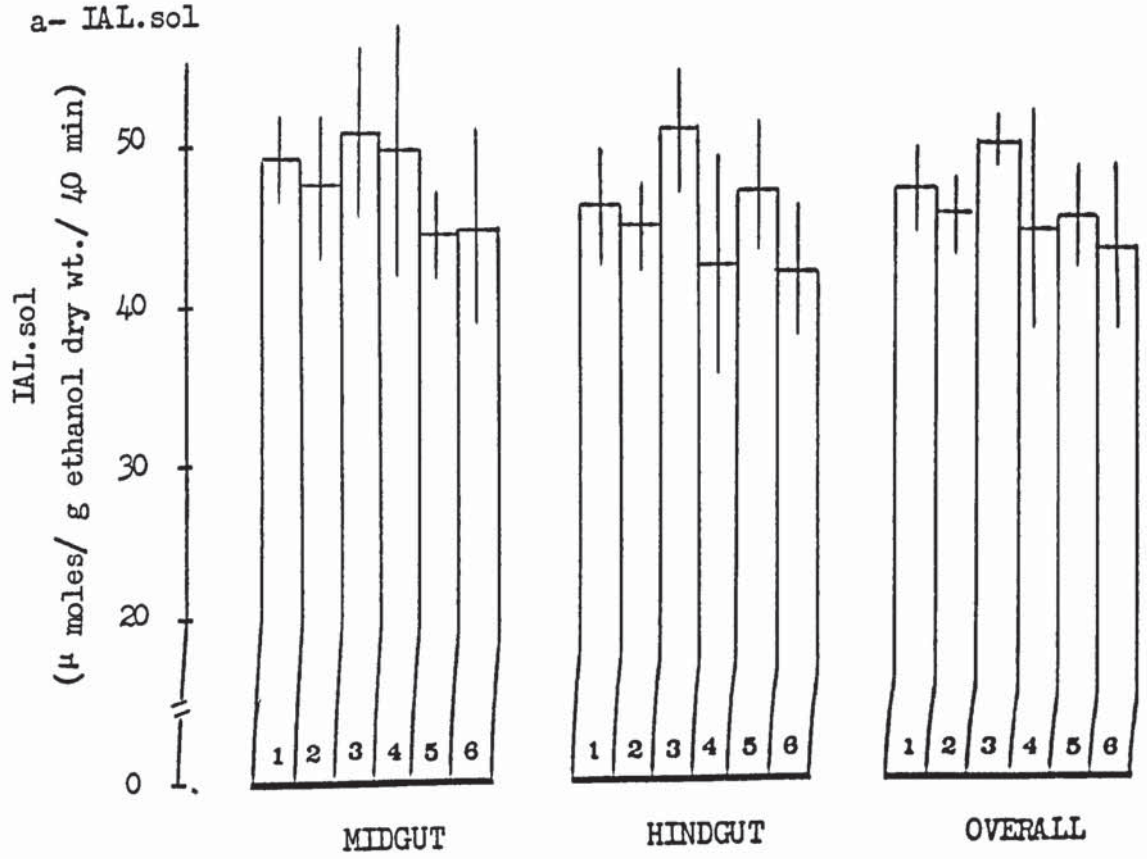
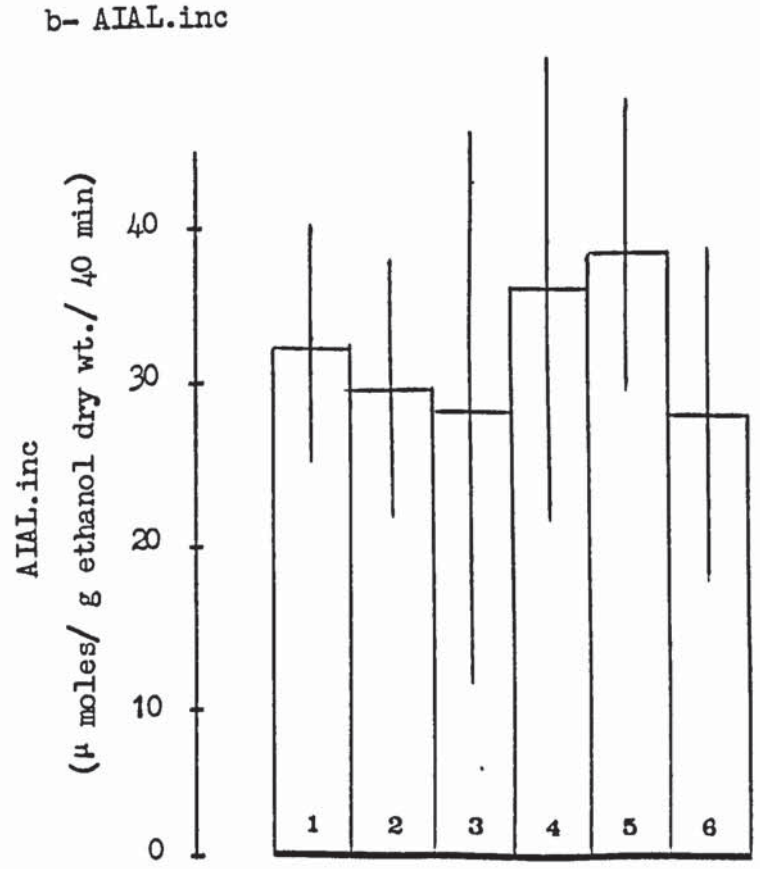


g

Fig. 8.4 Intestinal accumulation of solute leucine (IAL.sol) and apparent intestinal accumulation of incorporated leucine (AIAL.inc).





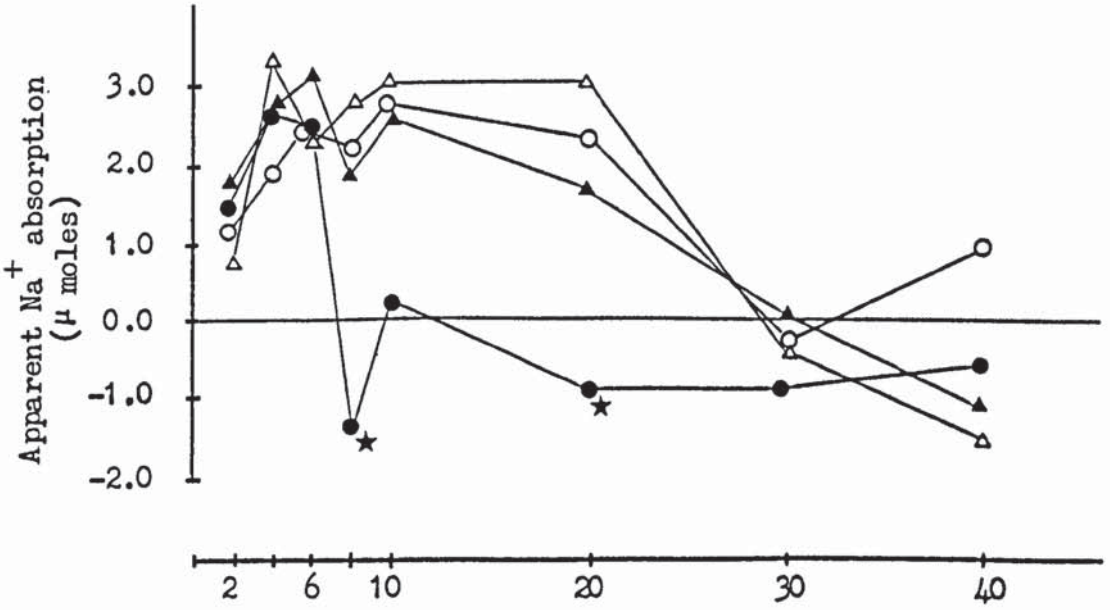
n= 4 ± S.E.

