FRUCTOSE METABOLISM IN THE LIVER

A Thesis submitted by

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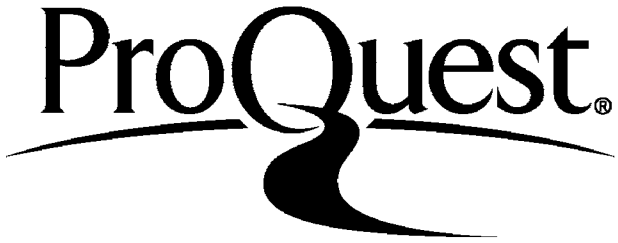

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ABSTRACT

An investigation of the metabolism of fructose by rat liver has been carried out with particular reference to control by sex hormones and the possible relationship in humans between dietary sucrose, blood triglycerides and heart disease.

The incorporation of label from ^{14}C-fructose and ^{14}C-glucose into liver triglycerides has been studied: the former sugar was observed to produce the greater incorporation.

Fructokinase activity in liver tissues from male and female animals at various stages of development has been measured and sex and age differences were noted. Testosterone injections elevated the level of fructokinase activity in male and female rats and produced similar effects with liver slices. Estrone, however, lowered the activity of the enzyme in male animals and increased it in females. The mode of action of testosterone has been investigated.

Fructose phosphate aldolase activity is higher in liver from male rats than from females and this may be related to the relatively rapid transfer of label from ^{14}C-fructose to trioses when the ketose is incubated with male liver extracts. Testosterone injections produce no change in the levels of the aldolase.

Some related, preliminary work on the purification of liver fructokinase and the analysis of glyceride fatty acids from various rat tissues is also described.

CONTENTS

ABBREVIATIONS

α-Gly-P	α-Glycerophosphate
DHAP	Dihydroxyacetone phosphate
F-1-P	D-Fructose 1-phosphate
F-6-P	D-Fructose 6-phosphate
F-1,6-P_2	D-Fructose 1,6-diphosphate
G-1-P	D-Glucose 1-phosphate
G-6-P	D-Glucose 6-phosphate
Gly-3-P	Glyceraldehyde 3-phosphate
PP_i	Pyrophosphate
PPO	2,5-Diphenyloxazole
POPOP	1,4-Di-2-(5-phenyloxazolyl)-benzene
UTP	Uridine triphosphate
UDPG	Uridine diphosphate D-glucose
Km	Michaelis constant
WHO	World Health Organisation

Abbreviations which refer to Results & Discussion and Experimental Sections only.

^{14}C-fructose	^{14}C-(U)-fructose
^{14}C-fructose 1-phosphate	^{14}C-(U)-fructose 1-phosphate
^{14}C-glucose	^{14}C-(U)-glucose

I N T R O D U C T I O N

Atherosclerosis: the disease process

The commonest cause of coronary heart disease is coronary atherosclerosis. Atherosclerosis tends to increase progressively with age but sometimes occurs with moderate or intense severity even in young adults. However, coronary heart disease, the clinical entity, may be absent even with moderately severe coronary lesions if the blood flow is not significantly diminished. It is, therefore, important to distinguish between coronary atherosclerosis, a pathologic term, and coronary heart disease due to atherosclerosis, the clinical term (1).

The word arteriosclerosis (2) is a generic term associated with thickening and hardening of arteries. If this occurs and (a), there is an absence of scarring of the intimal coat (produced by infections such as syphilis or tuberculosis); (b), no inflammation of the coronary artery; and (c), no thromboangitis (inflammation of the intima of a vessel, with thrombosis) or polyarteritis, then the condition is known as atherosclerosis (3). This is the association of a sclerosising process with the accumulation of fatty material.

To facilitate the standardization of pathological criteria and terminology in respect of artherosclerosis the W.H.O. proposed (W.H.O. Tech.Rep.No. 143, 1958) the following definition: "Atherosclerosis is a variable combination of changes of the intima of arteries (as distinguished from arterioles) consisting of a focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes".

The diagnosis of coronary heart disease is based on evidence of an old myocardial infaction, or of angina pectoris in a patient with no evidence of syphilis or valvular heart disease. Coronary atherosclerosis is the disease most frequently associated with angina pectoris. At least one major coronary vessel shows either extreme narrowing or complete obstruction. Angina pectoris is much more common in males than in females and increases in frequency with age, especially above the age of fifty. Diabetes mellitus, hypertension, hypercholesterolemia and

familial lipemia predispose to angina pectoris probably because of their relation to coronary atherosclerosis.

The cardiac pain of angina pectoris is similar to that of myocardial infarction in which it is known that blood flow has been interrupted by a coronary thrombosis. The essential difference is the greater severity and more prolonged duration of pain in myocardial infarction, which is explained by the suddenness, completeness and irreversibility of obstruction of a major coronary artery (1).

Myocardial infarction should not be regarded as identical with coronary thrombosis or occlusion. The commonest cause of the onset of angina pectoris is probably a coronary occlusion, but one that, because of the adequacy of collateral circulation, does not result in a prompt myocardial infarction. However, since in most cases of angina pectoris the underlying disease is severe coronary atherosclerosis or aortic valvular disease, heart failure frequently occurs as an eventual result of myocardial damage.

Acute myocardial infarction is a clinical syndrome resulting from sudden and persistent curtailment of the myocardial blood supply. Thrombosis, secondary to atherosclerosis, is the cause in more than 75 per cent of the cases of myocardial infarction. A fissure in an atherosclerotic plaque with intimal hemorrhage is often the initial factor in the occlusion. The thrombus occurs usually over a short distance (5mm or less) and involves a major coronary artery in its proximal position. The occurrence and extent of infarction depends in large measure on the distribution of blood supply from the various coronary arteries and on the site of thrombosis. The latter determines the collateral circulation available from branches of the occluded artery proximal to the occlusion and from neighbouring coronary arteries. Thus, it is possible for collateral vessels to maintain viability and prevent infarction, despite a coronary thrombosis. The coronary occlusion may result in infarction or only in potential myocardial ischemia (angina pectoris), or may cause no clinical symptoms. If infarction occurs, it may extend through the entire thickness of the ventricular wall (transmural infarction), or it may cause more limited necrosis, which is usually situated predominantly in the subendocardium. The subendocardial area is more

likely to suffer from a lack of blood supply than the subepicardial.

There is little theoretical or experimental evidence that arterial thrombosis can be prevented by the use of anti-coagulants. Clinical studies suffer from the relative paucity of cases or unsatisfactory controls, the latter due to problems in matching patients to be treated and those who serve as controls. However, despite the absence of scientific proof of their value, the use of anti-coagulants in acute myocardial infarction appears to be justified, because the vast majority of studies in every part of the world have consistently disclosed a significant reduction in mortality and an impressive diminution in the frequency of thromboembolism in patients treated with anti-coagulants.

Whereas anti-coagulants are given with the aim of preventing thromboembolic complications or extension or recurrence of coronary thrombosis, fibrinolytic agents are designed to induce lysis of the clot already present. In animals, fibrinolytic agents have been observed to hasten lysis of induced coronary thrombi, to reduce the size of infarct and to promote healing by diminishing capillary congestion and edema and eliminating microthrombi.

Arterial Hypertension (4) in general, rises with age in urban communities. The rise in diastolic (expansion of heart vessels) and systolic (contraction of heart musculature) pressures is greater in females at the menopause than at the corresponding age in males. The features it shares with all other hypertensive manifestations are those due to the increased load put upon the heart and the blood vessels in different territories. A main finding is the increased incidence of atheroma, both experimentally and pathologically. The increased pressure leads to hypertrophy of the left ventricle and a tendency to angina pectoris and myocardial infarction.

The combination of hyperinsulin response, insulin resistance and impaired glucose tolerance is now being frequently recognized in patients with atherosclerosis. Also in those with increased concentrations of the serum lipids, particularly triglycerides, commonly associated with atherosclerosis (5). The sex hormones affect both the amount and the distribution of fat. Women, for example, have a greater amount of subcutaneous fat than men. The relationship between parity

and obesity and the frequent menopausal gain in weight , although difficult to document, suggests a possible role of female sex hormones in the regulation of fat metabolism. The obesity of eunuchs suggests further that absence of male sex hormones also promotes adiposity. The hypogonad obesity that occurs in children just prior to adolescence disappears with puberty, at which time the adult distribution of fat takes place (5).

Atherosclerosis is more common in acquired than in inherited obesity and in the male type than in the female type. The incidence of atherosclerosis is much lower in pre-menopausal females, but 10 years after the menopause approaches that of males. No single agent has been pinpointed as the causal agent of the atherosclerotic plaque. The lesions consist of subendothelial accumulation of cholesterol, triglyceride and phospholipids that partially and at times completely occlude the lumen of the artery. Biochemical studies have demonstrated that cholesterol and triglyceride constituents are mainly derived from plasma lipids, whereas the phospholipids are at least partially synthesised in the vessel wall. The association between atherosclerosis and increased concentration of β lipoproteins and very low density lipoproteins, suggests that such triglyceride-rich macromolecules are particularly likely to become ensnared in the vessel wall.

A certain amount of lipid exchange between intima and plasma is normal, and such lipid is metabolized by the vessel wall. However, either because the entry of lipid becomes too great or because the metabolic processes by which it is normally removed are impaired, lipid accumulation becomes excessive and plaque formation ensues. The lipid is at first present in foam cells. The early lesions consist chiefly of triglyceride and, according to some workers, cholesterol is not a prominent constituent of the early plaque. With time the triglycerides and phospholipids are metabolized leaving behind cellular debris and the non-metabolizable cholesterol as the chief constituents of the older plaque. Böttcher et al (6) reported that a high percentage of triglycerides from the coronary arteries, as opposed to the aorta, predominated over all the other lipid fractions in the early stages of atherosclerosis. This percentage of triglycerides was higher than the sterol esters in the latter stages of the disease, in spite of

the higher percentage of the latter in the more severely diseased arteries. The percentage of phospholipids and of free fatty acids were not significantly different at the two stages. Eventually fibrous tissue and calcium replace the lipids.

The localization of atherosclerotic plaques at sites of injury and at points of bifurcation of arteries, and the greater incidence of atherosclerosis in hypertensive persons demonstrate that local injury and pressure relationships are important factors in plaque formation.

An alternative theory attributes plaque formation to intravascular coagulation initiated by increased platelet stickiness and platelet agglomeration or by a shift in the balance between fibrin formation, with secondary enmeshment of circulating lipids (7).

Three important biochemical findings have been identified in patients with coronary artery disease : (i), elevation of serum cholesterol concentration, (ii), increase in serum triglycerides and associated very low density lipoproteins, and (iii), impaired carbohydrate tolerance.

Myocordial Infarction

From a pathological point of view, the clinical opinion that angina pectoris is a manifestation of myocardial ischemia, usually from coronary atherosclerosis, is so strongly supported as to be axiomatic (8).

Platelet thromboemboli may be important not only in the development of some forms of atherosclerosis and occlusive thrombi, but also in initiating disturbances of the microcirculation (9).

Epidemiological studies of myocardial infarction, genetic and environmental factors, show that each of these variables can be modified and that coronary disease and myocardial infarction does not run an invariant course (10).

Epidemiological studies, and observations on animals, stimulated by these studies have indicated several factors involved:
(a) Ethnic differences. Persons with cholesterol and lipid levels greatly below those seen in the more affluent countries in the world have a reduced incidence of atherosclerotic disease in general; this is reflected in a reduced amount of coronary atherosclerosis and myocardial infarction (11-13). Ethnic differences in coronary heart disease among males in U.S.A. revealed a higher incidence for whites than for blacks (14-17). In contrast differences by social class found in the prevalence survey among white males were not found in the incidence study (14). Further, no differences were found by ethnic group for females; however within ethnic groups white males had a rate five times higher than white females, while the ratio for black males to females was only 2 to 1 (14). Yudkin (18) found a mortality rate two to three times greater in males than in females in Britain.
(b) Diet. The proportion of calories derived from saturated and unsaturated fat and the number of calories consumed per day have an effect on the lipid content of the plasma in man (19). Experiments have been carried out to elevate serum cholesterol in pigs by the addition of dietary lard or tallow (but not vegetable oil) to the diet (20-22). Serum cholesterol tended to increase with increasing degree of saturation of the fats tested. Similar trends have been reported with rats (23), chickens (24) and with humans (25).

Hutagalung (26) reports that the addition of 5% lard, beef tallow

or sheep tallow to a 12% protein diet resulted in increased cholesterol in serum, l.dorsi muscle and liver tissue and increased total lipid in the serum, whereas the addition of 5% corn oil elevated only serum total lipid. No significant differences in cholesterol levels were observed amongst pigs given these dietary treatments. The addition of 12.5% lard to diets containing 13 or 19% protein, elevated serum, muscle and liver cholesterol and serum total lipid, and depressed cholesterol in the backfat. Increasing the dietary protein level from 13% to 19% tended to reduce cholesterol in serum and muscle tissue, and markedly depressed total lipid in the serum and cholesterol in liver tissue. The addition of 1% cholesterol to the diet resulted in a trend toward increased total lipid in serum, and cholesterol in muscle and backfat and markedly elevated cholesterol in serum and liver tissue. Addition of a combination of 1% cholesterol and 12.5% lard to a 13% protein diet resulted in increases in serum cholesterol values significantly greater than the additive effects resulting from single additions of dietary cholesterol and lard. Yudkin (18) found no relationship between coronary mortality and consumption of vegetable fat or animal fats, nor was the consumption of hardened fats related to coronary mortality. In his survey of the years 1928-54 consumption of hardened fats increased considerably while coronary mortality fell.

(i) Dietary alternations of the Lipoprotein Levels

Ockner (27) observed the effect of various intraduodenally infused long chain fatty acids on the very low density lipoproteins (V.L.D.L.) in intestinal lymph of rats. He found that, compared with controls, all fatty acids (palmitic, oleic, linoleic) resulted in significant increases in chylomicron ($S_f > 400$) triglyceride. In addition palmitic acid resulted in a twofold increase in VLDL (S_f20-400) triglyceride, whereas with the absorption of oleic or linoleic acid VLDL triglyceride did not change significantly. Although the absolute amount of endogenous cholesterol in intestinal lymph was not significantly affected by lipid absorption under these conditions, its lipoprotein distribution differed substantially among the lipid infused groups. The studies demonstrated that dietary long chain fatty acids differ significantly in their effects upon the transport of triglyceride and cholesterol by lipoproteins.

These findings together with the observed differences in rates of removal of chylomicrons and VLDL from plasma (the half time of plasma survival of VLDL cholesterol-^{14}C was twice that of chylomicron cholesterol-^{14}C), suggest that variations in lipoprotein production at the intestinal level may be reflected in differences in the subsequent metabolism of absorbed dietary and endogenous lipids. Gofman (28) found that the β-lipoproteins were raised in four out of five subjects when the carbohydrate in the diet was raised, the nature of the carbohydrates not being stated. Gofman put forward the view that the S_f 20-400-β-lipoproteins are controlled by the level of dietary carbohydrate.

Studies on the effect of variation in the fibre content of the diet on the serum triglycerides and total cholesterol concentration of a community of Cistercian monks, showed no change in the serum-cholesterol with an enhanced cereal-fibre content. In the control group, where dietary fibre came entirely from non-cereal vegetables, the serum-cholesterol fell, but began to increase after eight weeks. Increasing the fibre content of the diet reduces the serum triglyceride concentration ($P<0.05$) in men under the age of 40. The values do not differ statistically for the men over 40 (29).

Stead (30) claims that the amount of sucrose in the diet has measurable but not marked effects on the cholesterol and total lipid content of the plasma. However, Ahrens et al (31) advanced the hypothesis that hyperlipemia could be carbohydrate-induced. This was confirmed in diabetics when the isocaloric substitution of carbohydrate for fat in the diet resulted in an increase in the serum lipid level (32). Corn starch as the main carbohydrate in a low fat diet of healthy men lowered the total lipid concentration over a period of 25 days, whereas sucrose under similar conditions had the reverse effect on the same subjects (33). Antar and Ohlson (34) confirmed this in both men and women but Macdonald (35,36) found no rise in the total serum lipid of pre- and post-menopausal women in response to dietary sucrose. In a series of fat-free diets given to men for 5 days, no change in the level of total serum lipids was found when the dietary carbohydrate was cornstarch or sucrose and a fall was observed when a partial hydrolyzate of starch, maltose or glucose was the dietary carbohydrate (37). It must be remembered that the total lipid concentration in the serum is composed

of several fractions whose levels can be altered independently of one another, and that the serum is only a reflection of metabolites "in transit" and gives but little idea of the contribution of the various metabolically active sites to the serum mix (38).

(ii) The effect of diet on cholesterol levels

Macdonald (38) gives a detailed history of the investigations of the effect of different carbohydrates on the cholesterol levels in animals. He draws the conclusion that there are too many variables which seem to be important in the regulation of serum cholesterol. These include the species of animal (cf 39) and within a species; the age and sex of the animal; the extent of cholesterol in the experimental diet; the extent and type of lipid in the diet; weight changes (which are known to influence serum cholesterol concentrations (40); and the number of meals ingested during 24 hours. Thus in animals, dietary carbohydrate can alter the serum cholesterol level but it is probably of minor importance.

Manning and Clarkson (41) were able to induce atherosclerosis in test cats fed a high fat cholesterol-containing diet for 12 months; no atherosclerosis was seen in the control cats fed the same diet without added cholesterol. Test cats did not develop sustained hypercholesterolemia when the diet contained 0.5% cholesterol. However, marked hypercholesterolemia occurred when the added cholesterol content was increased to 2%.

Luginbuhl et al (42) using 10 female pigs (12-14 years old) from breeding stock which had been a consistent source of animals with more or less advanced atherosclerosis of aortas, extra-mural coronary, cerebral artéries and other sites, separated them into two groups of 5 animals. One was fed the customary garbage ration and the other a garbage ration supplemented by dried egg-yolk in amounts which supplied 35% of the calorific intake. Egg-yolk feeding for 10 to 14 months was associated with significantly less aortic and extra-mural coronary atherosclerosis. Intramural coronary and cerebral atherosclerosis was not measurably different in the two groups. They concluded that factors other than egg-yolk feeding are responsible for the progress of atherosclerosis.

As serum-cholesterol concentration can be changed by the type of

carbohydrate ingested, the mechanisms responsible require more investigation. The results of Seethanathan and Kurup (43) clearly indicated that carbohydrate metabolism, both glycolysis and glycogen synthesis, is affected in rats fed a high fat-high cholesterol (hypercholesterolaemic) diet.

Fairhurst and Waterhouse (44) observed what appears to be a fat-withdrawal effect on cholesterol esters in man. In a diet containing 60-76% of calories as partially hydrolyzed starch the level of cholesterol ester fell, due mainly to a fall in cholesterol linoleate.

Pleshkor (45) suggests that the rapid rate of absorption of sucrose from the gastrointestinal tract was responsible for the elevation in the serum cholesterol of 30 patients with atherosclerosis of the coronary vessels, fed on extra dietary regime of 50g. sucrose/day. Winitz et al (46) observed a fall in the serum cholesterol of 18 healthy subjects on a fat-free diet with glucose as the sole carbohydrate, but when 25% of the glucose was replaced by sucrose the serum cholesterol level again rose rapidly.

Macdonald and Braithwaite (33) in a study on normal subjects on a low-fat diet (22-58g/day) observed a fall in serum cholesterol with starch as sole carbohydrate but when this was replaced by sucrose the serum cholesterol level rose. In pre- (35) and post-menopausal women (36) the serum cholesterol did not rise in response to sucrose. Macdonald (37) found that cornstarch, partially hydrolyzed starch, maltose, and glucose all caused the serum cholesterol level to fall after 5 days on a fat-free diet, whereas no significant change was seen with the sucrose-containing diet. Fructose as opposed to glucose in a similar study did not affect serum cholesterol levels (47).

Dunnigan et al (48) examined the effect of isocaloric exchange of dietary starch and sucrose on the serum lipids in 9 middle-aged men. These results indicated no change in the serum cholesterol and triglyceride levels within the range of starch and sucrose intakes of the normal Western diet; they also agreed with their earlier conclusions (49) and were similar to the findings of Grande et al (50), Lees (51) and Antonis (52). They are at variance with those of Macdonald and Braithwaite (33), Groen et al (53) and Antar and Ohlson (34). Changes in serum lipids may occur in gross hyperlipaemia which are not evident in normolipaemic

or even moderately hyperlipaemic subjects, Anderson (54); Grande (55).

However Naismith et al (56) raised the sucrose intake, at the expense of starch-rich foods, in the diet of 23 healthy male students and found an increase in the plasma concentrations of triglycerides, total cholesterol and phospholipids at the end of the high-sucrose period.

In a normal individual it seems likely that dietary carbohydrate can affect the serum cholesterol level only if the diet contains some fat, and doubtless the type of fat would have a more marked influence on serum cholesterol than the type of dietary carbohydrate (38).

(iii) Glyceride and phospholipid level changes through dietary modification.

Lyon et al (57) found that oral glucose resulted in an increase in the capacity of the liver to convert added acetate to fatty acids in fats. Bragdon et al (58) found a decrease of serum glycerides 1 hr. after the acute feeding of sucrose, and similar findings were separated after intravenous glucose in man (59). 35-40 days after rats had eaten a diet containing potato starch (9%) as the carbohydrate the serum glyceride concentration was elevated (60). Macdonald and Roberts (61) found no increase in serum glycerides in rats after 12 weeks on a fat-free diet containing glucose, fructose or sucrose as the carbohydrate. However they found evidence of adaptability in the serum-lipid response to fructose and sucrose, as opposed to glucose as the dietary carbohydrate. Using ^{14}C-labelled carbohydrate incorporation studies, more dietary sucrose and fructose than glucose was incorporated into serum glycerides with time. However, the one unknown factor in incorporation studies is the pool size. Hence, if as is quite possible, the pool size of fructose is extremely small compared to that of glucose, the dilution of the latter may lead to the wrong interpretation regarding rate of incorporation of ^{14}C-carbohydrate.

Coltart and Crossley (62) observed the effects of a ^{14}C-sucrose meal in baboons. Before the sucrose diet the blood fructose levels following a ^{14}C-sucrose meal were the same in both male and female baboons, an observation similar to that found with man. After 13 weeks on the sucrose diet, a ^{14}C-sucrose meal led to higher levels of blood fructose in male baboons than in females, and therefore resulted in greater

incorporation of fructose into triglyceride in the male animals. This is parallel to the situation in men and pre-menopausal women. Coltart et al suggest that this sex difference operates at a level of absorption rather than at one of metabolism.

Bailey et al (64) found higher hepatic enzyme activities of those enzymes thought to be associated with lipogenesis, in rats receiving dietary fructose or sucrose than in those fed diet 86, or glucose. Zakim et al (65) found a greater incorporation of acetate into lipid by homogenates of liver from rats which had been fed a sucrose rich diet than from those given a starch rich diet. The results of Bailey et al (64) showing an increased enzyme activity produced by fructose and sucrose could not be explained by greater intake of the two carbohydrates.

Hill (66) observed that in young rats fructose increased the serum phospholipids without elevation of triglyceride, while in mature rats hyperglyceridemia was followed by elevation of serum phospholipid. The increase in serum and liver triglyceride suggests a difference in the metabolism of carbohydrate or triglyceride, or both, with age.

During 25 days on a high carbohydrate diet, 7 men showed significant falls in serum phospholipid levels when the carbohydrate was starch, but no fall when sucrose replaced the starch (33). Young women showed a fall in phospholipid concentrations with both starch and sucrose diets (35) as did some post-menopausal women (36). Serum phospholipids were significantly reduced in both men and women on high-cereal diets and increased with a high "simple" carbohydrate diet (34). In 5 patients with hyperglyceridemia a high sugar intake tended to raise the serum phospholipids, whereas carbohydrate as starch lowered the phospholipid level (67). 19 male subjects previously on an isocaloric sucrose free diet exhibited a rise in fasting serum phospholipid levels after resuming a surcrose-containing diet (68). At the end of a sucrose enhanced diet (at the expense of starch-rich foods) 23 healthy male students showed a rise in plasma phospholipid, cholesterol and triglyceride concentrations (56).

Chevalier et al (69) showed that dietary fructose, as compared to glucose, enhanced lipogenesis from fructose but did not affect lipogenesis from glucose or acetate. They found (70) that with weanling and mature rats fed a diet containing 70.1% glucose, starch, sucrose, or fructose;

the dietary fructose or sucrose increased serum triglyceride levels in the mature but not in the weanling rats.

Dalderup et al (71) studying the life-span of Wistar strain albino rats, found that male animals with 30Cal/100Cal sucrose in their food had a shorter life-span than controls receiving 14.5Cal/100Cal, whereas the life-span of the females was not affected. They repeated the experiment (72) with weanling male animals and found no difference between the control animals and those with an extra sucrose enhanced diet, thus indicating that there are other factors besides the sucrose consumption to be considered.

Adams (73) reported a lower survival rate in BHE strain rats fed a diet containing 25% cooked dried whole egg and 39% sucrose, than in rats fed 100% cooked whole egg. Allen Durand et al (74) observed the effects of different carbohydrates in rats fed a 25% cooked egg diet. Sucrose-fed rats had larger livers at 150 and 350 days than rats fed cornstarch or glucose. At 150 days of age, sucrose-fed BHE rats had 37% total lipid as compared with 30% in the glucose fed rats. Wistars had 24% total lipid and 21%, respectively, for sucrose and glucose fed animals.

Ahrens et al (75) report that in a study on the effect of dietary carbohydrate source on the responses of rats of two different ages to forced exercise, exercise significantly reduced food intake, body weight gain, liver weight, the concentration of lipid and cholesterol in the liver, serum insulin level and the fat content of the carcass. In both studies, among the exercised animals, those fed the diet containing a mixture of starches and sugars commonly found in the human diet tended to gain more body fat than those fed a cornstarch diet. Among the sedentary animals those consuming the cornstarch diet tended to gain more body fat than those fed the carbohydrate mixture.

