SEX HORMONES AND TRIOSE METABOLISM

A Thesis submitted by

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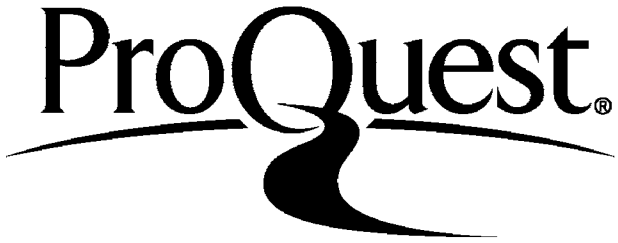

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ABSTRACT

In man there is a direct relationship between the consumption of sucrose and the concentration of blood triglycerides and high blood triglyceride levels are believed to be related to atherosclerosis and coronary heart disease (CHD).

From about 35 years of age onwards the incidence of CHD in men is high relative to the incidence in premenopausal women. In men dietary fructose leads to higher fasting blood triglyceride levels than in premenopausal women but in this respect post-menopausal women resemble the men and they also have a high incidence of CHD. Hence it is possible that CHD is related to fructose intake and subsequent conversion to fats and that this whole process is under the control of sex hormones.

The sex relationship between dietary fructose and the level of blood fats has also been observed in a number of animals other than man.

The trioses, glyceraldehyde and glycerol, are on a metabolic pathway from fructose to triglycerides and hence the effect of sex hormones on the metabolism of these compounds has been investigated.

The enzymes involved in the conversion of glyceraldehyde to L-glycerol-3-phosphate were compared in liver tissues from adult male and female rats and an attempt has been made to change the activities by castration and/or administration of exogenous sex hormones.

NAD-dependent glycerol dehydrogenase was found to be equally active in both male and female rat livers. Injection of testosterone, estrone or estradiol into animals of either sex did not affect the activity. Similar results were obtained with the NADP-dependent glycerol dehydrogenase.

Glycerol kinase activity was observed to be 30-40% higher in male rat liver than in the female tissues.

Injection of testosterone into male animals had little effect on the liver kinase but decreased activity was observed with the antiandrogens, estradiol-17β and cyproterone. Castration of male animals lowered the kinase activity and testosterone injection was observed to restore activity. Hypophysectomy also lowered liver kinase activity in males but, in this case, the activity could not be restored by male hormone injection.

In female rats neither testosterone not estradiol-17β affected the liver glycerol kinase level.

In vitro incubation of liver slices from rats of both sexes with testosterone resulted in increased kinase activity.

Attempts were made to investigate the mode of action of testosterone in the in vitro system.

The investigation suggests that glycerol kinase is under control of sex hormones and this, in part, may account for the sex differences observed in the conversion of fructose to triglycerides.

CONTENTS

ABBREVIATIONS

AMP	Adenosine 5'-monophosphate
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-Triphosphate
EDTA	Ethylene diamine tetra-acetic acid
Pi	Inorganic phosphate
NADP	β-nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced)
NAD	β-nicotinamide adenine dinucleotide (oxidized)
NADH	β-nicotinamide adenine dinucleotide (reduced)
IMP	Inosine 5'-monophosphate
Tris	Trihydroxymethyl methylamine

INTRODUCTION

ATHEROSCLEROSIS

The disease

Atherosclerosis (Gr. athero, mush) refers to a lesion of the arterial wall characterised, inter alia, by accumulation of lipid in the intima. The term was first suggested in 1904 by Marchand (217). The predominant form of this arterial defect is arteriosclerosis, a generic term for any vascular degeneration that leads to progressive thickening and loss of resiliency of the arterial wall. One type of arteriosclerosis is atherosclerosis, which refers to specific vascular alterations, such as atheromas or plaques characterized by a combination of fatty accumulation in the intima and an increase in connective tissue in the subintimal layers of the arterial wall (218). It is this form of arteriosclerosis that appears to be the major public health problem of most socio-economically advanced countries because it affects those vessels, such as the aorta and the coronary and cerebral arteries, that are extremely important in providing the necessary blood supply for the heart, brain, and other vital organs (218, 219).

Atherosclerosis is then the vascular disorder that underlies most 'arteriosclerotic heart disease' or 'coronary heart disease' (sometimes also called 'ischemic heart disease') and also plays a major rôle in cerebrovascular disease (218).

Almost half of all human beings die of arteriosclerosis and approximately two thirds of the

deaths are caused by thrombosis of one or more coronary arteries and the remaining one third by thrombosis or hemorrhage of vessels in other organs of the body - especially the brain, kidney, liver, gastrointestinal tract (220). The overall figures for deaths from heart disease in the U.S.A. are over a million.per annum. A very high proportion of these deaths are due to coronary diseases in male subjects in the prime of life. In this respect, atherosclerosis must be viewed as a disease that, sooner or later affects us all (218), working often unnoticed over the years from early childhood, it gradually destroys the arteries, ultimately preventing the exchanges of gases and nutrients which are necessary to keep organs, tissues, and cells alive and functioning normally (218).

As the disease is characterized by specific vascular alterations, caused in part by fatty accumulation in the intima layers of arteries, the metabolism of lipids in relation to the physiology and pathology of blood vessel walls has become a subject of vital importance (219).

Atherosclerosis is principally a disease of the large arteries in which protein and lipid deposits called 'atheromatous plaques' appear in the subintimal layer of the arteries. These plaques contain relatively large amounts of cholesterol and often are simply called cholesterol deposits. They usually are also associated with degenerative changes in the arterial wall. In a later stage of the disease, fibroblasts infiltrate the degenerative areas and cause progressive sclerosis of the arteries. In addition, calcium may precipitate with the

lipids to produce calcified plagues. When the two latter reactions have occurred, the arteries become extremely hard, and the disease is then called 'arteriosclerosis', or simply 'hardening of the arteries' (220).

Morphology

As a result of pathological, histochemical and microanalytical studies it has been possible to subject the vascular lesions occurring in the arterial system to a fairly detailed chemical analysis. The early fatty flecks or streaks found in the vessels of adolescents are largely composed of a lipid mixture the composition of which is somewhat similar to the lipid composition of blood plasma (218, 221). These observations have given support to the concept that the fatty streaks are generated by the passive deposition of plasma lipids in the vessel wall.

In order to produce a biochemical basis for the complicated pathological situation embracing atherosclerosis it is necessary to explain the appearance of fatty flecks in blood vessels with increasing age of the animal, the appearance of the fibrous plagues and, finally, the formation of the complex ulcerative type of atheromatous plagues. Since the accumulation of lipids in the intimal layer of the vessels has been regarded by many as an early event in the process of atherosclerosis, it is important that this event should be considered at the outset.

Etiopathogenesis

From morphological and biochemical points of view the natural history of atherosclerosis has been a subject

of controversy for over a century (218) and the cause of the arterial changes has been the subject of a number of theories.

Rokitansky (224) considered that the thickening of the intima was due to the deposition of material derived from blood. In 1856 Virchow, attributed the vascular lesions to a manifestation of inflammatory processes which were followed by fatty degeneration (222).

Some 85 years later the view that the process of atheromatosis was due to a type of fatty infiltration of the vessels was put forward by Leary (226).

The arterial intima slowly thickens throughout life (218) and since this arterial layer in males appears to thicken faster than that in females, Wilens (228) suggested that this might explain the difference between the sexes in their presentation with the clinical manifestation of atherosclerosis. He also considered that the appearance of lipid-laden plaques was a natural consequence of age-dependent intimal thickening (226, 229-231).

It is known that the lipid composition of the plasma in contact with the plaque resembles the lipid composition of the intima (232). Isotopic evidence also supports the concept that circulating plasma lipid can be deposited within the atheromatous material (233). The theory of lipid infiltration in which the severity of the vascular lesion is related to the concentration of certain lipids in plasma, would be consistent with the hypothesis that by lowering the plasma lipid level the lipid contained in the lesions might be made to regress. There

is evidence that certain experimental animals can be rendered atherosclerotic by elevation of the plasma lipids by dietary means. The degree of atheromatosis can then be reduced by dietary reversion to normal plasma lipid levels (234). Furthermore, in man it is often observed that the macroscopic appearance of the arteries of individuals dying of 'wasting diseases' is relatively free from superficial lipid-laden streaks (235).

There are many points of weakness in the lipid infiltration theory of atherosclerosis and these have been discussed by Duguid (227, 236, 237), who proposed the thrombogenic theory.

Subjects with atherosclerosis have been reported to possess an abnormal blood clotting mechanism and to be insensitive to heparin (234). Similarly, the blood platelets in these subjects exhibit an increased stickiness to glass (238).

The abundant evidence suggesting that atheromatous lesions are rich in both protein and lipid presents a major problem in attempts to reconcile all the variants of the lipid infiltration theory, with the thrombogenic theory. This has led many to accept a compromise explanation, in which the appearance of fatty flecks and lipid-laden streaks is ascribed to passive lipid deposition or lipid infiltration, whilst thrombus formation is considered to be a different process but causally related to the vascular lipid changes. Lipid lesions may then be considered as a site for the 'trigger reaction' involved in the blood clotting process by some mechanism as yet unexplained (234).

The histological interpretation by supporters of the thrombogenic theory is that the thrombus is first formed and then undergoes "fatty degeneration"; however this is difficult to explain on a metabolic basis.

As a result of atherosclerosis further activation of the clotting mechanism as well as promotion of platelet adhesion can occur because of the abnormal surfaces produced in the blood vessels (234). The atheromatous process seems to have a multicausal origin, with a complex sequence of events involving various factors which may operate at widely different periods throughout the life-span of the individual.

It is generally established that the fatty streaks appear in the arteries during the first decade of life and continue into the second decade, the timetable of the onset of lipid deposition varies depending on the vessel considered (239). In the coronary arteries lipid deposition starts in the second decade, and in the cerebral arteries in the third decade (239).

Yet from the standpoint of etiology and intimate pathogenesis the basic nature of the disease remains obscure and debatable (218).

Experimental Atherosclerosis

Besides analysis of the various morphological and biochemical components of the lesions, the timetable of appearance, their location, and relationship to the internal and external environment of the body, attempts have been made to produce atherosclerosis in laboratory animals.

In animals it is possible to induce aortic fatty streaks using various dietetic manipulations. Some arterial intimal lesions in experimental animals are chemically and histologically similar to early lesions found in man (223). Although, observations with laboratory animals may not always relate to man (218) they must be used in most experimental work in an endeavour to discover factors which may prevent or can be used to cure atherosclerotic lesions.

It is now recognised that, although man, other primates, birds, and swine are the only animals which seem to acquire atherosclerosis regularly and spontaneously, there is also a number of other species which can be made to develop arterial lesions similar to those found in human vessels by dietary manipulations of lipid metabolism (223, 219).

A structural similarity between rat and human aorta is known to exist and both animals are resistant to experimental atherosclerosis (1).

Risk Factors

Atherosclerosis is undoubtedly a disease with multiple causes. Severe forms of the disorder occur prematurely and result in a high mortality rate amongst the middle-aged. The phenomenon is common in most economically developed countries and is closely related etiologically to long-term living habits including patterns of overnutrition, physical inactivity and excessive cigarette smoking. Hence the causation of significant atherosclerotic disease involves complicated

interrelations between many exogenous (environmental) and endogenous (host) factors. Amongst the host factors hormonal influences may be very important especially when abnormalities interact synergistically with the environmental factors.

The epidemiology of atherosclerosis has been studied extensively for a number of decades and the literature that has accumulated on this subject even in the last few years is enormous.

The high mortality rate due to coronary heart disease in middle age, indicates that age is an important factor involved in the development of atherosclerosis.

The fact that in the U.S.A. the incidence of coronary heart disease is five times greater in males than in females in premenopausal age-groups, whereas in Japan the ratio is only 1:1 may be indicative that dietary factors are as important as the sex of the individual in determining the difference between males and females (140).

Although support for the belief that coronary heart disease "runs in families", is based on indirect evidence, considerable efforts have been made to determine more precisely the nature of this factor (218).

These investigations have tended to support the thesis that racial and many so-called hereditary factors can be cancelled by environmental and/or dietetic factors, for example, an individual belonging to a community in which the prevalence of this disease is low may very rapidly acquire the almost identical disease rate of the group in which he elects to live (224, 131).

However some well established genetic disorders do appear to be connected with coronary heart disease.

Diabetes is an example of an inheritable disease, which is expressed primarily in alterations of carbohydrate metabolism but also involves changes in lipid metabolism and a high incidence of cardiovascular mortality.

Another example is familial hyperlipoproteinemia which is also involved in atherosclerosis.

Amongst other diseases that predispose to atherosclerosis are hypothyroidism, hypertension and obesity (224).

Plasma Lipids and Atherosclerosis

The relationship between plasma lipids and atherosclerosis has been extensively investigated for many years. The spherical lipid particles circulating in the blood, called chylomicrons, mainly consist of triglyceride. Chylomicrons, however, only represent a small fraction of the total lipid present in normal fasting plasma. Most of the lipids in plasma occur in the form of α- and β-lipoproteins i.e. lipids combined with α- and β- globulins, respectively. The lipid portion of lipoprotein molecules consists of cholesterol, cholesterol esters, phospholipids, triglycerides and other lipids. Plasma β-lipoproteins are also referred to as low density lipoproteins: in addition there are pre-β-lipoproteins or very low density lipoproteins, α-lipoproteins are high density lipoproteins. In most mammalian species both α- and β-lipoproteins are present in plasma, but the ratio of these two constituents varies

considerably from species to species[+] (234).

Five different types of hyperlipaemia are recognised in man. Type 1 and type 5 are rather rare: type 2 and type 4 are the principal forms of this disease (195).

In type 2 lipoprotein disorder, the level of plasma cholesterol and triglyceride are elevated but the elevation of plasma triglyceride is much greater than plasma cholesterol (234, 238).

In type 4 plasma triglyceride is elevated, and the disease is endogenous and carbohydrate-induced.

In general, abnormal levels of plasma triglycerides occur in all types of hyperlipoproteinemia (234, 238).

The level of triglycerides in the blood serum is quite variable depending on the alimentary state of the individual, physical activity, and the general metabolic level. The quantity of triglycerides found in the serum is probably a better reflection of the dynamic state of metabolism than is the level of cholesterol, since the triglycerides can contribute freely to the energy pool of the body, but cholesterol cannot (241).

There are clear indications that there is a correlation between serum lipid concentration and the age of the animal.

Keyes and co-workers (70) have examined data published by Page et al (69) and noted that middle aged males generally have higher serum triglyceride levels than

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[+] The agerage : ratio in man is about 30:70, in the rabbit 50:50, while in the rat it is 90:10 (234). The ratio is reduced in subjects with coronary artery disease.

younger men.

Similar conclusions have been reached by Carlson (2).

With increasing age there appears to be no significant increase in serum free fatty acids or glycerol in male rats. Triglyceride levels according to Carlson (25) however, increase slightly from one to four months with a mean value of 0.8 mmol/l in the fourth month. They then rise steeply to a mean of 2.5 mmol/l at nine months and then remain constant. Serum cholesterol shows a similar trend.

Bottcher et al (242) compared the total lipid contents of different arteries in various stages of atherosclerosis. They found that the lipid content of the coronary artery was higher than that of the brain artery.

In addition there was striking difference in the composition of the lipids: in the coronary arteries the percentage of triglyceride was much higher than in the aorta.

Bassett et al (129) suggested that the high fasting serum triglyceride level in Japanese men in Hawaii could be related to the increasing incidence of coronary heart disease in this ethnic group.

In a somewhat similar study in Sweden, Carlson and Bottiger (130) reported a good correlation between plasma triglycerides and ischemic heart disease.

Clofibrate, a drug which especially lowers plasma triglycerides decreases the mortality from sudden death (127, 128).

SUCROSE AND CORONARY HEART DISEASE

Amongst the risk factors for atherosclerosis currently being investigated is the common component of all human diets, sucrose. On epidermiological grounds and the apparent relationships between this disaccharide and hyperglyceridaemia (85, 125, 126, 215) sucrose may well be an important risk factor for coronary heart disease.

Dietary Sucrose

Cultivation of plants as a source of sucrose began some 2,500 years ago and now the total production of this sugar is over 60 million metric tons *per annum* which means there is an average global *per capita* consumption of about 15 kg. The amount of sucrose utilized varies considerably depending on the affluence of the country. In wealthy states the average consumption is about 50 kg per head *per annum*, whilst only one tenth of this weight is consumed in developing countries where the average income is very low (85).

The percentage increase in 1958, compared with pre-war sucrose production shows, that it has increased over twice as much as wheat and other grains.

The approximate contribution of sucrose to the daily carbohydrate consumption in Europe is estimated to be 140 g (together with 200 g other carbohydrates, mainly starch) (85). Sucrose is the second most common dietary carbohydrate; in most countries it is superceded by starch which on digestion is converted almost entirely to glucose.

Sucrose and Blood Lipids

Higgins in 1916 (97) from a study of changes in the respiratory quotient in man, concluded that fructose, and hence sucrose, showed a greater tendency to change into fat, than did glucose.

The observation of Higgins was confirmed by Allen and Leahy (98) who found rats on a sucrose or fructose diet developed more body and liver fat than those animals on a glucose diet.

Similar findings were reported in the case of rats by Allen et al (99) and in baboons (48). Macdonald and Roberts (48) on the basis of their feeding experiments with baboon postulated that the fructose part of the sucrose molecule is responsible for the different response in the serum glycerides and that the liver may be the site of this metabolic differentiation.

In man, the different effects of sucrose and glucose on serum triglyceride levels was recently investigated by Mann et al (245). They found that after a formula meal containing fat and either glucose or sucrose as carbohydrate, the degree of lipaemia was less in glucose-fed individuals.

Kuo and Bassett (33), from their feeding experiments on the effect of sucrose and starch on plasma tri-glyceride concentration in normolipemic as well as familial hypercholesteremic patients, concluded that a high dietary sucrose intake may well be the most important factor in the production of the hyperglyceridemia.

Later it was shown by Nikkila and Pelkonen (55) that alimentary hypertriglyceridemia was enhanced in man by administration of fructose.

Studies on the effect of sucrose feeding on hepatic lipogenesis in man by Cahlin *et al* (27) revealed that the disaccharide enhanced triglyceride synthesis in the livers of normolipidemic patients with gallstone disease and raised the plasma triglyceride levels.