1; intact male
2: intact female
3: sham-operated ♂
4: sham-operated ♀
5: castrated
6: ovariectomized

Differences are not statistically significant.

Fig. 8.5

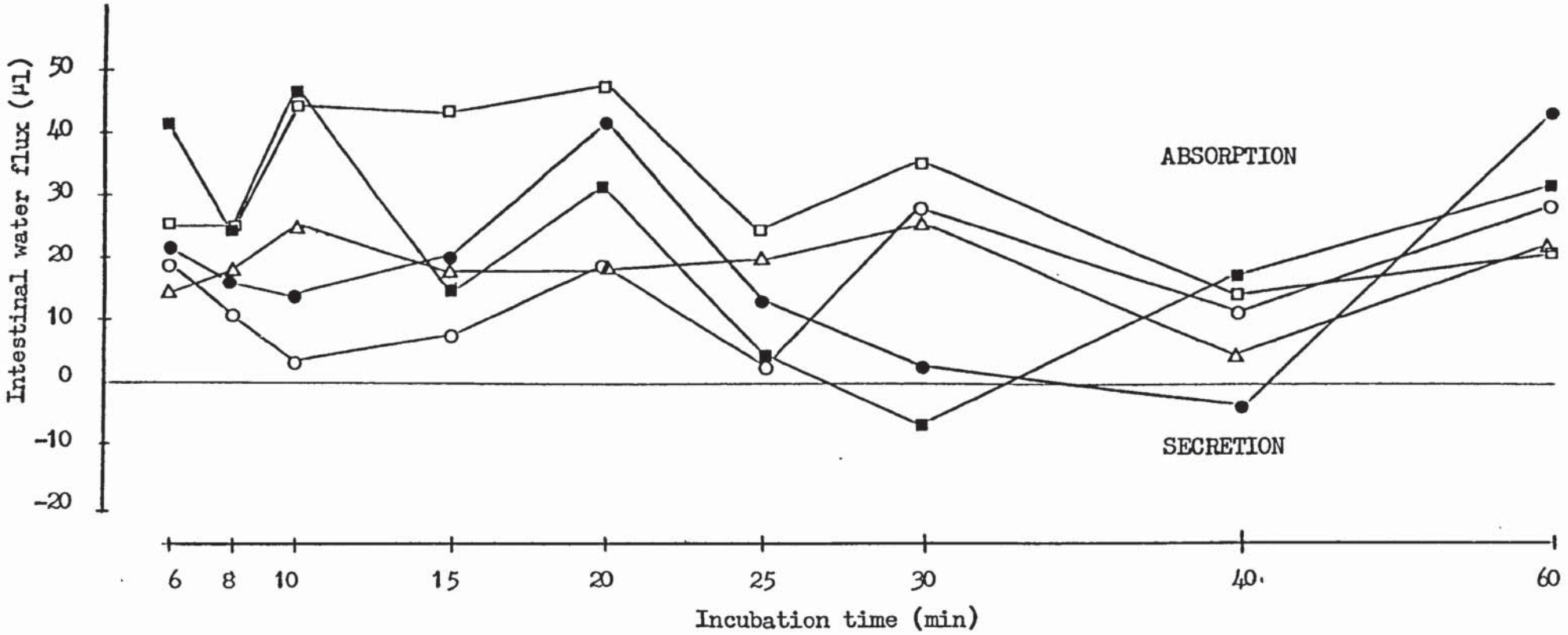
Apparent Na^+ absorption in the sham-operated and gonadectomized fish.



Values represent mean of 2 observations, each made up of pooled samples taken from 2 experimental groups. (★) indicates significant ($P<0.05$) variations between sham-operated male and female. Mean ethanol extracted dry weight of intestinal strips are (g); sham-operated male, 0.0285; sham-operated female, 0.0245; castrated male, 0.029; ovariectomized, 0.031. ●——● sham-operated male, △——△ sham-operated female, ▲——▲ castrated male, ○——○ ovariectomized. Standard errors are not included for clarity, see table 8.4 for variations. (concentration difference = initial concentration , 133.05 - concentration after time t).

Fig. 8.6

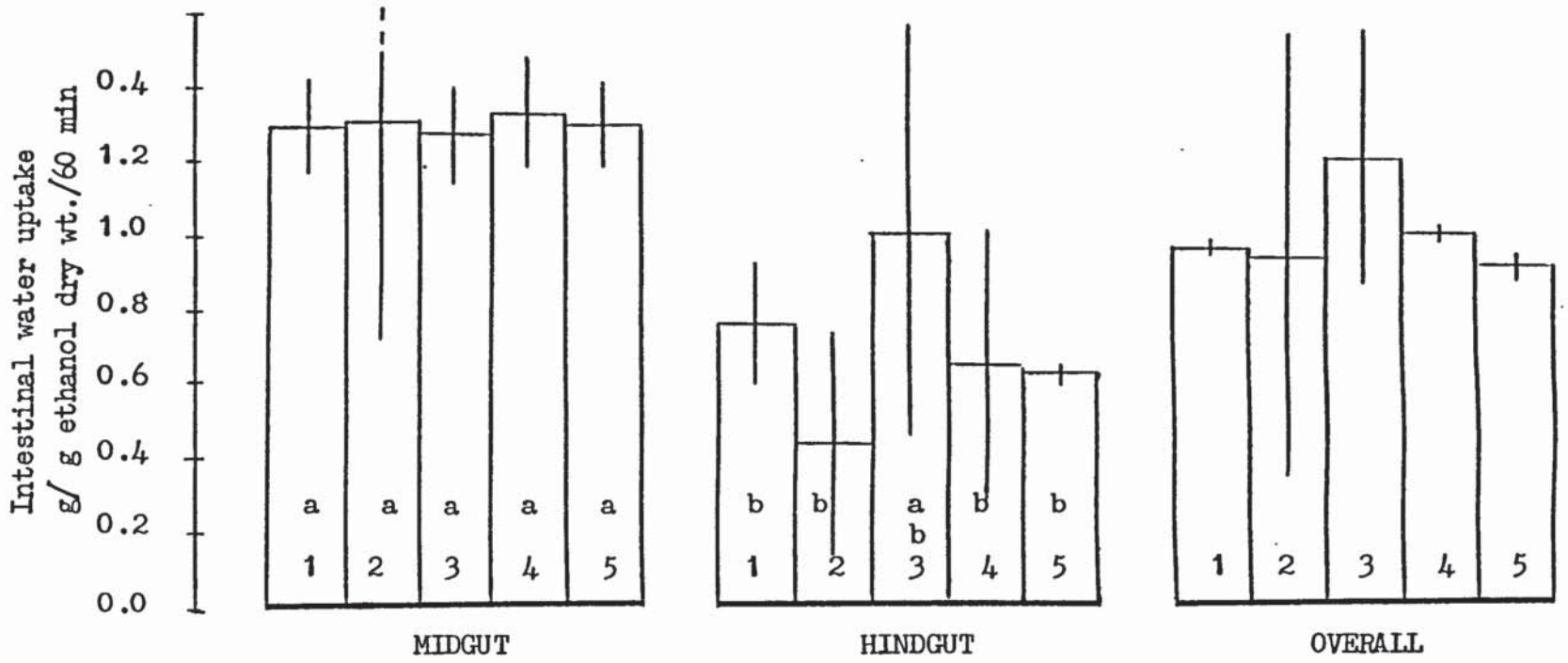
Intestinal water flux in male, female, sham-operated and gonadectomized fish



Values are mean of 2 observations. Standard errors are not included for clarity (see table 8.7). Differences are not statistically significant.■——■intact male;□ □intact female;●——●sham-operated male;○——○sham-operated female; △——△gonadectomized.

Fig. 8.7

Intestinal water uptake in male, female, sham-operated and gonadectomized fish.