Serum glycerides in man are of considerable interest because hyperglyceridemia is closely associated with ischemic heart disease (76,77). Harvel (78) found a reduction in serum glyceride levels in man after hourly glucose doses for 4 hr. comparable to changes in the rat. Swan et al (79) reported a fall in the free fatty acid (FFA) concentration in men given 100g. of glucose, sucrose or starch, the level remaining depressed longer with starch than with sucrose or glucose.

Volunteer prisoners who consumed a low-fat, high-carbohydrate diet for 39 weeks showed immediate and significant rises in their fasting serum glycerides, with peak values after 5 weeks on the diet (80). These high levels then subsided slowly and took 32 weeks before the glyceride level returned to the base level in some subjects. Gillman et al (81) using ^{14}C-labelled glucose to discover the origin of the serum glycerides found that the activity was confined to the glycerol moiety of the glycerides and that the fatty acid was mobilized in the depots or synthesised from a precursor other than the labelled glucose. Partially hydrolyzed starch (75% total calories) and fruit juice caused a marked rise in fasting serum glycerides compared with a high (daily) fat diet (82). In the same subjects the fasting FFA was at a lower level than the glycerides.

Waterhouse et al (83) suggest that the glyceride pool produced by high-carbohydrate feeding is more static than it is under the influence of low-calorie or high-fat diets. Sucrose elevated the serum glycerides of men on a low-fat high-carbohydrate diet (33). Maltose and glucose caused a fall in serum glycerides in a fat-free diet and sucrose was associated with a significant rise in serum glycerides, whereas cornstarch and partially hydrolyzed starch evoked no response (37). Macdonald (47) postulated that the elevation was due to the fructose in sucrose.

Bender & Thadani (84) reported depressed lipogenesis in both liver and adipose tissue in rats fed sucrose as cf. starch. Hepatic lipogenesis differed in different strains of rats. The sucrose effect was equally marked in fed and 24 hr. fasted rats. However Delboca et al (85) report that fasting leads to a marked impairment of lipogenesis, and even in the presence of large amounts of insulin the conversion of glucose to fat in adipose tissue cannot be restored to normal values. Brice et al (86) confirm that the pattern of eating can influence the metabolic response to a diet in man.

Froesch & Ginsburg (87) find that adipose tissue metabolizes fructose slightly less rapidly than glucose and independently of it. Fatty acid synthesis is stimulated above the expected level in the presence of both hexoses than with individual hexoses. Insulin stimulates fructose uptake only in the absence of glucose.

Paul et al (88) reported that myocardial infarct or coronary disease patients had consumed more sucrose than a control group but that the difference was less striking than reported by Yudkin and Rodday (89). Evidence suggests (90) that both hypercholesterolaemia and hypertriglyceridemia are associated with coronary heart disease, and that both lipids should be measured in coronary risk detection. Mounting evidence indicates that abnormalities in carbohydrate metabolism are in some way associated with ischaemic heart disease. Epidemological studies are consistant with such an hypothesis. Thus, studies in subjects with chronic coronary heart disease show a high incidence of carbohydrate intolerance which is not simply due to the presence of obesity (91).

Carlson and Bottiger (92) in a follow-up study of 3168 men found that the rate of ischaemic heart disease (I.H.D.) increases linearly with increasing fasting concentration of plasma-triglycerides and plasma cholesterol. Plasma triglycerides and cholesterol are risk factors for I.H.D. independent of each other, and a combined elevation of these two plasma lipids carries the highest risk for I.H.D.

There is some doubt as to whether fructose is the carbohydrate mainly responsible for serum glyceride elevation. Kaufmann et al (93) report a transient decrease in serum triglycerides in 11 normal subjects and 8 patients with carbohydrate induced hypertriglyceridemia. 4hr. after the fructose load, no significant differences were found in peripheral blood levels of fructose, glucose, pyruvate and lactate. Their results refute the concept that intestinal absorption of fructose is abnormal in patients with carbohydrate-induced hypertriglyceridemia. Walker (94) reached the conclusion that such evidence as is available does not incriminate sucrose as the causative agent in coronary heart disease (C.H.D.). Bearing in mind the multifactorial aetiology of C.H.D. it was questionable whether, within a given context, major incrimination of sucrose was possible.

Jourdan (95) found that intravenously injected fructose disappeared more rapidly than glucose from the serum of male baboons than from that of female baboons. He (96) also recorded that intravenously injected ^{14}C-fructose was more rapidly incorporated into the glycerides of male

baboons than female baboons. After glucose injection the specific activity in the glycerides was significantly less than after fructose injection, and no difference between the sexes was detectable.

^{14}C-(U)-fructose was incorporated three times more rapidly than ^{14}C-glucose into the triglyceride-glycerol of 7 patients who had had a myocardial infarct (97). The radioactivity of triglyceride glycerol was 10-20 times greater than that of triglyceride fatty acids.

Male Wistar rats were fed for 7 months on a diet containing 72% fructose, glucose or starch and they were then fasted for 18 hr. and given an intraperitoneal injection of 2.5g. fructose plus 2.5g. glucose per kilogram body weight. The animals were then either killed immediately or 30 min. after injection. These experiments showed that the fructose diet produced the poorest growth, but animals had the heaviest livers with high nitrogen contents. The cholesterol blood level produced by fructose was significantly higher than that of the animals on the other diets. The concentration profiles of the metabolites in the livers after fasting and after receiving the fructose/glucose injection showed that the fructose fed animals were best adapted for rapid metabolism of this monosaccharide and that the livers of these animals contained most α-glycerophosphate (98).

Hill et al (99) fed rats with glucose or fructose as sole carbohydrate for 3 days and then examined their response to orally administered glucose (glucose tolerance test). In the case of the fructose-fed animals there was an impaired capacity to utilize glucose.

The glucose tolerance (G.T.) curves in the fructose-fed rats resembled those observed with fasted (96hr.) rats. Their evidence indicated that the decrease in G.T. observed in the fructose-fed animals was the result of decreased liver glucokinase activity. These findings were considered to be a manifestation of enzymic adaptation to diet.

Inverted intestinal sacs of the guinea pig, but not of the rat, convert fructose to glucose during its passage through the intestinal wall (100). Ginsburg and Hers (100) conclude that in the guinea pig the conversion is mediated, as in the liver, by fructose 1-phosphate and triose phosphate intermediates and that the inability of the rat intestine to convert fructose to glucose is due to the absence of glucose-6-phosphatase (see scheme).

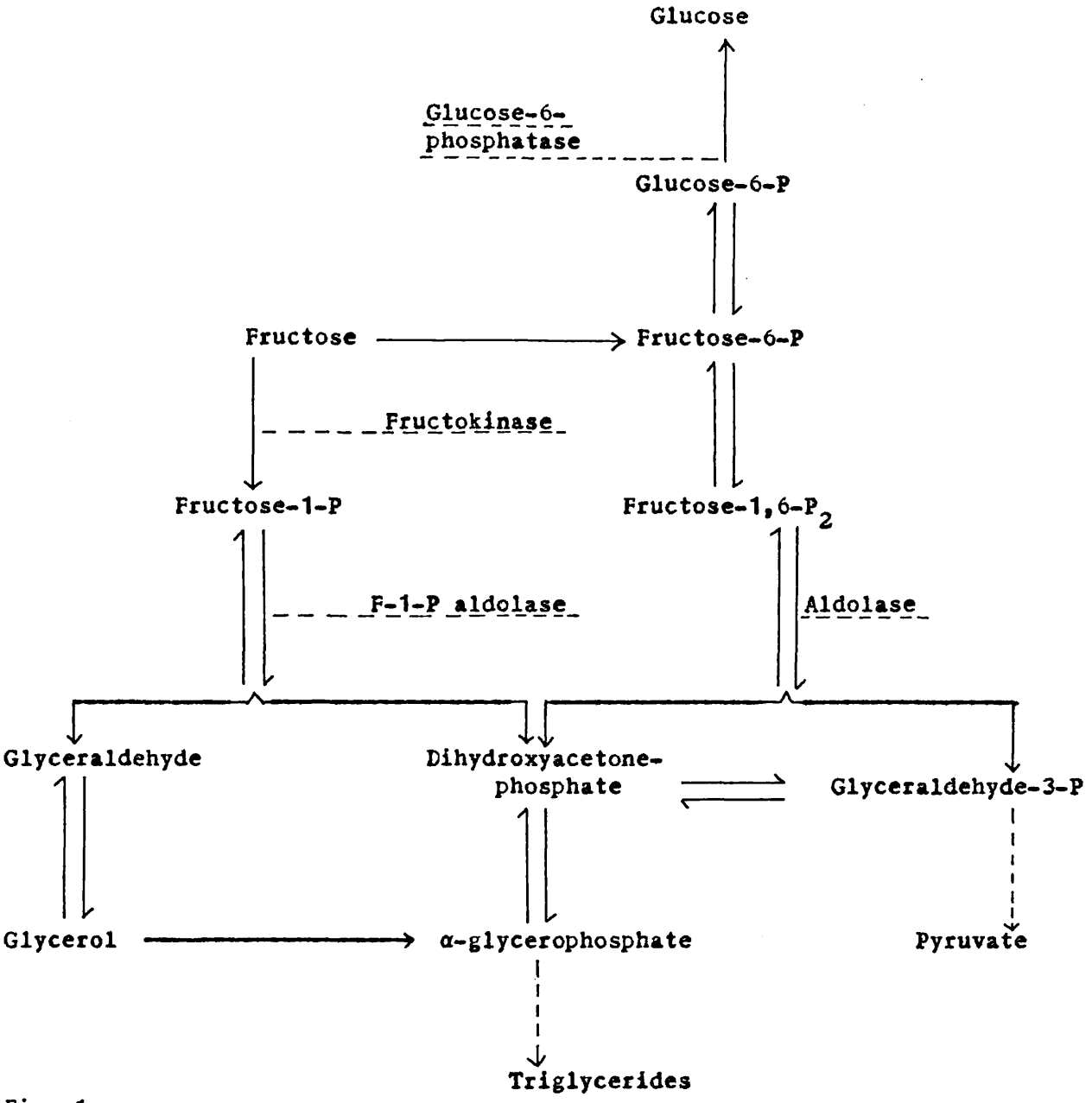


Fig. 1

Fructose to Glucose Conversion in Rat Liver.

Ballard (101) showed that in liver from fetal rats younger than 20 days, glucose is formed from both 1-^{14}C and 6-^{14}C-fructose without cleavage of the molecule by the aldolase reaction. The rate of glucose formation from fructose in the fetuses is approximately half that of the adult liver rate, and the direct conversion is not via sorbitol as in seminal vesicles. Liver slices incubated with ^{14}C-(U)-fructose gave similar intermediate products (refer scheme) whether from liver of 18-day foetal, newborn or adult rats.

Ballard and Oliver (102) report that the activity of fructokinase (ketohexokinase) is very low, or even absent (103) in fetal rat liver, and that the rate of incorporation of fructose into glycogen is low in liver slices from fetal rats (104). Walker (105) suggests that fructokinase reaches adult levels of activity 7-10 days after birth.

Fructokinase develops after birth in both fructogenic and non-fructogenic species and this may represent another example of an enzyme responding to hormonal changes occurring as a result of parturition (106).

In a study of jejunal enzymes (107) fructose had a specific adaptive effect upon fructokinase (E.C.2.7.1.3) and fructose 1-phosphate aldolase, whilst glucose exerts its adaptive effect upon hexokinase and glucokinase. Fructose 1,6-diphosphate aldolase activity is increased by sucrose, fructose, and glucose. Fructose had little effect on hexokinase, glucokinase and aldose reductase whereas glucose caused a maximal change in these enzymes. Sucrose, except for fructose 1,6-diphosphate aldolase changes, gave results intermediate between fructose and glucose.

The addition of calories in the form of casein to the diet of fasted rats caused a non-specific increase in the activity of all jejunal glycolytic enzymes studied. The changes in the rat jejunal glycolytic enzymes due to diet reflect those in rat liver.

The rate of incorporation of fructose into plasma and liver lipids of guinea pigs was 4 to 6 times that of glucose after intraperitoneal or oral administration (108).

Landau et al (109) found that in subjects without hereditary fructose intolerance (HFI-a disease characterized by a genetic deficiency of fructose 1-phosphate aldolase) the conversion of fructose to glucose appeared to be solely through Fructose 1-phosphate; hence in these subjects, and presumably in man in general, fructose is not converted via sorbitol to glucose in the liver.

A large increase of liver fructose 1-phosphate was reported after a large intraperitoneal injection of fructose (110). This is interpreted as the result of the inadequacy of fructose 1-phosphate aldolase activity to complement fructokinase activity and hence the aldolase is

a limiting factor in this pathway. It is suggested that the hexokinase route predominates when the system is flooded with fructose, whereas fructokinase is more active when fructose levels are low.

Muntz and Vanko (111) observing the effects of intraportally injected 1-^{14}C and 6-^{14}C fructose found appreciable amounts of α-glycerophosphate derived from both C_1 and C_6 of the fructose. This supports the view that glyceraldehyde is rapidly converted to α-glycerophosphate after fructose-1-phosphate scission. Unlabelled glyceraldehyde injected together with fructose did not reduce the label appearing in the products isolated. However, as aldehydes are particularly liable to isomerization and dehydration, no significant emphasis should be placed on this observation (112-115).

One third of the men in a sample study given a diet high in sucrose developed an increase in the immunoreactive insulin level in the blood (116). In the same men this was accompanied by a considerable increase in weight and increase in platelet adhesiveness. It has been established that the increase of liver size in rats induced by the substitution of sucrose for starch is caused by an increase in the number of cells, or at least in the number of nuclei. With fructose instead of starch, the effect is due to an increase in both cell number and cell size (117).

Insulin injected into rats has been shown to increase the deposition of lipid into the aortic wall (118).

The fact that only a proportion of men show "sucrose-induced hyperinsulinism" suggests that only certain people may be susceptible to the effects of sucrose in producing atherosclerotic disease (119).

Naismith et al (56) conclude that the hyperlipedemia (expressed as elevated plasmatriglycerides, total cholesterol and phospholipids) which resulted from the consumption of sucrose-rich diets was induced by sucrose itself and not by any change in voluntary calorie intake. Roberts (120) showed that a slight fall in fasting serum glyceride concentrations occurred during a sucrose-free period in 19 male subjects, but there was a significant rise in the glyceride concentration when the sucrose diet was resumed. This reached a peak at 8 weeks, followed by a return to the pre-dietary level over the following 16 weeks. Serum phospholipid rose after resuming the sucrose-containing diet, but there

were not significant changes in weight or serum cholesterol.

Sunflower-seed oil in the diet produced a significant fall in the fasting serum triglyceride, irrespective of the type of carbohydrate ingested or the sex of the individual (121). In men, cream caused a significant rise in fasting serum triglyceride when fed with fructose-starch or glucose-starch carbohydrate mixtures, but not with fructose-glucose mixtures. In women, cream was not associated with any significant change in fasting serum triglyceride levels.

An increase in the activity of fatty acid synthetase in male rats on diets of sucrose and fructose as opposed to corn starch, maltose or glucose has been reported (122).

Chevalier et al (69) suggest that a diet containing a high percentage of fructose causes a shift in the site of lipid synthesis from adipose tissue to the liver. Thus, while fatty acid synthesis in adipose tissue of the fructose-fed animals decreased, enzyme synthesis in liver increased.

α-Glycerophosphate stimulated fatty acid synthesis in vitro (123). Glucose and fructose in normal animals, and fructose in diabetic animals that lack insulin, produce a substance that stimulates the production of enzymes involved in fatty acid synthesis (124). The substance may be α-glycerophosphate which serves to remove a feedback inhibitor such as long chain acyl-CoA. This in turn could lead to the derepression of synthesis of these enzymes. Alternatively, but less likely, α-glycerophosphate may act as an inducer.

Zakim et al (65) found a higher concentration of α-glycerophosphate in the livers of rats fed a normal "purina" chow diet than with a high carbohydrate diet. In contrast, the amount of fatty acids synthesized from 1-^{14}C acetate by liver slices in chow fed animals was less than $^{1}/_{10}$th of that from tissues from carbohydrate-fed animals. Comparing concentrations of α-glycerophosphate the highest α-glycerophosphate concentration was present in the chow fed group and was associated with the lowest incorporation of isotope into fatty acids. α-Glycerophosphate concentration in rats fed high carbohydrate diets were essentially the same in all groups. These experiments suggest that contrary to what might be expected from results with systems

in vitro (123-125), the hepatic concentration of α-glycerophosphate does not correlate directly with fatty acid synthesis. It is possible that increased fatty acid synthesis removes α-glycerophosphate as glyceride and α-glycerophosphate accumulates where fatty acid synthesis is primarily decreased. α-Glycerophosphate then appears to play a passive rather than an active role in fatty acid synthesis. Under some circumstances a low concentration of α-glycerophosphate could limit the rate of fatty acid synthesis, a high α-glycerophosphate concentration does not seem to be a sufficient stimulus for increasing hepatic lipogenesis. It seems, therefore, that carbohydrate induction of fatty acid synthesis must be explained other than by the formation of an increased concentration of α-glycerophosphate.

Thus, it has been shown that fructose causes an increase of hepatic fatty acid synthesis and that fructose metabolism leads to an increased concentration of α-glycerophosphate in rat liver. These increased concentrations of fatty acids and α-glycerophosphate would presumably lead to increased triglyceride synthesis.

A comparison of pre-menopausal women on a low-fat diet containing either cornstarch or sucrose showed that with the latter carbohydrate a fall in serum glycerides occurred (35). In similar experiments with men sucrose was associated with a rise in serum glycerides (33), similarly, post-menopausal women on the sucrose diet exhibited an increase in serum glycerides (36). Macdonald (47) found that men and post-menopausal women on a fat free diet including fructose exhibited a rise in serum glycerides, whereas in pre-menopausal women there was a fall in serum glycerides. Jourdan (96) reports a greater incorporation of injected fructose into glyceride in male than in female baboons. The incorporation of glucose into glycerides in these animals was significantly less than in the case of fructose, and no difference between sexes was detectable in this instance.

The influence of the subject's sex on dietary carbohydrate lipid interrelationships is in accord with the incidence of ischemic heart disease, namely a high incidence in men and post-menopausal women and a low incidence in pre-menopausal women. The fact that men and post-menopausal women show similar glyceride responses would suggest that estrogens or progesterone, or both are influencing the metabolic handling

of dietary fructose (38).

Reinke et al (126) studied 10 female subjects and showed that during the luteal phase of the menstrual cycle (ie. at the highest progesterone level) there was a statistically significant increase of free fatty acids found in the blood which was accompanied by a distinct rise in free glycerol. Seng et al (127) found that with females using hormonal contraceptives, blood levels of glyceride-glycerol and triglycerides were significantly augmented. The same results were obtained in principle, when they investigated late pregnancies (128). Combined estrogen/progestagen oral contraceptives give rise to increased fasting serum triglyceride and, to a lesser extent, increased fasting serum cholesterol levels (129-133). Elevated mean fasting serum-triglyceride levels were reported in women on all oral contraceptive preparations (134), the effect being greater with increasing estrogen content of the pill. The most estrogenic pills gave the highest triglyceride values, and those with the most progestagen gave the highest cholesterol values.

Several reports show no significant effect on serum-lipid levels in men receiving progesterone (135-137), whilst very small amounts of orally active androgens can reverse the estrogenic effect on serum cholesterol and lipoproteins (137). Since the 19-nor-steroids (synthetic progestagens) used in oral contraceptives are closely re ated chemically to orally active androgens, an effect of such progestagens on serum-cholesterol is not unexpected (134). Wynn et al (133) showed that the changes in serum lipids and lipo-proteins produced by oral contraceptives resemble the pattern des-cribed by Fredrickson as type-IV hyperlipoproteinemia (138). There is as yet no agreement that there is a direct connection between type-IV hyperlipoproteinemia and the accelerated development of athero-sclerosis but there is evidence of a positive association (139-143).

Most of this work has been carried out on males, but since oral contraceptives tend to change the lipoprotein pattern of pre-menopausal women to one resembling that of men and post-menopausal women, the findings with men are indeed relevant. Tendencies towards obesity and impaired glucose tolerance, which are additional factors in the development of atherosclerosis, should be carefully considered

since both are associated with the use of oral contraceptives (144).

A study of 20 surgically castrated women and 65 female controls suggest that women who have been castrated for at least 5 years have no more coronary atherosclerosis than their ovary bearing, age-matched peers (145) and that castration does not adversely influence the risk of coronary artery disease created by coronary risk factors. However, Renaud (146) found that Premarin (a conjugated estrogen) when added to the diets of rats fed a butter-rich hyperlipemic diet, was effective in reducing both the serum cholesterol and triglyceride levels, and the severity of the atherosclerotic lesions. The lower dosage used in these experiments was the most effective in prolonging coagulation and in preventing thrombosis. When a higher dosage was used, however, a marked increase in the plasma corticosterone level and a decreased serum albumin level was observed.

Cherondache and Blinstruh (147) in a study of 240 elderly subjects (age range 65 to 95 years) reported that women showed a higher incidence of coronary artery disease than the men. In the atherosclerotic patients there was a high concentration of total serum cholesterol and a lower level of cholesterol-esterifying activity than in the healthy elderly subjects. These differences are more significant in women than in men.

With oral contraceptives an increased platelet adhesiveness has been described in a small number of patients (148). In another investigation (149) platelet behaviour in women receiving oral contraceptives resembled that of patients with arterial disease, in that there was an increase of platelet sensitivity to adenosine diphosphate. Cutts (150) found that in some cases (12%) a direct platelet effect on arterial walls could be produced experimentally by administering estrogens to a group of rats.

Despite the above evidence, the Framingham study (151) suggests that clinical coronary heart disease may be at least 10 times more frequent in men than in pre-menopausal women of a similar age group, and Stamler (152) has summarized clinical and autopsy evidence which strongly suggests that estrogen has a protective effect against heart disease. Experimentally, a decreased incidence of cholesterol-

induced coronary atherosclerosis in chicks receiving estrogen is well established (153).

It is paradoxical that on the one hand a positive relationship between estrogen-containing oral contraceptives and myocardial infarction exists and on the other hand there is evidence to suggest a role for estrogens in guarding women against infarction. The explanation may be that normally estrogen is a factor in preventing or retarding the atherosclerotic process but that it accelerates thrombogenesis and resultant myocardial infarction in the relatively rare susceptible group of individuals.

Certain clinical trials with estrogen appear to substantiate this explanation. Robinson et al (154) treated 57 survivors of myocardial infarction with high doses of estrogens and found a 38 per cent increase of new cardiovascular events. They also found a persistent lowering of total serum cholesterol and the ratio of cholesterol to phospholipid. In a study of patients with cerebral vascular disease a higher incidence of cerebral and myocardial thrombotic events were found in the group treated with estrogens (155). Stamler et al (152), however, concluded that estrogen therapy is beneficial in long-term survival of patients with coronary heart disease, despite the unexpectedly high incidence of fatal and non-fatal cardiovascular, renal and thromboembolic complications of recent survivors of myocardial infarction receiving a high dose (10mg. daily) of estrogen.

(c) Population distribution. Although populations from various countries have widely diverging incidence rates (12-14), the atherosclerotic disease process in all susceptible individuals at the time of infarction is of similar proportions. This same finding holds true when one compares the coronary arteries in young and old persons who have had a myocardial infarction. When an infarct is present; the amount and gross distribution of the atherosclerosis is the same in the young and older hearts (10).

(d) Extent of Atherosclerosis. Although the incidence of myocardial infarction increases with the amount of atherosclerosis in the coronary arteries, at any level of atherosclerosis only a proportion of the hearts will have developed an infarct; less than 50% of acute myocardial

infarctions are associated with fresh thrombi in the coronary arteries. The exact sequence leading to muscle death still has to be determined. Coronary atherosclerosis gives the setting in which muscle death becomes more likely. It is clearly not the entire cause.

(e) Hypertension. The pressure in the arterial tree (arteries around the heart) ie hypertension, accelerates arterial disease in man (4, 156-160) and in cholesterol, or fat-fed animals (161,162). In general, the influence of hypertension is more pronounced in diseases accompanied by hyperlipidemia, but arterial disease is observed even in the absence of elevation of serum lipids.

(f) Diabetes. The increased incidence of myocardial infarction among diabetic patients is well recognised (163-165).

(g) Smoking and Obesity. In males, the incidence rate of coronary heart disease (C.H.D.) in white non-cigarette smokers was 52.7/1000 and among blacks, 9.8/1000; among white cigarette smokers the rate was 101/1000 but in blacks only 32.5/1000. The incidence rate of C.H.D. increased with increasing obesity, and smokers run a substantial risk of developing C.H.D. increasing with increase in overweight (166).

(h) Physical Activity. The relationship between physical activity and morbidity or mortality from C.H.D. remains a subject of considerable controversy. Despite extensive research on the subject (167-182), there is no concensus as to whether sustained physical activity is protective against C.H.D. Cassel et al (183) in a seven-year follow up study of a county population put forward the proposition, based on their own observations and those of other workers, that there was a modest inverse relationship between physical activity and either the incidence prevalence of, or mortality from C.H.D. (171,172,177,181), and that there was little evidence (178,179,182) to suggest that physical activity may be protective against C.H.D. above some critical threshold value.

Two other reports (184,185) state that there is no important difference in either incidence of, or mortality from C.H.D. between active and sedentary men working in the same industry, even though the active men expend over 50% more calories than the sedentary. This would suggest that under such circumstances the threshold would have to be greater than 50% above the caloric expenditure of sedentary industrial workers. Cassel et al (183) conclude that the suggestion

by Rose et al (182) that physical activity leads to an increased caliber of the coronary vessels thus making atherosclerosis less consequential, is consistent with the known facts and may provide a plausible biological explanation for the role of physical activity. That atherosclerosis has many possible causes is very evident from the information already presented. That the dietary regimen is of crucial importance in the etiology of the disease is also evident.

Elevated levels of plasma cholesterol certainly appear to be a fairly accurate indicator of incidence prevalence for C.H.D., more so as cholesterol appears as a major component of the fat content of the arterial plaques in the more advanced stages of the disease.