Investigations of the withdrawal of sucrose from the diet of patients with ischaemic heart disease (43) and 'normal' men with a raised fasting serum triglyceride level (30) both showed that triglyceride levels were lowered. In men where the pre-diet triglyceride concentration was normal withdrawal of sucrose had little effect (30).

In a study with healthy young people, on a sucrose-free diet, Roberts (30) reported no significant fall in fasting serum glyceride concentration but he noted that when a normal sucrose-containing diet was resumed a significant rise in fasting serum glycerides occurred, reaching a peak at eight weeks, followed by a return to the pre-dietary level over the following sixteen weeks.

Macdonald and Braithwaite (46) studied seven male subjects on sucrose or maize starch diet for a period of 25 days. They observed that the high sucrose diet resulted in an abnormal increase in serum lipids, mainly glycerides,, whereas the starch diet produced a fall in serum lipids. Macdonald (47) confirmed his finding by a further, somewhat similar, study with male subjects, where he detected a rise in the triglyceride fraction of fasting serum lipid in response to dietary sucrose. The fasting serum response to starch regimen was a fall in total cholesterol only.

Akinyanju *et al* (28) examined the fasting levels of serum cholesterol and triglycerides in human voluntiers, on a

in part, to the inability of fructose to indirectly activate lipoprotein lipase and, hence, allow triglycerides to accumulate in the circulatory system.

METABOLISM OF FRUCTOSE AND GLUCOSE

Effect of age

In rats age, as well as some other factors, such as sex of the animal, duration of the experiment and presence of dietary fatty acids, are reported to effect various changes in body composition resulting from ingestion of starch and sucrose (36).

Hill (34) who carried out feeding experiments with young and mature male rats, showed that fructose produced elevation of the triglyceride level in the liver and blood serum of fed, mature rats, but not in young animals. With female rats 10% fructose in the drinking water decreased the liver triglycerides in mature animals but no change was observed with young rats (34).

These observations were further confirmed by Chevalier et al (190), who fed weanling and mature rats with synthetic diets containing 70% glucose, starch, sucrose or fructose. Their results clearly showed that dietary fructose or sucrose increased serum triglyceride levels in the mature rat but not in the weanling animal.

Age dependent changes in the concentration of plasma cholesterol, triglycerides and phospholids is reported in man (2). Cholesterol and phospholipids in the α, lipoproteins increased significantly with age: this was not the case with these same lipids in the β-lipoprotein fraction. Stern and collaborators (134) also observed that with increasing age the

seven day starch diet followed by a seven day sucrose diet (group I) and vice versa (group II). They report that in group I the serum triglyceride level fell in the second week after a significant rise in the first seven days. In group II, however, the rise in triglyceride level occurred in the second week. No significant changes in cholesterol or phospholipid were noted under these experimental conditions.

Nikkila and Ojala (3) studied the effect of feeding fructose on plasma triglycerides, to decide whether hypertriglyceridemia is an adaptive phenomenon or whether it is caused by the direct effect of fructose on triglyceride synthesis release or utilization. They concluded that in rats both fructose and glucose are able to increase the overall removal rate of triglycerides in the fasting state but are without measurable effect when given as large acute loads to previously fed animals. They further excluded any inhibition effect on triglyceride removal by either hexose. Recently, however, Cryer and his associates (279) showed that the effect of intragastric glucose, fructose and sucrose on the plasma insulin levels of male rats could be related to the activity of adipose lipoprotein lipase. Insulin increases the activity of the latter enzyme (Wing & Robinson (281) and hence, glucose which promotes insulin secretion raised the level of lipoprotein lipase in these experiments. Fructose had little effect on plasma insulin and, hence, in the case of these sugars the lipoprotein lipase remained at the same levels as the control. The effect of fructose and glucose on serum triglyceride concentrations in the rat could, therefore, be due, at least

level of plasma triglycerides rose in men but not in women (cf Bender et al (157))

The importance of these changes which occur with age is obvious when one remembers that atherosclerotic cardiovascular diseases, are features of old age and appear to be intimately related to elevated plasma lipid levels.

Sex differences

Sex differences in carbohydrate metabolism were reported as early as 1932, when Deuel and Gulick (72) noted that the extent of ketonuria is much greater in women than in men. Later the rate of absorption of glucose was shown to be significantly higher in female rats than in male aminals (73). Al-Nagdy (36) suggested that the sex of animals affected various changes which resulted from dietary starch and sucrose. Castration of male rats was shown to delay the fatty infiltration of the liver produced by a change in dietary carbohydrate (74).

The serum triglyceride concentration in male and female baboons after intravenous administration of glucose and fructose was studied by Jourdan (243). He observed a fall in serum triglycerides after glucose administration in both sexes. Whereas after fructose administration no change in triglyceride concentration was observed in male baboons but a significant reduction occurred in the female animals.

Jourdan (124) also showed that fructose, after rapid intravenous injection, disappeared from the serum of male baboons more rapidly than from the serum of female baboons. Glucose was not metabolized at different rates by the two sexes. Later, from a study on the rate of triglyceride formation

following intravenous injection of labelled fructose in male and female baboons (41), it was demonstrated that the specific activity of the glyceride fraction was greater over a 5 h. period in the male than in the female animal. The specific activity of the triglyceride fraction after injection of ^{14}C glucose was less than after fructose injection and no difference between the sexes was detectable.

Macdonald (56) examined the hyperglyceridemic effect of glycerol (a breakdown product of fructose) on men and premenopausal women. It was clearly demonstrated that whilst glycerol feeding increases the level of triglycerides in men, it does not alter the triglyceride level of premenopausal women. Macdonald (42) demonstrated that a 4 - 5 day fat-free fructose-starch or fructose-glucose diet did not increase the fasting serum triglyceride level of premenopausal women in comparison with women on a fat-free control diet. However, a significant increase in the level of fasting triglycerides was observed in men and postmenopausal women on fat-free fructose-glucose and fructose-starch diet when compared with the fat-free control diet. Such changes were not observed with a fat-free glucose-starch diet.

Fat-free-high carbohydrate diets have been reported to produce a sex dependent change in the level of the plasma triglycerides during a 16 day period (44). Macdonald (45) examined the response of men and women to a high sucrose diet:: higher fasting serum triglyceride levels were observed in the men than in the women. This difference was not observed in the case of a high starch diet. Since postmenopausal women showed a similar response to men on various dietary

carbohydrates (49-50), Macdonald, postulated that the ability of sucrose to increase the serum for glyceride fraction was influenced by ovarian hormones (49).

Hill (34) reported that the rise in serum triglycerides observed in mature male rats produced by fructose in the drinking water did not occur in mature female rats. The hyperlipaemic effect of sucrose was examined in male and female rats by Bruckdorfer et al (31). These investigators report higher concentrations of plasma triglyceride in male animals than in the female animals (confirmed by H. Williams (1975) Royal Holloway College unpublished results). No sex difference was observed in the serum cholesterol level or in the activity of lipoprotein lipase. They further concluded that the low concentrations of triglyceride in female animals cannot be explained by differences in the activity of fatty acid synthetase or lipoprotein lipase (cf Cryer et al (279)). Sex differences in the levels of plasma triglyceride and not the plasma cholesterol, have also been investigated thoroughly by Stern et al (134) in man.

Effects of sex hormones

Differences in the levels of lipids in animals of different sex and the effects of dietary carbohydrates on these levels are undoubtedly related to steroid hormone balances although, so far there is no clear picture of how they are involved.

Watkins et al (136) working on perfused liver from male and female rats noted a sex difference in the output of

triglyceride, which was greater in the case of the female organ: they subsequently suggested that the hepatic output of triglyceride can be regulated by gonadal or gonadotrophic hormones. In rats, production of glycerol 3-phosphate, a lipid precursor, and lactate in uteri from glucose and glucose 6-phosphate is shown to be strongly reduced by ovariectomy and increased to about 200% of the control values after administration of estradiol-17β. In addition, the same investigators, noted a reduction in the level of lactate and glycerol 3-phosphate after subsequent administration of anti-estrogenic agents (nafonidine hydrochloride and ethamoxytriphetol) or progesterone (143).

Hill and Martin (144) studying the effect of estrogens on rat serum lipoproteins, observed a significant increase in the level of very low density lipoproteins after administration of estradiol-17β, or ethynylestradiol. In further analysis of the VLDL fraction they found a higher level of serum triglyceride glycerol in ethynylestradiol- or estradiol-treated female rats. Administration of exogenous estrogen to male and female Japanese quails however, decreased the total lipid levels in the blood, but testosterone had no effect (146). Similarly, Coltart and Macdonald (68) showed that the increase in the plasma triglyceride levels produced by feeding male baboons with sucrose could be prevented by estradiol injections. Testosterone had little effect on the triglyceride levels of the female animals.
At this point it is interesting to note that certain estrogenic compounds including estradiol, when administered to male rats causes a drop in the level of circulating testosterone (149). In that respect estradiol acts in the same manner as

cyproterone in rat (158) or cyproterone acetate in man (159).

It has been known for some time that oral contraceptive pills, which are generally a combination of an estrogen and a progestogen, increase the level of triglycerides in plasma (140, 141). These findings have recently been confirmed by Lunell et al (138). They found that the level of plasma triglyceride increased after the first month of use of an oral combined contraceptive and remained elevated and unchanged during the second month of treatment. Sixty six per cent of the women treated longer than ten months with an oral combined contraceptive had higher triglyceride levels than normal, whilst the cholesterol level did not change (152). Mestranol, the estrogen component of the contraceptive, increased the triglyceride level promptly. Kekki and Nikkila (53), from their study on triglyceride levels, in women during the use of an oral contraceptive, concluded that the increased level observed resulted from an enhanced rate of synthesis.

The effect of an oral contraceptive on lipase activity was studied by Rossner et al (165) in an attempt to explain its hyperlipemic effect; it was observed to decrease lipase activity.

An interesting observation with women on various oral contraceptives was reported by Briggs and Briggs (148). They found that there was a rise in the plasma testosterone level which was independent of the type of oral contraceptive used. An increase in the concentration of corticosteroids is also recorded by these investigators. In contrast to the above observations data presented by Aftergood et al (147), shows a decrease in serum and liver triglycerides following oral

administration of small doses of an anovulatory drug to female rats. While estrogens are reported to be responsible for increased plasma triglyceride in women, administration of ethynyl estradiol to 100 survivors of myocardial infarction resulted in uniform correction of the abnormal circulating lipid concentration (154). In a similar study Marmorston et al (155) report that number of deaths due to myocardial infarction decreases after estrogen therapy in men. Effect of long term estrogen therapy in men with coronary heart disease has also been investigated by Robinson et al (156). Apart from side effects of estrogen therapy, they recorded a decrease in cholesterol/phospholipid ratio. Bender and associates (157) demonstrated that the plasma level of estradiol is lower in male subjects with coronary artery disease (who also have higher triglyceride levels) than in normal individuals and that there was a close reciprocal relationship between the levels of estradiol and triglycerides in blood from men.

Naturally occurring estrogens were at one time used in medicine as anti-hyperlipidemic agents. Modern drugs for reducing hyperlipemias all tend to be synthetic compounds with weak estrogenic effects which if necessary can be overcome by compounding a mixture of the drug and an androgen.

The synthetic compound p-chlorophenoxyisobutyric acid (CPIB) one of the first compounds to be used theraputically, in conjunction with androsterone, is an anti-hypertriglyceridemic and anti-hypercholesterolemic agent (197).

Clofibrate (Atromid-S), the ethyl ester of CPIB (ethyl 2-(p-chlorophenoxy)-2-methylpropionate) is now used extensively for lowering serum lipids in particular for patients with atherosclerosis.

Clofibrate (Atromid) was introduced for the first time to lower serum cholesterol and triglyceride levels in the monkey (198), and in man (199, 200).

The finding of the Newcastle and Scottish trials (257) was that clofibrate which lowers plasma triglycerides, and to a lesser extent cholesterol, also decreased the mortality from sudden death particularly in patients who were suffering from angina pectoris: this supports the theory that triglycerides play a vital role in the prognosis of atherosclerosis.

Although clofibrate has been used freely for more than 12 years as a lipid lowering agent, the mechanism of action of the drug is still unknown (203). A decrease in the release of triglycerides into the plasma is apparently one of the mechanisms by which this drug might operate (203). Clofibrate is also reported to compete with free fatty acids for the binding sites on albumin (201). Presumably this would prevent the acids from being 'carried' in the circulation to regions of triglyceride synthesis.

Galton (202) reported that in rats fed with Atromid for 6 days the hepatic synthesis of cholesterol from ^{14}C-acetate was reduced by about 70% although no effect was observed when the drug was added directly to isolated liver preparations. Atromid did not significantly inhibit the synthesis of triglycerides or phospholipids by the liver.

Relationships between sex hormones and enzyme activity

Although sex hormones have an obvious and profound effect on the metabolism of carbohydrates and lipids there has been relatively little work on the exact relationships between sex hormone and enzyme levels in aminal tissues.

It is generally assumed that some protein and, hence, enzyme synthesis is partially controlled by steroid hormones particularly in sex organs (282) although recent studies by Roy et al (280) suggests that the liver is also a 'target organ' for sex hormones as it possesses a specific protein receptor for dihydrotestosterone and estradiol.

Following the reports on sex differences in carbohydrate metabolism, many attempts have been made to elucidate the enzymic basis of these differences. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (174), an acetylcholinesterase (175), and β-glucuronidase (176), for example, have been shown to be more active in female than in male animals.

The discovery of the anabolic action of androgens was another guideline to encourage investigators using various sex hormones to attempt to manipulate the activity of the enzymes.

Kochakian and Emdahl (161) recorded an increase produced by androgens, of aspartic-glutamic and alanine-glutamic transaminases in temporal muscle of male guinea pig but only in proportion to increasing muscle weight. Later several enzymes of the Krebs cycle were shown to behave in a similar fashion (102).

An esterase was reported to be present in mature male mouse kidney, which was absent from female and immature male kidneys. The absence of this enzyme in castrated male mice

was demonstrated by Shaw and Koen (163) who also showed that testosterone induced higher levels of the esterase.

Alkaline phosphotase activities of plasma membranes isolated from rat liver were shown to be higher in male than female animals and the enzyme activity could be manipulated by castration and injection of sex hormones (168): such a sex difference was not observed with whole liver homogenates or brain tissue (169). In male rat liver, fructokinase is reported to be more active than in the female organ and the activity of this enzyme is also increased by injecting animals with testosterone (173).

Stifel et al (214) studied the effects of oral testosterone, estradiol, diethylstilbestrol and progesterone on jejunal glycolytic enzymes of male and female rats. These investigators reported that testosterone raised the levels of jejunal phosphofructokinase and pyruvate kinase activities as did estradiol, diethylstilbestrol and progesterone to a lesser extent. In female rats, in contrast estradiol and diethylstilbestrol were more effective than testosterone in increasing phosphofructokinase and pyruvatekinase activities. They further concluded that progesterone and testosterone are antagonistic to estradiol in the female rat jejunum, whilst estradiol is antagonistic to testosterone in the male rat jejunum. Lufkin et al (276) observed a low activity of jejunal pyruvatekinase in testosterone deficit (hypogonadal male) animals which was compensated after administration of testosterone.

Tepperman and Tepperman (164) reported that estrogen increased the NADP — dependent dehydrogenases in the

pentose phosphate pathway in male rats. The activity of glucose-6-phosphate dehydrogenase was reported to be reduced following ovariectomy (166). Replacement of either progesterone or estradiol restored the activity to the level of the intact animal.

The glucocorticoid-inducible enzymes, tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase are significantly increased in female rat liver following administration of estradiol. Their level of tryptophan oxygenase was lower in male than female rats, but the reverse was found with tyrosine aminotransferase. Although orchidectomy had no effect on tryptophan oxygenase activity, the increase in enzyme activity in orchidectomized males treated with estradiol was significantly higher than the response in intact male (167). A reduction in tryptophan oxygenase activities was reported to occur in ovariectomized rats and to result from testosterone treatment or adrenalectomy of female rats. These investigators, from their experience with adrenalectomized- and estrogen-treated animals, suggest that, for the full expression of the effect of estrogen upon tryptophan oxygenase and tyrosine aminotransferase and for the induction of alanine aminotransferase, the presence of adrenocorticosteroids is necessary.

In view of the weak estrogenic activity of clofibrate (see p.31), the action of this lipid lowering agent might also be mentioned here.

Pereira and Holland (203) working with rat reported that clofibrate stimulates the synthesis of mitochondrial L-glycerophosphate dehydrogenase. These investigators further

concluded that as a consequence of this stimulatory mechanism triglyceride synthesis is decreased by clofibrate (204).

Bowley et al (205) studying the effect of CPIB and similar compounds on human liver homogenates noted that whilst 20mM CPIB inhibited esterification of glycerol 3-phosphate by palmitate a concentration of 1mM stimulated esterification.

Brindley and collaborators (256) reported that clofibrate at the concentration of 20mM inhibits the synthesis of phosphatidate from glycerol 3-phosphate and palmitate in human liver homogenates. Clofibrate had also been shown to decrease the activities of jejunal glucose 6-phosphate dehydrogenase, fructose 1, 6-diphosphate aldolase and fructokinase without affecting the activity of hexokinase (244).

Stimulation of the hydroxylating system of liver microsomes occurs with clofibrate (206). The significance of this induction process is in the metabolism of drugs as well as some steroid hormones such as testosterone. Clofibrate will, therefore, encourage hydroxylation and conversion of these compounds to more polar derivatives.

Enhancement of cholesterol, progesterone and estradiol hydroxylation has also been reported in clofibrate treated rats (207, 208).

Digestion and Absorption of glucose and fructose

Sucrose is a disaccharide in which a molecule of glucose is glycosidically bonded to a molecule of fructose by a (1 → 2)-linkage. In this disaccharide the anomeric carbon atoms of both hexoses are involved in the glycosidic link and, thus, the molecule is non-reducing. Chemical or enzymic hydrolysis of sucrose releases the two combined monosaccharides.

Dahlqvist and Borgstrom report that the majority of dietary disaccharides are absorbed intact in different parts of the intestine and then subsequently undergo intracellular hydrolysis: in the case of sucrose this occurs in the distal jejunum and in the ileum (62, 63).

Sucrose is sensitive to acid hydrolysis but so far there is no evidence that this occurs in the stomach (209).

The small intestine contains a number of glycosidases. Only the brush border enzymes are responsible for the digestion of dietary disaccharides whilst soluble and lysosomal intestinal enzymes probably have other functions. Disaccharidases are localised in the brush borders of the mucosal epithelial cells and exert their function in this location. They are not secreted into the intestine. The activity of disaccharidases determines the ability of the subject to digest and absorb the disaccharides (209).