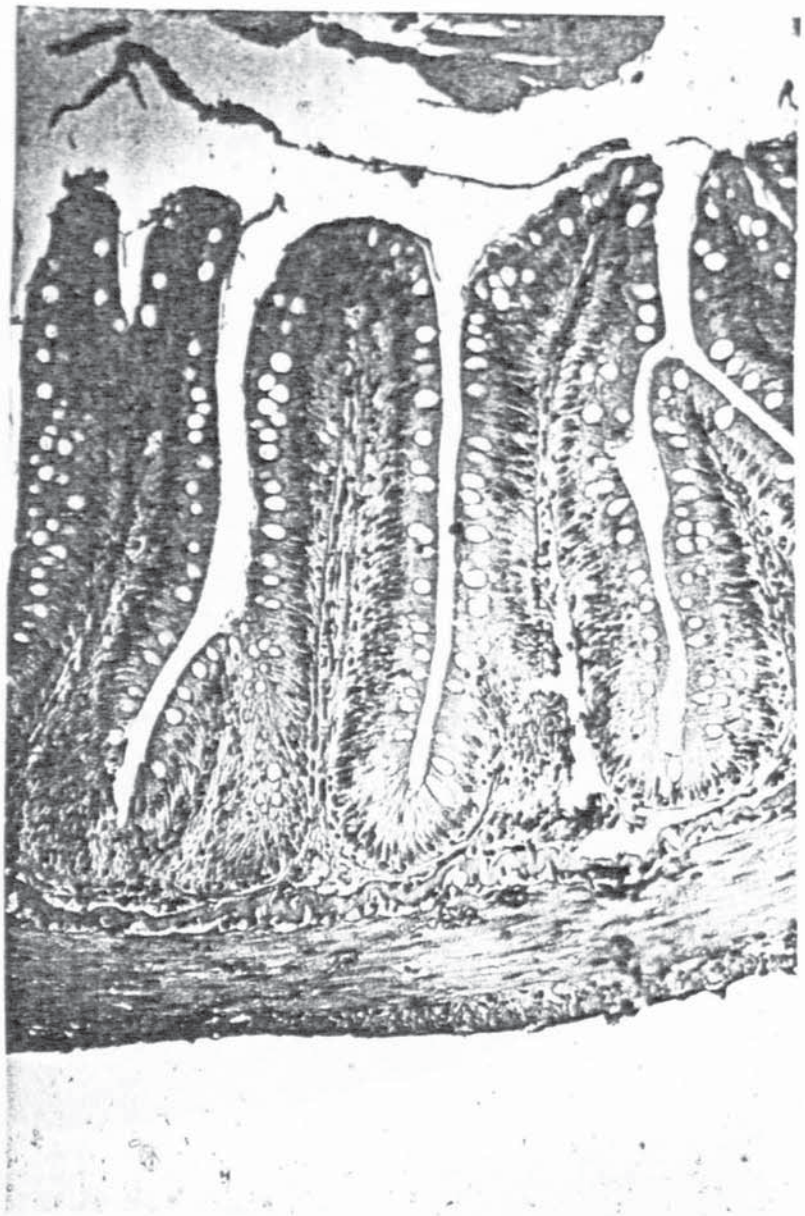
Values are mean +/- S.E. of 2 observations. Columns containing dissimilar symbols are significantly different ($P<0.05$). 1, male; 2, female; 3, sham-operated male; 4, sham-operated female; 5, gonadectomized.