Triglyceride, however, is present in greater proportions in the arterial plaques in the earlier stages of the atherosclerosis disease process in the coronary arteries. As sucrose and fructose are readily converted to triglyceride in the liver, the presence of the di- and mono-saccharide in the diet is of importance. The increase of serum glycerides in men after fructose ingestion, not observed in pre-menopausal women, is similar to the incidence prevalence of C.H.D. to which the male sex is more prone. It would appear therefore, that a sex difference in the metabolism of sucrose might be an important factor in the etiology of C.H.D.

Sucrose Metabolism in the Intestine and Liver

Carbohydrates, proteins and lipids are the three major basic constituents of the human diet. The proportions of different carbohydrates in the diet have been changing fairly rapidly in the more affluent countries in the last hundred years, with the result that sucrose is assuming more prominence at the expense of starch. A national food survey in the United Kingdom in 1967 indicated the following approximate carbohydrate contributions to the daily diet; Starch 175g; sucrose 140g; lactose 20g; other sugars (almost entirely fructose and glucose) 15g; (186).

Dietary sucrose is hydrolyzed by the enzyme sucrase (α-D-fructofuranosidase; E.C. 3.2.1.26) in the brush border of the intestinal mucosa to give equal proportions of glucose and fructose. The main site of sucrase activity is located in the mucosal cells of the small intestine and not in the gut lumen (187-191). The majority of the disaccharide is not hydrolyzed in the intestine, but is, instead, absorbed intact, and then hydrolysed by the intracellular enzyme (190).

Sucrase appears to be induced by a sucrose rich diet (192).

Gray and Ingelfinger (193) found the same intestinal absorption rates for fructose and glucose no matter whether sucrose or an equivalent mixture of fructose plus glucose was fed.

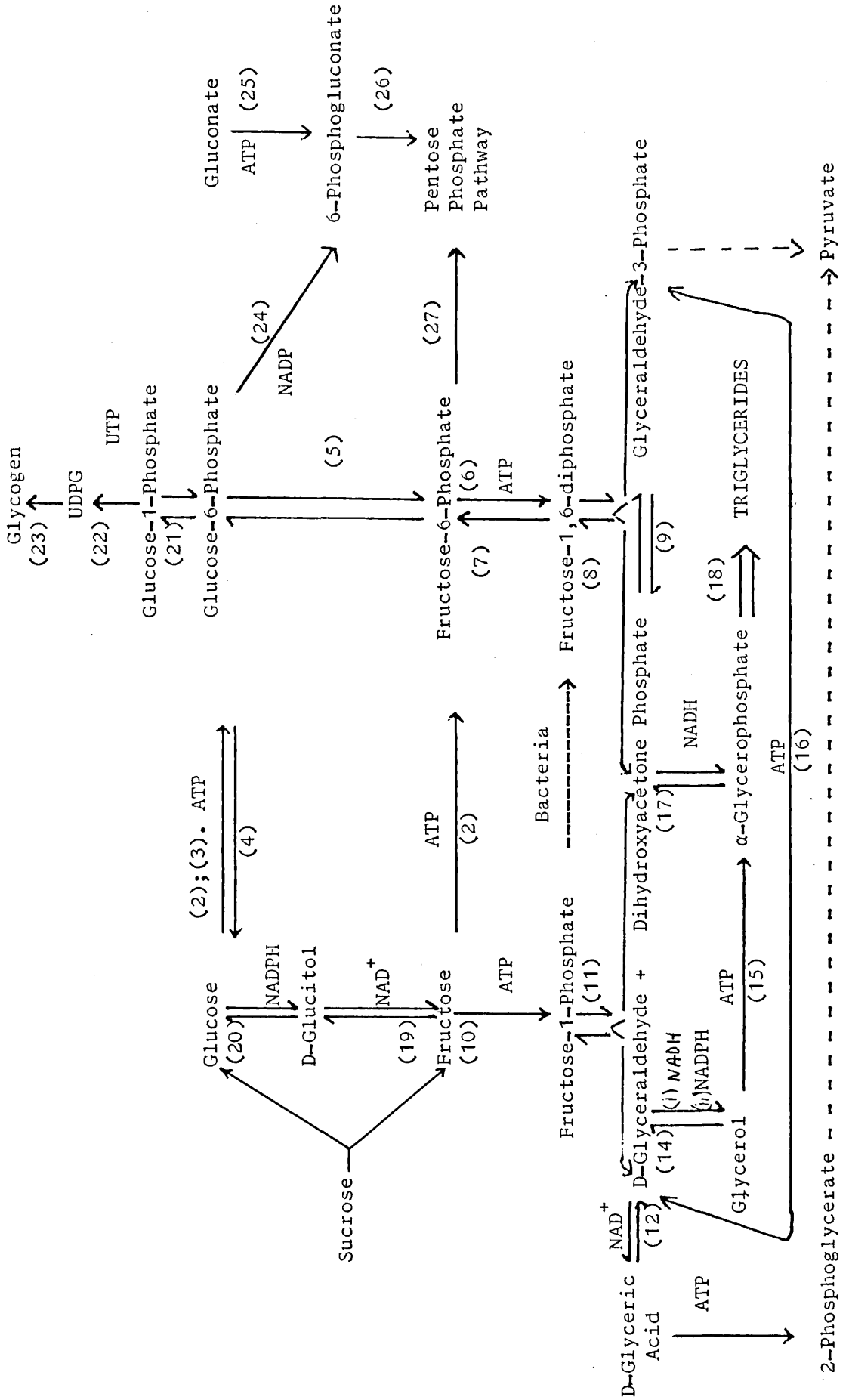
This means that it is not the individual absorption rates of glucose and fructose but the hydrolysis of sucrose which determines the latter's rate of absorption.

There is interconversion of glucose to fructose via glucitol in the seminal vesicles (194) but this has not been reported to occur in the intestine or liver (195,196). The main metabolic pathways for glucose and fructose are shown in Fig.2. Major differences in the pathways can be found at the phosphorylation and triose formation stages.

In the metabolism of glucose in liver the first stage is the phosphorylation of the hexose to glucose 6-phosphate by hexokinase (ATP: D-hexose 6-phosphotransferase; E.C. 2.7.1.1) or glucokinase (ATP: D-glucose 6-phosphotransferase; E.C. 2.7.1.2).

Hexokinase is relatively unspecific and utilizes ATP as a phosphate donor converting a number of different hexoses to the corresponding 6-phosphates.

Fig. 2 Pathways of Sucrose Metabolism



The Enzymes Involved in Sucrose Metabolism

(1) Sucrase or Invertase (E.C.3.2.1.26)
(2) Hexokinase (E.C.2.7.1.1.)
(3) Glucokinase (E.C.2.7.1.2)
(4) Glucose 6-phosphatase (E.C.3.1.3.9)
(5) Glucosephosphate isomerase (E.C.5.3.1.9)
(6) Phosphofructokinase (E.C.2.7.1.11)
(7) Hexose diphosphatase (E.C.3.1.3.11)
(8) Fructose 1,6-diphosphate aldolase (E.C.4.1.2.b)
(9) Triosephosphate Isomerase (E.C.5.3.1.1)
(10) Fructokinase or Ketohexokinase (E.C.2.7.1.3)
(11) Fructose 1-Phosphate aldolase (.E.C.4.1.2.7)
(12) Aldehyde dehydrogenase (E.C.1.2.1 3)
(13) Glycerate kinase (.E.C.2.7.1.31)
(14) Glycerol dehydrogenase (E.C.1.1.1.6)
(15) Glycerol kinase (.E.C.2.7.1.30)
(16) Triokinase (E.C.2.7.1.28)
(17) α-Glycerophosphate dehydrogenase (E.C.1.1.18)
(18) Glycerolphosphate acyltransferase (E.C.2.3.1.15)
(19) Glucitol dehydrogenase (E.C.1.1.1.14)
(20) Aldose reductase (E.C.1.1.1.21)
(21) Phosphoglucomutase (E.C.2.7.5.1)
(22) UDPG-pyrophosphorylase (E.C.2.7.7.9)
(23) Glycogen synthetase (E.C.2.4.1.11)
(24) Glucose 6-Phosphate dehydrogenase (E.C.1.1.1.49)
(25) Gluconokinase (E.C.2.7.1.12)
(26) Phosphogluconate dehydrogenase (E.C.1.1.1.44)
(27) Transaldolase (E.C.2.2.1.2) and Transketolase (E.C.2.2.1.1)

This probably occurs by a direct transfer of the phosphoryl group from the donor to the acceptor without a phosphoryl-enzyme intermediate (196). All enzymes transferring phosphate groups from nucleotide triphosphates require Mg^{2+} ions (197). Hexokinase activity is controlled by the cellular level of glucose 6-phosphate and glucose utilisation is dependent on the removal of this hexose-phosphate (198).

Soluble hexokinase is more sensitive to product inhibition at low ATP and less sensitive at high ATP concentrations than is the mitochondrial enzyme (199). Differential centrifugation and localization techniques have suggested that the bound hexokinase is located on the outer mitochondrial membrane (200). However Wilson (201) reports an intraparticle location of the enzyme but offers no clear physiological interpretation. Appreciable amounts of hexokinase in latent form have been reported in the rat (202,203) and guinea pig (204), however the metabolic significance is presently unknown.

Grossbard & Schimke (205) report the occurrence of three hexokinases in various rat tissues. The three enzyme types I (brain and kidney), II (skeletal muscle and epididymal fat pad) and III (liver) have different electrophoretic and chromatographic properties, as well as different low Km values when glucose is used as substrate, are similar with respect to pH optimum, molecular weight, hexose and nucleotide specificities, Km value for fructose, and the qualitative nature of inhibition by ADP and glucose 6-phosphate.

Casein non-carbohydrate diets have been shown to decrease the intestinal hexokinase activity in comparison with starved animals (206). High fructose diets do not increase hexokinase activity whereas high-glucose or sucrose diets increase hexokinase activity but only in comparison with the casein-fed animals.

Rat hepatic hexokinase activity increases after weaning (21 day old animals) to a maximum at 40-45 days before falling to pre-weaning values (70 days). Sucrose has little effect on this enzyme (207).

Rat kidney hexokinase activity increases after birth to a maximum at 30 days of age, thereafter falling to reach adult values at 50 days (208). These results are an extension of the findings of Sydow (209).

Glucokinase is a much more specific enzyme and appears to be restricted to the liver (210).

The enzyme from rat liver which has been purified (211) does show absolute specificity for glucose as substrate. Hence 2-deoxy glucose and mannose are both phosphorylated but they exhibit high Km values. However, in view of the low Km value, glucose is the only substrate likely to be phosphorylated by glucokinase under physiological conditions. The enzyme is inhibited by high concentrations of glucose 6-phosphate but glucokinase does not possess the special allosteric site for inhibition by product as do many mammalian hexokinases. ADP is an inhibitor but here the degree of inhibition depends on the Mg^{2+} ion concentration.

Sols et al (212) report that glucokinase appears in rat liver only some time after birth and its presence in the adult liver depends on induction by insulin. Insulin induction of glucokinase has been confirmed (213-215) and as a corollary the kinase activity in rats depends on the amount of glucose present in the diet (216). Glucokinase activity is decreased on feeding rats a fructose diet; this may be interpreted as a consequence of the inability of fructose to stimulate the release of insulin from the pancreas (217, 218).

Glucose 6-phosphate can be reconverted to glucose by glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase E.C. 3.1.3.9) or it can be isomerized to fructose 6-phosphate by glucosephosphate isomerase (D-glucose 6-phosphate ketolisomerase E.C. 5.3.1.9).

Glucose 6-phosphatase catalyzes the following reaction:

D-Glucose 6-phosphate $+H_2O \rightarrow$ D-glucose + orthophosphate.

In rat liver, enzyme activity is localized in the endoplasmic reticulum and nuclear envelope; the other cytoplasmic organelles possess no activity (219-223).

Inorganic pyrophosphate-glucose phosphotransferase activity appears to be identical with glucose-6-phosphatase, eg.

Glucose + $PP_i \longrightarrow$ Glucose-6-phosphate + P_i, and this same protein also appears to possess inorganic pyrophosphatase activity.

In rats there is a slow build up of hepatic activity to 18 days gestation followed by a marked increase at term. Thyroxine, glucagon,

epinephrine and cyclic-AMP are known to cause premature development of activity (224-227).

Rats fed diets in which protein, fat, galactose or fructose is substituted for a direct glucose source show a marked increase in liver glucose 6-phosphatase specific activity. This response to diets low in glucose is a metabolic adaptation expressed through increased synthesis of an enzyme directly involved in glucose biosynthesis (228).

Glucosephosphate isomerase catalyses the interconversion of glucose 6-phosphate to fructose 6-phosphate. No substrates other than these two phosphates are known (229). The enzyme is present in most tissues at very high levels (230) and has been purified from bovine mammary gland: this isomerase has a molecular weight of 48,000 (231).

The Michaelis constants for both hexose phosphates are very small (fructose 6-phosphate, 1.0×10^{-5}M; (glucose 6-phosphate 3.0×10^{-5}M). This results from the fact that the reaction velocity with substrate excess is approximately the same in both directions, even though equilibrium substantially favours glucose 6-phosphate formation (230).

In the case of isomerases from rabbit brain and muscle, and human erythrocytes, ATP, phosphoenol pyruvate, 6-phosphogluconate, phosphate, pyrophosphate and high concentrations of Mg^{2+} ions are known inhibitors (229,230).

Fructose 6-phosphate may be converted to fructose 1,6-diphosphate by phosphofructokinase (ATP D-fructose 6-phosphate 6-phosphotransferase; E.C. 2.7.1.11) as follows:

Fructose 6-phosphate + ATP $\longrightarrow$ fructose 1,6-diphosphate + ADP.

This enzyme has quaternary structure and is regulated by a variety of agents, including substrates, substrate analogues, AMP and cyclic 3',5'-AMP (232,233). The catalytic activity of the enzyme is inhibited by high concentrations of ATP, which specifically inhibit the binding of fructose 6-phosphate and this inhibition is reversed by inorganic phosphate. Citrate, a structurally unrelated compound, increases the affinity of the enzyme for ATP and decreases its affinity for AMP and fructose 6-phosphate (234).

Phosphofructokinase from brain and muscle is inhibited *in vitro* by phosphocreatine, 3-phosphoglycerate, phosphoenolpyruvate, 2-phosphoglycerate and 2,3-diphosphoglycerate (235). This inhibition is most

pronounced at neutral pH values, in the presence of inhibitory levels of ATP and non-activating fructose 6-phosphate levels and *in vivo* the whole phenomenon is probably important for the regulation of glycolysis.

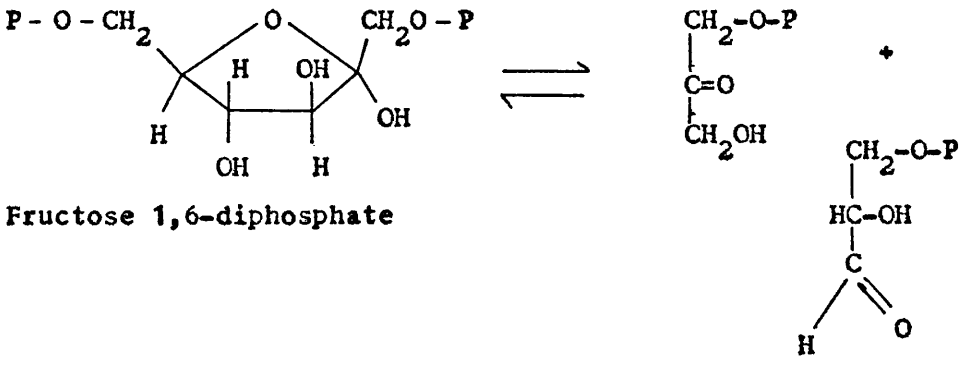
Paetkau and Lardy (236) have purified rabbit muscle phosphofructokinase and shown that it requires K^+ ions for maximal activity and is inactive at pH 7 unless the Mg^{2+} ion concentration exceeds the total ATP concentration. Enzymic activity is proportional to the number of reduced SH groups and is reversibly lost by oxidation. The smallest fully active form of the enzyme has a molecular weight of 3.8×10^5 and can be reversibly dissociated into units one-half or one-quarter this size.

Fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase; E.C. 3.1.3.11) with Mg^{2+} ions as cofactor will catalyse the reconversion of fructose 1,6-diphosphate to fructose 6-phosphate.

This enzymic step is presumed to be obligatory for glyconeogenesis in order to bypass the highly exergonic phosphofructokinase reaction (237).

Fructose 1,6-diphosphatase is inhibited by AMP, the degree of inhibition depending upon the concentration of fructose 1,6-diphosphate and ATP. At low fructose 1,6-diphosphate concentrations AMP is less inhibitory than at higher concentrations. The inhibition by AMP is partially reversed by ATP (238). The AMP/ATP ratio in the cells must, therefore, effect a delicate control of the fructose 6-phosphate-fructose 1,6-diphosphate interconversion by regulating both phosphofructokinase and fructose 1,6-diphosphatase.

The enzyme aldolase (Fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate lyase; E.C. 4.1.2.b) will cleave fructose 1,6-diphosphate yielding dihydroxyacetone phosphate and glyceraldehyde 3-phosphate as follows:



Fructose 1,6-diphosphate

Dihydroxy acetonephosphate

Glyceraldehyde-3-phosphate.

Claims have been made that the aldolase activities of liver with fructose 1,6-diphosphate and fructose 1-phosphate are due to different enzymes (239) although the studies of other investigators indicate that a single enzyme can account for both activities (240-244).

Crystalline fructose diphosphate aldolase preparations obtained from rabbit muscle and bovine liver exhibit different enzyme kinetics (245). However, in spite of these distinctive catalytic properties the two protein molecules have similar molecular weights and numbers of sulphydryl groups (243,246-248), although they differ in amino acid composition (240) and immunochemical properties (101,240-244,249-254). Treatment of the muscle enzyme with carboxypeptidase yields a protein which has a similar substrate specificity and catalytic activity to the liver enzyme (251).

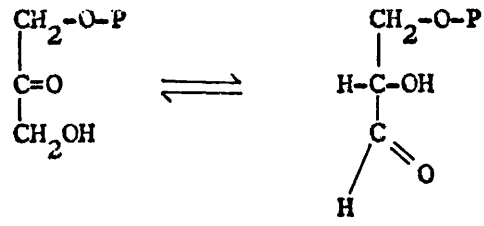
Rabbit muscle aldolase has a molecular weight of 160,000, and in cold alkaline borate solution dissociates into 4 sub-units of molecular weight 41,400-42,000 (254).

Normal and carboxypeptidase-treated muscle and liver aldolases are competitively inhibited by fructose 6-phosphate (K_I 1-2mM) with fructose 1,6-diphosphate as substrate (241).

In vitro rat muscle aldolase is competitively inhibited by ATP> ADP> AMP, and Mg^{2+} ions reverse the ATP inhibition. However liver aldolase is inhibited by AMP$\gg$ ADP and is not affected by ATP (209).

Liver aldolase activity in female rats is constant in 21-40 day old animals, rises to a maximum at 50 days and then falls until 70 days. Sucrose produces some elevation of enzyme activity at all these stages of development (207).

The two products of fructose 1,6-diphosphate cleavage, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate can be interconverted by <u>triose phosphate isomerase</u> (D-glyceraldehyde 3-phosphate ketol isomerase; E.C. 5.3.1.1):



This enzymic reaction was a high catalytic activity and is one of the connecting links between the main fructose metabolising and the gluconeogenic pathways.

The equilibrium constant $\frac{\text{Dihydroxyacetone phosphate}}{\text{Glyceraldehyde 3-phosphate}}$ is 22(255,256).

Phosphate ions inhibit the enzyme (255,256).

The first enzymic step in the metabolism of fructose in the liver involves fructokinase (ATP: D-Fructose 1-phosphotransferase; E.C.2.7.1.3). There is some confusion over the terminology of this enzyme. It is referred to as both fructokinase (E.C.2.7.1.3) or ketohexokinase (E.C. 2.7.1.3), however, in this thesis the former nomenclature will be used.

In the presence of ATP, fructokinase phosphorylates fructose to fructose-1-phosphate. The enzyme has an absolute requirement for Mg^{2+} ions (or Mn^{2+}) and is strongly activated by high concentrations (1-2M) of K^{+} ions (257,258). p-Chloromercuribenzoate is an inhibitor of fructokinase which is stable up to 50-55^{o} (206).

Fructokinase can be assayed in the presence of hexokinase first by partially destroying the hexokinase by acid treatment followed by inhibition of the remainder with 2-acetamido 2-deoxy-D-glucose (259).

Liver fructokinase acts on the three ketohexoses: fructose, sorbose and tagatose (257,260). Aldohexoses and fructose-6-phosphate are not phosphorylated. The affinity for fructose is very high , the K_s value being $< 5 \times 10^{-4}$M.

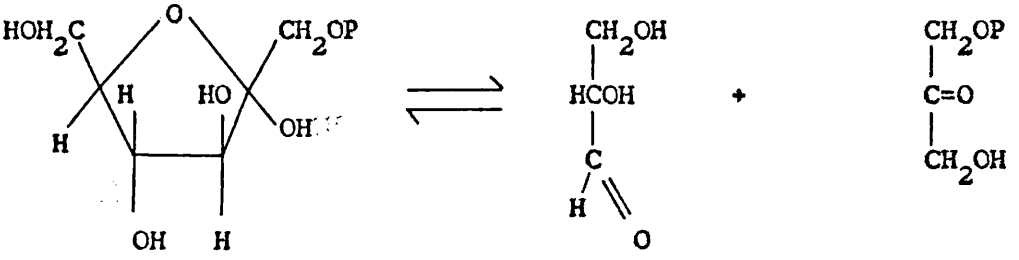
Rat liver fructokinase has been purified to a near homogeneous state (206).

Fructokinase is not found in fetal rat livers (103) but Ballard and Oliver (102) suggest that it may be present at a very low level. However, Ballard (101) observed high conversion rates of fructose to glucose in fetal rat liver which is probably due to the action of hexokinase, an enzyme known to be present in neo-natal animals.

Liver fructokinase activity is increased in rats on a fructose-enriched diet (99) or when fructose is administered intraperitoneally (261). Fasting rats lose their enzyme rather rapidly - 50-60% total activity remains after a 3-day fasting period (261) Normal fructokinase activity in fasted male rats is restored by feeding fructose: recovery of activity occurs within a 4- to 8- hr. period following a lag of about

16 hr., and roughly parallels increase in liver weight. Complete recovery occurs after 24 hr. Fructokinase activity of adrenalectomized rats on a normal diet is the same as the fasting level of normal rats and is neither lowered further by fasting nor increased by subsequent feeding of fructose or glucose (262). Long term feeding of fructose to normal rats results in a maintained, considerably higher enzyme level than that in animals on a high fat or high protein diet (262).

The cleavage of fructose 1-phosphate by fructose 1-phosphate aldolase (ketose 1-phosphate aldehyde lyase, E.C. 4.1.2.7) produces glyceraldehyde and dihydroxyacetone phosphate.



The role of this aldolase in the metabolism of fructose 1-phosphate in the liver was recognised in 1952 by Hers et al (195,264) and Leuthardt et al (265,266). The enzyme may be the same aldolase which reacts with fructose 1,6-diphosphate in the glycolytic pathway (240-244).

The reaction with fructose 1-phosphate is freely reversible, however the equilibrium favours the fructose ester (263).
Spolter et al (241) reports that the kinetic behaviour of rabbit muscle and liver aldolases towards fructose 1-phosphate in the presence of glyceraldehyde, a product of the reaction, indicates that the cleavage of the phosphate by both native and partially degraded (carboxypeptidase-treated) forms of the liver enzyme is an ordered reaction in which glyceraldehyde is released before the enzyme-dihydroxyacetone phosphate complex is dissociated. With the native or carboxypeptidase-treated muscle enzyme it is a random reaction with no obligatory sequence in the release of either product from the enzyme.

Fructose 1-phosphate aldolase activity decreases to about one-half of its normal total activity when male rats are fasted for 48-72 hr. and activity is completely restored in 24 hr. by feeding fructose (262); feeding glucose has a similar effect. As with fructokinase, there is a 16 hr. lag period on feeding fructose before aldolase activity is restored to normal.

Long term fructose feeding results in considerably higher aldolase levels than in the case of high fat or protein diets (262); this is again similar to the behaviour of fructokinase.

Alloxan-diabetic rats have similar levels of aldolase to normal animals, as do fed, adrenalectomized rats. In the latter case fasting produces a rapid decrease in activity which is not alleviated by subsequent feeding of fructose or glucose. The same pattern was found with hypophysectomized as with adrenalectomized rats (262).

Heinz et al (267) report ratios of aldolase activity, measured with fructose 1,6-diphosphate and with fructose 1-phosphate, of 2.62 (n=5, S.D=0.24) for rat liver and 1.67 (n=11, S.D=0.16) for human liver. These figures indicate that there are differences between the two aldolases. This observation is in agreement with those of Anstall, Lapp and Trujillo (268) who showed that the aldolase isozyme patterns were different in human and rat livers.

Hereditary fructose intolerance (H.F.I.) is due to a lack of fructose 1-phosphate aldolase. Children with this condition thrive in the absence of a dietary source of fructose, but in the presence of the monosaccharide, fructosemia, fructosuria, hypoglycemia, aminoaciduria, glycosuria, proteinuria and acidosis may occur (269).

Cain and Ryman (270) report a high liver glycogen level in a case of hereditary fructose intolerance in humans. At necropsy the liver enzyme estimations showed that fructose 1-phosphate aldolase activity was absent and the fructose 1,6-diphosphate aldolase activity reduced. (cf Hers and Joassin (271)). Cain and Ryman (270) have questioned whether fructose 1-phosphate aldolase and fructose 1,6-diphosphate aldolase activities are due to two separate catalytic proteins or whether they represent two activities of the same enzyme protein one of which is totally, and the other partially, lost in H.F.I.

Glyceraldehyde is the centre of a metabolic 'crossroads' as shown in the figure. (Fig.2a).