Intestinal sucrase is really an α-glycosidase which hydrolyses sucrose, maltose and some other α-D-glucosides.

Isomaltase is another intestinal disaccharidase which is closely associated with sucrase. The relationship between these two enzymes is not fully understood: they are separate protein molecules and they seem to be controlled by a common gene (210).

Patients with a congenital lack of intestinal sucrase are unable to digest and absorb sucrose (210, 211).

The effect of disaccharides on the activity of disaccharidases is believed to be very little (209). However Deren (87) reported that intestinal sucrase activity is stimulated by dietary sucrose.

It has been suggested that the hydrolysis of sucrose determines the rate of absorption of this disaccharide in the intestine as mixtures of glucose and fructose are absorbed less rapidly than the corresponding molar quantity of sucrose (89, 93). The fact that $^{14}CO_2$ production from labelled sucrose is greater than from labelled fructose or glucose supports this contention (92).

Crane et al (212) report that there is a spatial relationship between the sucrase and a specific membrane carrier for glucose released from sucrose during hydrolysis. Such glucose possess a kinetic advantage and is absorbed more readily than free glucose. The absorption of the fructose component of the sucrose molecule was thought to be 'passive' although a recent publication suggests that there is an active transport mechanism for fructose in rat (241).

Ockerman and Lundborg (90) observed an increase in the level of glucose in mesenteric veinous blood when fructose was injected into the jejunum of patients with duodenal ulcers by means of catheters. They concluded that the jejunum converted up to 70% of the absorbed fructose to glucose. White and Landau (246) suggested that the percentage conversion was much smaller. Macdonald and Turner (247) showed that significant increase in blood fructose occurred when fasted men and women were given sucrose or an equivalent mixture of constituent monosaccharides. The intestine of rat apparently cannot convert fructose to glucose (60, 84).

Sucrose itself enters the blood stream when fed in large amounts. When absorbed without prior digestion, the disaccharide is treated as a foreign substance and appears unchanged in the urine (88).

It is clear that the fructose moiety of sucrose is responsible for elevation of serum triglycerides in a number of species (cf (48)). It is, therefore, of importance to review the metabolism of fructose and the pathways for the conversion of this ketose to triglycerides and to compare these pathways with those for glucose. The fact that effects of dietary sucrose and of fructose on the levels of many enzymes are indeed the same, and the finding that glucose has little or no effect on these enzyme levels indicates that fructose follows a different metabolic pathway from that of glucose (91). For example, whilst the level of glucokinase is elevated more by dietary glucose than by fructose (95), the level of many other enzymes e.g. glucose 6-phosphatase phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase, aldolase, ketohexokinase, malic dehydrogenase, and α-glycerophosphate dehydrogenase are increased by dietary fructose but not glucose (77, 78).

The major known differences in fructose and glucose metabolism occur initially in the phosphorylation stages and later in the aldolase-catalysed cleavage of the phosphorylated hexoses.

ENZYMES OF FRUCTOSE METABOLISM

A summary of the major routes of fructose metabolism is given in Fig. 1 (4-6, 19, 22, 86).

As shown, the first step in fructose utilization is phosphorylation to fructose 1-phosphate by the action of ketohexokinase and ATP (101-104).

Ketohexokinase (ATP: D - fructose 1-phosphotransferase; E.C. 2.7.1.3., also called fructokinase).

Ketohexokinase occurs in the liver, intestinal mucosa and kidney of mammals. The enzyme from rat intestinal mucosa has a high affinity for D-fructose (Km 7 X 10^{-4} M; pH optima 6.5 at 30°C) (86). Ketohexokinase requires Mg^{2+} and K^{+} ion activity and ADP inhibits the reaction. Rat liver ketohexokinase also has a relatively high affinity for D-fructose (Km 4 X 10^{-4}M) and a broad pH optima (5.5-8)(108).

In liver cells, ketohexokinase is localized in the cytosol (18). Heinz (86) reports that the enzyme activity in the liver of the rat is 2.2 U/g wet weight and 1.7 U/g wet weight in human liver at 37°C.

The enzyme was reported to be absent from fetal rat liver (79) but Ballard and Oliver (80) suggest that it may be present at a low level. However, Ballard (81) observed high conversion rates of fructose to glucose in fetal rat liver which is probably due to hexokinase activity.

A diet rich in fructose increases rat liver ketohexokinase activity (112): activity also increases when fructose is administered intraperitoneally (110).

Fasting reduces the activity of rat liver enzyme and it can be subsequently restored by feeding fructose (110).

Hexokinase (E.C. 2.7.1.1. ATP D-hexose-6 phosphotransferase).

Hexokinase is a rather nonspecific enzyme which can phosphorylate D-glucose, D-fructose and D-mannose. Hexokinase utilizes ATP and requires Mg^{2+}; the corresponding hexose 6-phosphate is produced.

The activity of hexokinase towards glucose in the liver of man and other animals is believed to be about 0.4 U/g fresh weight (86).

Three hexokinase isoenzymes can be identified by electrophoresis which are designated I, II and III (86). Types I and II are found in soluble and particulate liver fractions (250), whereas type III is only detected in the cytosol.

It is suggested that phosphorylation of fructose by hexokinase *in vivo* is negligible in adult animals (188). Fructose 1-phosphate, the product of ketohexokinase activity is cleaved by the action of aldolase to yield dihydroxy acetone phosphate and D-glyceraldehyde. The role of this enzyme in the metabolism of fructose 1-phosphate was first recognized by Hers *et al* (26, 111).

Ketose 1-phosphate aldolase (phosphofructoaldolase; E.C. 4.1.2.7., Ketose 1.phosphate aldehyde lyase).

Aldolase 1-B, one of the three forms (isoenzymes) of this enzyme, is found in the liver (26, 114).

Aldolase 1-B of human liver, has a Km value of 7.5×10^{-3}M for fructose 1-phosphate. The thermodynamics

of the reaction favour fructose 1-phosphate formation (115). ATP degradation products such as allantoin and uric acid inhibit the reaction (116). The activity of aldolase in human liver is 2.08 U/g wet weight and in rat liver 1.63 U/g.

The aldolase reaction is thought to be a rate-limitihg step in the metabolism of fructose (10, 11, 116).

D-Glyceraldehyde the product of the aldolase reaction, may be metabolized in three different ways.

One of the highest activities in human liver is that of glycerol dehydrogenase (86). This occurs in NAD- and NADP-dependent forms.

Glycerol dehydrogenase (NADP-dependent), (E.C. 1.1.1.2. glycerol:NADP oxidoreductase.

This enzyme is highly specific for the reduction of D-glyceraldehyde to glycerol /Km (rat liver enzyme) for D-glyceraldehyde, 6.2×10^{-4} M and for glycerol, 0.63 M/ (21). The activity is 3.6 U/g wet weight liver in man and 0.38 U/g in rat liver.

In view of the high Km value for glycerol the reaction strongly favours the reduction step hence the name aldehyde reductase or glyceraldehyde hydrogenase is proposed for this enzyme (21).

The enzyme was first reported by Moore (21), who was able to purify it about 50-fold by using DEAE-Cellulose chromatography.

Glycerol dehydrogenase (NAD-dependent) E.C. 1.1.1.6. glycerol:NAD oxidoreductase.

The Km of the rat liver enzyme for D-glyceraldehyde is very high and hence reduction of glyceraldehyde would not be favoured as in the case of the NADP-dependent enzyme (20). The activity of this dehydrogenase in human liver is again relatively high (3.1 U/g wet weight) and in rat liver somewhat lower (1.64 U/g).

Glycerol kinase (E.C. 2.7.1.30. ATP:glycerol phosphotransferase or L-triokinase).

This enzyme catalyzes the ATP-dependent phosphorylation of glycerol to L-glycerol 3-phosphate.

The name L-triokinase (46) has been used because the enzyme also phosphorylates L-glyceraldehyde. Glycerol kinase was first isolated from rat liver by Bublitz and Kennedy (13). The asymetrical 'treatment' of glycerol by glycerol kinase was demonstrated by Bublitz and Kennedy (40). Reports of the Km for glycerol vary between 10^{-4} and 10^{-6} M (255). The activity of the enzyme in rat liver is 2.08 and in human liver 0.62 U/g wet weight (86).

The enzyme was also detected in muscles of rabbit and rat (262) and in adipose tissue (16, 61) where it was found to be more active in obese than in lean animals. ADP and L-glycerol 3-phosphate are inhibitors of the enzyme (120). The latter compound can induce the enzyme in E. coli (17) and recently the catalytic and allosteric properties of glycerol kinase from this bacterium was investigated and fructose 1,6-diphosphate shown to be an inhibitor (15).

The glycerol 3-phosphate produced by the glycerol kinase reaction can be utilized for triglyceride synthesis by reaction with acyl CoA derivatives, removal of phosphate and further acylation.

Glycerol 3-phosphate can also be converted to dihydroxyacetone phosphate by oxidation.

Glycerol phosphate dehydrogenase (E.C.1.1.99.5., L-glycerol 3-phosphate:NAD oxidoreductase).

This enzyme is specific for L-glycerol 3-phosphate and dihydroxyacetone phosphate. Glycerolphosphate dehydrogenase is widely distributed in various tissues, e.g. skeletal muscle, liver, kidney, heart, brain and blood cells (252). Relatively high glycerol phosphate dehydrogenase activities are found in the main body organs.

Soluble glycerol phosphate dehydrogenase is located in the cytosol whereas glycerol phosphate oxidase (E.C. 1.1.2.1., L-glycerol 3-phosphate:cytochrom C oxidoreductase) is confined to the mitochondria. The two enzymes are involved in the glycerol 3-phosphate cycle (Bucher Cycle) (253).

The equilibrium of the reaction catalyzed by soluble glycerol phosphate dehydrogenase favours the formation of glycerol 3-phosphate ($K = 5.5 \times 10^{-12}$ at $25^{o}C$, pH 7.0). The Km value of the soluble enzyme for glycerol 3-phosphate is 1.1×10^{-4} M and for dihydroxy acetone phosphate, 4.6×10^{-4} M at pH 7.0 (254).

Dihydroxy acetone phosphate can be converted to glyceraldehyde 3-phosphate by the action of triose phosphate

isomerase. Glyceraldehyde 3-phosphate is one of the key intermediates in glycolytic pathway (see Fig. 2) and hence can also be readily converted to fats serving as both a source of glycerol and fatty acids.

A second possible fate for D-glyceraldehyde (see p.) is phosphorylation.

D-Triokinase (E.C.2.7.1.28. ATP: D-glyceraldehyde 3-phosphotransferase).

This enzyme phosphorylates D-glyceraldehyde using ATP to give D-glyceraldehyde 3-phosphate. The Km for D-glyceraldehyde is relatively low and varies between 8×10^{-6} and 35×10^{-6}M depending on the ATP-Mg^{2+} concentration (19, 86). Optimum activity is obtained at pH 7.4 (251).

Triokinase is a cytoplasmic enzyme found in liver (109), kidney, and intestinal mucosa (jejunum) (117). Its activity in human liver is 2.07 U/g wet weight.

Perhaps the least important reaction in vivo for metabolism of D-glyceraldehyde is direct oxidation.

Glyceraldehyde dehydrogenase (E.C.1.2.1.3. Aldehyde: NAD oxidoreductase).

Utilizing NAD this enzyme can convert D-glyceraldehyde to D-glyceric acid.

Glyceraldehyde dehydrogenase occurs in cytoplasmic and mitochondrial fractions of liver, kidney and intestinal mucosa (86). The Km value for the aldehyde is 4×10^{-4}M (86) and the optimum pH for the reaction is 9-10 (75).

The activity in rat liver is 0.6 U and in human liver 1.04 U/g wet weight.

From the pharmacological point of view, it is suggested that the enzyme may be important in oxidation and detoxification of biologically active aldehydes derived from endogenous sources, such as acetaldehyde and formaldehyde (76, 86).

Any D-glycerate resulting from the activity of glyceraldehyde dehydrogenase could be phosphorylated.

D-glycerate kinase (E.C.2.7.1.31., ATP:D-Glycerate-2-phosphotransferase).

This enzyme phosphorylates D-glycerate, using ATP, to give 2-phospho D-glyceric acid.

The enzyme is found in the liver, kidney and intestinal mucosa of the rat (18, 118, 119) and is equally distributed between the mitochondrial and cytoplasmic fractions (18). Only traces of this enzyme can be detected in human liver (0.13 U/g wet weight; Km (glycerate) 2.5-3.0 X 10^{-3}M) (4,274), kidney and intestinal mucosa of man (117). Accordingly, it probably does not play an important role in fructose metabolism in humans (86). It is inhibited by ADP.

In rat liver the activity is somewhat higher (3.14 U/g wet weight) and the enzyme has a lower Km value for glycerate (3 X 10^{-4}M) (18,86,119).

For comparative purposes the relative activities of the major liver enzymes involved in fructose metabolism in man and rat are given in Table I.

Table I. Comparison of the activity* of liver enzymes involved in fructose metabolism in man and rat.

Tissue	Ketohexo-kinase	Fructose 1-phosphate aldolase	Glycerol dehydrogenase (NAD)	Glycerol dehydrogenase (NADP)	D-Trio-kinase	D-Glyceraldehyde dehydrogenase	D-glycerate kinase	Glycerol kinase
Rat liver	1.80	1.20	1.30	0.31	1.34	1.14	3.60	1.6
Human liver	1.20	2.50	4.0	4.6	2.0	1.3	0.07	0.72

*Activities expressed as Units of enzyme activity per 100 mg protein. From Heinz et al (4, 58).

ENZYMES OF GLUCOSE METABOLISM

The pathways for glucose metabolism are well-known and differ from those of fructose in the initial phosphorylation and subsequent aldolase-catalyzed reaction. The reactions will only be briefly summarized here.

Glucose after being converted to glucose-6-phosphate by glucokinase (or hexokinase) can be isomerized to fructose 6-phosphate by the action of glucose-6-phosphate isomerase. Phosphorylation of fructose 6-phosphate at C-1 followed by aldolase cleavage gives rise to dihydroxy acetone phosphate and glyceraldehyde 3-phosphate (Fig. 3). (The latter product is replaced by free glyceraldehyde in fructose metabolism). After triose formation the metabolism of glucose and fructose is similar. It should also be mentioned that triose formation from glucose 6-phosphate is possible via the pentose phosphate pathway but this pathway is much less important than the Embden-Meyerhof in liver.

PATHWAYS OF GLUCOSE AND FRUCTOSE CARBON *in vivo*

Liver, kidney and small intestinal mucosa have high metabolic capacities for fructose.

In human liver, the total capacity for fructose metabolism is calculated to be about 0.5 g/min at 37°C (12, 123). There is excellent evidence from *in vitro* experiments to support the *in vivo* observations (86).

Rat liver can metabolize about 1g of fructose/g liver/h (6).

A very recent study performed by Sestoft (188), indicates that perfused rat liver can take up 6.6 mol fructose/min/g tissue (i.e. about 1g/min/g liver). It has been calculated that the liver possess 77% of the total capacity for the metabolism of fructose in man (the corresponding values for kidney and small intestinal mucosa are 14% and 9% of the total capacity, respectively (86).

Due to this high metabolic capacity of animal tissues for fructose and the fact that the ketose cannot be stored, fructose loading of tissues causes an elevation of many intermediary metabolites as well as end-products of metabolism (10, 11, 116, 122).

Macdonald and Roberts (48) compared the serum triglyceride level in 48h fasted baboons after ingestion of 40g of glucose, sucrose or a partial hydrolysate of starch all equally and uniformly labelled with ^{14}C. In their study, they measured the triglyceride content of venous blood at half hourly intervals over a period of 3h and then at hourly intervals up to 5h. They found that the rise in serum triglyceride concentration was greater in the case of sucrose-fed animals when compared with that produced by the starch

hydrolysate or glucose. The levels of serum cholesterol esters and phospholipids were also measured but no significant differences was noted.

Gale and Crawford (5) studied the different rates of incorporation of $[^{14}C$ - U] fructose and $[^{14}C$ - U] glucose into lipids in guinea pigs after oral or intraperitonial administration of the sugars. They found that the fructose contribution to the triglyceride fraction of the lipid in plasma was 4-6 times higher than that of glucose.

Ojala (37) attempted to study triglyceride metabolism in rats given fructose - or glucose - supplemented diets but no significant difference in the liver triglyceride levels of the animals on these diets was observed. However, in comparison with the control animals both sugars produced an increase in triglyceride. Plasma triglycerides only showed a significant increase in the animals on the fructose-rich diet. In this latter group injection of $[^{14}C]$ palmitic acid resulted in significant labelling of the plasma triglycerides within 30 mins. The incorporation of ^{14}C was much lower in the case of the glucose-fed and control animals. These investigators concluded that "fructose hypertriglyceridemia" was the result of an increased rate of production of plasma triglycerides without a corresponding increase in their removal rate.

In a study to compare the effects of glucose and fructose on the hepatic secretion of very low density lipoproteins, Topping and Mayes (177) observed an important metabolic difference between fructose and glucose when they were compared on a mole-for-mole basis. Their data from

experiments with perfused rat livers supports previous reports (172) that fructose enhances the secretion of triglycerides as very-low-density lipoprotein.

Liver slices from male rats incubated with [^{14}C-U] glucose showed lower incorporation of radioactivity into triglyceride glycerol and triglyceride fatty acid fractions than did slices incubated with [^{14}C-U] fructose. Similar results were obtained with liver slices from female rats (216).

Baron and Stein (38) administered [^{14}C] fructose and [^{14}C] glucose to male rats intragastrically. In the case of glucose the ratio of incorporation of the ^{14}C label into serum triglyceride fatty acids and triglyceride glycerol was approximately 1:5 2h after loading. When fructose was used there was a much greater incorporation into the glycerol moiety and the ratio changed to approximately 1:16. Similar trends were observed with the components of the liver triglycerides.

Cohen and Teitelbaum (29) fed rats for 65 days with diets containing 72% of either fructose or glucose. They then measured the rate of incorporation of [^{14}C] acetate (intraperitoneal) into total lipid and triglyceride in liver and serum. They found no statistically significant differences between the glucose- and fructose-fed animals or between the rates of incorporation of acetate into liver or serum triglycerides. Their results indicate that lipogenesis from acetate is not affected by dietary glucose and fructose. However, incorporation of [^{14}C] glycerol into liver triglycerides increased on feeding fructose in comparison with glucose (34). After fasting, rats incorporated label from

acetate into liver and serum triglycerides at a reduced rate and feeding fructose to these animals had no significant effect.