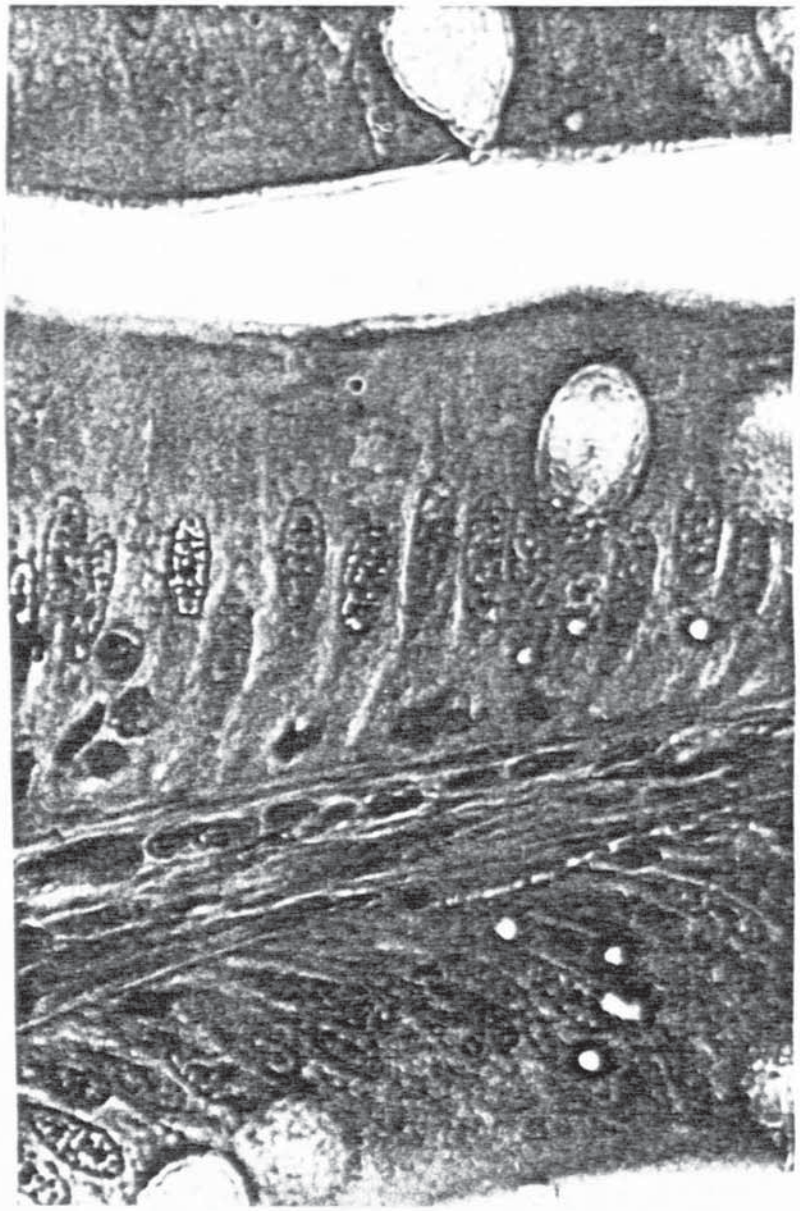
Fig.8.8

30 MONTHS TROUT x 8

9-10 MONTH TROUT x 20

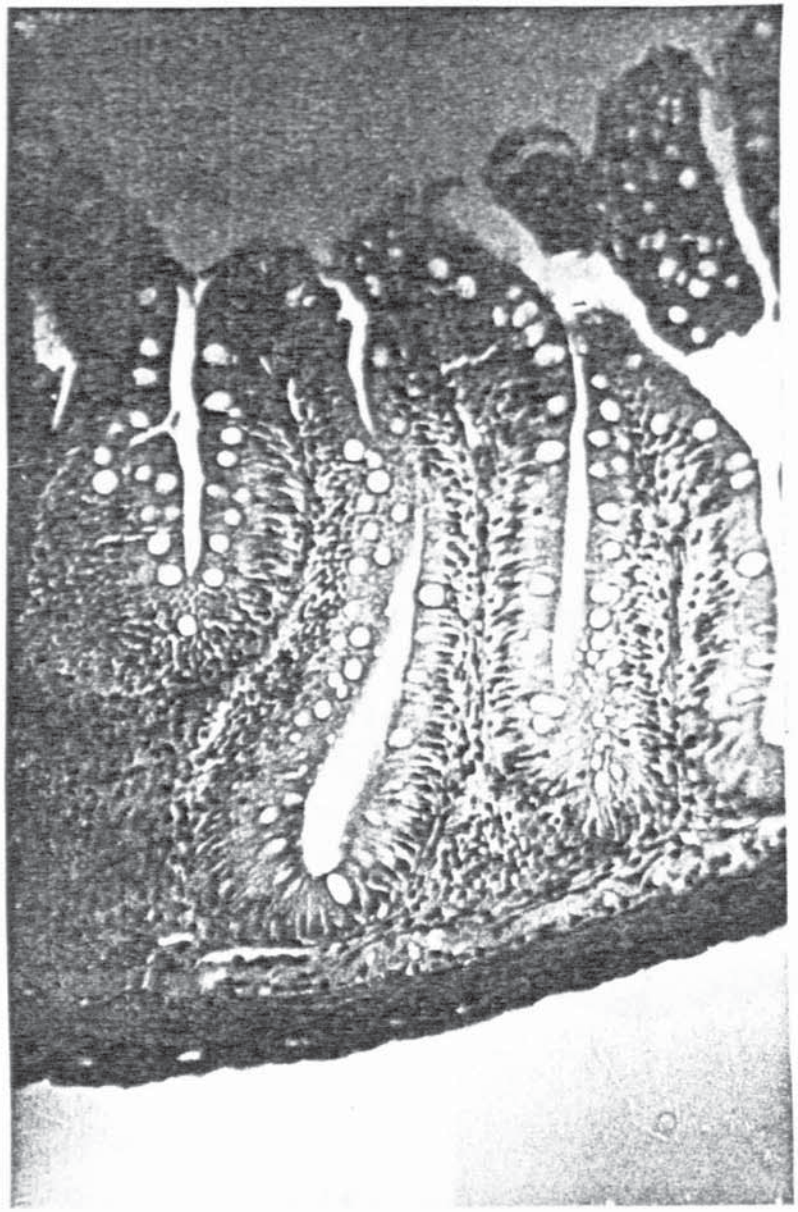
Fig.8.9

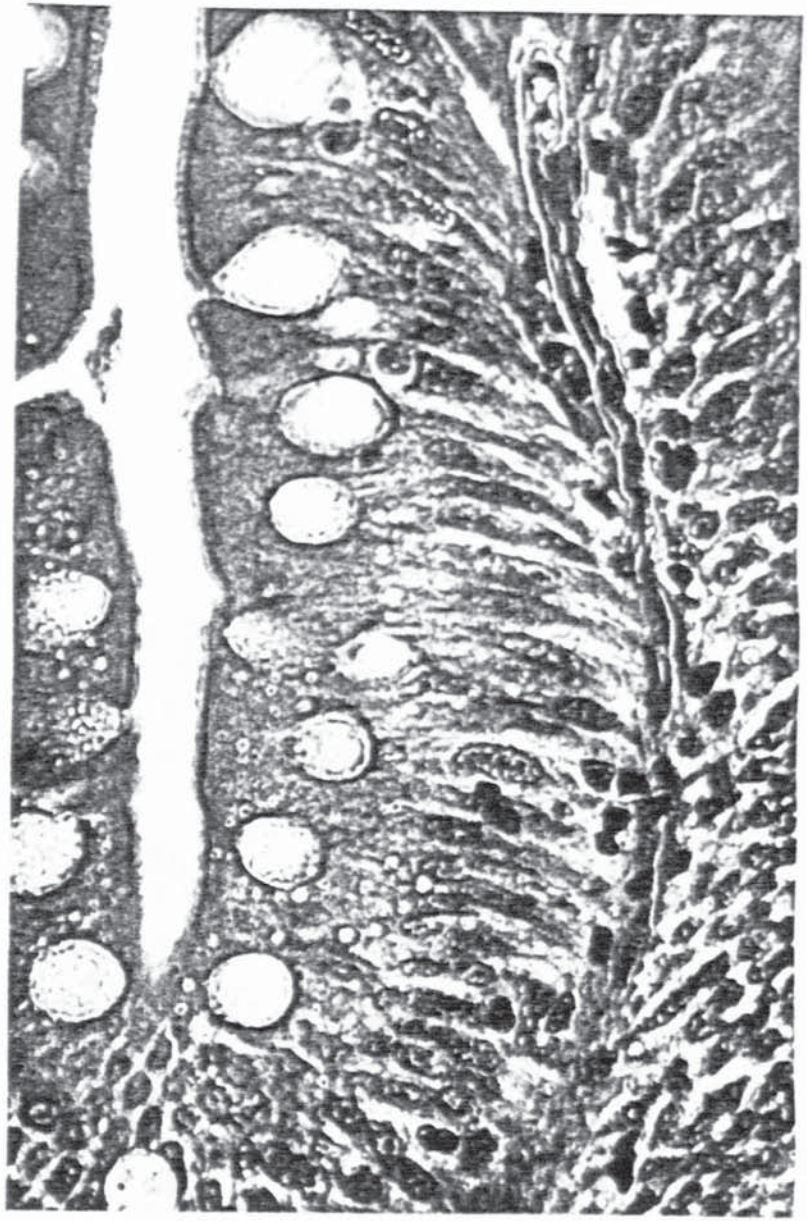




X 16 INTACT MALE X 80

Fig.8.10



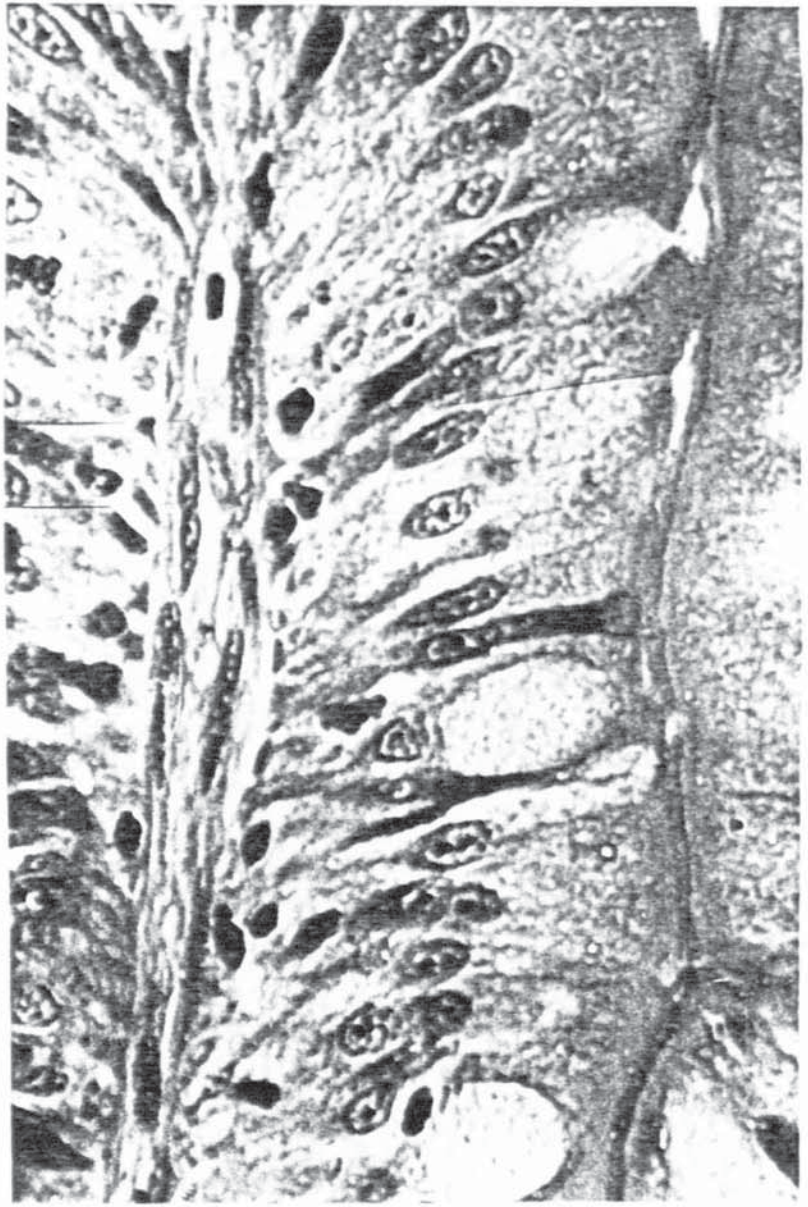


X 16 INTACT FEMALE X 80

Fig. 8.11

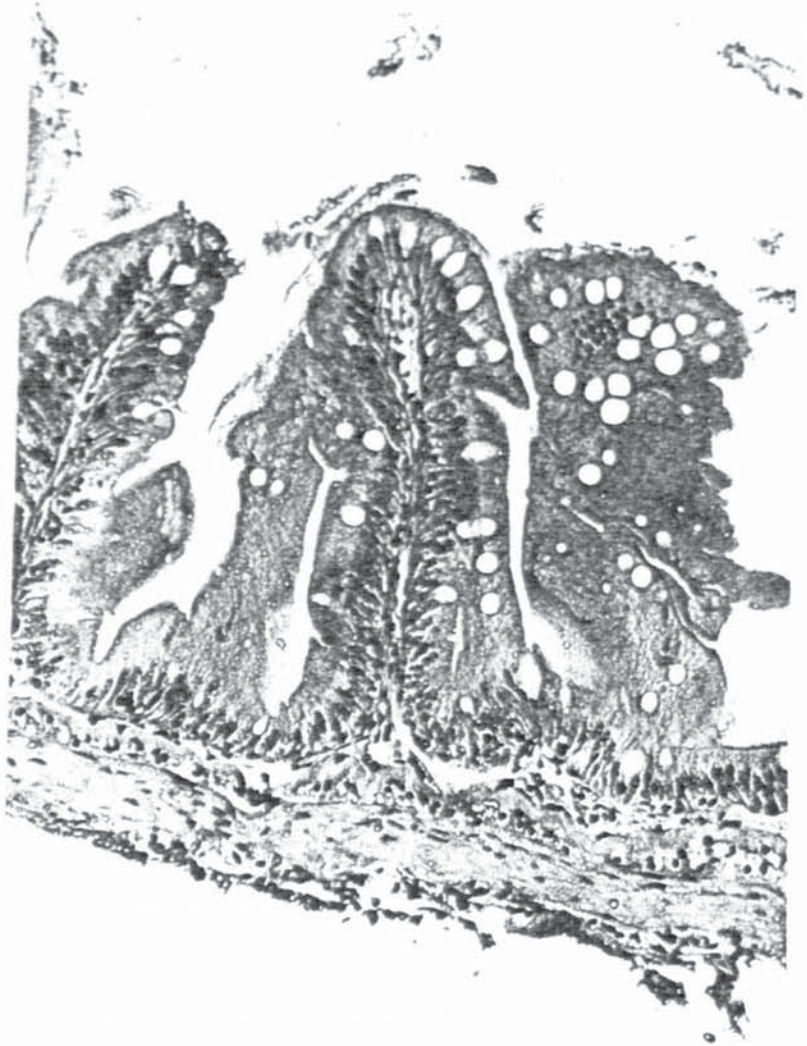
X 16

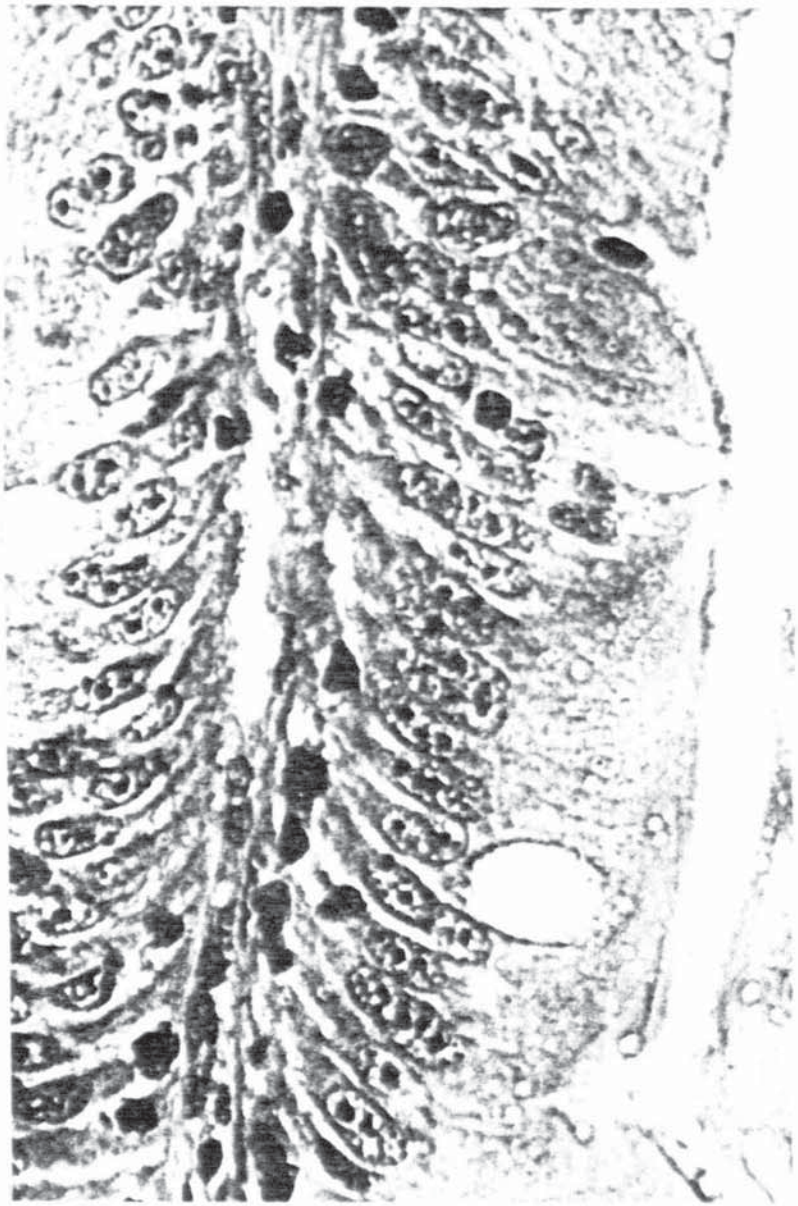
CASTRATED



X 80

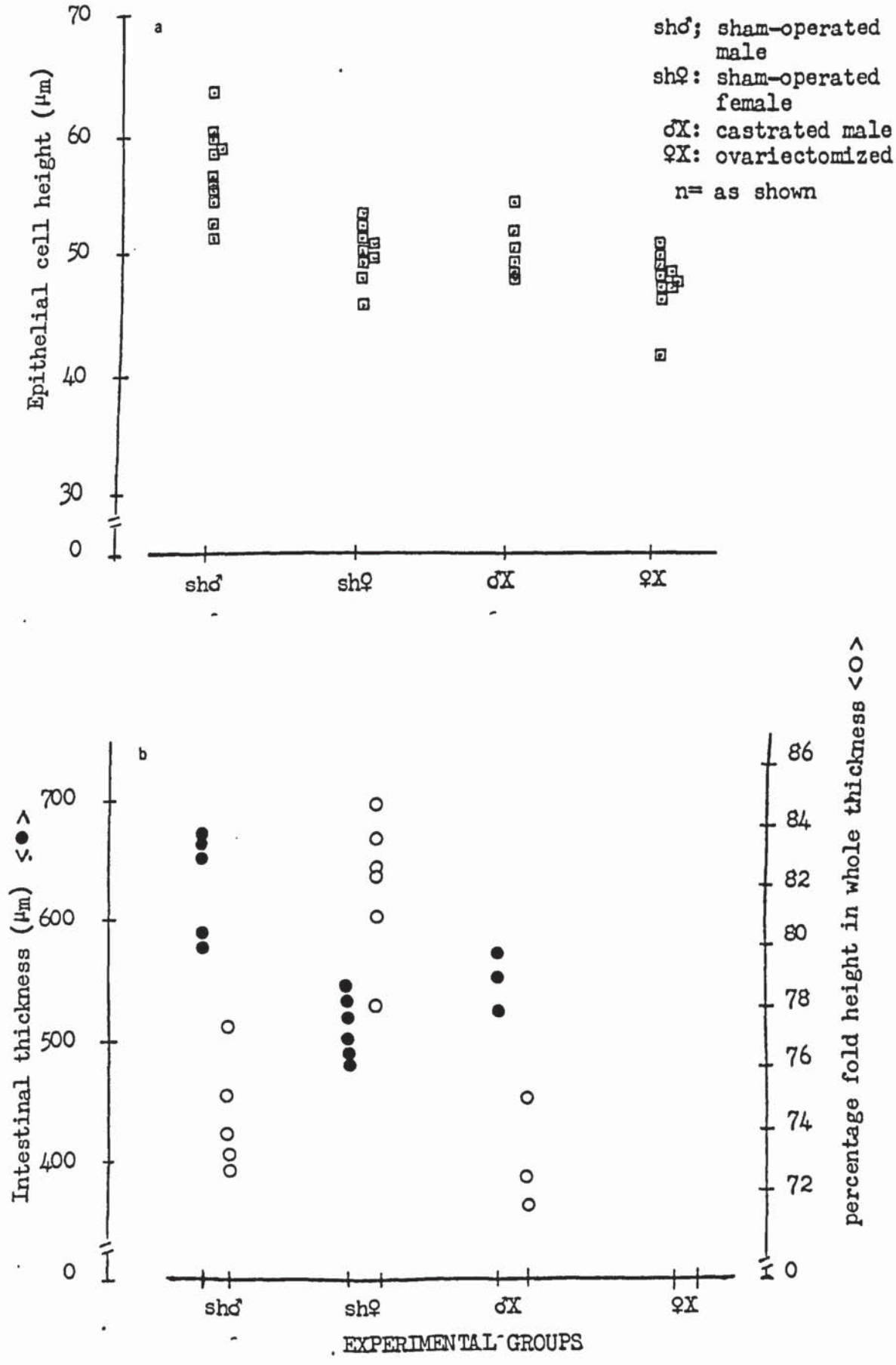
Fig. 8.12





X 16 OVARIECTOMIZED X 80

Fig. 8.13 Intestinal epithelial and fold heights



9. General conclusions

Section 9

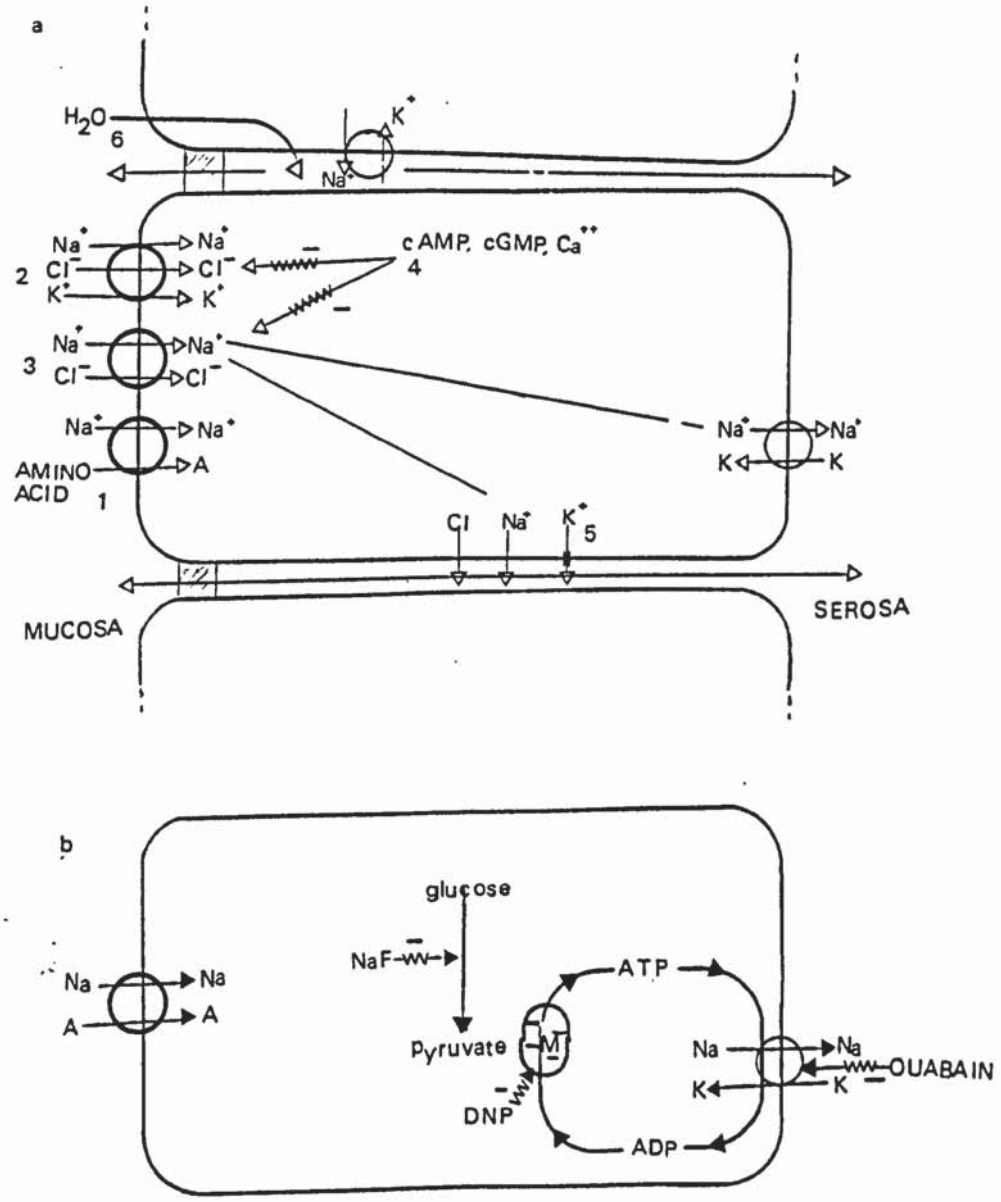
The present study has demonstrated that MT significantly enhances the intestinal transport of leucine, both when injected for 10 days and when administered in vitro by addition to incubation media. Although it remains uncertain if the long-term effects of the steroid were caused by a direct action on the intestinal epithelial cells, it is clear that the short-term stimulation of leucine transport was the consequence of a direct effect on the enterocytes, as was evident from the results obtained following luminal administration of the steroid. Furthermore, it has been shown that, while addition of MT and KT to incubation media increased leucine absorption, administration of E2 in vitro was without a significant effect. These observations, although do not constitute proof that the androgen-induced amino acid absorption was the result of a specific action on the permeability of the apical membrane, they rule out the possibility of a non-specific action of the steroids on membrane permeability. In this context however, an influence on membrane permeability alone is unlikely to be the only mechanism responsible, since MT-stimulated leucine transport was abolished by impairment of Na+,K+,ATPase activity, and to a great extent reduced by blocking oxidative phosphorylation, indicating the involvement of energ tic processes in the mechanism of action of MT. KT-stimulated leucine absorption was also significantly reduced following inhibition of the Na "pump" and oxidative phosphorylation, but to a lesser extent. Thus, it is suggested that the short term effects of the androgenic steroids, especially MT, might be partly mediated through the activity of Na+,K+,ATPase, although the mechanisms of the early action of MT (5-20 min) remain less certain.