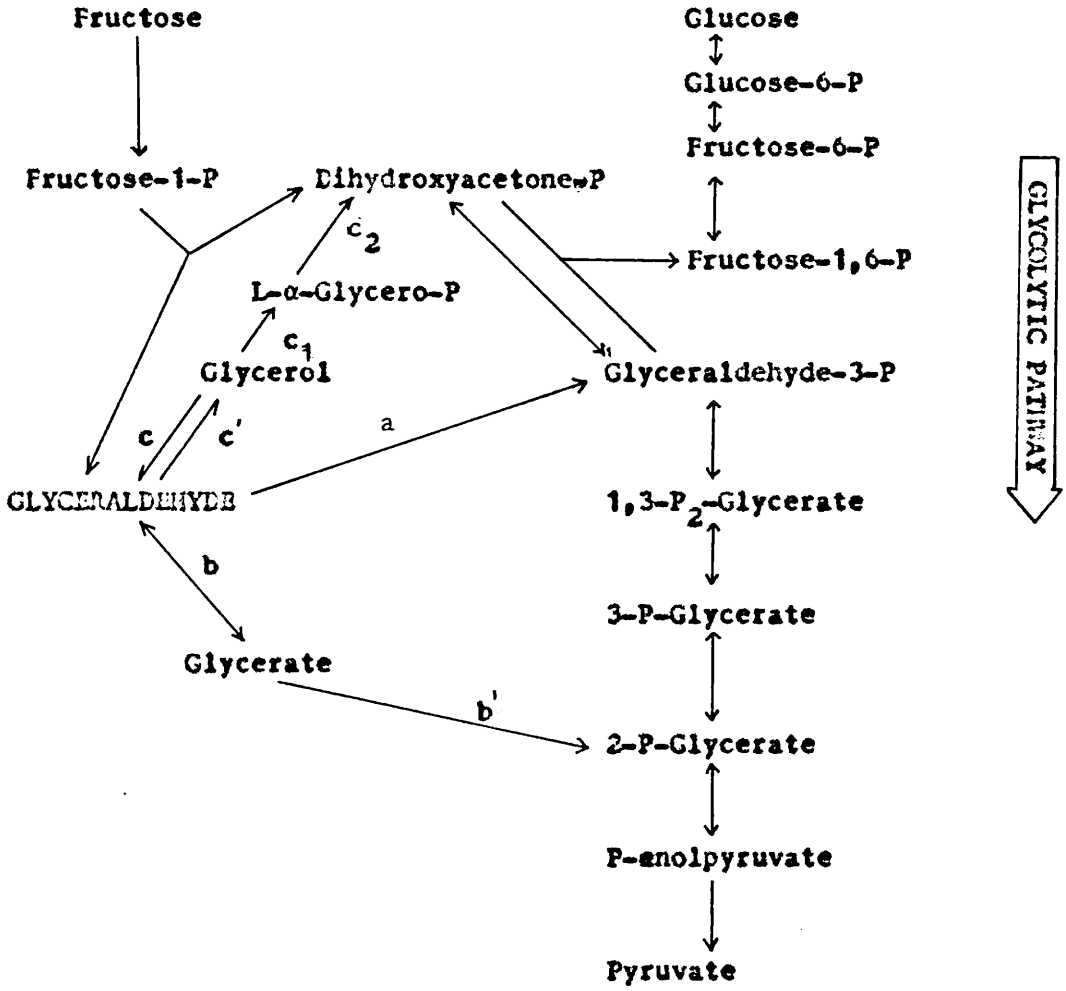


Fig. 2a

see Sillero et al (217)

Fructose metabolism in liver and glyceraldehyde 'crossroads'.

a - triokinase; b - aldehyde dehydrogenase; c - c'alcohol dehydrogenase

There are three postulated pathways for the further metabolism of glyceraldehyde by the glycolytic pathway: (a) it can be converted to glyceraldehyde 3-phosphate by means of a triokinase (195); (b) it can be converted to 2-P-glycerate via glycerate, the reactions being catalysed by aldehyde dehydrogenase (266, 272,273) and glycerate kinase (274-276); and (c) an alcohol dehydrogenase (277-279), glycerol kinase (280,281) add glycerolphosphate oxidase (282) can together effect dihydroxyacetone phosphate production.

Sillero et al (217) on the basis of a study of female rat liver enzymes with animals on different regimens of nutrition conclude that the triokinase pathway (a) prevails for fructose metabolism. The name triokinase (ATP:D-glyceraldehyde 3-phosphotransferase; E.C. 2.7.1.28) was given to the enzyme which converts D-glyceraldehyde to the corresponding 3-phosphate and dihydroxyacetone to the 1-phosphate (195), in the presence of ATP and Mg^{2+} ions. The rate of phosphorylation is approximately the same for both triose substrates, neither reaction being inhibited by glycerol. L-Glyceraldehyde is not phosphorylated by the enzyme (283).

In human liver, the activity of triokinase is 2.7 units/g. (267) therefore direct phosphorylation of D-glyceraldehyde can probably occur in this tissue.

A second possibility is that glyceraldehyde can be phosphorylated indirectly to α-glycerophosphate by successive reaction with alcohol dehydrogenase and glycerol kinase. This is perhaps unlikely, however, as there is a low level of the latter enzyme in human liver (267).

Rat liver possesses a significantly lower level of triokinase and a higher level of glycerol kinase than the human tissue.

Aldehyde dehydrogenase (Aldehyde: NAD (P) oxidoreductase E.C. 1.2.1.3.): This enzyme catalyses the conversion of D-glyceraldehyde to D-glycerate. The enzyme oxidizes a number of substrates viz. formaldehyde, acetaldehyde and propionaldehyde. It is a sulphydryl enzyme with a sharp pH optimum at 9.3 (284).

The activity of aldehyde dehydrogenase in human liver is three-quarters of that found in rat liver (267).

Glyceric acid enters the Emden-Meyerhof glycolytic pathway after phosphorylation to 2-phosphoglycerate which is catalysed by Glycerate kinase (ATP:D-glycerate 2-phosphotransferase; E.C. 2.7.1.31).

Purified rat liver glycerate kinase is completely inactive towards L-glycerate and has a Michaelis constant for D-glycerate of about one-tenth of that found for the horse-liver enzyme (275). Mg^{2+} ions cannot be replaced by Mn^{2+} ions, and kinetic studies suggest that an ATP-Mg^{2+}-ATP complex could be the substrate for the reaction (285).

Glycerate kinase activities for human and rat livers were 0.13 u/g and 3.19 u/g, respectively, (267). The authors (267) believe that in

human liver the capacity of the kinase is too low to play any physiological role. If D-glyceraldehyde were oxidized to D-glyceric acid, this substrate would accumulate in human liver and escape into the blood.

Glycerol Dehydrogenase (Glycerol:NAD oxidoreductase, E.C.1.1.1.6)

Wolf and Leuthardt (286) reported the presence of NAD^+ dependent glycerol dehydrogenase in rat- and pig- liver extracts which catalysed the reduction of DL-glyceraldehyde to glycerol. The equilibrium for this reaction strongly favours glycerol, and dihydroxyacetone is a poor substrate for this enzyme.

Moore (279) detected $NADP^+$ specific glycerol dehydrogenase in rat liver with a total activity one-eighth that of the NAD^+ dependent enzyme. The equilibrium again favours glycerol formation, and the enzyme is strongly inhibited by p-mercuribenzoate.

A $NADP^+$ specific glyceroldehydrogenase was also found, predominantly in the non-particulte fraction of the cell, in skeletal muscle (287). Dihydroxyacetone is reduced at 5% of the rate of D-glyceraldehyde by this enzyme. The rate of reaction with NADH is only 10% of that with NADPH. The pH optimum stated is 7.0 for D-glyceraldehyde reduction, and 9.0 for glycerol oxidation. This enzyme has been purified by Karmann et al (288) who showed that it has a molecular weight of $34{,}000 \pm 1000$.

NAD^+-dependent glycerol dehydrogenase appears to be present only in liver whereas the NADP dependent glycerol dehydrogenases are found in placenta, liver, kidney, heart, skeletal muscle, small intestine, brain, lung and adipose tissue (289). Kinetically, however, there are several major differences between the NADP-dependent glycerol dehydrogenases from different organs, and they can also be distinguished on the basis of inhibition by fluoride (289). Toews (289) suggests that the reaction mechanism for skeletal muscle glycerol dehydrogenase is probably an ordered mechanism, where the first product leaves the enzyme before the addition of the second substrate.

Glycerolkinase (ATP: glycerol phosphotransferase; E.C.2.7.1.30) catalyses the reaction.

$$ATP + Glycerol \longrightarrow L\text{-glycerol-3-phosphate} + ADP$$

Hers (285) uses the name L-triokinase which describes adequately the catalytic properties of the enzyme; the term glycerolkinase is more

descriptive of its presumed physiological function.

Boblitz and Kennedy (280) purified the rat liver enzyme 170-fold and crystallization by Wieland and Suyter (281) gave a 580-fold purification.

The purified enzyme catalyses the phosphorylation of dihydroxy acetone and L-glyceraldehyde twice as fast as that of glycerol. The product of this phosphorylation can be acylated in the liver to give phosphatidic acids which in turn are further acylated to triglycerides.

Glycerol is a molecule in which the C-2 carbon atom can be represented thus:

Where the two CH_2OH groups are not geometrically equivalent but bear a mirror-image relationship to each other. The C-2 carbon atom has been termed a meso-carbon. Enzymes which catalyse reactions involving compounds bearing meso-carbon atoms invariably discriminate between the two non equivalent but chemically identical groups. Thus the phosphorylation of glycerol by glycerolkinase results exclusively in the formation of L-α-glycerophosphate rather than the DL-mixture that would otherwise result (290).

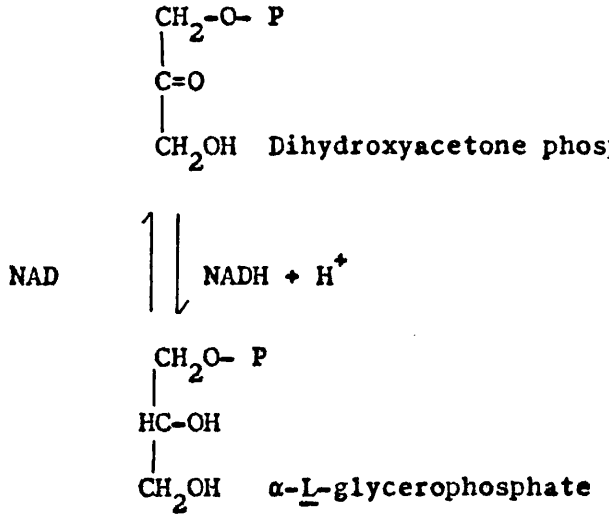
Maximum activity of glycerolkinase is obtained with a ratio ATP/Mg^{2+} of 2. Mn^{2+} ions can replace Mg^{2+}, but Ca^{2+} is inhibitory. UTP can serve as phosphate donor but the reaction rate is then half that obtained with ATP (280).

Adelman et al (262) report a total triokinase activity of about one-half of normal in male rats that had been fasted for 48-72 hr. This was also observed by Kampf et al (291). Adelman et al (262) report normal levels of triokinase activity in fed adrenalectomized rats, and a sharp decrease in activity on fasting, which does not recover on subsequent glucose or fructose feeding. Essentially the same pattern is found for hypophysectomized as for adrenalectomized rats (262). However, Kampf et al (291) stated that there was a decrease in enzyme activity after adrenalectomy, but an increase after cortisol substitution of the adrenalectomized animals.

Triokinase activity, reduced on starvation, is restored to normal after feeding fructose for 24 hr; the recovery occurring within a 4- to 8- hr. period following a lag of about 16 hr. (262). Feeding fat for the first 3 days lowers the enzyme activity, but increases it after 3 days (276). Long term feeding of fructose produces a considerably higher level of enzyme in comparison with high fat or high protein diets (262). Glucose feeding of previously fasted animals only partially restores the enzyme activity (262).

An increase of α-glycerophosphate formation in female ovariectomized rats given estradiol-17β was reported by Lea et al (292). Significant increases were observed 4hr. after injection with a maximum rate occurring after 16 hr. These increases were significantly reduced by anti-estrogenic agents and progesterone. Actinomycin and cycloheximide resulted in almost completed inhibition of the induced increases of α-glycerophosphate.

α-Glycerophosphate is formed either by phosphorylation of glycerol by the enzyme glycerokinase or by the action of a NADH - requiring α-glycerolphosphate dehydrogenase (L-glycerol 3-phosphate : NAD oxidoreductase, E.C. 1.1.1.8). This enzyme reacts with dihydroxyacetone phosphate in the following way



A soluble glycerolphosphate dehydrogenase has been reported (293) and also a particulate enzyme, localized in the outer part of the inner membrane of the mitochondrion (294).

There appears to be a constant ratio in different tissues between the activities of mitochondrial α-glycerolphosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase, one of the key glycolytic

enzymes (295). Thus, in highly oxidative tissues such as cardiac and red skeletal muscle, the α-glycerolphosphate dehydrogenase (mitochondrial) level is low (296); whereas in glycolyzing tissues like white skeletal muscle this enzyme is very active (295).

A high activity of mitochondrial α-glycerol phosphate dehydrogenase is also found in rat and hampster brown adipose tissue (297,298).

It is apparent from Figure 2 that a major difference in the metabolism of fructose and glucose is that the former is converted to glyceraldehyde and dihydroxyacetone phosphate; whereas glucose is catabolized to the dihydroxyacetonephosphate and glyceraldehyde 3-phosphate. This difference may account for the fact that fructose is a better precursor of triglycerides than glucose. For example, glyceraldehyde may be converted more rapidly to α-glycerophosphate than either glycoraldehyde 3-phosphate or dihydroxyacetone phosphate. α-Glycerophosphate, of course, can accept acyl groups from acyl Co A derivatives and is hence a major precursor of triglycerides.

The Sex Hormones and their effect on Carbohydrate and Lipid Metabolism

Sex hormones have been found to influence the development of atherosclerosis. Estradiol improves the condition by correcting abnormal lipid levels. Testosterone on the other hand aggravates the disease by being antagonistic towards estrogen and by increasing cholesterol levels (114).

Once secreted by endocrine glands the steroid hormones, bound to specific hormone-binding proteins, use the blood stream to reach their target cells. These hormones penetrate the cells, in contrast to the action of certain peptide hormones which act at the level of the external membrane. In the cell the hormones act to change certain enzyme levels and as a consequence the metabolic pathways of the cell will be modified.

The activities of enzymes (Δ^4-5α-hydrogenase, steroid hydroxylases, 3α- and 3β-hydroxysteroid dehydrogenase and 20-ketoreductase) concerned with the metabolism of both male and female hormones were studied in rat liver with respect to their dependence on age and sex over a period of 1-180 days after birth (299). A uniformly increasing activity, with no difference between the sexes was observed during the infant phase (the first 30 days). Sexual differentiation of steroid metabolizing enzymes in the rat liver is a process which starts at birth (300). The experimental data of Denef and De-Moor (301) shows that the potential development of the steroid metabolizing enzymes is basically feminine in the newborn male rat. Testosterone, which appears within a few critical days after birth, primes a masculine differentiation. The hormone at that moment acts as an organizer so that at the age of puberty (30 days), a masculine pattern of metabolism comes to expression and is retained at a significant level during the whole lifespan, even in the absence of any further source of male sex hormones *eg.* after castration and adrenalectomy.

Schriefer *et al* (299) similarly report that the infant phase in rats is followed by a phase of variable length during which sexual differences in metabolism are first apparent (puberty). This is followed by the phase in which enzyme activities remain at a sex-specific plateau (full sexual maturity). Finally, in the so called ageing phase, there is

a decrease in Δ^4-5α-hydrogenase activity: levels of other steroid-metabolizing enzymes do not, apparently, change during this phase.

The biosynthetic pathways for the biosynthesis of sex hormones have been well established and these are summarised in Fig. 3.

Estrogens

The estrogens in combination with other hormones, are responsible for the development and maintenance of the female sexual organs. Estrogens are produced in the gonads, the placenta and the adrenal cortex. The main site of formation in the female is normally the ovary, but during pregnancy the quantity synthesised by the placenta increases until in late pregnancy this contribution may be a thousand times greater than that of the ovary. Of the three main estrogens in humans, estradiol-17β is biologically more potent than estrone or estriol, as judged by bioassays.

Estrone is produced in a sulphated form by the adrenal cortex (302) and this derivative is reduced to estrodiol-17β 3-mono sulphate which in turn is hydrolysed by microsomal enzymes to estradiol-17β. Estrone sulphate is more readily converted to estradiol-17β than is estrone itself (303). This is presumably because sulphate groups protect estrogens from biological inactivation (304).

The studies of Means and O'Malley (305) indicate that estradiol-17β, when it first appears in the uterus, binds to a cytoplasmic receptor protein (Fig.4). This complex is then modified in some manner and transferred to the nucleus. A stimulation of synthesis of nuclear RNA ensues and this leads to enhancement of protein synthesis, some of which appears in the cytoplasm, and some in the nucleus. It is possible that the induction phase may result in an amplification phase; (i.e., increased synthesis of nuclear RNA which in turn is transported to the cytoplasm for the overall regulation of uterine protein synthesis).

Estrogen induces a profound oviduct growth in chicks (306), primarily by hyperplasia (increased amount of DNA). Progesterone alone has little or no effect on oviduct growth, however, in combination with estrogen it antagonizes the estrogen-induced hyperplasia of oviduct, but synergizes with estrogen in eliciting cellular hypertrophy. In gonadectomized rats exposure to a constant physiological dose of labelled

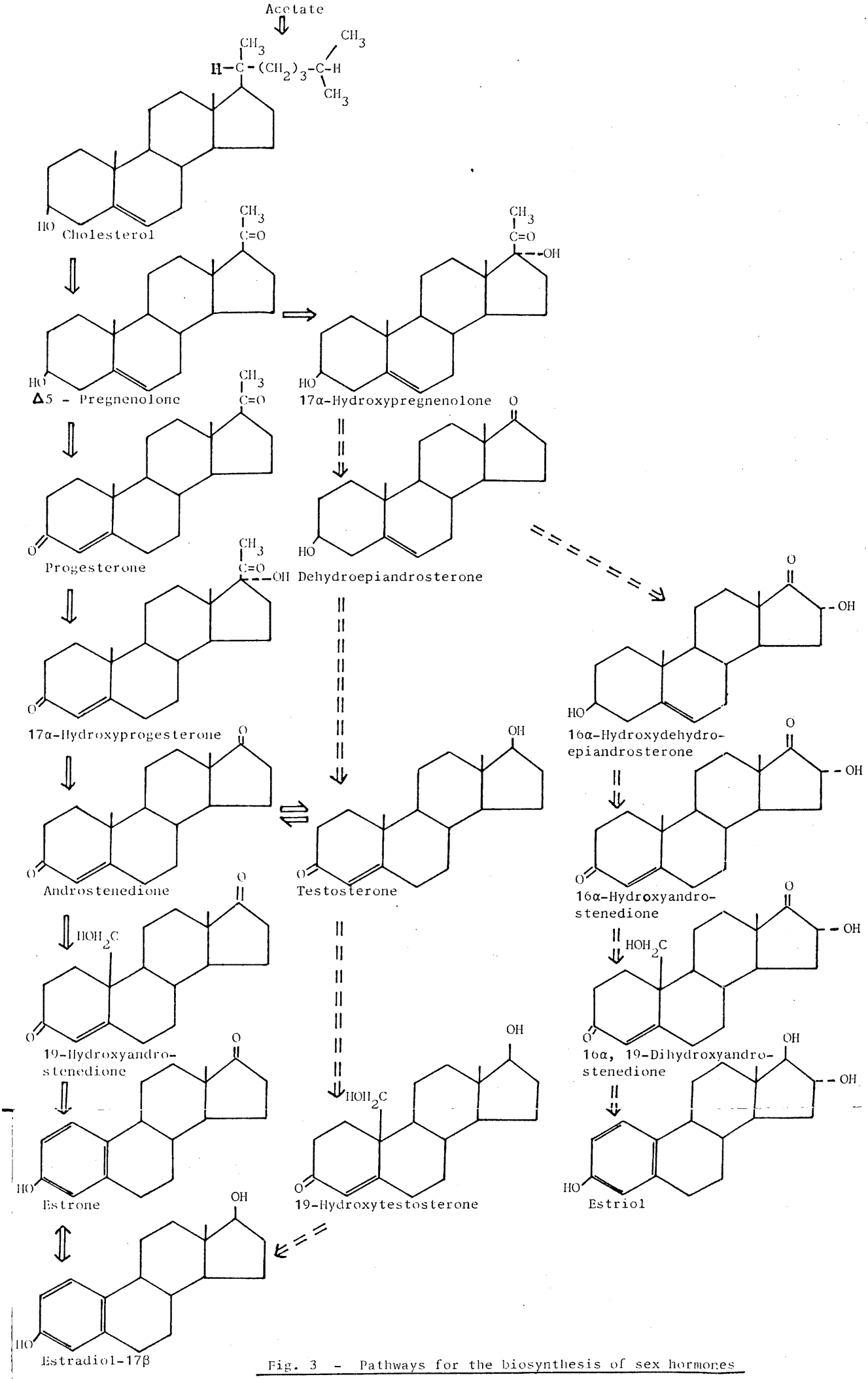
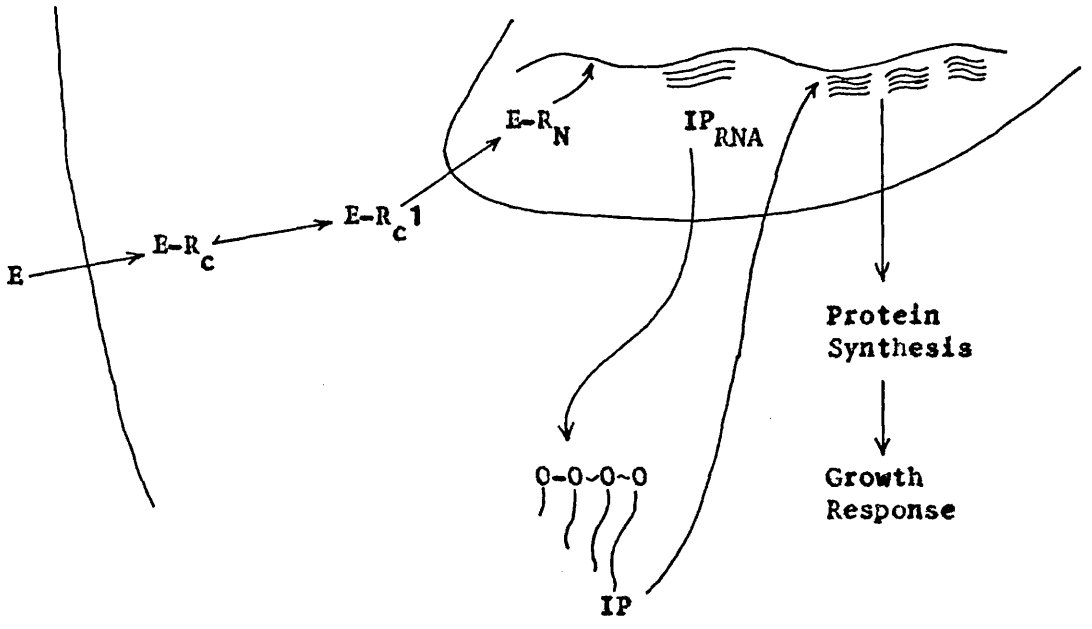


Fig. 3 - Pathways for the biosynthesis of sex hormones

(ex "Estrogens" in Hormone Chemistry, Ed. Butt,W.R., Ch.13, p.280)



E - Estrogen

R_c - Cytoplasmic receptor protein

R_N - Nuclear receptor protein

IP - Cytoplasm

IP_{RNA} - Nuclear Ribonucleic Acid

Fig. 4 The biochemical effects of estrogens on rat uterus

estrogen for a few days results in a highly significant increase in retention of the label by rat uteri. Progesterone administration during final phases of estrogen treatment greatly reduces the retention of the latter in the target tissues (307). These observations are consistent with the known synergisms of the two hormones.

The effect of the administration of estrogenic hormones to males depends on the dosage of the hormone, the duration of treatment and the age and species of animal; the simultaneous administration of androgens also influences it. The principal action of estrogens is anti-androgenic, ie they can inhibit and antagonize the effects of androgens (308).

Vermeulen (309) found that both estrogen treatment and pregnancy increases the testosterone binding capacity of testosterone binding protein (T.B.G. [globulin]). He quoted evidence to suggest that T.B.G. bound testosterone is biologically less active than free testosterone. Indeed, high testosterone plasma levels in pregnancy or during estrogen

treatment are not accompanied by signs of hyperandrogenism. Moreover the metabolic clearance of testosterone decreases when T.B.G. is increased.

Vertes and King (310) confirm observations of previous workers that androgenization affects the hypothalamus and that it may exert its effect by altering the binding of estradiol to receptors in this region of the brain. It remains uncertain whether androgenization also directly affects estradiol binding in the adenohypophysis and uterus; or whether this is a secondary effect governed by the primary reaction at the hypothalamus.

Administering estrogen in combination with progesterone enhances the activities of phosphofructokinase, aldolase and fructose 1,6-diphosphatase in female chicks (311). Furthermore, estrogen treatment alone results in a lesser, but significant, response in the case of phosphofructokinase and aldolase activities and no change in fructose 1,6-diphosphatase activity. Progesterone alone has no observable effect on any of these enzymes.

Estrogen or progesterone induces significant increases in liver glycogen concentration, combinations of the two hormones exhibit a synergistic effect (306).

Female rats pretreated with estradiol, on fasting lose their liver glycogen at a lower rate than rats pretreated with testosterone. Fasting rats injected with cortisol were able to accumulate liver glycogen at a higher rate when pretreated with estradiol than with testosterone propionate. Large doses of estradiol, but not of testosterone, provoke increased accumulation of liver glycogen in fasting normal rats, but not in fasting adrenalectomized animals (312). In rat uterus, enzymes which occur at branch points of metabolism, viz. particulate bound hexokinase, glucose 6-phosphate dehydrogenase and ribulose 5-phosphate isomerase show a five-fold increase in activity following estradiol treatment. Of the other enzymes in the glycolytic and pentose phosphate pathways the increase in activity after estradiol treatment was only two-fold. It is suggested (313) that the large increases observed in the above 3 enzymes is in accordance with the concept that control occurs at branch points of metabolism (314).