Maruhama (35) in his study on patients who had suffered myocardial infarct, compared the conversion of orally administered [^{14}C] glucose and [^{14}C] fructose to triglyceride glycerol and triglyceride fatty acids over a period of 4h. He found that the radioactivity of the glycerol was 10-20 times greater than that of the fatty acids after both glucose- and fructose-feeding. From his data it can be seen that, whilst the specific radioactivity of the glyceride-glycerol reaches a peak 3h after ingestion of glucose, the increasing specific activity of the glycerol after ingestion of labelled fructose is linear up to 4h at which time it is 3-4 times greater than that obtained with [^{14}C] glucose.

Pereira and Jangaard (6) who compared the rate of incorporation of ^{14}C into intermediary metabolites of carbohydrate and lipid metabolism (such as lactate, pyruvate and fatty acids) showed that it was 3 times faster from [^{14}C] fructose than from [^{14}C] glucose.

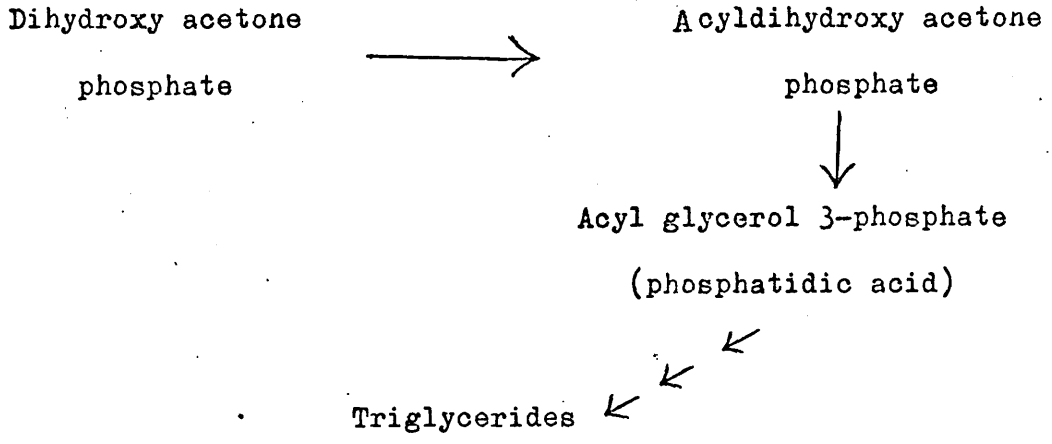
The studies of Zehner et al (7) with perfused livers from fasted rats suggest that fructose metabolism may be more complex than was originally envisaged. An examination of metabolites produced from specifically labelled fructose in the perfusate and in the tissues indicates that two compartments for metabolism of the ketose by the liver may exist. One utilizes fructokinase and produces lactate from C4, C5 and C6 of fructose : gluconeogenesis also occurs in this compartment. The other compartment initially phosphorylates fructose in the 6-position (hexokinase) and possesses only glycolytic activity.

In all these experiments isolated glycerol-3-phosphate had a relatively low specific activity. Perhaps this is to be expected with tissues from starved animals. The comparative study on fructose administration to fed and starved rats and the resulting differences in the levels of metabolites should be noted in this connection (6).

In the biosynthesis of triglycerides, glycerol 3-phosphate is mono-acylated at C-1 by reactions involving coenzyme A esters of the fatty acids.

This produces the so-called phosphatidic acids which are subsequently dephosphorylated and further acylated to the triacylderivatives of glycerol.

However, there is evidence (269) for the existence of a second pathway from trioses to triglycerides starting with dihydroxy acetone phosphate which is then acylated :



The biosynthesis of acyldihydroxy acetone phosphates in mitochondrial and microsomal fractions of rat liver has been studied by La Belle and Hajra (270). Hajra and Agranoff (269) showed that acyl dihydroxy acetone phosphate can be reduced to 1-acyl glycerol 3-phosphate by NADH prior to its conversion to

triglycerides. Later, Manning and Brindley (271) using a mixture of [2-^{3}H] glycerol and [1-^{14}C] glycerol claimed that only 40-50% of glycerol incorporated into lipid by rat liver slices proceeded via the glycerol 3-phosphate route with the remaining 50-60% via acyl dihydroxy acetone phosphate pathway. However, very recently Rognstad, Clark and Katz (261) carrying out a similar study with isolated rat liver cells concluded that the pathway involving reduction of acylated dihydroxy acetone phosphate conversion plays a minor role in the biosynthesis of glycerolipids.

It is clear from the foregoing discussion that fructose is readily converted to liver and blood triglycerides and, over short periods of time, the greater proportion of the sugar is used for the formation of triglyceride glycerol rather than triglyceride fatty acid.

The details of the pathway involved in the biogenesis of triglycerides from fructose can be elucidated from a knowledge of tissue enzymes and their activities (see previous section) and by the use of labelled compounds.

There is little doubt that a major fate of fructose *in vivo* is C-1 phosphorylation by ketohexokinase (but see Zehner *et al* (7) p. 53). Both feeding experiments with rats (11) and perfusion experiments with rat livers (116, 150) have shown that fructose 1-phosphate rapidly accumulates in liver tissues following loading with fructose. In turn, this results in loss of ATP and inorganic phosphate.

Further metabolism of fructose 1-phosphate undoubtedly occurs via the action of fructose 1-phosphate aldolase which cleaves the hexose chain between C-3 and C-4 giving

dihydroxy acetone phosphate and D-glyceraldehyde. Little has been published on this reaction as it occurs in vivo but kidneys perfused with fructose release glyceraldehyde into the circulating medium (170).

When fructose is being actively phosphorylated in liver the depletion of ATP and the resulting increase in the deamination of adenine nucleotides to IMP, uric acid, etc. causes inhibition of the aldolase(86).

Three enzyme systems occur in mammalian tissues for the further metabolism of D-glyceraldehyde.

The occurrence of NAD- and NADP-dependent glycerol dehydrogenases (see p. 43) would allow the conversion of the aldehyde to glycerol. The evidence to support the reduction occurring in vivo in the liver is not strong, however. Rat liver perfused with $/^{14}C/$ fructose has been reported to incorporate 10-20% of the total activity into glycerol and there is rapid equilibration between the glycerol and fructose 1-phosphate pools (150). Some support for direct reduction of glyceraldehyde appears in publications by Gericke et al (258), Kupke et al (51, 58), Landau and Merlevede (60) and Antony et al (54). Landau and Merlevede (60) suggested that rat liver possessed two different compartments: one for the utilization of glyceraldehyde derived from fructose and one for glyceraldehyde derived from exogenous sources. Fructose perfusion of kidney is also said to produce glycerol (170). The main reason for believing that the dehydrogenases function in vivo is based on the rapid appearance of glycerol 3-phosphate in tissues following the administration of fructose. Glycerol 3-phosphate in this case is assumed to arise from

glycerol by a glycerol kinase-catalysed phosphorylation. Rat liver perfusion experiments (64) with fructose (20 mM) increased the molar ratio of glycerol-3-phosphate/dihydroxy acetone phosphate from a normal value of 7:1 to 22:1 hence there appears to be little equilibration between these two trioses and most of the glycerol-3-phosphate supposedly must have been produced via glyceraldehyde and glycerol and not from reduction of dihydroxy acetone phosphate.

Burch et al (11) demonstrated the formation of glycerol 3-phosphate after intraperitonal injection of fructose into rats. Intraportal injection of $[^{14}C]$ fructose into rats (9) also resulted in glycerol-3-phosphate formation and in this case the phosphate was shown to have a higher specific activity than glycerol, hexose phosphates, phosphoglycerate or lactate. Administration of glyceraldehyde together with labelled fructose reduced incorporation into glycerol-3-phosphate, this suggests that the aldehyde is on the pathway from fructose to glycerol-3-phosphate. This could not be substantiated by Wusteman and Macdonald (278) who treated male rats intragastrically with $[^{14}C]$ fructose with or without 'cold' glyceraldehyde. The glyceraldehyde in this case had no effect on incorporation of label into the liver triglycerides. The chemical stability of the glyceraldehyde in the alimentary tract is open to question in this particular experiment, however.

Zakim and Herman (8) showed that intravenous administration of fructose to rat, increased the hepatic glycerol-3-phosphate concentration faster than glucose. The peak of fructose response was also shown to occur 5 min

after injection whilst the response to glucose developed more slowly. This rapid rate of conversion of fructose in comparison with glucose, to glycerol-3-phosphate was confirmed by Pereira and Jangaard (6).

Exogenous glycerol is metabolised rapidly by the liver: labelled triglycerides can be derived from glycerol (37, 57) and glycerol feeding is reported to produce hypertriglyceridemia in rat (53) and in man (55, 56).

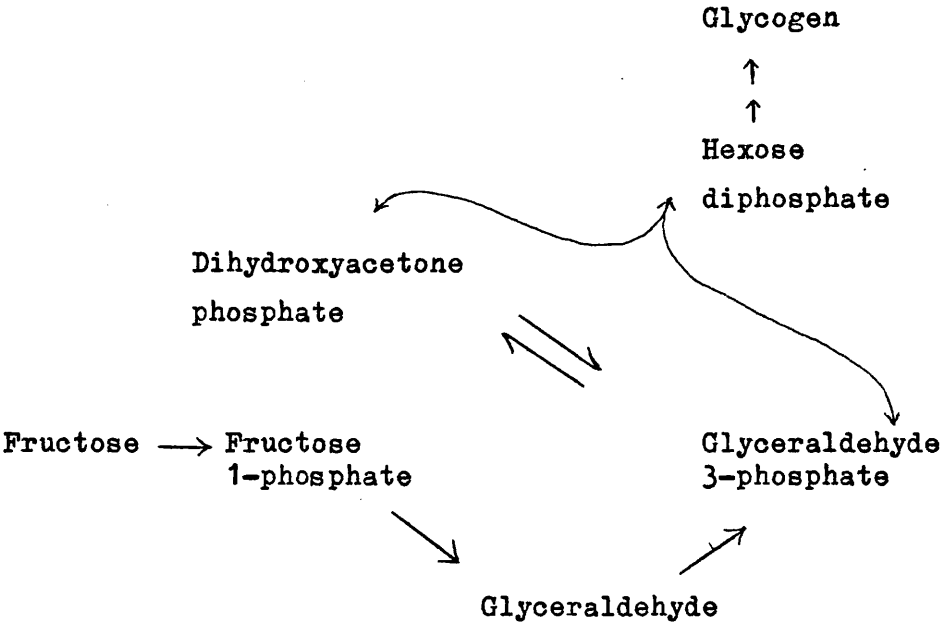
The conversion of glycerol to glycerol-3-phosphate has been demonstrated by several investigators in vivo and with perfused livers. Woods and Krebs (183) showed that rat livers perfused with 10 mM glycerol accumulated glycerol-3-phosphate and Burch and his collaborators (11) showed that intraperitoneal injection of glycerol into rats raised the glycerol-3-phosphate level: the glycerol-3-phosphate/dihydroxy acetone phosphate molar ratio in liver tissues was increased from 18:1 to 670:1 and in kidney from 18:1 to 1070:1 by the injection.

Hagen (52) studied glycerol metabolism in rabbits and reported that there was a good agreement between the V_{max} for the glycerol kinase reaction (0.045 mol/g liver/min) and the rate of utilization of glycerol by liver slices (0.065 mol/g liver/min). It was concluded that the major route of metabolism of glycerol in liver was via the glycerol kinase reaction.

It has been claimed that fatty acid esterification to triglyceride is regulated by the availability of glycerol 3-phosphate (178, 179, 180). When the concentration of glycerol 3-phosphate was low in livers from fasted rats, perfusion with [^{14}C] oleate gave only half the incorporation

of label into triglycerides that occurred with livers from fed animals (150, 172, 181, 182).

Many believe that glycerol dehydrogenases only play a minor role in the metabolism of glyceraldehyde derived from fructose. For example Hue & Hers (171) and Sillero et al (19) in tracer studies on glycogen synthesis say that phosphorylation of glyceraldehyde to glyceraldehyde 3-phosphate involving triokinase is more important.



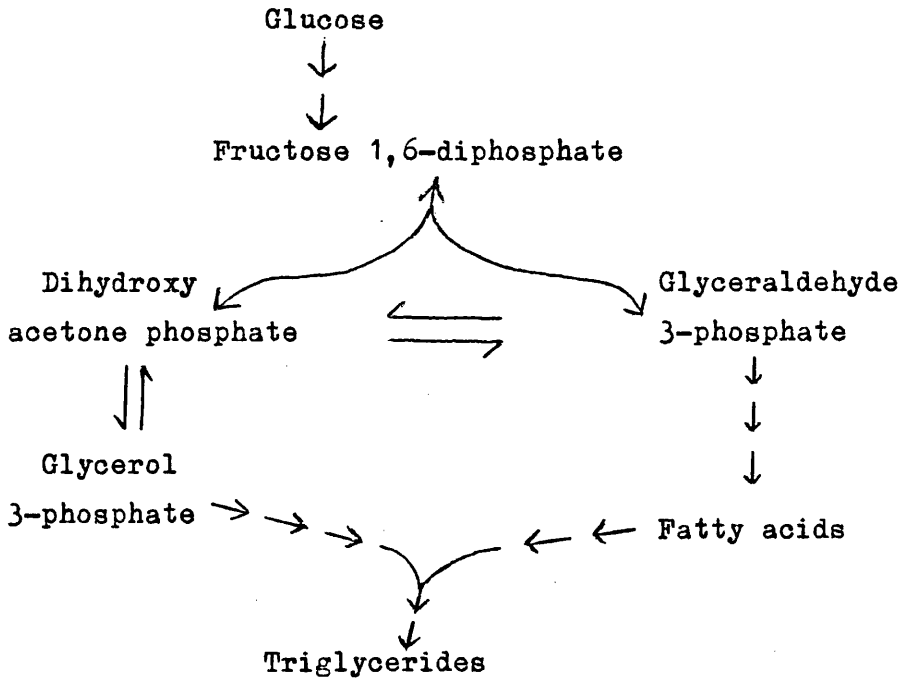
However, in the short period the conversion of fructose to glycogen appears to be minor in comparison to conversion to triglycerides and, hence, to extrapolate from work on glycogen synthesis to triglyceride synthesis is questionable. It should also be noted that glucose is converted more rapidly to glycogen than fructose (171).

Rauschenbach and Lamprecht (272) in 1964 claimed that triokinase had no functional significance in liver and that carbon was transferred from glyceraldehyde to glycogen via D-glycerate and D-glycerate-2-phosphate. This has been refuted by Hue and Hers, however, on the basis of [^{14}C] tracer studied (171).

All the major pathways for glucose and fructose carbon that have been discussed in this section are summarized in Fig. I .

In summary it seems possible that one route for the conversion of fructose to triglycerides in liver involves glycerol and that this trihydric alcohol is not significantly concerned with triglyceride formation from glucose.

Both the glycerol and fatty acid moieties of triglycerides produced from glucose are derived from the two triose phosphates resulting from aldolase action on fructose-1,6-diphosphate :



The glycerol moieties of triglycerides derived from fructose presumably arise from an initial aldolytic cleavage of fructose 1-phosphate to glyceraldehyde and dihydroxy acetone phosphate. These trioses could then be converted to triglyceride-glycerol by any of the pathways shown in Fig. 1.

Glyceraldehyde could be incorporated via glycerol and

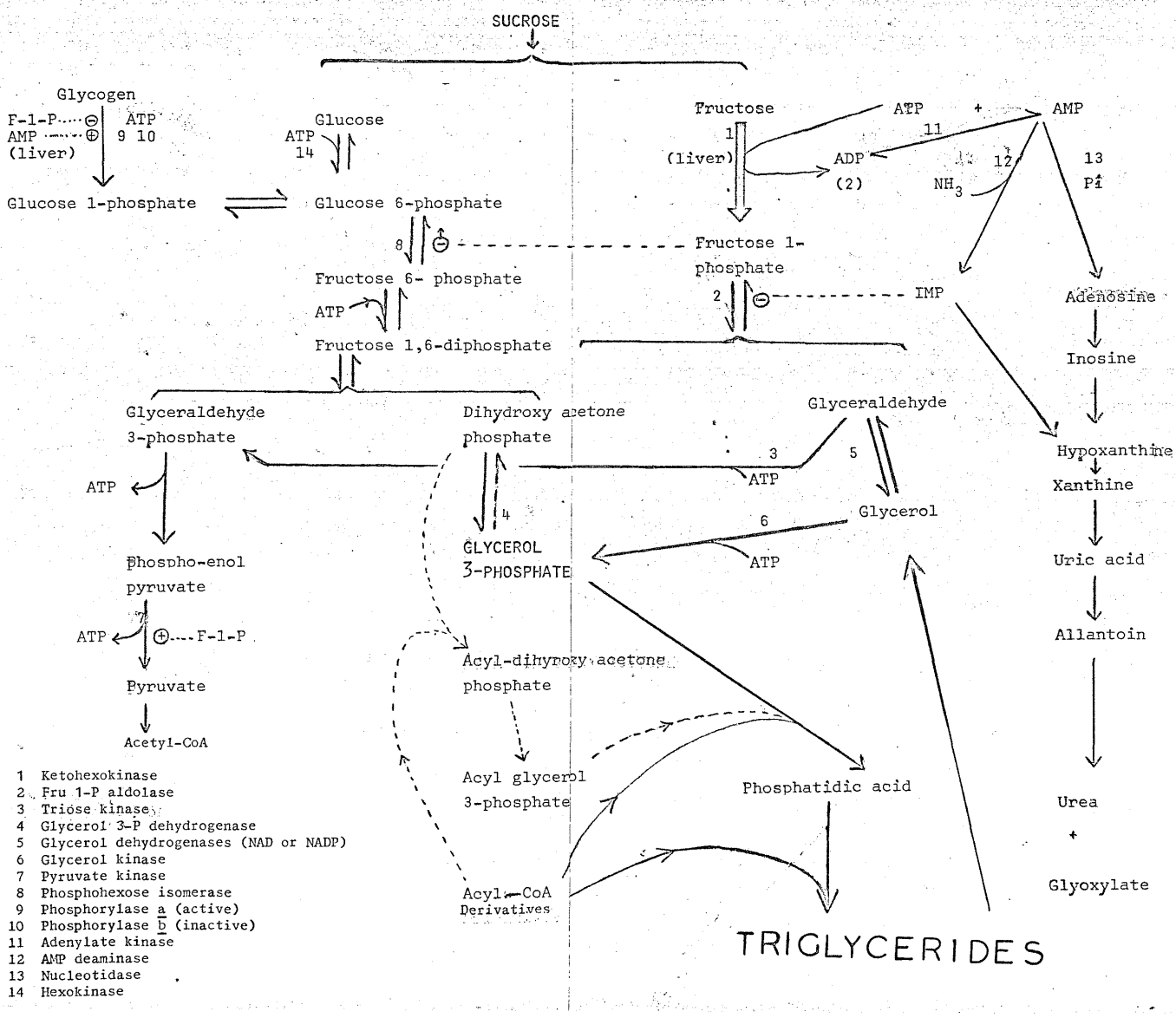
glycerol 3-phosphate or phosphorylated to glyceraldehyde 3-phosphate and then proceed via dihydroxy acetone phosphate. The alternative pathway from glyceraldehyde via glyceric acid and glyceric acid 2-phosphate is not generally favoured (4, 86, 117, 171).