The enhanced intestinal absorption of leucine by MT, suggests that steroid-induced growth promotion in fish may be partly a consequence of increased efficiency of intestinal function which supports the earlier suggestion by Yamazaki (1976) that "at certain dosage-levels MT induces a higher rate of digestion or absorption of the food". However, the study of the physiological role of gonadal hormones on intestinal function revealed that, in immature rainbow trout, sex steroids do not apparently play a significant role in the control of intestinal amino acid absorption. This suggests that the observed effects of androgens on the intestine have a pharmacological basis, possibily distinct from their normal actions in vivo. Furthermore, in contrast to the stimulatory actions of pharmacological doses of the androgens on intestinal transport, 'apparent' Na+ absorption was lower in intact immature male than in female trout. In this context, it would appear that the difference in Na+ transport resulted from either a reduced rate of absorption, or an increased rate of Na+ secretion in males, rather than an increased absorption of Na+ by the intestine of female fish. In addition, contrary to the reported hypertrophy of intestinal granular cells following oral treatment with MT for two weeks in trout (Yamazaki, 1976), the present findings indicated no significant variations in intestinal granule cell density between gonadectomized or normal male and female trout. However other histological studies revealed that, while total intestinal thickness and enterocyte heights were higher in males, the percentage fold height was greater in females. The suggestions put forward for the possible mechanisms of action of methyltestosterone on the

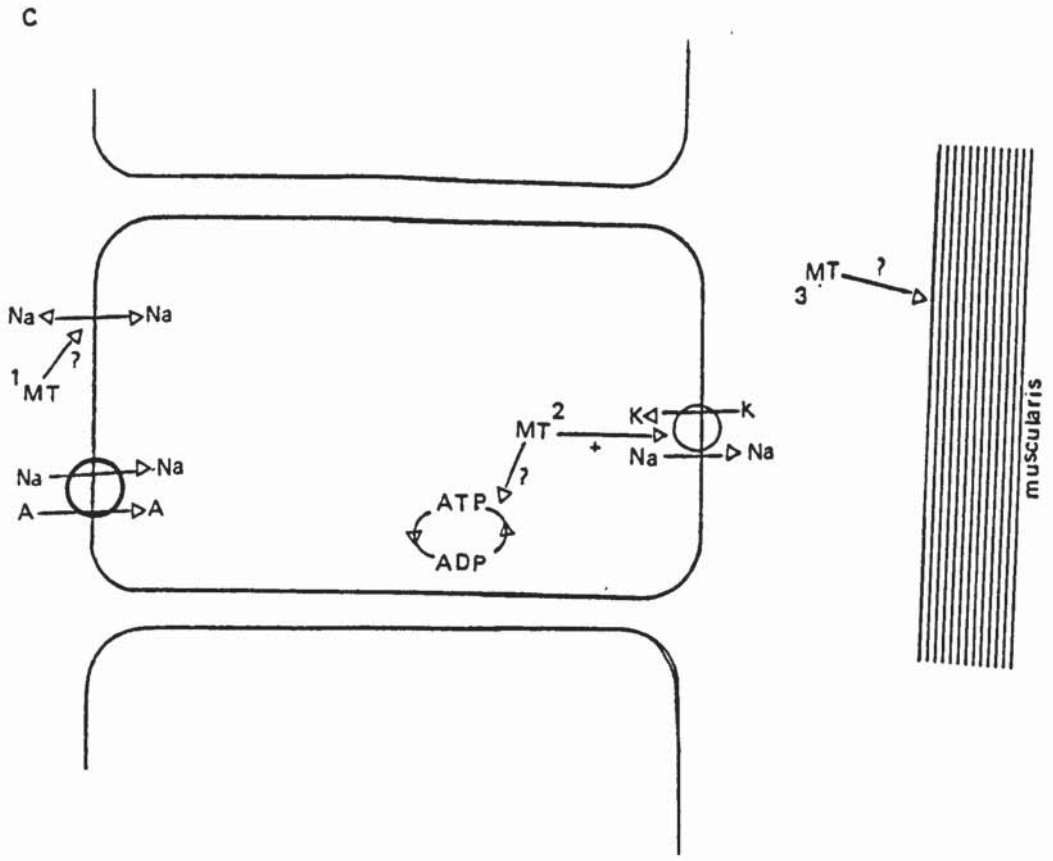
intestinal transport of leucine are summarised in Fig. 9.1.

Finally, in the context of long-term steroid effects on intestinal absorption of amino acids, it might be of relevence to further investigate the effects of androgenic steroids and withdrawal over a longer period of time, since, it has been shown that, although high doses of androgenic-steroids promote growth rates during short experimental periods (days), similar dosage of the steroid might exert a relatively smaller growth response over a longer period of time (Higgs, et al., 1982).

Fig. 9.1.



9.1.a Illustration of the current views on the absorption of water and solutes by the intestine of fish: 1) Smith (1970), Ingham and Arme (1977), Munck (1981); (2) Musch, et al (1982); (3) Field, et al (1978), McKay and Lahlou (1980); (4) Field, et al (1980); McKay and Lahlou (1980); (5) Moreno and Diamond (1974); (6) Schultz (1981).
9.1.b Site of action of inhibitors used (ouabain, DNP and NaF)



9.1.c. Illustration of the suggested mechanisms of action of MT on intestinal leucine transport. (1) possible membrane action during the initial 20 min, influencing ionic permeability, thus rapidly effecting the electrochemical gradient across plasma membrane (Cytosol vs luminal fluid); (2) Stimulating effect of MT on Na+,K+,ATPase after 20-30 min time-lag, resulting in a more negative cytoplasm with respect to luminal fluid. Hence, increased Na-amino acid co-transport (influx); (3) possible long term effect of MT on smooth muscle (or some striped muscle), stimulating uptake of amino acid into muscle cells and incorporation of amino acid into protein (Matty and Cheema, 1978).