Seng et al (315) report that synthetic estrogens caused slight but significant increases in the levels of triglyceride, glyceride-glycerol,

acetoacetate and pyruvate in 12 normal young women. No significant changes were observed in other lipids.

In male, weanling rats the specific activity of diglyceride acyltransferase is decreased by estradiol pretreatment in both castrated and normal animals. The activity in castrated animals was increased by testosterone (316).

Hence, in conclusion, it is apparent that estrogens can regulate protein synthesis and, to some extent, the metabolism of carbohydrates and fats.

Androgens

The androgens are synthesised in the Leydig or interstitial cells of the testis under the influence of the gonadotrophins and in the adrenal cortex under the influence of corticotrophin. Gonadotrophin secretion is regulated by androgens in a feed back system acting on the hypothalamus (317).

Kniewald et al (318) suggest that the adrenal gland of normal male rats is capable of producing testosterone, and that the synthesis of this hormone by the adrenals is increased immediately after castration. Moreover, the adrenal gland also contributes to the biosynthesis of testosterone in an indirect fashion, ie by providing the testis with an essential precursor, possibly progesterone.

The two main pathways for testosterone biosynthesis are shown in Fig. 5.

Coffey et al (319) have shown that in vitro the conversion of progesterone to testosterone in the testis, a functional capacity acquired during fetal life (320) remains intact in animals up to maturity.

In view of the relative potencies of the three androgens, testosterone, androstenedione and dehydroepiandrosterone, and the low metabolic clearance rate of testosterone as compared with the other two hormones, the androgenic activity of adult male plasma is presumably due mainly to the presence of testosterone (321).

In vitro studies have demonstrated 5α-dihydrotestosterone (5αDHT) formation from testosterone in the nuclear fraction of kidneys obtained from both male and female rats (322). Recently 5αDHT has been found to be more firmly bound than testosterone to nuclear chromatin material in androgen target tissues such as prostate (323).

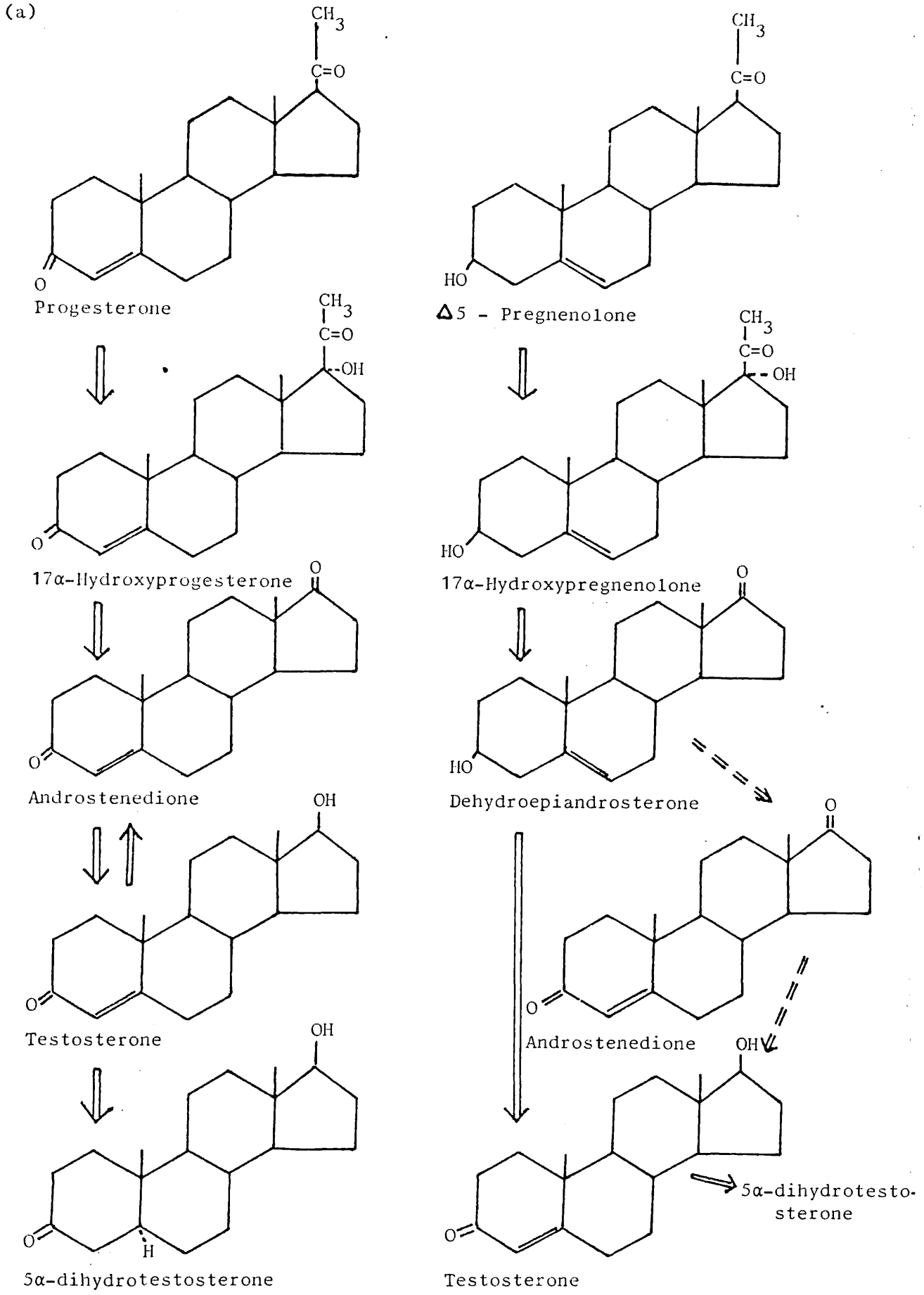


Fig 5 The main pathways for Testosterone biosynthesis

It is suggested that 5αDHT may be the active androgen in these tissues, and that testosterone is the main transport form in the blood (324).

Unbound testosterone in plasma is more biologically active whilst the protein-bound fraction serves as a reservoir and transport form of the hormone. The biologically active 17β-hydroxysteroids are largely bound to a specific β-globulin, which has a high affinity but low binding capacity for testosterone, and albumin, which has a low affinity but high capacity for testosterone (309,325).

Variations in the proportions of unbound testosterone and 5αDHT could influence the expression of biological activity. Clarke et al (325) observed a significantly lower per cent binding of both hormones for normal men than for normal women. The levels were elevated in men receiving estrogen, in pregnant women and in women receiving oral contraceptives.

Jungblut et al (326) found that female and male secondary sexual organs contain cytoplasmic receptors for estradiol and 5αDHT, both receptors exhibiting differences in their steroid binding specificity. A 10^3-fold excess of estradiol completely inhibits the attachment of 5αDHT to the androgen receptor. Competition by a 10^3-fold excess of 5αDHT results in only a 42% reduction of receptor-bound estradiol. It is suggested that the deprivation of androgen receptors could suppress the growth promoting effect of androgenic hormones.

In addition to the androgenic effects of testosterone, it also has powerful metabolic activity and causes retention of nitrogen, potassium and phosphorus. It increases body weight particularly by growth of bone and muscle, and administration of the hormone is followed by a very early increase of nuclear RNA synthesis in the kidney (327). Few specific biochemical effects appear to have been observed with androgens.

Progesterone

Progesterone is secreted by the corpus luteum of the ovary and is responsible for preparing the endometrium for pregnancy. Its biosynthesis in vitro from ^{14}C-labelled acetate via cholesterol has been demonstrated using human corpora lutea (328) (see Fig. 3).

The hormone was observed to have no significant effect on lipids and lipoproteins (114), however, serum cholesterol, triglycerides and

phospholipids all rise steadily in pregnancy as the estrogen and progestogen output increases (329,330). Recently Stokes and Wynn (134) reported that the most progestational oral contraceptive pills were associated with the highest cholesterol values in women.

Bolton et al (149) report that contraception by continuous administration of a progestogen compound such as chlormadinone is not accompanied by changes in the platelet electrophoretic behaviour (p.e.b.) (p.e.b. is abnormal in patients with occlusive arterial disease) and may, therefore, not be associated with vascular disease.

Prostaglandins

The prostaglandins are a class of C_{20} fatty acids containing a cyclopentane ring. They are unusual among highly active biological compounds in that they lack nitrogen in which respect they resemble the steroid hormones.

The precursors of prostaglandins are the essential fatty acids and in man the biosynthetic pathway is shown in Fig. 6.

There is evidence to suggest that prostaglandins may be involved in the genesis and prevention of atherosclerosis and thrombosis (331). Kloeze (332) has demonstrated in vitro that platelet adhesion and aggregation induced by ADP and by collagen can be prevented or counteracted by PGE_1. A tentative theory is that PGE_1 influences atherosclerosis by permeating and/or coating the vessel wall which in turn influences the initial thrombus development.

PGE_1 can also cause vasodilation and hence decrease the blood pressure, a further important factor implicated in atherosclerosis (331).

Vergroesen et al (333) showed that the contractile force of an isolated perfused heart is stimulated by prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$. These compounds, which are mainly synthesised in lung tissue, and then immediately transported to the heart, could, therefore, also influence coronary thrombosis and myocardial infarction.

Maxwell (334) reports that PGE_1 and $PGF_{2\alpha}$, have contrasting properties. He noted (334-335) that PGE_1 and $PGF_{2\alpha}$, separately, cause considerable cardio-acceleration; given together, however, they have relatively little effect on heart rate. The hypotensive effect (vasodilation) of PGE_1 is not abolished by $PGF_{2\alpha}$(334), in so far as a decrease in systemic pressure is always found when the two fatty acids

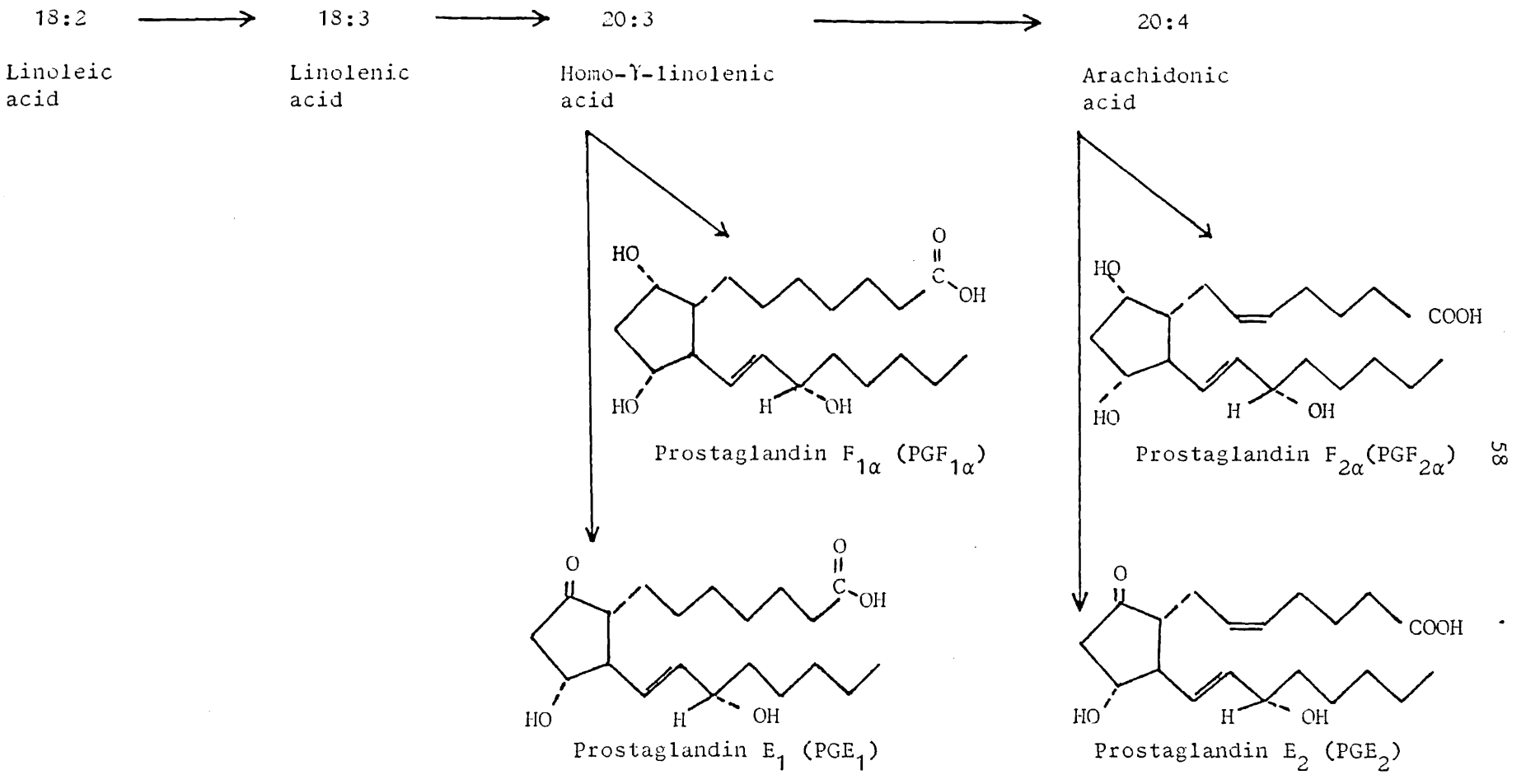


Fig. 6 The biosynthetic pathway of the prostaglandins

are administered together. $PGF_{2\alpha}$ increases pulmonary arterial pressure (334), whereas PGE_1 has little effect (335). The pulmonary hypertensive effect of $PGF_{2\alpha}$ is apparently abolished if PGE_1 is given concurrently. Similarly, the increase in cardiac output which occurs with PGE_1 no longer occurs if stoichiometric amounts of $PGF_{2\alpha}$ are given at the same time.

The suggested role of prostaglandins in the development of atherosclerosis fits in well with the filtration theory advanced by Constantinides (336) concerning the influence of dietary fat on the disease. He suggests that a normal diffusion of lipoproteins occurs from the lumen into the adventitia across the arterial wall. These lipoproteins may be trapped in the intima, causing the development of atherosclerosis. This process would be promoted when the blood lipid level is increased. In addition, the blood pressure and the permeability of the vessel wall is assumed to play a part. Hence the hypotensive effect of PGE_1 and its prevention of platelet aggregation would appear to counteract the lipid penetration and retard the development of the atherosclerotic process.

RESULTS AND DISCUSSION

There is a considerable amount of literature concerning differences in carbohydrate metabolism in men and women and many attempts have been made to link these observations to a theory of atherogenesis. Of particular importance is the fact that sucrose and fructose, but not glucose or starch, can increase the levels of fasting blood serum triglycerides in men and post-menopausal women, but not in pre-menopausal women (33,35,36,47). Triglycerides are the most abundant lipids in the arterial plaques in coronary arteries in the early stages of atherosclerosis (6). Hence there could be a relationship between dietary sucrose and heart disease. An obvious hypothesis is that fructose is a better precursor of triglycerides than glucose.

This project was undertaken to examine in greater detail the metabolism of fructose in the liver and to investigate possible control of the metabolism of this sugar by sex hormones.

Initially it was intended to investigate in detail the conversion of ^{14}C-fructose to triglycerides in rat liver slices and to examine possible sex hormonal control of this complex pathway.

Preliminary experiments were designed to measure the relative incorporation of label into the glycerol and fatty acid moieties of the triglycerides and to compare the fates of fructose and glucose. The liver is the major organ for triglyceride synthesis and hence liver slices were chosen for this study.

Conversion of fructose to triglycerides

Liver slices from both male and female animals were incubated in Krebs-Ringer Bicarbonate containing either ^{14}C-(U)-fructose or ^{14}C-(U)-glucose. Incubation was followed by maceration of the tissues and removal of non-saponifiable lipids with Bloor's Solvent. The residual extract was then saponified and resolved into fatty acid and glycerol fractions. The radioactivity in the two fractions was determined by liquid scintillation counting and the percentage incorporation of label calculated. The results are summarised in Tables I, II and III.

TABLE 1. Incubation of male rat liver slices with ^{14}C-(U)-fructose and ^{14}C-(U)-glucose for 1 hr. at 37°C.

^{14}C-(U)-fructose c.p.m./5g. wet wt. liver tissue					
Fructose total c.p.m.(a)	Residual* fructose c.p.m.(b)	Glyceride glycerol		Fatty acids	
		Total c.p.m.	% incorporation ≠	Total	% incorporation
570,000	266,000	28,120	9.3	2,100	0.69

^{14}C-(U)-glucose c.p.m./5g. wet wt. liver tissue					
Glucose total c.p.m.(a)	Residual glucose c.p.m.(b)	Glyceride glycerol		Fatty acids	
		Total c.p.m.	% incorporation	Total c.p.m.	% incorporation
750,000	325,000	28,180	6.6	1,626	0.38

* Labelled fructose remaining in incubation medium plus tissue washings

$$\neq \frac{\text{Total c.p.m. x 100}}{\text{Total (a) - Total (b)}} \%$$

TABLE II. Incubation of male rat liver slices with ^{14}C-(U)-fructose and ^{14}C-(U)-glucose for 1 hr. at 37°C

^{14}C-(U)-fructose c.p.m./5g. wet wt. liver tissue								
Fructose total c.p.m.	Residual fructose c.p.m.	Total lipids c.p.m.	Free glycerol		Glyceride glycerol		Fatty acids	
			Total c.p.m.	% Incorporation	Total c.p.m.	% incorporation	Total c.p.m.	% incorporation
760,000	208,500	21,000	34,000	6.2	8,400	1.5	560	0.1

^{14}C-(U)-glucose c.p.m./5g. wet wt. liver tissue								
Glucose total c.p.m.	Residual glucose c.p.m.	Total lipids c.p.m.	Free glycerol		Glyceride glycerol		Fatty acids	
			Total c.p.m.	% incorporation	Total c.p.m.	% incorporation	Total c.p.m.	% incorporation
760,000	387,000	13,080	19,000	4.9	4,200	1.1	410	0.1

TABLE III. Incubation of female rat liver slices with ^{14}C-(U)-fructose and ^{14}C-(U)-glucose for 1 hr. at 37°C

^{14}C-(U)-fructose c.p.m./5g. wet wt. liver tissue						
Fructose total c.p.m.	Residual fructose c.p.m.	Total lipid c.p.m.	Glyceride Glycerol		Fatty acids	
			Total c.p.m.	% incorporation	Total c.p.m.	% incorporation
760,000	445,450	28,703	17,200	5.3	1,020	0.31

^{14}C-(u)-glucose c.p.m./5g. wet wt. liver tissue						
Glucose total c.p.m.	Residual glucose c.p.m.	Total lipid c.p.m.	Glyceride glycerol		Fatty acids	
			Total c.p.m.	% incorporation	Total c.p.m.	% incorporation
760,000	594,240	13,800	6,100	3.7	1,200	0.72

It is clear from these experiments, that in all tissues both glucose and fructose give rise to a greater incorporation into triglyceride glycerol than fatty acid. Fructose, however, is a better source of label than glucose in both fat components. With male liver tissues the glycerol/fatty acid activity ratio is approximately the same with incorporation from both hexoses into glycerol being 10-17 times greater than into the triglyceride fatty acid. The same is true with female tissues incubated with ^{14}C-fructose, however with ^{14}C-glucose the activity ratio is significantly decreased to 5:1.

There appears to be a greater incorporation of label into the triglyceride fraction of the total lipids in male liver tissues when fructose is incubated in comparison with glucose (43 and 33%, respectively). A similar differential occurs with female liver tissues (65 and 52%).

This present study appears to confirm previous suggestions that triglyceride may be produced more readily from fructose than glucose (69,96, 97,108). This approach to the problem of fructose/triglyceride relationships was, however, discontinued in view of the difficulties of experiment interpretation which were likely to be encountered because of differences in the tissue pool sizes of glucose and fructose and their derivatives.

Instead it was decided to direct the investigation towards the individual enzymes likely to be involved in the conversion of fructose to triglyceride and to examine the possible sex hormone effect on the individual stages in this pathway (see Fig. 7).

Hormonal control of Fructose phosphorylation (fructokinase activity)

Initial experiments were designed to measure the rates of fructose utilisation in both male and female animals and to investigate whether these enzymes, and in particular fructokinase, were subject to control by estrogens and androgens.

The site of fructose phosphorylation in liver cells was first checked. A combination of differential and zonal centrifugation was used (as outlined in the scheme (Fig.8)) tofractionate the cell contents.

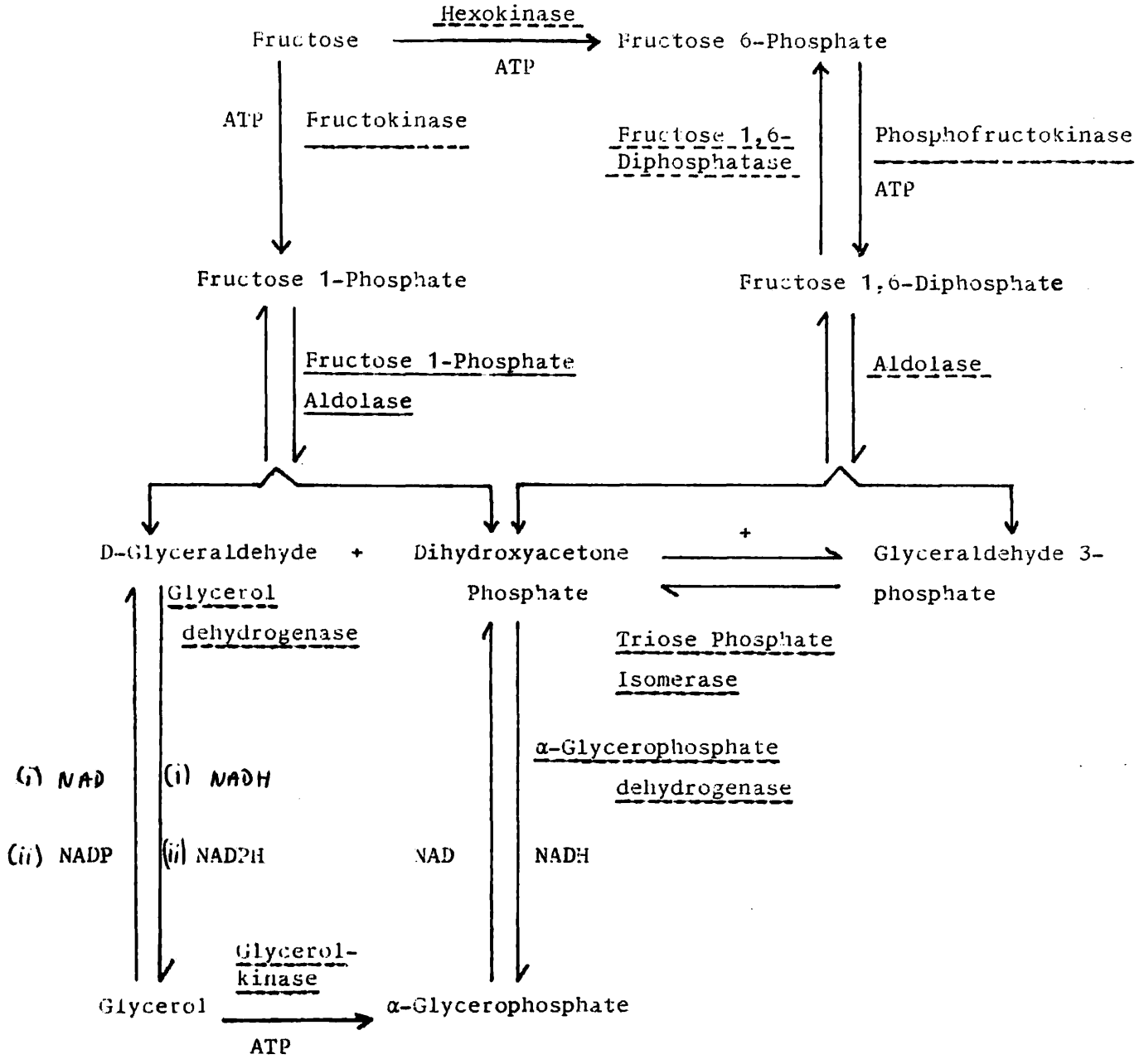


Fig. 7. Pathways involved in fructose metabolism

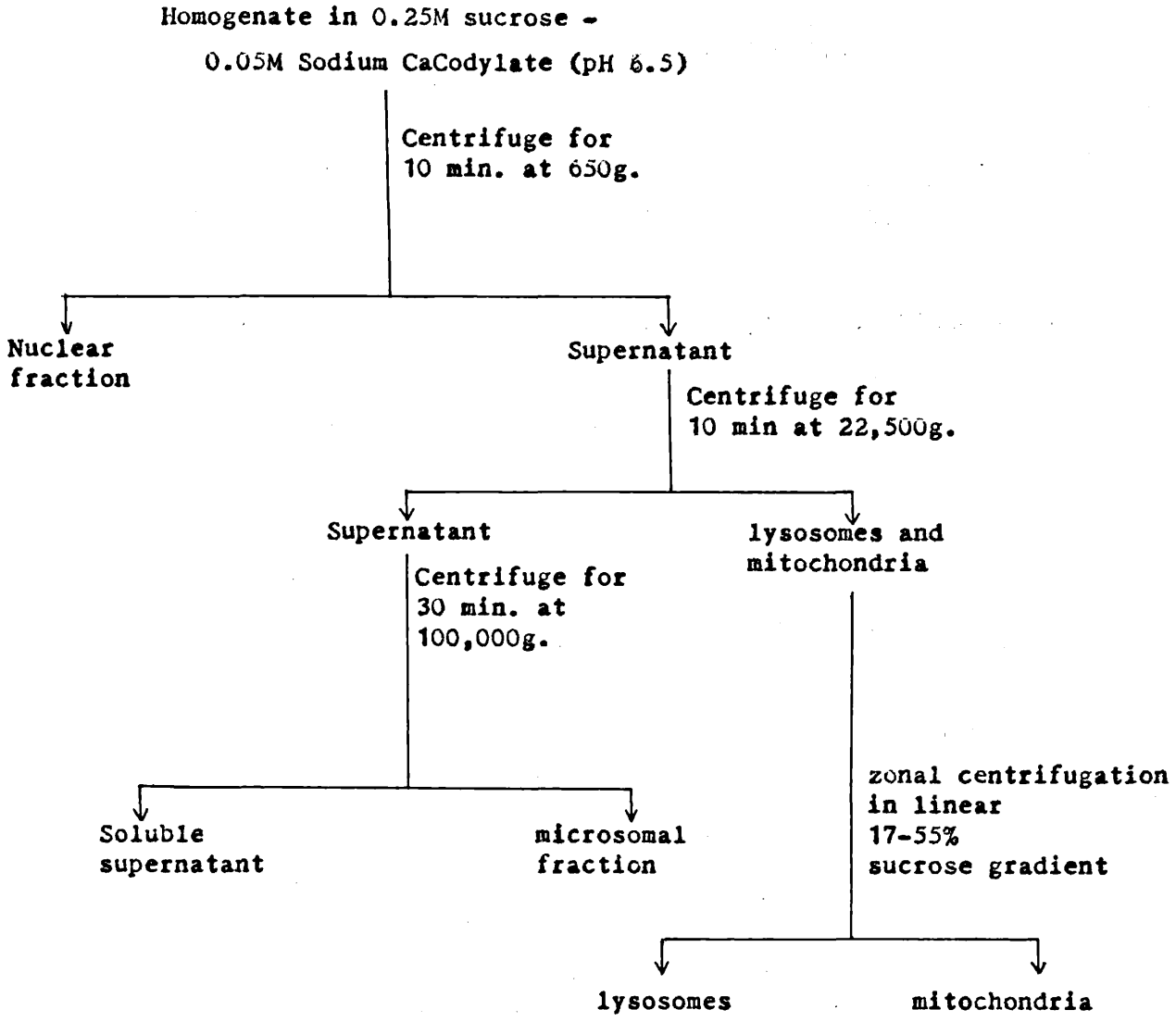


Fig. 8. Fractionation of liver cells

(see Schneider and Hogeboom (337), Brown and Brown (338))

The rate of phosphorylation of fructose by each fraction was measured by incubation with ^{14}C-fructose, F^- ions and ATP and separating the resulting fructose phosphates by paper chromatography. Radioactivity was then measured by a scintillation technique.