The importance of the acyldihydroxy acetone phosphate pathway is also in some dispute (59, 71, 82, 261).

The more rapid transfer of label from [^{14}C] fructose to triglyceride glycerol than from [^{14}C] glucose may be a result of the quite different pathways which exist for conversion of the two hexoses to triose or the answer may be quantitative rather than qualitative; for example, the initial phosphorylation rates for glucose and fructose could be the controlling factor.

The transfer of label from [^{14}C] fructose or [^{14}C] glucose to triglyceride fatty acid moieties must in both cases occur via glyceraldehyde 3-phosphate and further glycolysis reactions.

FIG. 1. SUCROSE METABOLISM



1 Ketohexokinase
2 Fru 1-P aldolase
3 Triose kinase
4 Glycerol 3-P dehydrogenase
5 Glycerol dehydrogenases (NAD or NADP)
6 Glycerol kinase
7 Pyruvate kinase
8 Phosphohexose isomerase
9 Phosphorylase a (active)
10 Phosphorylase b (inactive)
11 Adenylate kinase
12 AMP deaminase
13 Nucleotidase
14 Hexokinase

AIM OF INVESTIGATION

The foregoing discussion has shown that there are statistical links between the incidence of human ischaemic heart disease and (1) the level of intake of dietary sucrose; (2) the fasting blood serum triglyceride levels. The former has been disputed (283) but the facts warrant further, careful investigation at epidemiological, medical and biochemical levels, of dietary sucrose as a possible risk factor in ischaemic heart disease.

Of further interest is the high risk of ischaemic heart disease associated with men and post-menopausal women in comparison with young women and the related nutritional studies, particularly with human volunteers (47 and 49) and baboons (68), which suggest that high levels of blood triglycerides in males and older females may relate to the intake of dietary sucrose (or fructose) but not glucose. Hence, it could be postulated that sucrose or fructose is a causal factor in ischaemic heart disease and that its effect is mediated by triglycerides. Further, it could be suggested that the incidence of heart disease in men and women is different because biochemical events involving both sucrose and triglycerides are different in the two sexes. The relationship between dietary sucrose and high serum triglyceride levels could, of course, be due to a rapid conversion of the sugar to fat and/or low rates of 'clearing' of fat from the blood.

For the purposes of this thesis it was decided to examine aspects of the synthesis of triglycerides from fructose and, in particular, to concentrate on sex differences

in the conversion of D-glyceraldehyde (the triose derived from the catabolism of fructose but not glucose) to L-glycerol 3-phosphate, a major precursor of triglycerides.

The rat was chosen for the study in preference to primates (for obvious reasons) although at the time there was little information in the literature on sex differences in sucrose and triglyceride metabolism in this rodent. It was known, however, that mature male rats on chow, starch or sucrose diets had higher triglyceride levels than females (31 and 34) although the output of fats by perfused female rat livers was thought to be higher than that of male livers (136). It was also known that dietary sucrose, compared with starch, produced a greater percentage increase in fasting blood fat levels in females than in males although the absolute level was higher in males. Fatty acid synthetase activity in response to a sucrose diet showed a greater percentage increase in males than females although, again, the absolute levels were apparently reversed (31). Finally, initial studies by A.H.W. Hay (173, 216) in this Department had indicated that male rat livers had a higher glycerol 3-phosphate content and greater ketohexokinase and fructose 1-phosphate aldolase activities than female livers.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

In order to account for the apparent high contribution of fructose relative to glucose to triglyceride-glycerol synthesis an attempt was made to examine some of the likely enzymes to be involved in the conversion of glyceraldehyde to glycerol 3-phosphate and to see whether the levels were controlled by sex hormones.

Glycerol Dehydrogenases

Two enzymes, both dehydrogenases, may be involved in an initial reaction whereby glyceraldehyde is converted to glycerol (see p.). The two dehydrogenases differ mainly in their specificities towards pyridine nucleotide cofactors: one is specific for NAD and the other NADP. The activity of the former in rat liver is about four times greater than that of the latter and the Km values for glyceraldehyde are 1.1×10^{-2} M and 6.2×10^{-4} M, respectively.

The exact physiological significance of the two dehydrogenases is not known (86) but it is certainly possible that one or both are involved in the reduction of glyceraldehyde derived from fructose to glycerol *in vivo* (58). The availability of reduced nucleotides and the glyceraldehyde concentration would be important in determining which, if either, enzyme played the major role.

NAD-Dependent Enzyme

The first report of the occurrence of NAD-dependent glycerol dehydrogenase in rat liver appeared in 1954 (20):

The Km for glyceraldehyde is recorded as 1.1×10^{-2}M at 18°C and the reaction catalysed by the dehydrogenase strongly favours glycerol formation (24). At pH 9.0, using an aldehyde trapping agent, the reaction favours the formation of glyceraldehyde.

In this present study NAD-dependent glycerol dehydrogenase was determined by measuring the rate of reduction of D-glyceraldehyde at 340 nm.

Initial experiments were designed to compare the activity of the rat liver NAD-dependent dehydrogenase in crude soluble fractions from the livers of normal fed male and female rats. The results are summarised in Table II and it is clear from these experiments that there is no significant difference between the activity of this enzyme in male and female rat liver. Furthermore, it was shown Tables III and IV that treating male animals with exogenous male (testosterone) or female (estradiol) hormones produced no significant difference in dehydrogenase. Injection of female rats with testosterone (Tables V and VI) and estrogen also did not show any effect on the activity of the dehydrogenase Table VI.

The results of this investigation therefore suggest that the different rates of fructose metabolism observed in male and female animals cannot be attributed to differences in the levels of NAD-dependent glycerol dehydrogenase, at least in rats. Both sexes appear to possess relatively high levels of the enzyme but reduction of glyceraldehyde would be dependent on the availability of NADH which in turn would be affected by processes such as the Embden-Myerhof pathway and the rate of oxidation of the coenzyme by mitochondria.

The formation and oxidation of NADH might, of course, be sex controlled. In this connection it is interesting to note that injection of estradiol-17β into ovariectomized rats raised the levels of lactate and glycerol 3-phosphate in the uterus tissues (143) which would presumably tend to lower the NADH concentration.

NADP-Dependent Enzyme

This enzyme was first reported to be present in rat liver by Moore (21) in 1959.

In this present study NADP-dependent glycerol dehydrogenase was examined by measuring the rate of oxidation of NADPH at 340 nm.

Table VII shows the results obtained when comparing the activities of NADP-dependent glycerol dehydrogenase in male and female livers from rats on a normal chow diet. It is apparent from these data that the activity of this enzyme, like that of the NAD-linked dehydrogenase, is very similar in both male and female liver tissues. Administration of testosterone in low (0.5mg/kg bdy wt.) or high (5.0mg/kg. bdy wt.) doses to male rats did not affect the activity of the liver enzyme Table III. Male rats were also treated with estradiol-17 in a similar manner, and the results of these experiments are shown in Table IX. Again no significant difference in control and hormone-treated animals was observed.

Treatment of female rats with male (testosterone) or female (estrogen) hormones also produced no significant changes in the liver NADP-dependent dehydrogenase Tables X and Xa. Again, as in the case of the NAD-dependent dehydrogenase, any observed sex differences in the metabolism

Table II. Activity of NAD-dependent glycerol dehydrogenase in male (M) and female (F) rat liver.

Sex of animals	No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Specific activity (μmol/min/mg of protein) ± SD
F	8	210 ± 8.9	8.41 ± 0.36	1.15 ± 0.06
M	9	239 ± 6.5	9.34 ± 0.47	1.07 ± 0.03

D-glyceraldehyde reduction was followed by measuring the rate of oxidation of NADH spectrophotometrically (340 mn; 1 cm light-path, quartz cell) at 21°C over a period of 10 min.

The incubation mixture consisted of: D-glyceraldehyde (30 μmol); NADH (0.125 μmol); crude liver extract (100,000g supernatant; 20 μl); phosphate buffer, pH 7.0 (155 μmol); total volume 3.3 ml.

Table III. Effect of testosterone (T) and estradiol-17β(E) on the activity of NAD-dependent glycerol dehydrogenase in male rat liver.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt.)	Time interval (h) **	No. of injections	Specific activity ** (μmol/min/mg protein) ± SD
*3 Controls	232 ± 3.6	8.9 ± 0.53	Saline glycol	24	1	1.03 ± 0.02
3	225 ±5.6	8.6 ± 0.26	T 5.0	24	1	1.07 ± 0.02
3	241 ± 9.6	9.2 ± 0.46	E 5.0	24	1	1.04 ± 0.04
*3 Controls	248 ± 8.5	9.8 ± 0.53	Saline glycol	16	1	1.00 ± 0.02
3	235 ± 9.5	9.3 ± 0.7	T 5.0	16	1	1.03 ± 0.03
3	236 ± 11.5	9.4 ± 0.52	E 5.0	16	1	1.02 ± 0.03

* Control groups were treated with 0.5% propylene glycol in saline, (hormone vehicle).

** Between last injection and killing animal.

** The incubation mixture and assay of glycerol dehydrogenase are described in Table II.

Table IV. Effect of testosterone (T) and estradiol-17β(E) on the activity of NAD-dependent glycerol dehydrogenase in male rat liver tissue.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt.)	Time interval (h)**	Total No. of injections	Specific Activity* (μmol/min/mg protein) ± SD
*3 Controls	234 ± 11.8	9.7 ± 0.6	Saline glycol	24	5	1.11 ± 0.03
3	225 ± 11.3	9.5 ± 0.5	T 0.5	24	5	1.13 ± 0.03
*3 Controls	241 ± 11.1	9.8 ± 0.65	Saline glycol	24	6	1.08 ± 0.04
3	232 ± 10.4	9.5 ± 0.8	E 0.5	24	6	1.10 ± 0.04

* Control groups were treated with 0.5% propylene glycol in saline, (hormone vehicle).

** Between last injection and killing animal.

* The incubation mixture and assay of glycerol dehydrogenase are described in Table II.

Table V. Effect of testosterone on the activity of NAD-dependent glycerol dehydrogenase in female rat liver.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt.)	**Time interval (h)	No. of daily injections	*Specific activity (μmol/min/mg protein) ± SD
6 Controls+	216 ± 6.3	8.21 ± 0.41	Saline glycol	24	1	1.11 ± 0.05
5	211 ± 7.2	8.13 ± 0.45	5.0	24	1	1.13 ± 0.04
6 Controls+	207 ± 6.7	8.05 ± 0.44	Saline glycol	12	** 1½	1.07 ± 0.03
5	209 ± 7.1	8.13 ± 0.52	0.1	12	** 1½	1.17 ± 0.02

+Control groups were treated with 0.5% propylene glycol in saline, (hormone vehicle).

**Injections were made twice daily (every 12h).

**Between last injection and killing animal.

*The incubation mixture and assay of glycerol dehydrogenase are described in Table II.

Table VI. Effect of testosterone (T) and estradiol-17β (E) on the activity of NAD-dependent glycerol dehydrogenase in female rat liver tissue.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt.)	*Time interval (h)	Total no. of injections	Specific activity ** (μmol/min/mg protein) ± SD
*3 Controls	206 ± 8.7	8.4 ± 0.46	Saline glycol	24	10	1.06 ± 0.03
3	219 ± 34	8.8 ± 0.26	T 0.5	24	10	1.10 ± 0.02
*3 Controls	210 ± 37	8.1 ± 0.36	Saline glycol	24	10	1.11 ± 0.02
3	215 ± 40	8.5 ± 0.52	E 0.5	24	10	1.07 ± 0.02
*6 Controls	207 ± 9.2	8.4 ± 0.46	Saline glycol	24	8	1.08 ± 0.02
4	201 ± 6.0	8.20 ± 0.62	T 0.5	24	8	1.13 ± 0.04
*4 Controls	214 ± 9.5	8.30 ± 0.48	Saline glycol	24	5	1.12 ± 0.035
4	219 ± 7.4	8.1 ± 0.39	T 0.5	24	5	1.04 ± 0.02

* Between last injection and killing animal. ** The incubation mixture and assay of glycerol dehydrogenase are described in Table II.

* Control groups were treated with 0.5% propylene glycol in saline. (hormone vehicle).

of fructose cannot be attributed to differences in the levels of NADP-dependent glycerol dehyderogenase in male and female rat liver tissues. The availability of the reduced coenzyme needed to convert glyceraldehyde to glycerol should once more be considered and the possibility that NADPH levels in males and females might be different. An important source of NADPH is the pentose phosphate pathway and Tepperman and Tepperman (164) have shown with male rats that the dehydrogenases in this pathway are raised by injecting estrogen and this could presumably lead to higher levels of NADPH in the liver and hence favour dehydrogenase activity.

Table VII. Activity of NADP-dependent glycerol dehydrogenase in male (M) and female (F) rat liver tissue.

Sex of animals	No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Specific activity * (μmol/min/mg protein) ± SD
M	6	229 ± 16	8.6 ± 0.7	5.17 ± 0.30
F	6	207 ± 9	7.8 ± 0.8	5.15 ± 0.25

* D-glyceraldehyde reduction was followed by measuring the rate of oxidation of NADPH spectrophotometrically (340 mm, 1 cm light-path, quartz cell) at 21°C over a period of 10 min.

The incubation mixture consisted of: D-glyceraldehyde (2μmol); NADPH (0.125 μmol); crude liver extract (100,000 g supernatant; 100 μl); phosphate buffer, pH 7.0 (145 μmol); total volume 3.3 ml.

TableVIII. Effect of testosterone on the activity of NADP-dependent glycerol dehydrogenase in male rat liver tissue.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt.)	Time interval** (h)	Total No. of injections	Specific Activity (μmol/min/mg protein) ± SD
3 * Controls	248 ± 10.6	9.8 ± 1.0	Saline glycol	16	1	5.6 ± 0.36
3	235 ± 12.3	9.3 ± 0.53	5.0	16	1	5.2 ± 0.27
6 * Controls	237 ± 8.9	9.3 ± 0.60	Saline glycol	24	1	5.4 ± 0.3
6	242 ± 8.2	9.7 ± 0.55	5.0	24	1	5.2 ± 0.34
2 * Controls	229 ± 6.5	8.9 ± 0.55	Saline glycol	24	10	5.05 ± 0.1
3 Controls	238 ± 13.4	9.2 ± 0.33	0.5	24	10	5.15 ± 0.23

* Control groups were treated with 0.5% propylene glycol in saline.

** Between last injection and killing animal.

Table IX. Effect of estradiol-17β on the activity of NADP-dependent glycerol dehydrogenase in male rat liver.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Amount of Hormone injected (mg/kg body wt.)	Time interval ** (h)	Total No. of injections	Specific Activity ‡ (μmol/min/mg protein) ± SD
6 Controls*	234 ± 12.8	9.7 ± 0.58	Saline glycol	24	1	5.03 ± 0.12
5	225 ± 12.6	9.5 ± 0.49	5.0	24	1	5.10 ± 0.10
2 Controls*	220 ± 11.3	8.8 ± 0.70	Saline glycol	24	10	5.10 ± 0.05
3	229 ± 8.7	8.9 ± 0.65	0.5	24	10	4.98 ± 0.06
2 Control*	222 ± 7.1	9.2 ± 0.56	Saline glycol	12	2	4.97 ± 0.1
3	214 ± 5.3	8.7 ± 0.53	E (0.5)	12	2	5.05 ± 0.08
3 Control*	237 ± 10.8	9.6 ± 0.52	Saline glycol	16	1	5.10 ± 0.13
3	236 ± 11.5	9.3 ± 0.23	E (5.0)	16	1	5.03 ± 0.07

* Control group were treated with 0.5% propylene glycol saline. ** Between last injection and killing animal.

‡ The incubation mixture and assay of glycerol dehydrogenase are described in Table VII.

Table X. Effect of estradiol-17β on the activity of NADP-dependent glycerol dehydrogenase in female rat liver.

No. of Animals	Body Wt. (g)	Liver Wt. (g)	Hormone injected (mg/kg body wt.)	Time interval (h) ‡	Total No. of injections	Specific activity ** (μmol/min/mg protein)
3 * Control	201 ± 11.5	7.8 ± 0.5	Saline glycol	24	3	5.20 ± 0.40
3	207 ± 13	7.6 ± 0.7	0.5	24	3	5.30 ± 0.43
3 * Control	209 ± 10	7.9 ± 1.0	Saline glycol	24	5	5.10 ± 0.35
3	203 ± 9	7.6 ± 0.8	0.5	24	5	5.05 ± 0.25
3 * Control	199 ± 12	7.3 ± 0.6	Saline glycol	24	9	5.15 ± 0.40
3	207 ± 8	7.5 ± 0.6	0.5	24	9	5.20 ± 0.30
3 * Control	221 ± 12	8.1 ± 0.5	Saline glycol	16	1	5.11 ± 0.40
3	225 ± 13	8.0 ± 0.7	5.0	16	1	5.17 ± 0.38

* Control groups were treated with 0.5% propylene glycol in saline.

‡ Between last injection and killing animal.

** The incubation mixtore and assay of NADP-dependent glycerol is described in table VII.

Table Xa. Effect of testosterone on the activity of NADP-dependent glycerol dehydrogenase in female rat liver.