Appendices

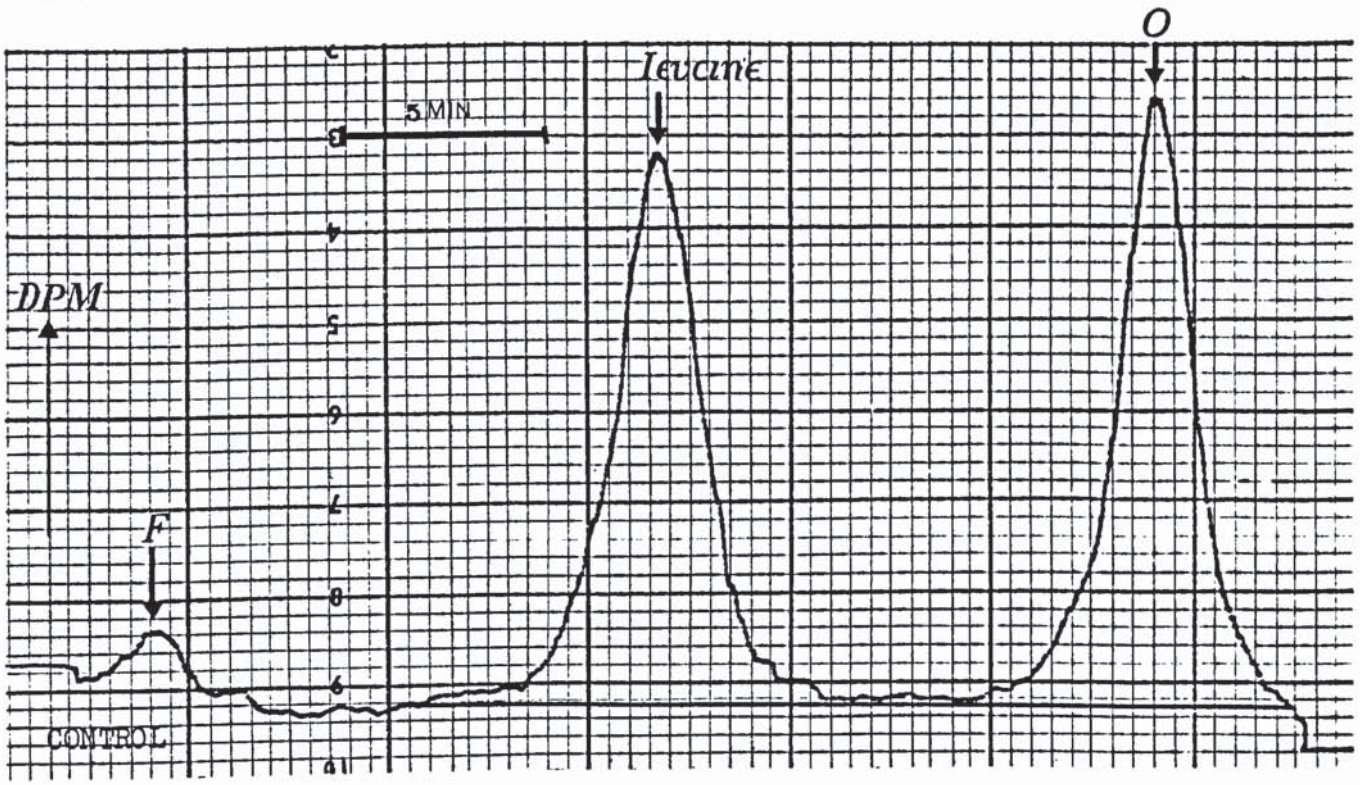
Section 10

Table 7.a.
Ci/Cf ratios for determination of tissue fluid flux.

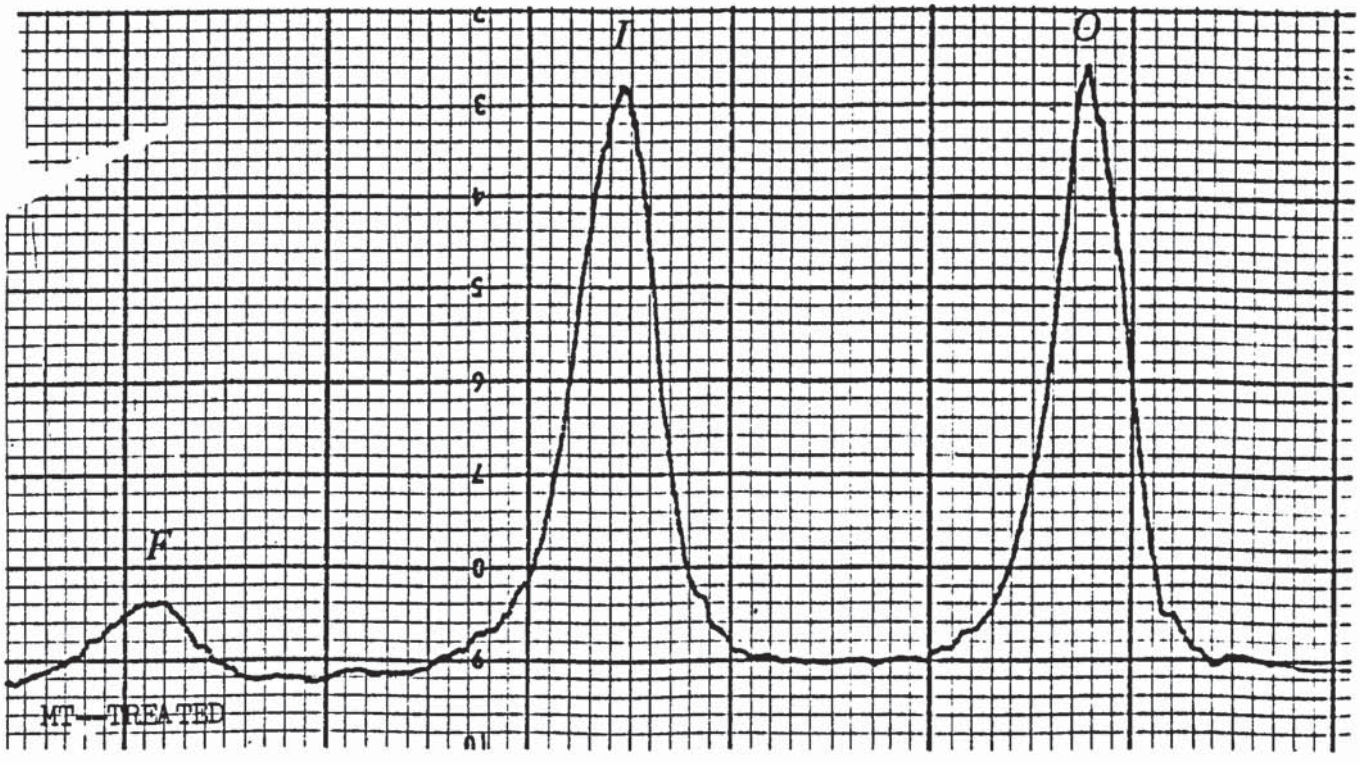
	SAMPLING TIME (MIN)					
TREATMENT	5	10	20	30	40	60
CONTROL	0.9980	0.9973	0.99421	0.9952	1.0056	0.9892
+/- S.D.	0.0110	0.0077	0.0153	0.0082	0.0169	0.0214
MT+	1.0032	0.9984	0.9971	0.9923	0.9938	0.9766
+/- S.D.	0.0057	0.0195	0.0200	0.0160	0.0190	0.0290
KT+	1.0088	1.0075	0.9920	0.9952	0.9928	0.9816
+/- S.D.	0.0140	0.0039	0.0206	0.0118	0.0068	0.0175
OUA+	1.0221	1.0164	1.0054	0.9880	0.9987	1.0192
+/- S.D.	0.0070	0.0115	0.0114	0.0171	0.0018	0.0113
DNP+	1.0039	0.9786	0.9923	1.0146	1.0076	0.9962
+/- S.D.	0.0054	0.0015	0.0183	0.0168	0.0326	0.0091
NaF+	1.003	1.0027	1.010	1.0025	1.0255	1.0195
+/- S.D.	0.0098	0.0122	0.0143	0.0339	0.0206	0.0288
MT+OUA	1.0014	1.0152	1.0014	1.0014	1.0308	1.0082
+/- S.D.	0.0019	0.0096	0.0057	0.0019	0.0115	0.0038
MT+DNP	0.9911	1.0091	0.9911	1.0077	1.0104	1.0026
+/- S.D.	0.0055	0.0091	0.0055	0.0071	0.0034	0.0108
MT+NaF	1.0000	1.0348	1.0124	1.0212	1.0393	1.0197
+/- S.D.	0.0000	0.0026	0.0118	0.0067	0.0098	0.0135
KT+OUA	0.9987	1.0028	0.9933	1.0041	1.0001	0.9836
+/- S.D.	0.0019	0.0038	0.0095	0.0057	0.0038	0.0106
KT+DNP	1.0050	0.9973	1.0129	0.9987	0.9859	0.9945
+/- S.D.	0.0144	0.0181	0.0105	0.0055	0.0130	0.0364
KT+NaF	1.0066	1.0079	1.0142	1.0003	1.0193	1.0537
+/- S.D.	0.0092	0.0111	0.0099	0.1109	0.0038	0.0121

The values for Control, MT, and KT are mean +/- S.D. of 6 observations. The remaining values ares mean +/- S.D. of 2 observations. For details see Section 7.

Fig. 7b



Determination of the percentage recovery of L-leucine (original trace record). Ordinate, decomposition per min (DPM); Abscissa, time, as indicated by bar. *O*, Origin; *F*, Front.



References

Section 11

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