TABLE IV. Fructose phosphorylation by liver cell fractions from a male rat

Liver wt. = 12.0g.					
Fraction	Volume ml.	Protein mg/ml.	Activity* /ml.	Specific ≠ Activity	Total Activity ($X\ 10^{-6}$)
Nuclear	13.3	16.0	602,500	505	8.03
Mitochondrial	6.3	3.5	58,000	122	0.37
Lysosomal	3.6	-	-	-	-
Microsomal	7.0	4.0	394,000	1,310	2.76
Soluble supernatant	52.0	11.75	1,400,000	1,570	72.3

TABLE V. Fructose phosphorylation by liver cell fractions from a female rat

Liver wt. = 6.9g.					
Fraction	Volume ml.	Protein mg/ml.	Activity* /ml.	Specific ≠ Activity	Total Activity ($X\ 10^{-6}$)
Nuclear	14.5	4.25	236,000	738	3.43
Mitochondrial	5.0		-	-	-
Lysosomal	4.2	0.2	-	-	-
Microsomal	6.8	0.2	105,700	7,040	0.72
Soluble supernatant	28	5.25	791,000	2,000	22.2

* c.p.m. fructose phosphate/ml.

≠ c.p.m. fructose phosphate/mg. sol. protein/min.

As can be seen in Tables IV and V the soluble supernatant fraction from both male and female livers possesses most of the fructose phosphorylating activity. This confirms previous studies (259,339). The small amount of activity occuring in the other fractions is probably due to contamination by the soluble enzyme.

In all the studies which followed the soluble supernatant solution was used as a source of enzyme and was prepared by centrifugation of homogenates first at 5000g. to remove gross cellular debris, and then at 100,000g. to sediment organelles.

The identity of the phosphate produced by incubation of ^{14}C-fructose with ATP and the supernatant fraction was examined by paper chromatography. Fructose 1-phosphate was the only hexose derivative which could be detected. This observation was confirmed by treating the enzyme preparation with acid (to pH 6.0) and then examining the rate of fructose phosphorylation in the presence of 2-acetamido-2-deoxy-D-glucose. Acid treatment destroys much of the hexokinase (but not fructokinase) activity and the remainder is inhibited by the amino sugar derivative (259).

TABLE VI. Fructose phosphorylation by acid treated enzyme preparations in the presence of 2-acetamido-2-deoxy-D-glucose.

No*	Sex	Incubation Time (min.)	Untreated enzyme preparation (Total c.p.m.) X 10^{-6}	Treated enzyme (Total c.p.m.) X 10^{-6}
I	Male	30	2.17	2.19
		60	3.50	3.48
		60	3.41	3.46
2	Male	30	0.37	0.38
		60	0.60	0.62
3	Female	30	0.34	0.35
		60	0.68	0.68

* Different radioactive concentrations were used in the three experiments

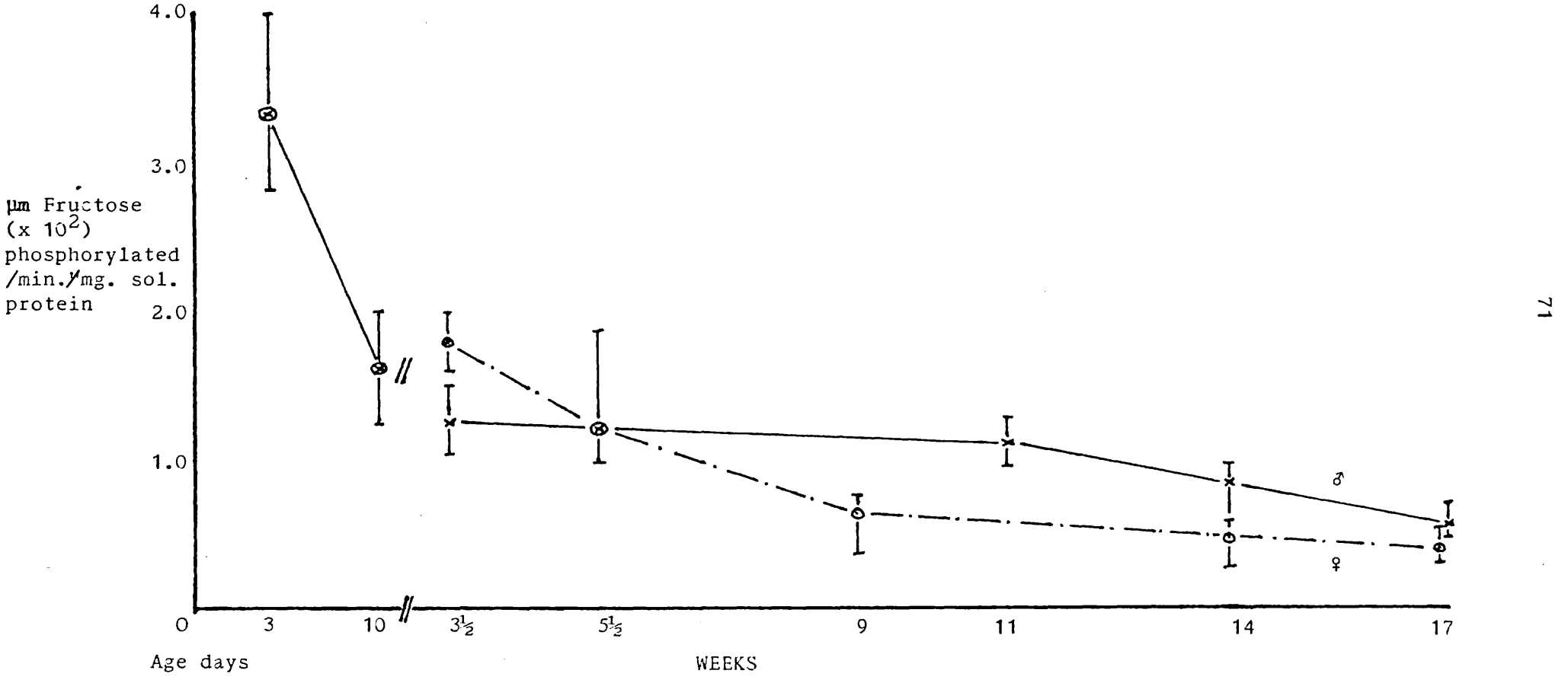
In this instance the phosphorylation rates using treated and untreated enzyme were almost identical (see Table VI) thus showing that fructokinase is the major phosphorylating enzyme in the liver preparations.

Although fructose 1-phosphate was observed to be the major product some triose phosphates (see P. 97) were also apparent when enzyme digests were examined on paper chromatograms. The radioactivity in these materials was only 5-10% of that present in the fructose 1-phosphate. In calculating rates of fructose phosphorylation the total counts in the fructose 1-phosphate and triose phosphates were used, however.

Having established that fructose was converted mainly to fructose 1-phosphate by liver tissue extracts the rate of fructose phosphorylation was measured in animals over a range of ages. The results expressed in μmoles fructose phosphorylated/min./mg. soluble protein or per total wt. liver tissue are shown in Figs. 9 and 10.

Walker (103) examined hepatic fructokinase in new-born guinea pigs liver and basing his results in terms of μmoles fructose utilised/min./g. wet wt. liver tissue (or 100g. body wt.) found that fructokinase activity appeared after birth and reached adult levels in 7-10 days.

Three day old rats exhibited a high fructokinase specific activity which fell sharply at 10 days. As the total activity per liver rose during this period (Fig. 10) the decrease in specific activity was presumably due to protein synthesis. After 10 days there was a more gradual decrease in fructokinase activity over a period of 4 weeks. At 3½ weeks female rats appeared to have a higher activity than the corresponding males from the same litter, although only two animals of each sex at this age were available for study. The difference in fructokinase activity between the sexes was not apparent at 5½ weeks, however in females the activity at 9 weeks was half that observed in males at 11 weeks. This latter value was only slightly lower than the value observed for 5½ week old animals. Enzyme activity continued to fall in both sexes after 11 weeks, but was less marked in the case of the female animals, however. At about 17 weeks the rate of fructose phosphorylation was approximately the same in both sexes.



♂ = male animals

♀ = female animals

Fig. 9. Fructose phosphorylation (fructokinase activity) by liver preparations from rats of different ages.

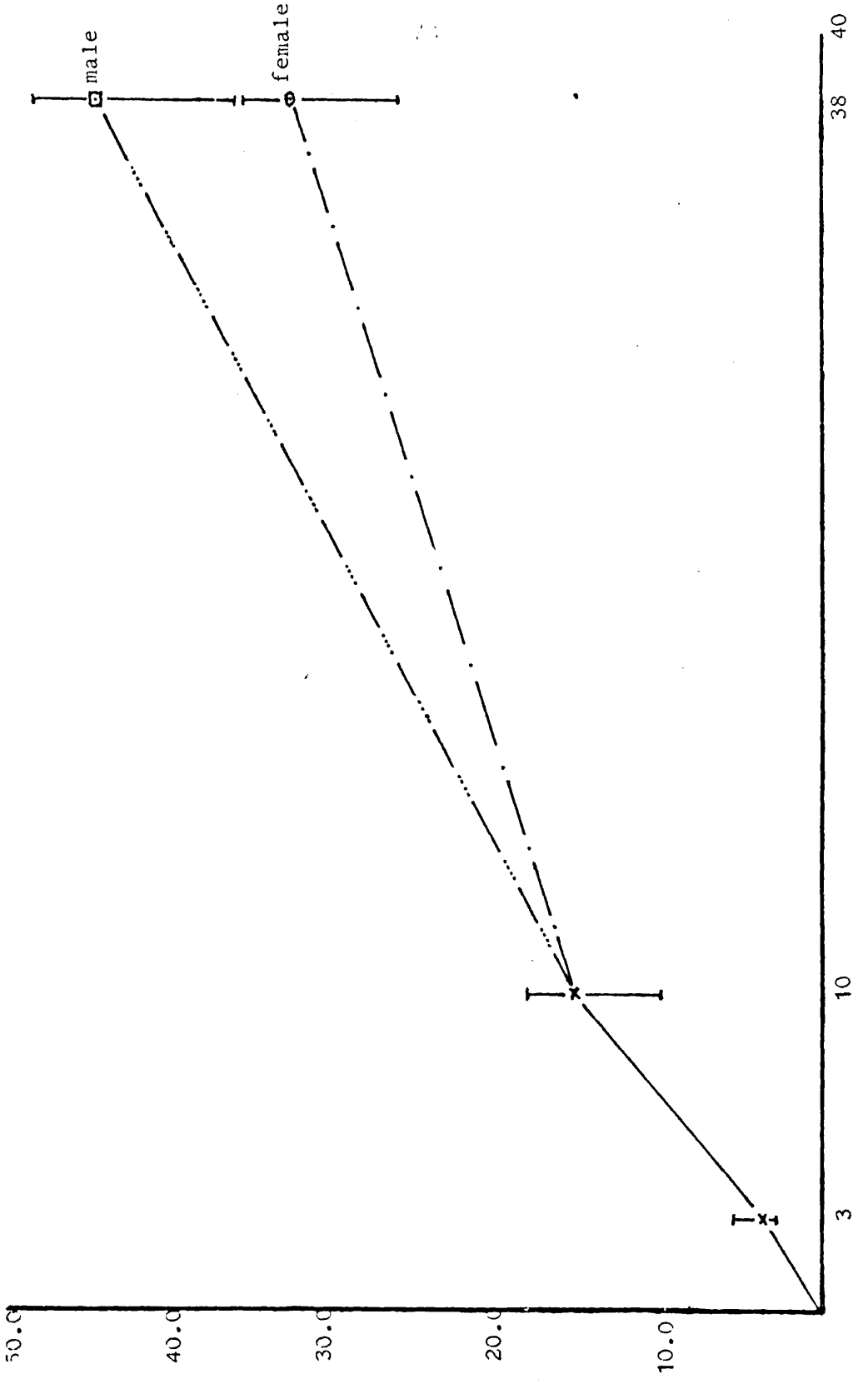


Fig. 10. Total fructokinase activity by liver preparations from rats of different ages

The difference in fructokinase activity in sexually mature animals is shown in Fig. 11 for males at 11 weeks and females at 9 weeks. Here it is quite clearly seen that the enzyme activity of the livers of male animals is twice that of female animals. Hence the effect of sex hormones was next investigated.

Effect of testosterone on fructokinase in female rats

In initial experiments injection of different doses of testosterone in a saline/propylene glycol mixture (3mg. and 24mg./Kg. body weight [Kg.b.wt.]) were investigated. The results are shown in Table VII.

TABLE VII Fructose Phosphorylation in female rats injected with testosterone (T)

^{14}C- fructose 1-phosphate c.p.m. x 10^{-3} /mg. sol. protein *			
Concentration / Time	Controls	3mg.T/Kg.body wt.	24mg.T/Kg.body wt.
12 hr.	139.4±4.0	160.2±4.7 **	190.1±11.9 ***
48 hr.		136.4±8.7 †	140.6±5.3 †

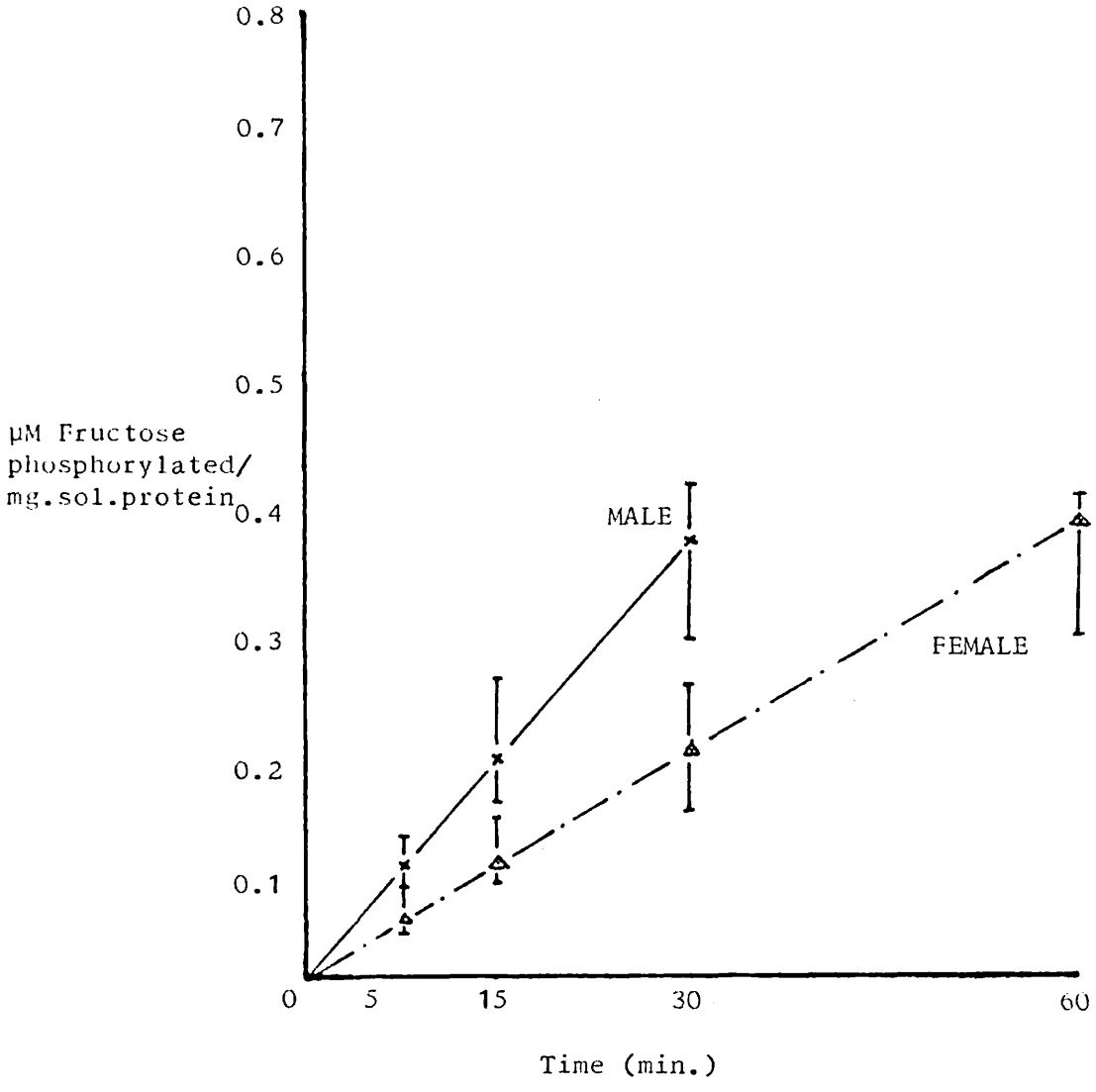
* 15 min. incubations. Arithmetical means plus standard deviations calculated using triplicate samples from 6 livers for each assay. Controls were injected with saline/propylene glycol, with the hormones for injection dissolved in this mixture.

** *** Statistically significant (**$P < 0.01$; ***$P < 0.005$) increase in fructokinase activity compared with control

† Not significantly different from control.

Twelve hours after a single injection of 3mg./Kg.b.wt. there was a 14% increase in activity which fell to the control level 48 hr. after injection. In the case of the larger dose there was a more marked rise (36%) after 12 hr. but again the level of activity fell after 48 hr. Both increases in activity after 12 hr. were significant.

Fig. 11. Fructokinase in adult male and female rats



Duplicate assays on livers from 6 animals were carried out. Hence each experimental point represents the mean of 12 determinations.

Fig. 12 shows the results of more detailed experiments where rate/time curves have been constructed for injections of single and double doses of testosterone into female animals. It can be seen that 24 hr. after a single injection (24 mg./Kg.b.wt.) of hormone the rate of phosphorylation approximately doubles in comparison with the female control and reaches the level of activity in livers from normal male animals. A second injection of hormone followed by a further 24 hr. period increased the fructokinase activity beyond that of the male control.

It is clear from these experiments that male hormone administered to female animals increases the rate of fructose phosphorylation in vivo. The level of hormone used in these experiments was high, but it is probable that only part of that injected was biologically active as solid residues were observed, when the animals were sacrificed, at the sites of injection in the sub-cutaneous tissues.

In the Introductory Section the role of sex hormones in promotion of protein synthesis was described and in view of the known facts it seemed probably that testosterone was affecting fructokinase activity by influencing the synthesis of liver proteins. Hence, in vitro experiments using liver slices were designed as a first stage in testing this hypothesis.

TABLE VIII Effect of testosterone (T) on fructose phosphorylation in female rat liver slices

μ moles fructose utilised/g.wet wt. liver tissue*	
Controls	1 mg.T./10ml. K.R. ‡
0.28±0.016	0.41±0.035**

For each assay, 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer bicarbonate solution was carried out for 2 hr. at 37°C. The rates of fructose phosphorylation were then measured with duplicate samples from each tube after incubation for 15 min.

* The arithmetical means plus standard deviations were calculated.

‡ Weight of testosterone/10 ml. Krebs-Ringer bicarbonate solution.

** Statistically significant ($P<0.01$) increase in fructokinase activity compared with control.

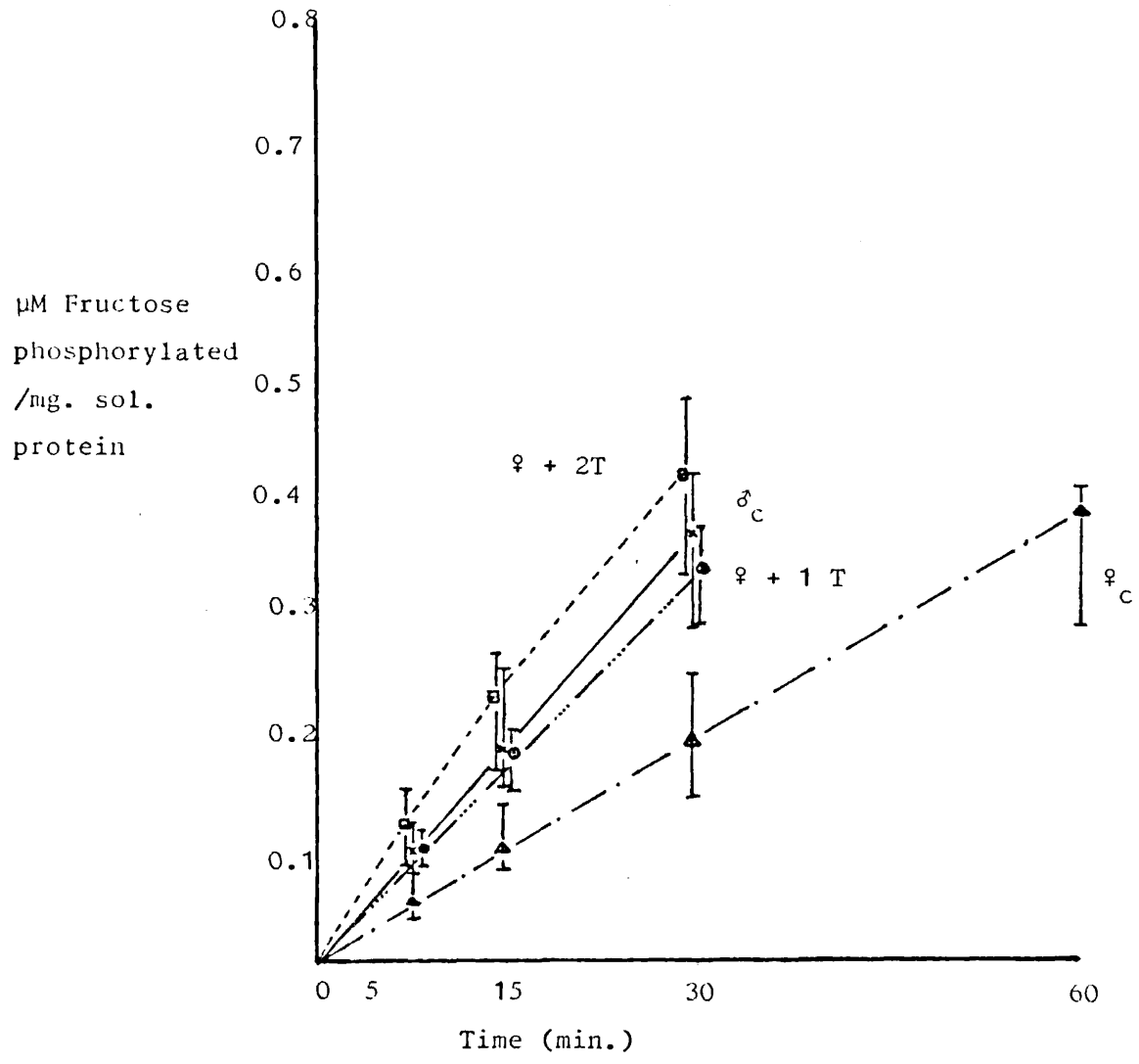


Fig. 12. Fructokinase in female rats injected with testosterone.

$♂_c$ = male rats injected with saline/propylene glycol and sacrificed after 24 hr.

$♀_c$ = female rats injected with saline/propylene glycol and sacrificed after 24 hr.

♀ + 1 T = female rats injected with testosterone (24 mg./Kg.b.wt.) in saline/propylene glycol and sacrificed after 24 hr.

♀ + 2 T = female rats injected twice with testosterone (24 mg./Kg.b.wt.) in saline/propylene glycol; the second injection given 24 hr. after the first and the animals sacrificed after a further 24 hr.

Duplicate assays on livers from 6 rats were carried out; therefore each experimental point represents the mean of 12 determinations.

TABLE IX Effect of testosterone (T) on fructose phosphorylation in female rat liver slices

^{14}C-fructose 1-phosphate formation (c.p.m. x 10^{-3}) /mg. soluble* protein	
Control	0.63 mg.T/10 ml. K.R. ∓
1893.4±1.11	** 2552.6±22.8

* For each assay, 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer Bicarbonate solution was carried out for 3 hr. at 37°C. The rates of fructose phosphorylation were then measured with duplicate samples from each tube after incubation for 15 min. The arithmetical means plus standard deviations were calculated.