No. of Animals	Body Wt. (g)	Liver Wt. (g)	Hormone injected (mg/kg body wt.)	Time interval (h) **	Total No.of injections	Specific activity ** (mol/min/mg protein)
3 * Control	208 ± 10	7.3 ± 0.8	Saline glycol	24	10	5.14 ± 0.35
3	201 ± 12	7.2 ± 0.6	0.5	24	10	5.20 ± 0.40
3* Control	203 ± 14	7.5 ± 0.7	Saline glycol	24	4	5.19 ± 0.20
4	198 ± 7	7.0 ± 0.4	5.0	24	4	5.15 ± 0.30

* Control groups were treated with 0.5% propylene glycol in saline (Hormone vehicle).

** Between last injection and killing animal.

** The incubation mixtore and assay of NADP-dependent glycerol is described in table VII.

Glycerol kinase

Glycerol would be the product of glycerol dehydrogenase activity in the liver and the triol would also arise from the action of lipases. Further metabolism of glycerol almost certainly involves glycerol kinase which transfers, stereospecifically, the terminal phosphoryl moiety of ATP to a primary hydroxyl group of glycerol, forming L-glycerol 3-phosphate. Thus glycerol can be utilized for triglyceride production, glyconeogenesis and for energy purposes via the glycolytic pathway. It is possible that the conversion of glycerol back to glyceraldehyde is an insignificant reaction in the liver because the Km for glycerol in the case of NADP-dependent glycerol dehydrogenase is 0.63M at 38°, pH 9.5 (21). There is no reported Km value in the literature for the NAD-dependent enzyme but Wolf and Leutherdt (23) state that the equilibrium in this reaction strongly favours glycerol formation.

The unavailability of a sensitive method for measuring the low activity of glycerol kinase in some tissues (e.g. muscle and adipose) seems to be one of the reasons why biochemists, until quite recently, have almost ignored the significance of this enzyme. Since the development of the sensitive radiochemical enzymic assay for glycerol kinase in 1967 by Newsholme and his collaborators (65) many attempts have subsequently been made to describe the role of glycerol kinase in carbohydrate and triglyceride metabolism.

As glycerol kinase appears to be the major enzyme involved in glycerol metabolism (96) it is not surprising to find that there are various mechanisms for the control of

its activity in the liver cell. The E. coli enzyme is allosteric and is inhibited by fructose 1,6-diphosphate (15, 121) but this is apparently not the case with the liver enzyme. Glycerol kinase activity in rat liver changes according to the nutritional state of the animal. Starvation and short term feeding with fats, for example, decrease the activity. The enzyme activity does not increase on feeding starved animals glycerol in other words substrate does not appear to induce activity (64, 96, 113, 135).

Contrary to this idea is the observation that the changing levels of glycerol in the blood of developing animals can be roughly correlated with the activity of liver glycerol kinase (137). The concentration of blood glycerol increases immediately after birth in male rats and then decreases in adulthood to a level which is similar to that at post-partum (137). Paralleling this is an increase in liver glycerol kinase activity starting in the fifth week after birth. The relationship between blood glycerol and liver glycerol kinase activity has been related by Kampf and co-workers (135).

Feeding fats, over a long term period, to rats, i.e. producing glyconeogenetic condition increases the level of glycerol kinase in the liver (135). This is thought to be due to the fatty acid moieties rather than glycerol (96). Cortisol also appears to induce glycerol kinase synthesis probably via hyperglycemia and insulin production (135).

In addition to the liver, adipose tissue also possesses glycerol kinase activity and the enzyme in the two tissues may relate to obesity: glycerol kinase is more active in adipocytes from obese mice than from the cells of

lean litter mates (139, 142, 240). In obese mice the level of glycerol kinase in adipose tissue increases with age but this does not occur with lean animals of the species (139). Koschinsky and co-workers (16) claim that the level of glycerol kinase in adipose tissue from ob^+/ob^+ genetically obese mice is directly related to the serum insulin concentration (via enzyme induction) and to the degree of insulin resistance. In other genetically obese mice and rats the adipose glycerol kinase level is not raised although hyperinsulinemia exists (240). In humans it has been reported that glycerol kinase activity in subcutaneous adipose tissue from obese individuals is higher than that in adipose tissue from normal persons (186).

Before starting to examine the levels of glycerol kinase in male and female rat livers the distribution of the enzyme in the cells was investigated. Liver cells of both sexes, were separated by differential centrifugation into nuclear, mitochondrial, microsomal and soluble fractions. In male and female tissues the soluble supernatant fraction was observed to contain 98% of the total activity (Table XII, cf 185).

A careful analysis of glycerol kinase activities of the 100,000g soluble fractions from 16 male and 13 female adult rat livers (Table XI) showed that in males the activity was 37% higher than in cells from the female animals. These results support the experiments of Vernon and Walker (137) who were examining the relationship between age and glycerol kinase activity and who noted with a limited number of 12 week-old rats, that there was a difference in the enzyme activity in males and females. Vernon and Walker (137) found no differences between the sexes in the case of immature rats

Table XI. Activity of glycerol kinase in male (M) and female (F) rat liver tissues.

Sex of animals	No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Specific activity* nmol glycerol phosphorylated/min/mg protein ± SD
M	16	230 ± 8.5	9.3 ± 0.30	39.9 ± 0.8
F	13	209 ± 7.2	8.1 ± 0.30	29.1 ± 0.7

* The activity of the enzyme was measured using [1-^{14}C] glycerol and the labelled glycerol 3-phosphate formed was separated from excess substrate by paper chromatography. Areas of paper containing unreacted glycerol and newly formed glycerol 3-phosphate were cut out and the radioactivities measured with a liquid scintillation counter. The incubation mixture (100 µl) consisted of tris (8.3 µmol), EDTA (0.16 µmol), NaF (2.2 µmol), mercaptoethanol (3.3 µmol), ATP (0.5 µmol), $MgSO_4$ (0.5 µmol), [1-^{14}C] glycerol (0.5 µmol); specific µCi/µmol activity 0.25 Ci/µmol), and crude liver extract (100,000 g supernatant, 10 µl). The reaction was stopped by addition of an equal volume of ethanol after 10 min.

which would, perhaps, be expected if glycerol kinase levels are controlled by the sex hormone balance.

In order to establish whether this sex difference in the activity of glycerol kinase could be directly related to sex hormones an attempt was made to manipulate the enzyme level by injecting male animals with various levels of testosterone in different vehicles.

From the results obtained (Table XIII) it can be seen that the activity of the liver enzyme from male animals does not change after loading the animals with male hormone.

When testosterone was administered to female rats (Table XIV) again there was no apparent increase in glycerol kinase activity. Following administration of a steroid hormone to rats, it has been shown that the concentration of the hormone reaches a maximum in the liver as well as in other organs after 30 mins and then starts to decline and reaches the endogenous level after about 6h (263). In view of this observation female animals were injected with relatively high doses of testosterone (12 mg/kg body weight/injection) every 12h and the enzyme activity was measured 12h after the third injection. The results of this particular experiment are also given in Table XIV : there was again no apparent increase in the level of liver glycerol kinase activity.

In further experiments to examine the possible role of testosterone on levels of glycerol kinase in male rat livers the effect of castrating the animals and then injecting testosterone was investigated.

These experiments revealed that the enzyme activity decreased significantly after castration (Tables XI, XVI, XVII, XVIII) and that subsequent treatment of castrated animals with various levels of testosterone (0.03 to 3.0 mg/kg body weight/ day) for 10 days appeared to increase the activity of glycerol

Table XII. Sub-cellular fractionation of rat livers.

Fraction (xg)	Specific activity (nmol/mg protein/min	
	Male	Female
650 (nuclei) (10 min)	0.32	0.34
20,000 (mitochondria) (10 min)	0.11	0.18
100,000 (microsomes) (60 min)	0.25	0.07
Supernatant solution	32.92	28.60

*The incubation mixture and assay of glycerol kinase are described in Table XI.

Table XIII. Effect of various levels of testosterone on the activity of glycerol kinase in male rat liver tissue.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt./ injection.	No. of daily injections	Time interval (h)*	Specific Activity** (nmol/min/mg of protein ± SD
Controls 2	347 ± 20	12.5 ± 1.3	Corn oil ⁑	10	24	40.4 ± 2.0
3	312 ± 29	11.8 ± 0.7	0.030	10	24	39.7 ± 1.6
3	291 ± 12	10.5 ± 0.7	0.30	10	24	42.5 ± 0.9
3	325 ± 4	11.2 ± 0.4	3.0	10	24	41.8 ± 1.1
Controls 4	333 ± 13.5	11.7 ± 0.35	Saline glycol***	10	24	41.1 ± 1.6
3	325 ± 14.3	11.4 ± 0.4	4.50	10	24	40.8 ± 1.5

* Time interval between last injection and killing animal.

** The incubation mixture and assay of glycerol kinase are described in Table XI.

*** Animals were injected with propylene glycol in saline (0.5%) (hormone vehicle).

⁑ Animals were injected with 0.1 ml corn oil (hormone vehicle).

Table XIV. Effect of testosterone on the activity of glycerol kinase in female rat liver tissue.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt./injection)	No. of daily injections	Time interval (h)***	Specific activity** (nmol/min/mg protein) ± SD
Controls 4	203 ± 9.0	7.9 ± 0.40	Saline glycol*	2	24	30.3 ± 1.20
4	208 ± 10.5	8.0 ± 0.55	0.5	2	24	29.5 ± 0.90
Controls 3	198 ± 7.0	7.7 ± 0.45	Saline glycol*	5	24	31.1 ± 0.90
3	203 ± 5.5	7.9 ± 0.40	0.5	5	24	30.2 ± 1.15
Controls 2	210 ± 5.5	8.1 ± 0.35	Saline glycol*	10	24	29.0 ± 1.2
4	215 ± 6.0	8.0 ± 0.40	0.5	10	24	31.1 ± 1.5
Controls 3	205 ± 8.5	7.8 ± 0.30	Saline glycol*	1½****	12	30.7 ± 1.0
4	213 ± 7.0	7.9 ± 0.45	12.0	1½****	12	31.1 ± 1.5
Controls 3	217 ± 8	8.3 ± 0.5	Corn oil ‡	2	24	29.8 ± 0.5
3	210 ± 9	8.0 ± 0.6	5.0	2	24	30.5 ± 0.8

* Animals were injected with 0.5% propylene glycol in saline (hormone vehicle).

** The incubation mixture and assay of glycerol kinase are described in Table XI.

*** Time intervals between last injection and killing animal.

**** Injections were made twice daily every 12h.

‡ Animals were injected with corn oil (hormone vehicle).

Table XV. Effect of castration of 9½ week-old male rats and testosterone replacement on the activity of glycerol kinase in liver tissue.

Source of the liver and no. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt./ injection)*	No. of daily injections	Time interval (h)***	Specific activity**** (nmol/min/mg of protein) ± SD
Controls intact 3	410 ± 21	19 ± 2.1	Corn oil **	10	24	44.8 ± 1.3
Castrated controls 3	358 ± 15	18 ± 1.2	Corn oil **	10	24	35.7 ± 2.5
Castrated and hormone treated 3	396 ± 18	19 ± 2.0	0.30	10	24	40.8 ± 1.0
Castrated‡ 3	405 ± 17	19 ± 1.5	Corn oil **	10	24	37.0 ± 1.2

* Hormone treatment started when the animals were 17-18 weeks old.

** Control animals received corn oil (hormone vehicle).

*** Time interval between last injection and killing the animal.

**** The incubation mixture and assay of glycerol kinase are described in Table XI.

‡ Animals were on fructose-free diet for 15 days before and during the experiment.

Table XVI. Effect of castration of 9½ week-old male rats and treatment with testosterone (T), progesterone (P) and estradiol-17β (E) on food consumption, total body weight and the activity of glycerol kinase in liver tissue.

Source of liver	No. of ‡ animals	Average Wt. gain in 11 days ± SD	Average food consumption/ g/rat/day	Final body wt. (g) ± SD	Liver Wt. (g) SD	Hormone injected (μg/kg body wt./ injection)	No. of daily injections	Time ** interval (h)	Specific Activity (nmol/min/mg protein) ± SD ***
Intact controls	3	35 ± 17	19.6	357 ± 14	14.1 ± 1.1	Corn oil*	10	24	43.8 ± 1.1
Castrated controls	3	63 ± 11	27.2	357 ± 6	18.0 ± 1.2	Corn oil*	10	24	33.3 ± 0.9
Castrated & treated	3	57 ± 8	26.6	334 ± 13	15.2 ± 1.3	$T_{(30)}$	10	24	39.1 ± 1.2
Castrated & treated	3	16 ± 3	21.3	320 ± 6	16.0 ± 1.2	$E_{(30)}$	10	24	31.2 ± 1.1
Castrated & treated	2	41 ± 1	26.3	351 ± 18	16.7 ± 0.9	$P_{(30)}$	10	24	38.2 ± 1.0

* Controls were injected with 0.1 ml of corn oil (hormone vehicle).

** Time interval between last injection and killing animal.

*** The incubation mixture and assay of glycerol kinase are described in Table XI.

‡ Hormone treatment started when the animals were 15-17 weeks old.

Table XVII. Effect of progesterone (P) and various levels of testosterone (T) on the activity of glycerol kinase in castrated male rats ($9\frac{1}{2}$ week-old) liver tissue.

Source of the liver	No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt./ injection) *	No. of daily injections	Time interval (h)**	Specific activity ‡ (nmol/min/mg protein) ± SD
Intact controls	3	358 ± 12.5	16 ± 1.5	Corn oil***	10	24	42.0 ± 1.50
Castrated controls	3	344 ± 40	15 ± 1.0	Corn oil ***	10	24	34.8 ± 1.30
Castrated controls****	3	331 ± 25	15 ± 1.5	Corn oil ***	10	24	36.0 ± 1.05
Castrated & treated	3	364 ± 12.5	16.7 ± 1.5	T (0.030)	10	24	38.4 ± 1.10
Castrated & treated	3	320 ± 23	16.2 ± 1.2	T (0.30)	10	24	42.2 ± 1.3
Castrated & treated	3	363 ± 22	15.2 ± 1.5	T (3.0)	10	24	48.1 ± 1.7
Castrated & treated	3	355 ± 18	16.2 ± 1.3	P (3.0)	10	24	40.7 ± 1.20

* Hormone treatment started when the animals were 15-16 weeks old.

** Time interval between last injection and killing animals.

*** Control animals received corn oil (hormone vehicle).

**** Animals were on a fructose-free diet for 30 days during the experiment and after castration.

‡ The incubation mixture and assay of glycerol kinase are described in Table XI.

Table XVIII. Effect of castration of 3-4 week-old male rats and testosterone replacement on the activity of glycerol kinase in liver tissue.

Source of liver	No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (μg/kg body wt./ injection) ‡	No. of daily injections	Time interval (h)**	Specific Activity *** (nmol/min/mg of protein ± SD
Intact controls	3	271 ± 10	12.1 ± 1.0	Corn oil*	9	24	41.0 ± 1.0
Castrated controls	3	287 ± 14	11.8 ± 1.3	Corn oil*	9	24	37.4 ± 4.0
Castrated & hormone treated	3	276 ± 13	11.7 ± 1.1	0.050	9	24	39.4 ± 2.3
Castrated controls	2	290 ± 20	14 ± 0.5	Corn oil*	1	4½	37.0 ± 0.5
Castrated & hormone treated	2	312 ± 8	12.7 ± 1.3	3.0	1	4½	42.9 ± 1.1

* Controls received corn oil (hormone vehicle).

** Time interval between last injection and killing animal.

*** The incubation mixture and assay of glycerol kinase are described in Table XI.

‡ Hormone treatment was started at the age of 11-12 weeks.

kinase beyond the normal level found in the intact control animals.

Administration of progesterone to castrated animals also raised the enzyme activity in the liver preparation. Perhaps because progesterone can be converted to testosterone. The formation of testosterone from progesterone is known to occur in testicular, adrenal cortical, ovarian and corpus luteum cells (196, 225, 267, 277).

The removal of the testes, the major source of testosterone, lowered glycerol kinase activity in liver preparation from male rats and, therefore, attempts were next made to effect the activity in intact male rats by injecting testosterone antagonists. Table XIX shows that the female hormone, estradiol-17β, lowers glycerol kinase activity as does the anti-androgen, cyproterone acetate (Table XX).

As an extension of the experiment with castrated male rats, hypophysectomized male animals were also examined. There was a marked reduction in the liver enzyme activity when the hypophysis was removed (Table XXI) which was to be expected if the gonadotrophic hormone produced by this organ stimulated the production of testosterone by the testes. However, treatment of hypophysectomized rats with testosterone did not restore the glycerol kinase activity. This could be interpreted as non-involvement of testosterone in the process or perhaps hypophysectomy removes other factors which are important for glycerol kinase activity and in their absence injected testosterone has no effect. It has been claimed, for example, that in man corticotropin, a product of the hypophysis, reduces blood triglycerides.(192,193). Could this effect be mediated by glycerol kinase? It should be noted, that

Table XIX. Effect of estradiol-17β on the activity of glycerol kinase in male rat liver tissue.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected (mg/kg body wt./ injection)	No. of daily injections	Time interval (h)*	Specific Activity** (nmol/min/mg of protein ± SD
Controls 3	243 ± 11.5	9.8 ± 0.30	Saline glycol***	2	24	39.7 ± 1.0
4	242 ± 9.3	9.7 ± 0.45	1.0	2	24	34.9 ± 1.21
Controls 2	229 ± 8.7	8.9 ± 0.40	Saline glycol***	5	24	40.2 ± 2.1
4	237 ± 9.5	9.2 ± 0.35	1.0	5	24	32.1 ± 0.90
Controls 2	243 ± 10.6	9.3 ± 0.45	Saline glycol***	1-	24	39.5 ± 1.80
4	235 ± 10.0	9.6 ± 0.50	1.0	10	24	30.8 ± 0.70

* Time after last injection and killing animal.

** The incubation mixture and assay of glycerol kinase are described in Table XI.

*** 0.5% propylene glycol in saline (hormone vehicle).

Table XX. Effect of cyproterone acetate on the activity of glycerol kinase in male rat liver tissue.

No. of animals	Initial body wt. (g)	Average food consumption (g)/rat/day	Final body wt. (g) ± SD	Liver wt. (g) ± SD	Hormone injected (mg/rat/injection	No. of daily injections	Time interval (h)*	Specific activity*** (nmol/min/mg protein) ± SD
Controls 3	229 ± 7	22.8	260 ± 10	10.2 ± 0.3	Corn oil**	10	24	42.2 ± 2.0
3	225 ± 10	19.9	228 ± 8	9.5 ± 0.5	1.00	10	24	34.1 ± 1.1

* Time intervals between last injection and killing animals.