∓ Weight of testosterone/10 ml. Krebs-Ringer bicarbonate solution.

** Statistically significant ($P < 0.025$) increase in fructokinase activity compared with control.

Table VIII shows that incubation of liver slices with testosterone produces a statistically significant elevation of fructokinase activity. In this series of experiments the enzyme activity was assayed by measuring loss of fructose from the medium using a resorcinol/thiourea reagent. The investigation was repeated using the routine procedure with ^{14}C-fructose as substrate and assaying the labelled phosphate formed; again (Table IX) it was obvious that the male hormone significantly activated the kinase in the female liver tissues.

Further studies including the use of varying concentrations of testosterone and varying incubation times from 1-12 hr. were made. In all cases activation was observed although there was a poor correlation between enzyme activity and the varying factors.

The wide spread in the results obtained from the *in vitro* studies are probably due to permeability differences, *ie.* uneven thickness of tissue slices.

Effect of testosterone on fructokinase in male rats

The experiments carried out with female rats were repeated with male animals.

TABLE X Fructose phosphorylation in male rats injected with testosterone (T)

^{14}C-fructose 1-phosphate formation (c.p.m. x 10^{-3})/mg. soluble* protein		
Time	Control	24mg.T/Kg.b.wt.
24h	111.8±6.6	** 152.0±5.2
48h		*** 130.3±4.9
72h		† 129.9±7.0

* 15 min. incubations. Arithmetical means plus standard deviations calculated using triplicate samples from 6 livers for each assay. Controls were injected with saline/propylene glycol, with the hormones for injection dissolved in this mixture.

** Statistically significant (** $P < 0.005$; *** *** $P < 0.05$) increase in fructokinase activity compared with control.

† Not significantly different from control.

TABLE XI Fructose phosphorylation in male rats injected with testosterone (T)

^{14}C-fructose 1-phosphate formation (c.p.m. x 10^{-3})/mg. soluble protein *				
Concentration / Time	Controls	3mg.T/Kg. b.wt.	12mg.T/Kg. b.wt.	24mg.T/Kg. b.wt.
12h	182.9±8.7	** 225.7±8.6	*** 256.1±10.2	**** 214.8±7.1
48h		∓ 195.5±11.3	∓ 195.8±14.9	∓ 178.7±7.5

* 15 min. incubations. Arithmetical means plus standard deviations calculated using triplicate samples from 6 livers for each assay. Controls were injected with saline/propylene glycol, with the hormones for injection dissolved in this mixture.

** *** **** Statistically significant (** $P < 0.01$; *** $P < 0.001$; **** $P < 0.025$) increase in fructokinase activity compared with control.

∓ Not significantly different from control.

TABLE XII Fructose phosphorylation in male rats injected with testosterone (T)

^{14}C-fructose 1-phosphate formation (c.p.m. x 10^{-3}) /mg. soluble protein *		
Concentration / Time(hr.)	Controls	12mg. T./Kg.b.wt.
1	102.3 ± 2.5	** 87.6 ± 0.9
3		† 100.7 ± 2.9
6		*** 123.0 ± 1.8
12		**** 118.3 ± 1.8

* 15 min. incubations. Arithmetical means plus standard deviations calculated using triplicate samples from 6 livers for each assay. Controls were injected with saline/propylene glycol, with the hormones for injection dissolved in this mixture.

** Statistically significant ($P < 0.01$) decrease in fructokinase activity compared with control.

† Not significantly different from control.

*** **** Statistically significant (***$P < 0.001$; ****$P < 0.001$) increase in fructokinase activity compared with control.

The results shown in Tables X - XII and Fig. 13 are all statistically significant and indicate that as with female animals, testosterone increases the fructokinase activity in male liver tissues. The optimum dose appeared to be 12 mg./Kg.b.wt. with a period after injection of from 6 to 12 hr. and the effect was greatest (Fig.13) when two injections of testosterone (24 mg./Kg. b.wt.) were given.

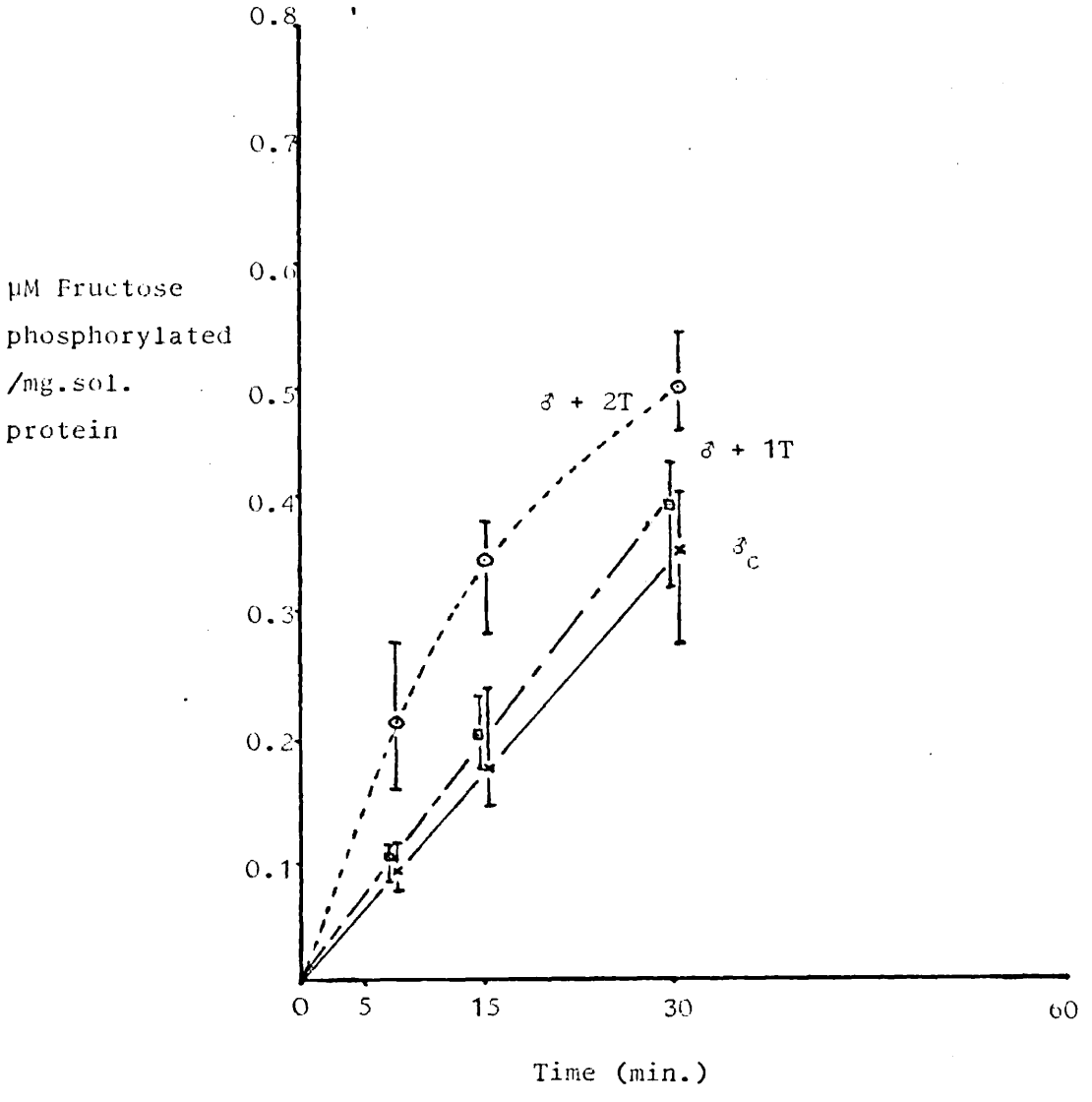
An in vitro investigation (Tables XIII and XIV using liver slices from male rats showed that testosterone was also able to increase the specific activity of the fructokinase in the male tissues.

In Table XIV where the results are based on g.wet wt. liver the decrease in specific activity in the controls is due to the gradual disintegration of the slices. However in the 3hr. incubations the level of fructokinase induced by the testosterone is significantly different from that in the control.

In order to investigate the probable involvement of protein synthesis in testosterone-treated slices the experiments were repeated in the presence and absence of cycloheximide (Table XV). Statistical treatment of these results shows that the increase actually produced by testosterone is nullified by the presence of cycloheximide. It may be assumed, therefore, that the hormone functions by activating fructokinase synthesis in male and presumably female tissues. A second but less likely possibility is that the synthesis of another protein (enzymic or non-enzymic) is enhanced which in turn activates the kinase. Such systems being well known, to take one example, in glycogen metabolism.

Effect of estrone on fructokinase in male rats

In view of the observed activation of fructokinase by testosterone the effect of estrone on the enzyme was next investigated. Injections of estrone (24mg/Kg.b.wt.) into male rats produced no effect after 24hr., however, a second injection 24hr. after the first produced a change in the kinetics of the enzyme after a further 24hr. The results are shown in Fig.14 and these have been confirmed by a repeat experiment. It can be seen that the initial rate of activity is normal up to 15 min. incubation, however after this period there is a sudden reduction of rate. This change in the gradient of the curve cannot be attributed to



$♂_C$ = male rats injected with saline/propylene glycol and sacrificed after 24 hr.

♂ + 1T = male rats injected with testosterone (24mg./Kg.b.wt.) in saline/propylene glycol and sacrificed after 24 hr.

♂ + 2T = male rats injected twice with testosterone (24mg./Kg.b.wt.) in saline/propylene glycol; the second injection given 24hr. after the first and the animals sacrificed after a further 24 hr.

Duplicate assays on livers from 6 rats were carried out; therefore each experimental point represents the mean of 12 determinations.

Fig. 13. Fructokinase in male rats injected with Testosterone

TABLE XIII Fructose phosphorylation in male rat liver slices incubated with testosterone (T)

^{14}C-fructose 1-phosphate (c.p.m. x 10^{-3})/ g.wet wt. liver tissues *		
Control	6 hr.	83.4 ± 6.5
** 12mg.T.	6 hr.	*** 125.2 ± 10.7
Control	12 hr.	29.4 ± 4.2
** 12mg.T.	12 hr.	† 27.2 ± 1.1

* For each assay 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer Bicarbonate was carried out for 6 and 12 hr. at 37°. The rates of fructose phosphorylation were then measured with duplicate samples from each tube after incubation for 15 min. Arithmetical means plus standard deviations were calculated.

** Weight of testosterone/10ml. Krebs-Ringer Bicarbonate solution.

*** Statistically significant ($P < 0.025$) increase in fructokinase activity compared with control.

† Not significantly different from control.

TABLE XIV Fructose phosphorylation in male rat liver slices incubated with testosterone (T)

^{14}C-fructose 1-phosphate (^{14}C-F-1-P) c.p.m. x 10^{-3}		
Sample	^{14}C-F-1-P / g.wet* wt.liver tissue	^{14}C-F-1-P / mg.sol.* protein
Controls Ohr.	48.1±2.9	166.7±7.8
Controls 3hr.	16.4±0.4	160.8±7.1
†0.63mg.T 3hr.	** 23.8±2.3	*** 223.6±15.6

* For each assay 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer Bicarbonate was carried out for 3 hr. at 37°. The rates of fructose phosphorylation were then measured with duplicate samples from each tube after incubation for 15 min. Arithmetical means plus standard deviations were calculated.

† Weight of testosterone/10ml. Krebs-Ringer Bicarbonate solution.

** *** Statistically significant (**P < 0.01; ***P < 0.005) increase in fructokinase activity compared with respective 3hr. controls.

TABLE XV Fructose phosphorylation in male rat liver slices incubated with testosterone (T) and cycloheximide (C)

^{14}C-fructose 1-phosphate (F-1-P) c.p.m. x 10^{-3}		
Sample	F-1-P/g.wet.wt* liver tissue	F-1-P/mg. soluble* protein
Control 0hr.	614.0±43.3	1874.0±99.0
Control 3hr.	245.6±27.3	1887.8±42.1
X 0.5mg.T 3hr.	** 744.1±112.8	† 5317.6±677.2
X 0.5mg.T; 4mMC 3hr.	*** 375.8±109.7	‡ 2843.6±810.0

* For each assay 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer Bicarbonate was carried out for 3 hr. at 37°. The rates of fructose phosphorylation were then measured with triplicate samples from each tube after incubation for 15 min. Arithmetical means plus standard deviations were calculated.

X Weight of testosterone/10ml. Krebs-Ringer Bicarbonate solution.

** Statistically significant ($P < 0.005$) increase in fructokinase activity compared with 3 hr. control.

*** Statistically significant ($P < 0.05$) decrease in fructokinase activity compared with testosterone incubation. (i)

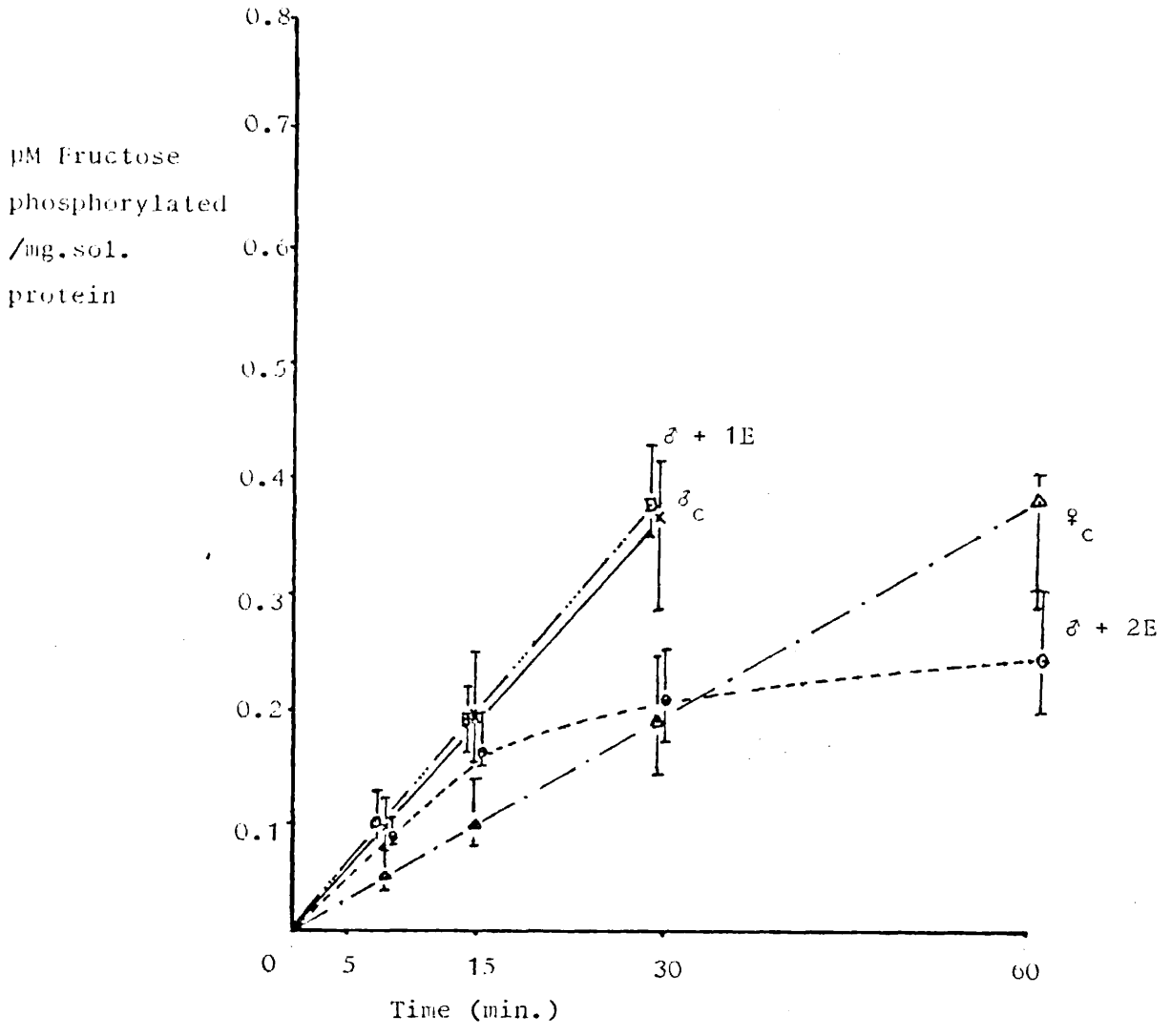
(ii) Not significantly different from 3hr. control.

† Statistically significant ($P < 0.001$) increase in fructokinase activity compared with 3hr. control.

‡ Statistically significant ($P < 0.05$) decrease in fructokinase activity compared with testosterone incubation. (i)

(ii) Not significantly different from 3hr. control.

Fig.14 Fructokinase in male rats injected with Estrone



$♀_C$ = female rats injected with saline/propylene glycol and sacrificed after 24hr.

$♂_C$ = male rats injected with saline/propylene glycol and sacrificed after 24hr.

♂ + 1E = male rats injected with estrone (24mg./Kg.b.wt.) in saline/propylene glycol and sacrificed after 24hr.

♂ + 2E = male rats injected twice with estrone (24mg./Kg.b.wt.) in saline/propylene glycol; the second injection given 24hr. after the first and the animals sacrificed after a further 24hr.

Duplicate assays on livers from 6 rats were carried out; therefore each experimental point represents the mean of 12 determinations.

depletion of substrate. Instead it suggests that the hormone in some way affects the stability of the kinase perhaps by interfering with the synthesis of some stabilising factor, possibly a nonenzymic protein. Alternatively, estrone may prevent the synthesis of an enzyme which activates fructokinase, or the hormone may enhance the synthesis of an enzyme which in some way lowers the activity of the kinase. A fourth possibility is that the hormone promotes the synthesis of an enzyme which destroys the product fructose 1-phosphate. In theory this could be a phosphatase which renders the reaction reversible. This seems unlikely, however, as a relatively high concentration of fluoride ions was added when the fructokinase was assayed.

When liver slices were incubated at 37^{o} in Krebs-Ringer bicarbonate to which varying concentrations of estrone had been added; a reduction of the level of fructokinase was observed. (Table XVI)

A concentration of 1mg.estrone/10ml. Krebs-Ringer bicarbonate produced the maximum effect. Levels resulting from treatment with higher concentrations were not significantly different.

The effect of incubation/time was also examined with the male rat liver slices using estrone (1mg./10ml.). Table XVII shows that the hormone effect is not apparent after 1hr.

Hence in male animals studies both in vivo and in vitro indicate that estrone has an inhibitory effect on fructose phosphorylation in the liver.

Effect of estrone on fructokinase in female rats

The one remaining question which arose as a result of the observations already described was whether estrogens exerted the same effect in female animals as they did in male animals

The effect of injections of estrone into female animals on fructose phosphorylation is shown in Fig.15. Injection of the natural female hormone increased the rate of fructose phosphorylation by the livers of these animals over and above that produced by testosterone (see Fig. 12).

Confirmation of this observed estrone effect was again obtained by incubating liver slices from female animals with the female hormone. The results of these studies are given in Table XVIII.

TABLE XVI Fructose phosphorylation in male rat liver slices incubated with estrone (E)

μM Fructose utilised / g.wet wt. liver tissue*	
Control	0.214±0.011
x 1mg.E	** 0.141±0.008
2mg.E	*** 0.163±0.013
4mg.E	† 0.157±0.010
8mg.E	≠ 0.143±0.021

* For each assay 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer bicarbonate was carried out for 2hr. at 37°. The rates of fructose phosphorylation were then measured with duplicate samples from each tube after incubation for 15 min. Arithmetical means plus standard deviations were calculated.

x Weight of estrone/10ml. Krebs-Ringer bicarbonate solution.

** Statistically significant ($P < 0.001$) decrease in fructokinase activity compared with control.

***
(i) statistically significant ($P < 0.025$) decrease in fructokinase activity compared with control.
(ii) not significantly different from 1mg.E.

† Statistically significant (†$P < 0.005$; ≠$P < 0.025$)
≠ decrease in fructokinase activity compared with control.

TABLE XVII Fructose phosphorylation in male rat liver slices incubated with estrone (E)

µM Fructose utilised / g. wet wt. liver tissue*	
Control	1mg.E[x] - Incubation for 1 hr.
0.156 ± 0.014	* 0.138 ± 0.01
Control	1mg.E - Incubation for 2 hr.
0.151 ± 0.024	** 0.094 ± 0.012

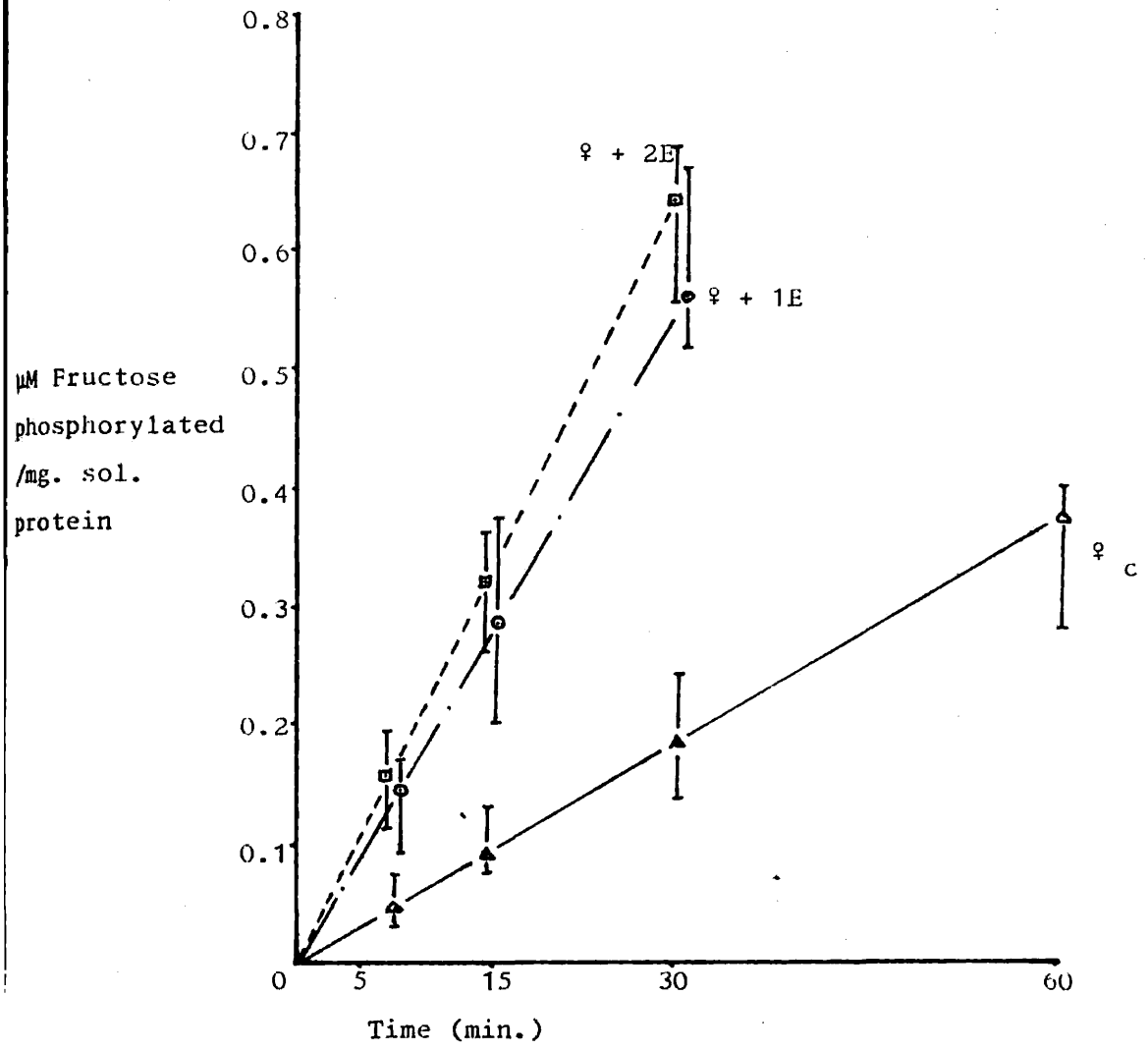
* For each assay 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer bicarbonate was carried out for 1 or 2 hr. at 37^{o}. The rates of fructose phosphorylation were then measured with duplicate samples from each tube after incubation for 15 min. Arithmetical means plus standard deviations were calculated.

x Weight of estrone/10ml. Krebs-Ringer bicarbonate solution

* Not significantly different from control

** $0.1 > P > 0.05$ with $P \simeq 0.055$ and therefore assumed to be a statistically significant decrease in fructokinase activity compared with the 2hr. control.

Fig.15 Fructokinase in female rats injected with Estrone



$♀_c$ = female rats injected with saline/propylene glycol and sacrificed after 24 hr.

♀ + 1E = female rats injected with estrone (24mg./Kg.b.wt.) in saline/propylene glycol and sacrificed after 24 hr.

♀ + 2E = female rats injected twice with estrone (24mg./Kg.b.wt.) in saline/propylene glycol; the second injection given 24 hr. after the first and the animals sacrificed after a further 24 hr.

Duplicate assays on livers from 6 rats were carried out; therefore each experimental point represents the mean of 12 determinations.

TABLE XVIII Fructose phosphorylation in female rat liver slices incubated with estrone (E)

μM Fructose utilised / g. wet wt. liver tissue*	
Control	0.118 ± 0.011
x 1mg.E	** 0.234 ± 0.025
4mg.E	*** 0.176 ± 0.009

* For each assay 6 tubes each containing 2 liver slices were used. Incubation with hormone or Krebs-Ringer bicarbonate was carried out for 2 hr. at 37^{o}. The rates of fructose phosphorylation were then measured with duplicate samples from each tube after incubation for 15 min. Arithmetical means plus standard deviations were calculated.

x Weight of estrone/10ml. Krebs-Ringer bicarbonate solution.

** *** Statistically significant (**$P < 0.005$; ***$P < 0.005$) increase in fructokinase activity compared with control.