** Control animals were injected with corn oil.

*** The incubation mixture and assay of glycerol kinase are described in Table XI.

Chandrabose and Bensadoun (187, 189) claimed that hypophysectomy of chickens increased liver glycerol kinase activity and that this was caused by a reduction in thyroxine production. The reason for this difference between chickens and rat is not clear.

The possible effects of female hormone on liver glycerol kinase in female animals was also examined. Subcutaneous injections of various doses of estradiol-17β failed to significantly affect the activity of the liver enzyme (Table XXII).

At this stage in the studies it was decided to investigate possible sex hormone control of activity at a lower level of biological organisation, i.e. using liver slices. In this way it was hoped that direct hormone effects on liver tissue could be examined in the absence of complicating *in vivo* factors such as the production of hormones by the endocrine system. On the other hand, it was realised that such *in vitro* systems produce new complications in the form of ischemia, lack of substrates and loss of essential controlling processes all leading to a possible breakdown of the system observed *in vivo* and to the development of artefacts.

In the first instance, the effect of testosterone on liver slices from male rats was investigated (Table XXIII). Incubation of the tissues with hormone at a concentration of 100 μg/ml, for 2h, for example , produced an apparent 30% increase in glycerol kinase activity. Much of this hormone was not taken up by the cells and appeared in the residue resulting from centrifugation of the homogenate. With increasing time of incubation with the steroid the activity decreased in both the control and testosterone-treated preparations: this

Table XXI. Effect hypophysectomy of male rats and treatment with testosterone (T) and progesterone (P) on the activity of glycerol kinase in liver tissue.

No. of animals	Body wt. (g)±S.D.	Liver wt. (g)±S.D.	Amount of hormone injected mg/Kg body wt./ injection	No. of daily injections	Time interval (h)***	Specific activity** n mol/min/mg protein±S.D.
Control intact (3)	362±18	16.33±1.8	Corn oil* 0.1 ml	10	24	45.11±1.81
Control hypophysectomized (3)	231±14	9.2±0.6	Corn oil* 0.1 ml	10	24	29.04±2.11
Hypophysectomized hormone treated (3)	241±2	9.6±0.5	Testosterone 0.06	10	24	31.01±1.20
Hypophysectomized hormone treated (3)	234±9	9.8±0.7	Testosterone 0.60	10	24	29.18±2.1
Hypophysectomized hormone treated (3)	232±18	8.5±1.5	Testosterone 6.0	10	24	29.08±1.71
Hypophysectomized hormone treated (3)	237±6	9.8±1.2	Progesterone 6.0	10	24	31.30±1.80

/continued.....

Table XXI (cont.)

* Animals were injected with 0.1 ml corn oil (hormone vehicle).

** The incubation mixture and assay of glycerol kinase are described in Table XI.

*** Time intervals between last injection and killing animal.

was observed in all the liver slice experiments. Incubation of male liver slices with cholesterol (Table XXIII) had no effect on the level of the enzyme in comparison with the control and neither did estradiol-17β or cyproterone, both of which appeared to decrease glycerol kinase activity *in vivo* (Table XXIII). Liver slices from castrated male rats would possess a lower endogenous level of testosterone in comparison with tissues from intact animals and, hence, the effect of exogenous testosterone might be expected to be greater. It was observed, however, (Table XXIV), that testosterone incubated with slices from a castrated animal produced an apparent activity of a similar order to that observed with tissues from an intact animal (Table XXIII).

Hormonal experiments were also carried out with liver slices from female rats. Tables XXV and XXVI show that incubation of slices with testosterone gives an apparent increase in glycerol kinase activity. This also occurs with 5α-dihydrotestosterone.

In the case of the *in vivo* studies with female rats (Table XXII) estradiol-17β and progesterone injections had no effect on the kinase activity but with the female slices estradiol only appeared to produce a small increase in the enzyme level (Table XXV): progesterone was presumably converted to testosterone and hence gave a relatively large apparent increase in activity. Androstenedione and Cyproterone in the *in vitro* system (Table XXVI) were without effect.

In the view of the apparent 30% activation of glycerol kinase in liver slices from female rats by testosterone an

Table XXII. Effect of estradiol-17β(E) and progesterone (P) on the activity of glycerol kinase in female rat liver tissue.

No. of animals	Body Wt. (g) ± SD	Liver Wt. (g) ± SD	Hormone injected mg/kg body wt./ injection	No. of daily injections	Time interval (h)*	Specific activity*** (nmol/min/mg protein) ± SD
Controls 2	232 ± 6	10.0 ± 0.5	Corn oil**	10	24	28.3 ± 0.3
3	233 ± 3	11.3 ± 0.7	E (0.60)	10	24	29.1 ± 0.2
Controls 3	217 ± 8	8.5 ± 0.5	Corn oil**	2	24	29.8 ± 0.5
3	213 ± 7	8.4 ± 0.4	E (6.0)	2	24	30.8 ± 0.4
3	221 ± 5	9.0 ± 0.5	P (6.0)	2	24	30.9 ± 0.6

* Time interval after last injection and killing animal.

** Animals were injected with corn oil (hormone vehicle).

*** The incubation mixture and assay of glycerol kinase are described in Table XI.

Table XXIII. Effect on glycerokinase activity on incubating male rat* liver slices with steroids and antiandrogen (cyproterone). 500±10 gm of slices were used. Glycerokinase activities were determined as described in Table XI. The activity values are given as means ± S.D. with the number of observations in parentheses.

Time of incubation with steroid (h) (1mg/10ml Krebs-Ringer)	Specific activity mol/min/g of tissue at 37°C				
	Control	Cholesterol	Cyproterone	Estradiol 17-β	Testosterone
2	2.14±0.05 (3)	1.94±0.09 (3)	2.03±0.21 (3)	1.92±0.12 (3)	2.79±0.15 (3)
3	1.56±0.09 (3)	1.57±0.11 (2)	1.81±0.2 (2)	1.67±0.13 (2)	2.07±0.08 (3)

* Slices were prepared from 17 week old (310g body weight) male rat.

Table XXIV. Effect on glycerokinase activity of incubating castrated male rat liver slices with testosterone. 400±10mg of slices were used. Glycerokinase activities were determined as described in Table XI. The activity values are given as means ± S.D. with the number of observations in parentheses.

Time of incubation with steroid (h) (1mg/10ml Krebs-Ringer)	Total activity /~mol/min/g tissue at 37°C	
	Control	Testosterone
3	1.29±0.05 (3)	1.69±0.07 (3)

Slices prepared from a castrated rat

Table XXV. Effect on glycerokinase activity of incubating female rat* liver slices with steroids. 600±10mg slices were used. ±Glycerokinase activities were determined as described in Table XI. The activity values are given as means ± S.D. with the number of observation in parentheses.

Time of incubation with steroid (h) (1mg/10ml Krebs-Ringer)	Total activity μmol/min/g of tissue at 37°C				
	Control	Testosterone	5α-Dihydrotestosterone	Progesterone	Estradiol 17-β
2	1.73±0.15 (3)	2.22±0.18 (2)	2.09±0.15 (3)	2.09±0.18 (3)	1.96±0.20 (3)
4	1.31±0.14 (2)	1.90±0.17 (3)	1.86±0.16 (2)	1.83±0.15 (3)	1.66±0.12 (2)

*Slices were prepared from 13 week old (228g body weight) female rat.

Table XXVI. Effect on glycerokinase activity of incubating female rat liver slices with various hormones. 500±10mg slices were used. Glycerokinase activities were determined as described in Table XI. The activity values are the average of two separate sets of incubations made with liver slices from two female animals (body wt. 280g and 265g).

Time of incubation with steroid (h) (1mg/10ml Krebs-Ringer)	Total activity /mol/min/g tissue at 37°C				
	Control	17β-Hydroxy-5β-androstan-3-one (Androsteredione)	5α-Dihydrotestosterone	Testosterone	Cyproterone
	1.81±0.01 (2)	1.77±0.09 (2)	2.06±0.07 (2)	2.20±0.04 (2)	1.73±0.09 (2)

initial attempt was made to see whether the hormone effect involved activation of the protein synthesizing machinery of the cells. The incubation times with testosterone were considered to be long enough for protein synthesis to occur and to increase the amount of glycerol kinase to a level high enough to account for the observed activity change (191). Female liver slices were incubated with testosterone and with testosterone plus a protein synthesis inhibitor and the kinase levels in both systems compared (Tables XXVII and XXVIII). Little effect was observed using cycloheximide, actinomycin D, ethidium bromide or puromycin. This would indicate that the apparent increase in glycerol kinase activity produced by testosterone is not due synthesis of new enzyme molecules or other proteins which in some way might be involved in activation of glycerol kinase. The possibility that penetration of the tissues by the inhibitors was poor, relative to testosterone, must, of course, also be considered as a reason why the increase in the level of the enzyme was not inhibited. The work of Ling and Dixon (133) suggest, however, that the preincubation time for at least actinomycin D and Cycloheximide, was quite sufficient to inhibit protein synthesis in the case of liver slices.

A further experiment which casts some doubt on the idea that testosterone *in vitro* increases glycerol kinase activity via acceleration of protein synthesis was carried out. Male rat liver slices were incubated with testosterone for 2h at 37^{o} then a 100,000g supernatant was prepared and glycerol kinase activity assayed. The homogenate was then allowed to stand at 4^{o} for various times up to 48h and the

Table XXVII. Effect on glycerokinase activity of incubating female rat liver slices with testosterone (in the presence of cycloheximide (C) or puromycin (P)). 500±10mg slices were used. Glycerokinase activities were determined as described in Table XI.**The activity values are given as means ± S.D. with the number of observations in parentheses.

Time of incubation with steroid (h) (1mg/10ml Krebs-Ringer)	Total activity /mol/min/g tissue at 37°C			
	Control	Testosterone	Testosterone+P	Testosterone+C
2(a)	1.56±0.05 (2)	2.05±0.1 (2)	1.89±0.04 (4)	1.99±0.07 (2)
2½(a)	1.47±0.04 (2)	1.79±0.08 (2)	1.84±0.05 (2)	1.68±0.07 (4)
2(b)	1.19±0.02 (2)	1.42±0.02 (2)	1.47±0.05 (4)	1.41±0.03 (2)
2½(b)	1.39±0.05 (2)	1.75±0.05 (2)	1.61±0.04 (2)	1.40±.04 (4)

(a) Slices were prepared from a 20 week old female rat (295g body wt.) and preincubated for 30 min with the inhibitors.

(b) Slices were prepared from a 20 week old female rat (313g body wt.) and preincubated for 15 min with the inhibitors.

The concentrations of cycloheximide was 4mg/10ml (145, 160) and puromycin 4mg/10ml (145) Krebs-Ringer solution.

Table XXVIII. Effect on glycerol kinase activity of incubating female rat* liver slices with testosterone propionate(TP) in the presence of cycloheximide (C), actinomycine D (A) or ethidium bromide (E). 500±10mg slices were used. Glycerol kinase activities were determined described in Table XI. The enzyme activity values are given as mean ± S.D. with the number of observations in parentheses.

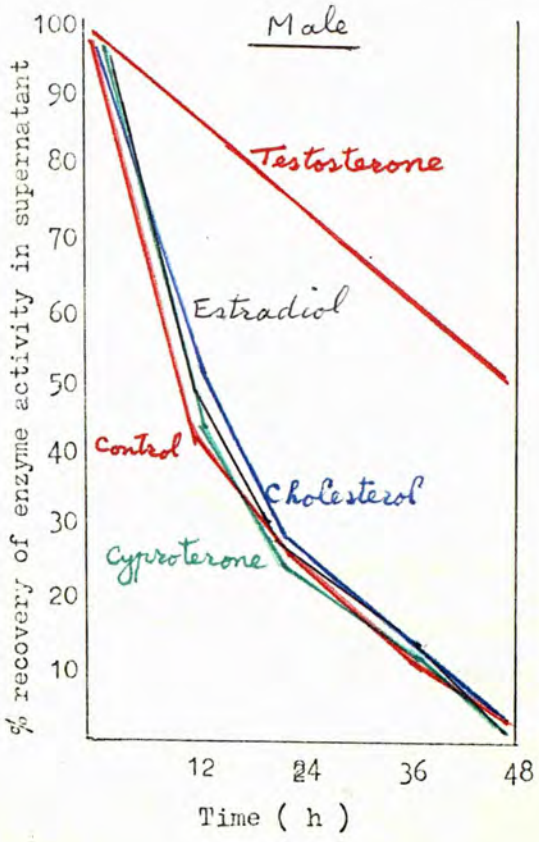
Time of incubation with steroid (1mg/10ml Krebs-Ringer)	Total activity /mol/min/g tissue at 37°C				
	Control	Testosterone Propionate	TP+C	TP+A	TP+E
2	1.86±0.10 (2)	2.11±0.09 (2)	2.13±0.11 (2)	1.98±0.16 (2)	1.93±0.08 (2)

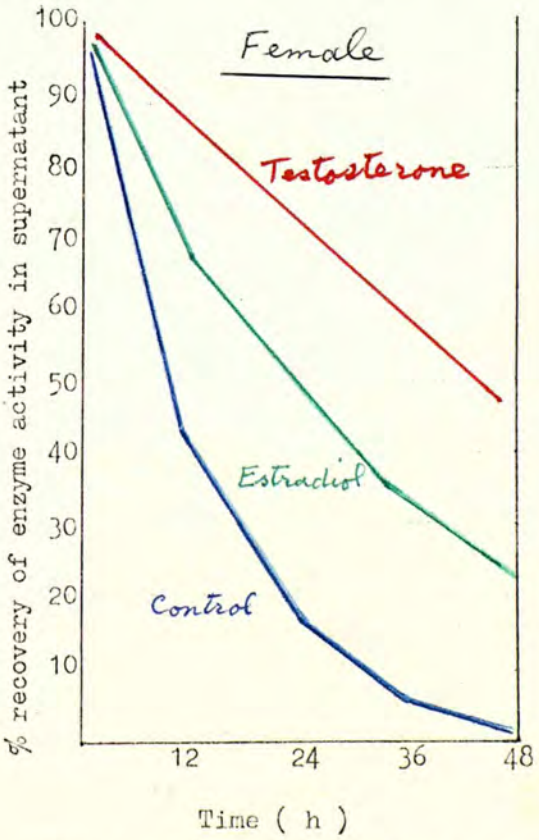
*Slices were prepared from the liver of an 18 week-old (280g body wt.) female rat. The protein synthesis inhibitors cycloheximide, actinomycin D and ethidium bromide were used in Krebs-Ringer solutions at concentrations of 4mg, 0.5mg and 0.04mg/10ml, respectively (145, 151, 153).

enzyme activity monitored. The results are shown in Fig II and it is clear that testosterone prevents decrease in activity which could be explained by one or more of the following phenomena: (a) a direct or indirect stabilising effect of the steroid on the enzyme; (b) inhibition (direct or indirect) of the enzymes causing degradation of glycerol kinase; (c) direct or indirect activation of glycerol kinase; (d) inhibition of the synthesis of kinase degrading enzymes and/or activation of the synthesis of degrading enzyme inhibitors. In the case of (d) it is assumed that the rate of protein synthesis is increased by testosterone in the slices despite the results obtained with protein synthesis inhibitors. Whatever reaction is in operation it must be considered to be relatively specific as estradiol, cholesterol and cyproterone, had no significant effect on the glycerol kinase level.

The experiments were repeated with female rat liver slices and again the results were similar but in this case estradiol possessed some 'stabilizing' effect but not as great as testosterone. The different behaviour of estradiol in these male and female in vitro systems cannot really be explained. It is possible in the experiment with female slices that estradiol was exhibiting a non-specific 'stabilizing' effect (as the structures of estradiol and testosterone are similar). But why was this not observed with the male tissue system? In relation to both systems it should be remembered that direct addition of testosterone to a 100,000g liver supernatant (for 1h at $0^{o}C$) did not alter the activity of glycerol kinase.

Fig. II

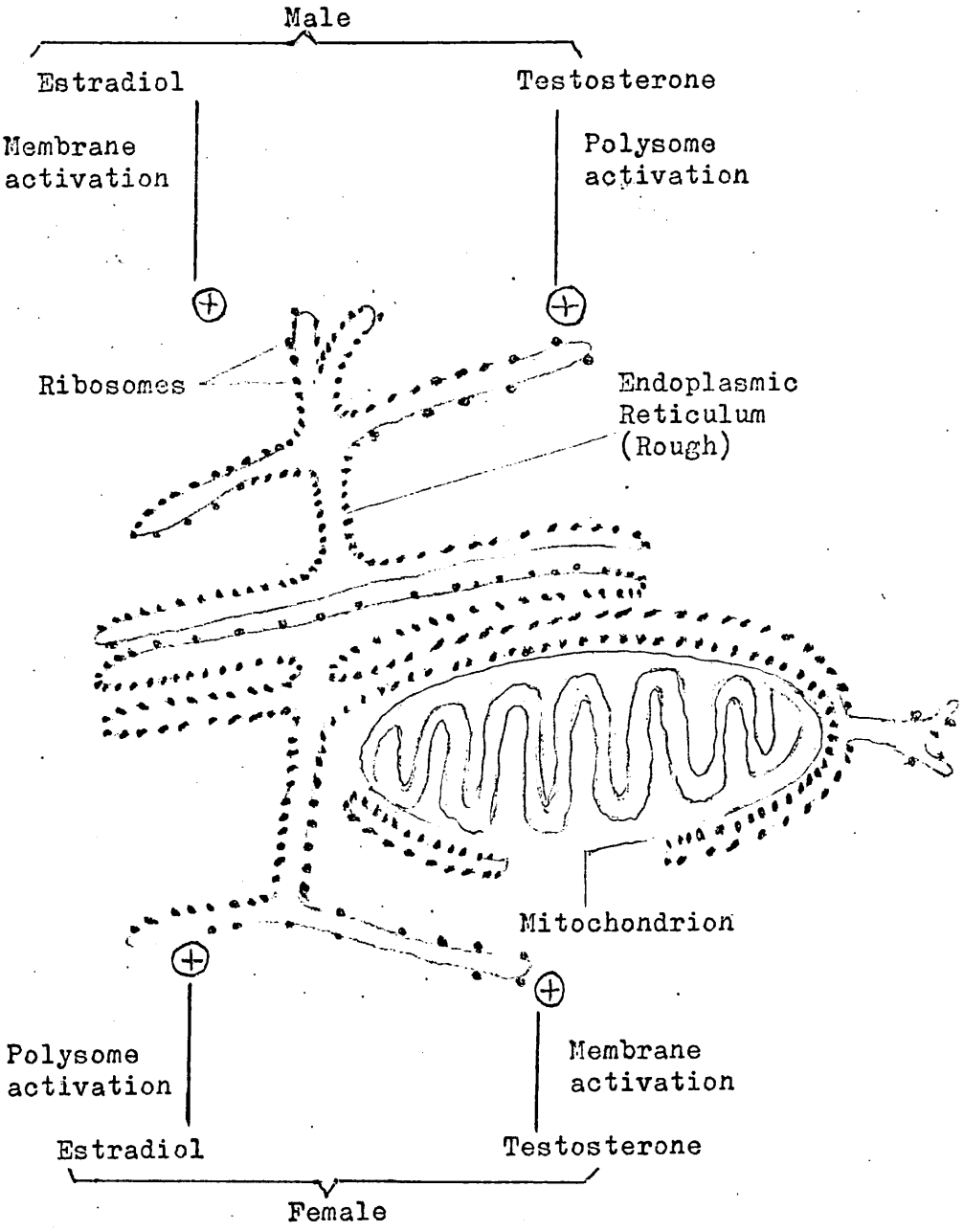




For details see section on Results and Discussion (pp 86)

Considering all the results of the *in vivo* and *in vitro* investigations of glycerol kinase activity in rat livers it is clear that the effect of steroid hormones on the enzyme is extremely complex. It is possible that we are dealing with physiological and non-physiological phenomena and with anabolic and catabolic processes. None of this preliminary work proves that sex hormones stimulate the production of glycerol kinase molecules. To prove this conclusively an increase in the specific protein would have to be demonstrated.

Assuming however, that the synthesis of glycerol kinase *de novo* is occurring in the systems studied and that this synthesis is controlled by sex hormones it is of interest to attempt to explain the experimental results in this thesis on the basis of two major facts already known about steroid control of protein synthesis. The studies of Blyth and co-workers (248) suggest that estradiol and testosterone are involved in the activation of polysomes and the process leading to the binding of polysomes to the smooth microsomal membranes. The hypothesis, which does not involve nucleic acid control of protein synthesis, is illustrated in the following digram:


Male
Estradiol
Testosterone
Membrane activation
Polysome activation
Ribosomes
Endoplasmic Reticulum (Rough)
Mitochondrion
Polysome activation
Membrane activation
Estradiol
Testosterone
Female

The ideas presumably relate only to membrane-bound polysomes which we believe to synthesize products which are transported out of the cell, for example, some glycoproteins (259). The hypothesis makes no comment on quantitative or qualitative differences in the protein produced by tissues from different sexes and as far as can be seen makes no real contribution to the studies on glycerol kinase.

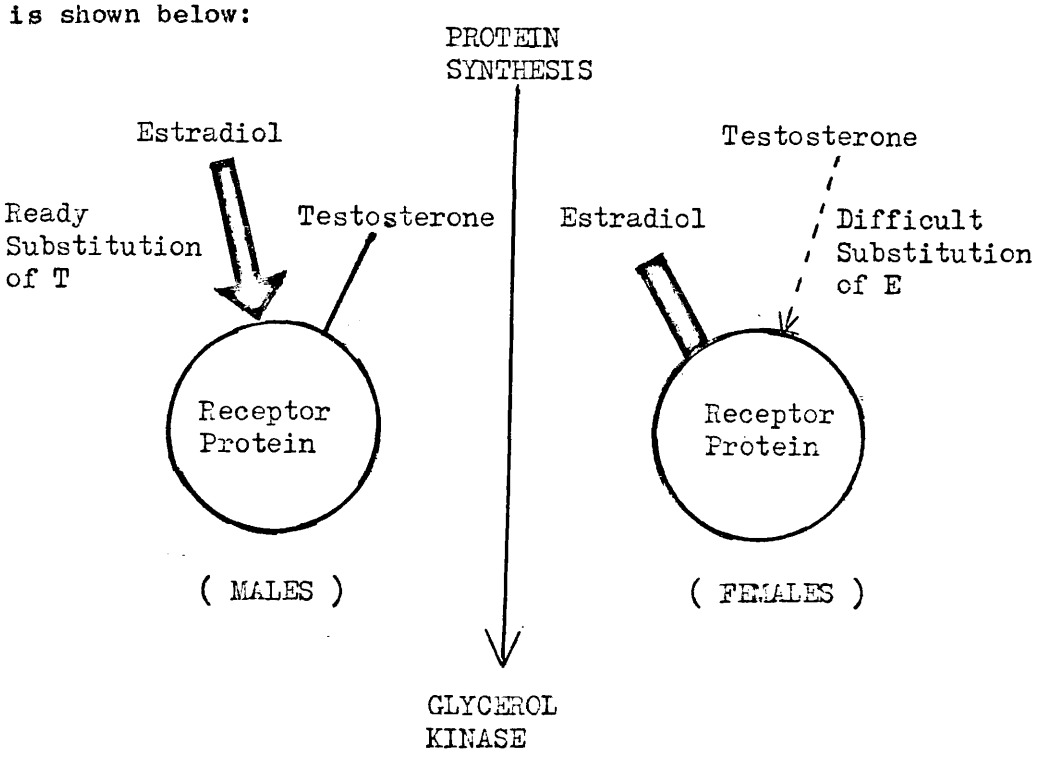
Roy and co-workers (280) have been interested in the synthesis of α_{2u}-globulin by rats. This is a protein only in the livers of males of the species and synthesis is undoubtedly an androgen-dependent reaction. The group have shown that the liver contains a receptor protein which binds both dihydrotestosterone (and testosterone) and estradiol-17β. At saturating concentrations the receptor binds 3 moles of dihydrotestosterone and 1 mole of estradiol. Estradiol strongly inhibits the uptake of dihydrotestosterone by the receptor but dihydrotestosterone only weakly interferes with estradiol binding. The level of androgen receptor closely follows the output of α_{2u}-globulin in the urin. Immature and senile males which do hot produce the globulin lack the receptor. Castration reduces both and injection of estradiol into adult male rats prevents receptor and globulin synthesis.

It is interesting to note that recently an androgen receptor has also been isolated from rat pancreas (260) and of course, their presence in sex organs has been known for sometime (264).

It is possible that this study by Roy *et al* (280) has some bearing on our work with glycerol kinase and that the synthesis of this enzyme is governed by a liver receptor protein

which controls the level of m-RNA, the generally accepted mechanism for the control of protein synthesis by sex hormones (249).

If the existence of a specific hepatic receptor which governs glycerol kinase synthesis and which has properties similar to those described by Roy et al (280) is assumed then many of the in vivo and in vitro studies with glycerol kinase can be explained. The basis of the hypothesis is shown below:



Hypothetical control of glycerol kinase synthesis involving a specific hepatic steroid acceptor protein with high binding capacity for estradiol and low capacity for testosterone (cf 280).

NB 5α-Dihydrotestosterone ("active" testosterone) may be bound to the receptor protein instead of testosterone : The former is readily converted to the latter in liver tissues (275).

Thus injection of adult male rats with testosterone would not raise the level of glycerol kinase (cf Table XIII) because sufficient testosterone would already be present to combine with the receptor and excess would presumably be combined with sex hormone binding globulin in the blood (265, 266). Testosterone would be likely to induce glycerol kinase in the case of castrated animals (Tables XV - XVIII) and male liver slices (Table XXIII) because of an initial low level of endogenous testosterone.

Administration of testosterone to female rats (Table XIV) did not increase liver glycerol kinase activity whereas the hormone did raise the activity when applied to liver slices from females (Tables XXV & XXVI). In the first instance this could be explained by assuming that a receptor with tightly bound estradiol was initially present in the tissues and that this estrogen could not be readily replaced by testosterone. In the slices, however, the receptor might not be saturated with estradiol and hence testosterone could more readily bind to the protein.

Explanations based on the receptor protein hypothesis in the case of estradiol treatments are not quite so satisfactory, however.

Estradiol injections lower the hepatic glycerol kinase activity in male rats (Table XIX) possibly because of the high affinity of this steroid for the receptor i.e. estradiol may readily replace testosterone. However with slices from male animals (Table XXIII) estradiol had no effect which at present cannot be explained. Administration of the estrogen to female rats (Table XXII) produces little

effect presumably because of an already existing high level of the receptor - estrogen complex which according to the hypothesis relates to a low or zero rate of glycerol kinase synthesis. With female liver slices estradiol produced a small increase in enzyme level (Table XXV) which was probably insignificant.

In summary, it is clear that high hepatic glycerol kinase activity is directly related to high levels of testosterone. High levels of estradiol, on the other hand are associated with[a] diminished kinase activity. Much of the investigation taken in conjunction with the main activity of steroid hormones would point to control of glycerol kinase synthesis by androgens and estrogens. However, the preliminary study with tissue slices and protein synthesis inhibitors does not support this theory. The work with slices also suggests that steroids may confer differing stabilities on glycerol kinase which complicate any attempted interpretation of the kinase -steroid relationship <u>in vivo</u>.

FINAL CONCLUSIONS

The aim of this investigation was described on page 63 and the work has shown clearly that there are no sexual differences in the activities of NAD- and NADP-dependent glycerol dehydrogenases in rat livers. In addition their activities could not be manipulated by exogenous testosterone or estradiol-17β. If synthetic reactions relate to the different responses of male and female animals to a sucrose or fructose load then it is unlikely that these dehydrogenases play any part.

Glycerol kinase activity however, may well be under some form of hormonal control with higher levels in male rat livers than in livers from females and there were indications that these levels could be influenced by testosterone and estradiol-17β. This in turn could lead to higher levels of glycerol 3-phosphate for fat synthesis in male rats and could, at least in part, account for elevated serum triglycerides in males in comparison with females on a variety of diets (cf 31 and 34). The substrate for this enzyme could be derived from fructose, from dietary glycerol or from lipolytic reactions.

Sex differences in the levels of glycerol kinase alone cannot, of course, be singled out as the only factor likely to produce different responses to dietary carbohydrate in male and female rats. It has been noted that male animals have higher liver ketohexokinase and aldolase activities than females (pp 173). In the former case, this should lead to a higher metabolic rate specifically for dietary fructose in male rats and in the case of aldolase,

to higher rates for dietary fructose and glucose as liver aldolase reacts with both fructose 1-phosphate and fructose 1,6-diphosphate.

Reactions leading to fatty acid components of triglycerides may also result in different responses to carbohydrate diets by male and female animals. Thus pyruvate kinase, which is involved in the Embden-Meyerhof pathway from carbohydrate to acetate, may be under testosterone control in rat jejunum (276), although liver enzymes have not been examined.

The dehydrogenases supply NADPHfor fatty acid synthesis, i.e. glucose 6-phosphate and 6-phosphogluconate dehydrogenases, appear to be much higher in female rat livers than in males and the apparent activities of these enzymes are clearly reduced in response to ovariectomy. Castration of males does not influence the dehydrogenase levels (284). This state of affairs cannot be directly related to the apparent serum triglyceride levels in vivo in male and female rats as the former are higher than the latter. However, it is interesting to note that liver triglycerides in animals on a normal chow diet are reported to be slightly higher in female rats than in males (285) and the triglyceride output of perfused female rat livers is also greater than that of male (136).

Paralleling the differences in dehydrogenase activities are the activities of male and female fatty acid synthetase which are significantly higher in livers from the females no matter whether the diet is normal chow or enriched with sucrose or glucose (S.A. Hashemi, Biochemistry Department, Royal Holloway College, unpublished results).

A similar but less significant trend was observed by Bruckdorfer et al (31).

Hence some sex differences in enzyme levels could favour higher serum triglyceride production from carbohydrate in males and some females. Obviously without further comparative kinetic data it is impossible to state which enzymes actually control fat synthesis and give rise to the serum levels observed in animals of both sexes.

So far, only the synthesis of fats has been considered. The phenomenon of clearing, catalysed by lipoprotein lipase, is perhaps of equal importance. Studies by Cryer et al (279) provide one explanation of why dietary fructose gives rise to higher serum triglyceride levels than glucose in male rats. These workers believe that insulin, the synthesis of which is stimulated by glucose and not by fructose, activates lipoprotein lipase and hence 'clearing' is more rapid in animals on dietary glucose than on dietary fructose. However, studies on sex differences in the levels of lipoprotein lipase have been limited. Bruckdorfer and co-workers (31) have presented data which suggest that the difference in enzyme levels in the adipose tissues of male and female rats is not large: no statistical information was given, however. Obviously further work is required on lipoprotein lipase in order to establish how important this enzyme is in connection with sex differences in response to dietary carbohydrates. Possible differences in the synthesis of insulin in male and female animals should also be borne in mind in relation to the role of the lipase.

MATERIALS AND METHODS

Materials

Analar grade chemicals were used whenever possible; other chemicals were of the best available purity.

Glass distilled deionized water was used for preparation of solutions.

Animals

Male and female Wistar-strain rats from 10-12 weeks of age were used for all experiments *in vivo* and *in vitro*. Castrated and hypophysectomized male rats were obtained from Anglia Laboratory Animals (part of Huntingdon Research Centre), Alconbury-Huntingdon, U.K. The latter animals were operated on 10 days before the experiment.

The animals were fed on a Commercial stock diet (diet 86 supplied from E. Dixon and Son, Ltd. Ware, Herts *ad libitum* and killed as needed by cervical fracture. All experiments were started between 7.30 and 9.00 a.m. in order to avoid any possible complications due to diurnal rhythms (96,100).

Hormone Treatments

Injections of hormones were by the subcutaneous method into the abdomen.

Preparation of Hormone Solutions for in vivo Studies

(a). A weighed amount of hormone was transferred to a test tube, a small amount of propylene - glycol was added, the mixture

was warmed to about 60°C and saline (0.9% NaCl at 60°) was added to give the desired concentration of hormone. The final concentration of propylene-glycol was not allowed to increase above 0.5%, v/v (105). The resulting mixture was passed through a glass homogenizer with teflon pestle (clearance 0.012 in) several times. The resulting fine suspension of hormone in propylene-glycol/saline was then sterilized by autoclaving at 120° for 30 min.

(b) In some experiments, as designated, the hormone was dissolved in corn oil without subsequent sterilization.

Hormone Studies with Liver Slices

Liver slices were prepared within 5-10 min. of sacrificing the rat. About 3-4 slices (total wt. 500± 10 mg) were incubated in Krebs-Ringer phosphate, pH 7.4 (118) with or without added hormone in glass stoppered test tubes. The incubation mixture was kept under a 5%/95% (O_2/CO_2) atmosphere at 37°C for the prescribed time. Hormones were dissolved in 0.5% prcpylene-glycol in 0.9% saline solution and 0.2-0.5 ml of these solutions made up to 10 ml with Krebs-Ringer phosphate solution. The final hormone concentration was 1 mg/10 ml and the propylene glycol concentration was not allowed to exceed 0.5%, v/v. Control digests contained the hormone vehicle with the same final glycol concentration as the hormone-containing digests.

Liver Enzyme Preparations

Livers were homogenized in the appropriate ice-cold solution (1:2, w/v) using a glass homogenizer with a teflon

pestle (clearance 0.012-0.015 in). NAD-dependent glycerol dehydrogenase preparations were made in 150 mM KCl-2 mM $KHCO_3$ (23), NADP-dependent glycerol dehydrogenase in distilled water (106, 107) and glycerol kinase in 150 mM KCl - 2 mM $KHCO_3$ pH 7.4 (113). The resulting homogenates were centrifuged at 100,000xg for 60 min at 0-4°C.

Enzyme Assays

These are described as foot-notes under Tables in the Results and Discussion section. They were all carried out with crude 100,000g supernatant solutions:

NAD-dependent glycerol dehydrogenase - Table II (23).

NADP-dependent glycerol dehydrogenase - Table VII (21).

Glycerol kinase - Table XI (65).

The specific activities quoted in the case of *in vitro* studies with liver slices relate to the total activities measured in the slice homogenates and the bathing media.

Paper Chromatography

Whatman No. 3 paper was used for separating $[^{14}C]$ glycerol 3-phosphate from unreacted, labelled glycerol in the glycerol kinase assay. Descending chromatography was employed (a modification of the method used by Benson *et al* (39)) using strips of Whatman No. 3 paper (2.5 cm width). The solvent system used was the second solvent employed by Crowley *et al* (32) and it was prepared as follows:

Solution A	Butane-1-ol	1246 ml
	water	84 ml
Solution B	Propionic acid	620 ml
	water	790 ml

Equal volume of solutions A and B were freshly mixed just before use.

Prior saturation of the atmosphere was not employed, but about 0.5 cm of solvent was placed in the bottom of the chromatography tank and separation was achieved at room temperature overnight (12-14 hr). After that time the strips of paper were removed from the tank, dried in air and further in a ventilated oven at 60°C. Using standard compounds it was shown that glycerol 3-phosphate and glycerol exhibited Rf values of 0.18 and 0.75, respectively, under the conditions described. These compounds were detected on the paper by method described by Smith (83). Areas of paper containing unreacted glycerol and synthesized glycerol 3-phosphate were cut out, transferred to a scintillation vial containing 10 ml of scintillant (7g Butyl PBD in 1L toluene), and the radioactivity measured in a Packard Tri-carb liquid Scintillation Counter.

Protein Estimation

The protein content of all enzyme preparation was measured spectrophotometrically (Unicam SP 500 spectrophotometer) by the method of Lowry et al (94) except that the Folin-Ciocalteu reagent was diluted with an equal volume of water. Human serum albumin was used as a standard.

Examination of Liver Cell Fractions for Glycerol Kinase Activity

Fractionation of male and female rat liver homogenates in 250 mM sucrose - 1 mM EDTA pH 7.0 (the ratio of tissue to buffer solution was 1:11, w/v) was achieved by centrifugation

at 650xg (10 min), 22,500xg (10 min) and 100,000xg (30 min) (194). The resulting precipitates were, in all cases, washed with sucrose - EDTA solution, reisolated by centrifugation and suspended in KCl-$KHCO_3$ buffer and sonicated (30 sec.). The disrupted organelle preparations and the original supernatant solutions were assayed for glycerol kinase activity as described in Table XI.

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