*** Not significantly different from 1mg.E.

These observations were unexpected and complicate the over simplified view that male hormones are responsible for increased triglyceride synthesis from fructose and female hormones responsible for a lower triglyceride synthesis from the ketose.

Estrone has been reported to have effects on metabolism which may relate to our observations in the case of female rats. For example, estrogen administered to fowls, or high levels of endogenous estrogen during egg formation, leads to a striking lipemia (340). It has also been suggested that the synthetic estrogens may be responsible for elevated triglyceride levels in pre-menopausal women (134,341). The so-called atherogenic index of women using oral contrceptives approaches that of men of comparable age (130), and the incidence of myocardial infarction (114) and of cerebral vascular accidents (342) is significantly increased as compared to pre-menopausal women not using these steroids. Kekki and Nikkila (341) suggest that the increased synthesis of plasma triglycerides in pre-menopausal women might be attributed to the estrogen moiety of the 'pill'. Stokes and Wynn (134) give confirmation of this in their findings that the most estrogenic oral contraceptive pills produced high triglyceride levels and the most progestational gave the highest cholesterol values. Kekki and Nikkila (341) observed that the rate of production of plasma triglycerides increased with the length of the treatment period up to 4 yr., while the rate of elimination was not related to the duration of contraceptive use. Compatible with this was the finding that the over production of plasma triglycerides was reversed only slowly after discontinuation of the therapy. Clearly the important relationship in female animals between estrogens and high triglyceride levels could operate, at least in part, through the metabolism of fructose. This would be in line with our original working hypothesis that facile triglyceride synthesis from fructose occurs via glyceraldehyde and that the rate of fructose phosphorylation is important for regulation of this process.

Hormonal Control of fructose 1-phosphate aldolase activity.

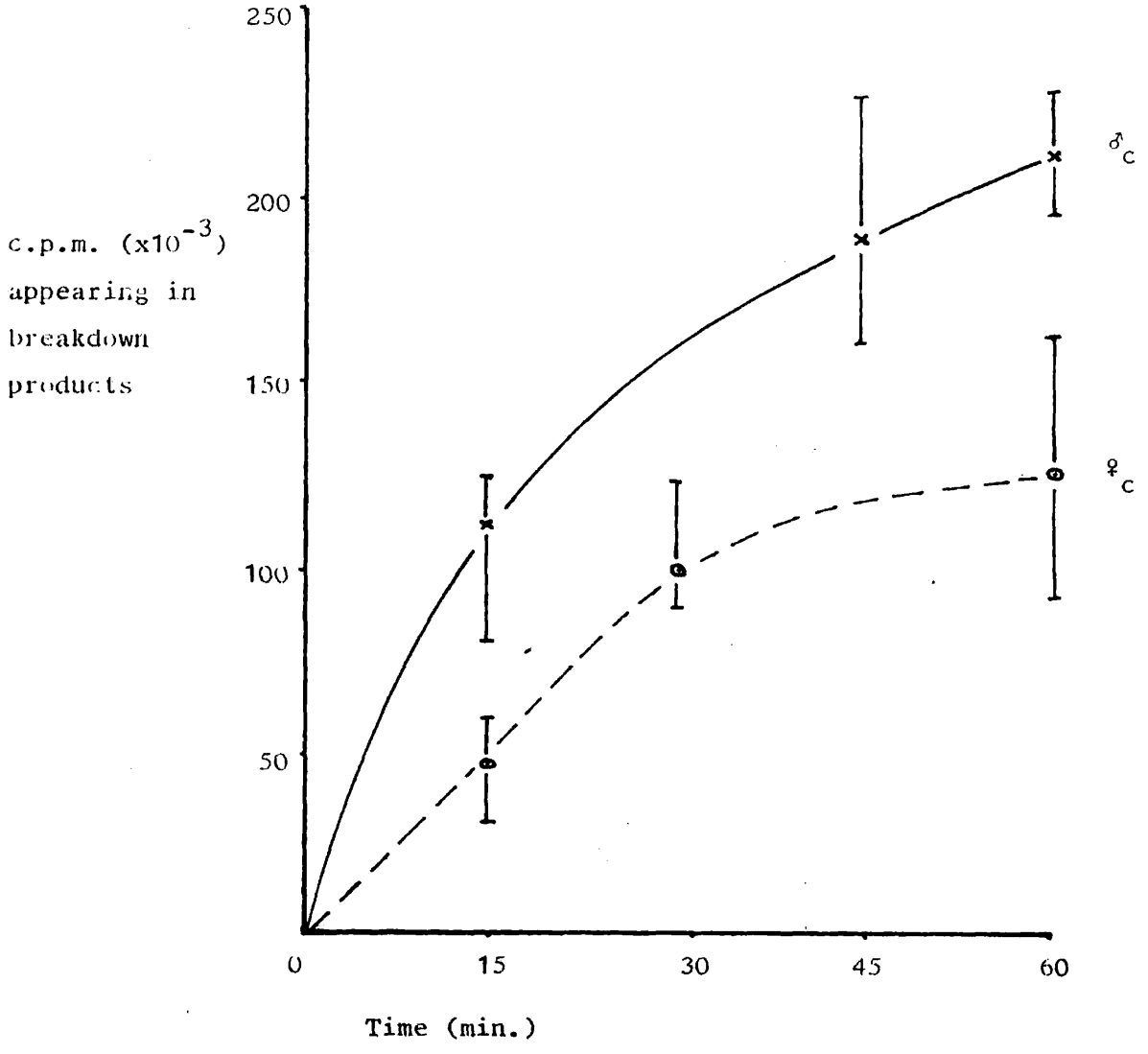
This enzyme catalyses the cleavage of the product of fructose phosphorylation, fructose 1-phosphate, with the production of dihydroxy-acetone phosphate and glyceraldehyde.

For these studies radioactive fructose 1-phosphate was prepared by incubating the 100,000g supernatant of a rat liver homogenate with ^{14}C-fructose. The ^{14}C-fructose 1-phosphate was isolated by preparative paper chromatography and checked for purity by chromatographic methods.

Fructose 1-phosphate aldolase activity was determined by incubation of enzyme preparations with the labelled fructose 1-phosphate in the presence of fluoride ions. This was followed by chromatographic separation of the products (more than 80% of which appeared to be triose derivatives) and the residual substrate and measuring radioactivities by a liquid scintillation technique. The radioactivity of all the products was taken as a measure of the aldolase activity. No fructose was produced in these reactions. In all cases the loss of fructose 1-phosphate corresponded approximately to the appearance of products: 90-95% of the radioactivity could be accounted for.

In preliminary experiments the level of aldolase in liver tissues from adult male and female rats (10 weeks old) was measured. Fig. 16 clearly shows that the activity of the aldolase in the male tissues is approximately double that in the female liver. When female rats were given a single subcutaneous injection of testosterone (12mg./Kg.b.wt.) and left for 12hr. before preparation of the liver enzyme no significant change in aldolase activity occurred (Fig. 17). In the case of male animals treated similarly with testosterone (Fig. 18) there appeared to be a reduction in activity; although the exact significance of the results are in doubt because of unexplainable variability of the readings obtained, particularly during the later stages of the incubations.

Obviously further experimentation with varying hormone doses and 'exposure' times are required but it is of interest to note that the difference in the normal levels of aldolase in male and female animals appear to facilitate fructose metabolism in the former. It should be remembered that a similar observation was made in the case of fructokinase activity in animals of the same age. One would assume that the different aldolase levels in males and females are a result of sex-hormone activities, on the other hand hormonal control normally relates to enzymes catalysing irreversible reactions (343).

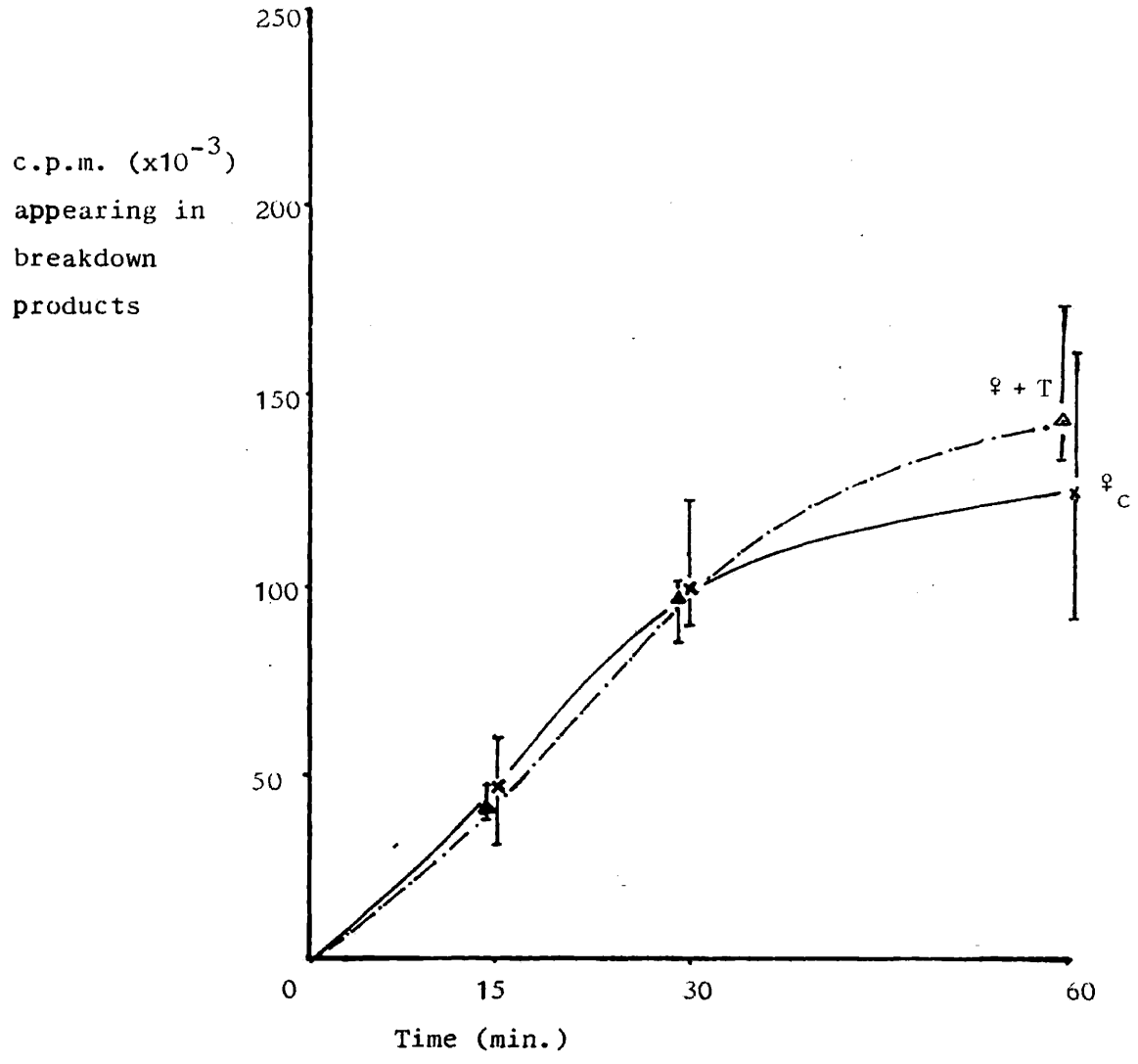


♂C = male rats injected with saline/propylene glycol and sacrificed after 12 hr.

♀C = female rats injected with saline/propylene glycol and sacrificed after 12 hr.

Duplicate assays on livers from 6 rats were carried out; therefore each experimental point represents the mean of 12 determinations.

Fig. 16. Fructose 1-phosphate aldolase in male and female rats.

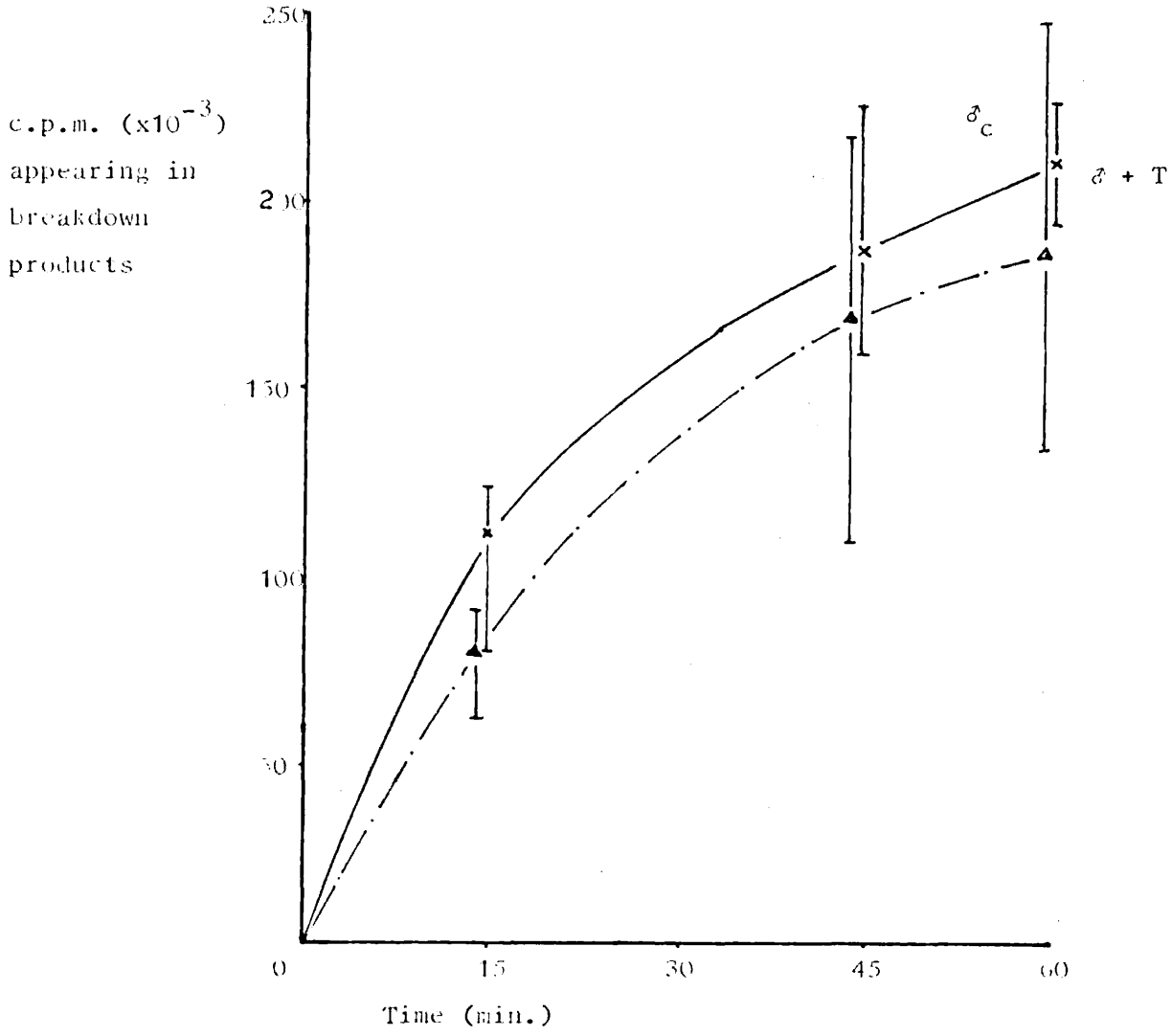


♀$_c$ = female rats injected with saline/propylene glycol and sacrificed after 12 hr.

♀ + T = female rats injected with testosterone (12 mg./Kg.b.wt.) in saline/propylene glycol and sacrificed after 12 hr.

Duplicate assays on livers from 6 rats were carried out; therefore each experimental point represents the mean of 12 determinations.

Fig. 17. Fructose 1-phosphate aldolase in female rats injected with testosterone



$♂_C$ = male rats injected with saline/propylene glycol and sacrificed after 12 hr.

♂ + T = male rats injected with testosterone (12mg./Kg.b.wt.) in saline/propylene glycol and sacrificed after 12 hr.

Duplicate assays on livers from 6 rats were carried out; therefore each experimental point represents the mean of 12 determinations.

Fig. 18. Fructose 1-phosphate aldolase in male rats injected with testosterone

It was mentioned that small amounts of labelled compounds other than the expected products of fructokinase and aldolase activities were produced during the assay of these enzymes. Further experiments were therefore designed to determine the nature and levels of these minor components and their relationships to the products of fructokinase and aldolase action.

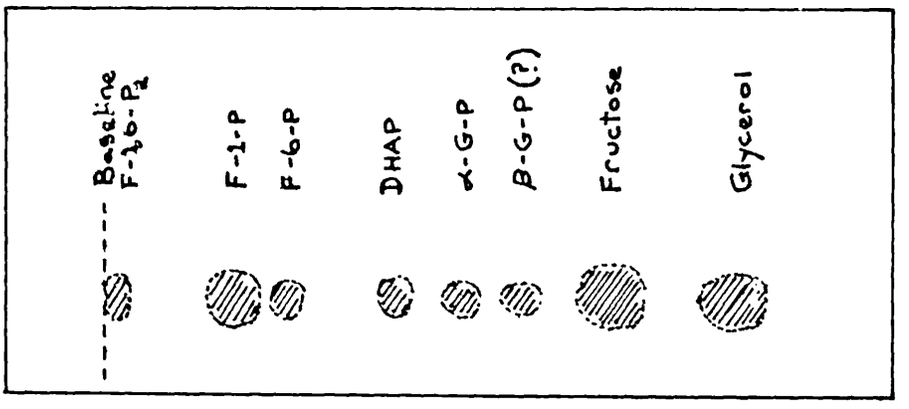
The identity of these various products produced by incubating high specific activity ^{14}C-fructose and ATP with liver preparations was investigated. This was achieved by paper chromatography using 2 different solvent systems and by paper electrophoresis. In this way it was shown (Fig. 19) that the enzyme preparations converted fructose to fructose 1-phosphate, dihydroxyacetone phosphate, α-glycerophosphate and fructose 1,6-diphosphate. Qualitatively, there was no difference with the preparations obtained from male and female animals. A compound which behaved as β-glycerophosphate in all the analytical systems was also observed although its identity is in some doubt as this compound has not been reported before as a metabolite.

The conversion of fructose to hexose phosphates and trioses was also investigated qualitatively. In initial experiments fructose at two concentrations and two activities was incubated with ATP liver preparations from male and female rats of different ages. The results of this study are summarized in Table XIX.

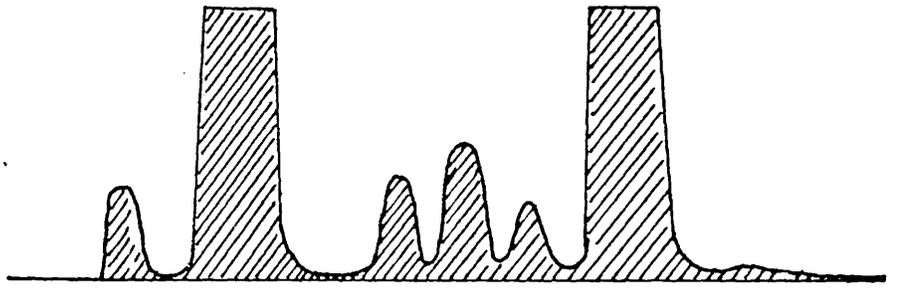
Considering the percentage incorporations of label into the products from fructose in the younger (12-13 wk.) animal preparations it is again clear that fructose phosphorylation activity in the male tissues is significantly greater than that in the female. This is also accompanied by an increase in trioses in the case of the male. In addition, there is probably a greater amount of fructose 1,6-diphosphate produced by the female enzyme preparation. It is possible to speculate that the larger proportion of fructose 1,6-diphosphate and smaller amounts of trioses produced by the female preparation is due to some rate limiting factor in the pathway to α-glycerophosphate and/or triglyceride. (In this connection it is interesting to note that Dr. A. Fathipour in this laboratory (unpublished) has observed that glycerol kinase activity is higher in male liver tissues than in female). (see Fig.20).

Fig. 19. Identification of the Products of ^{14}C-fructose metabolism by liver extracts

Paper chromatography (ethylacetate/acetic acid/ formic acid/ water; 18:3:1:4, v/v)

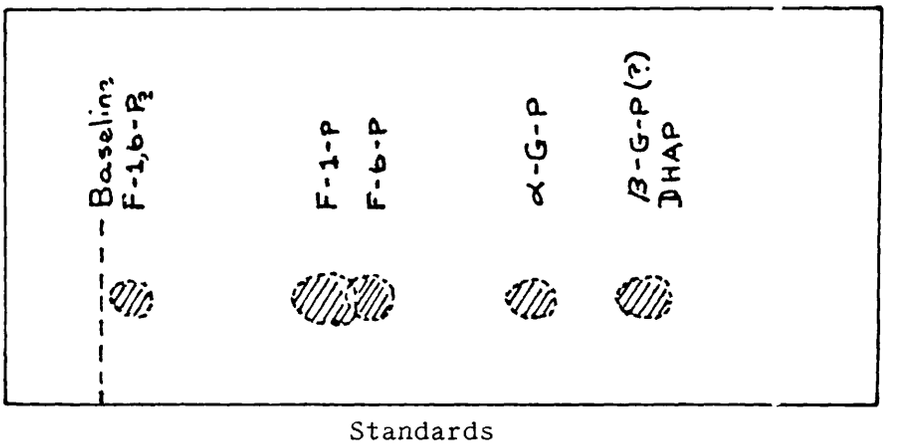


Standards



Labelled fructose metabolites

Paper chromatography (methoxy ethanol/methyl ethyl ketone/ 3N. Ammonia; 7:2:3, v/v)



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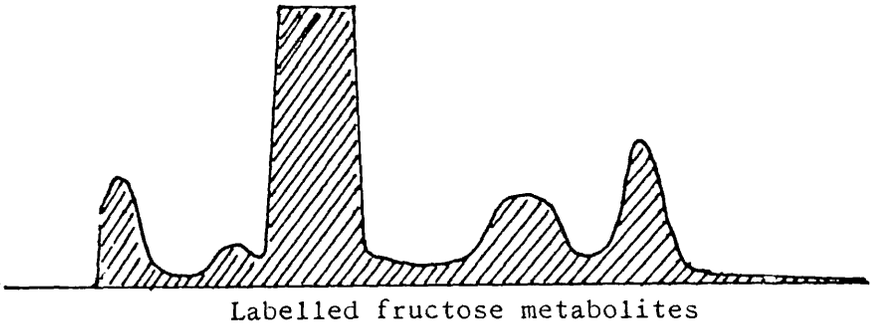
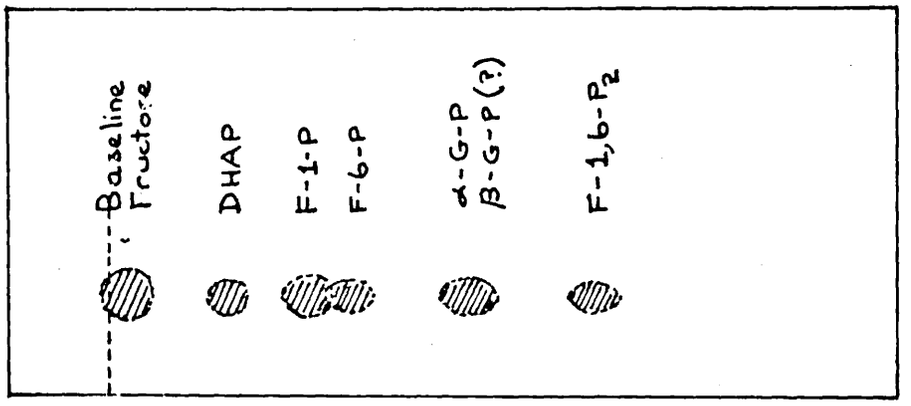


Labelled fructose metabolites

Fig. 19 continued

Paper electrophoresis (ammonium formate buffer; 0.1M, pH 3.8).



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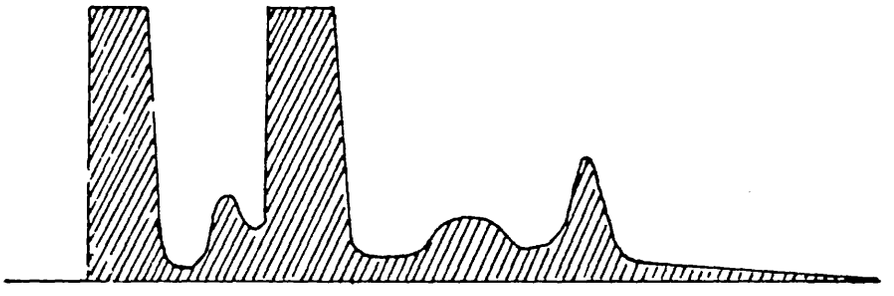


Labelled fructose metabolites

TABLE XIX. Fructose metabolism by liver extracts from male and female rats

c.p.m. x 10^{-4}/15 min./mg.sol.protein				
Products	* Male ≠	* Female ≠	** Male ≠	** Female ≠
Fructose 1-phosphate	240.0 (48%)	190.0 (38%)	51.0 (18.6%)	49.0 (18.0%)
Dihydroxy-acetone phosphate	15.2 (3.0%)	5.8 (1.2%)	1.65 (0.6%)	1.51 (0.55%)
α-glycero-phosphate	12.3 (2.5%)	5.0 (1.0%)	1.87 (0.7%)	1.25 (0.45%)
β-glycero-phosphate (?)			2.47 (0.9%)	1.31 (0.43%)
Fructose 1,6-diphosphate	8.0 (1.6%)	11.9 (2.0%)	1.30 (0.47%)	1.62 (0.59%)
Fructose	180.0 (36%)	230.0 (46%)	191.0 (70%)	192.3 (70%)

* 12-13 wk. old animals; initial fructose concentration 0.1M, 5.0×10^{6} c.p.m.

** 14-15 wk. old animals; initial fructose concentration 0.2M, 2.8×10^{6} c.p.m.

≠ Liver preparation was obtained from a single animal.

Numbers in parenthesis represent % incorporation of label.

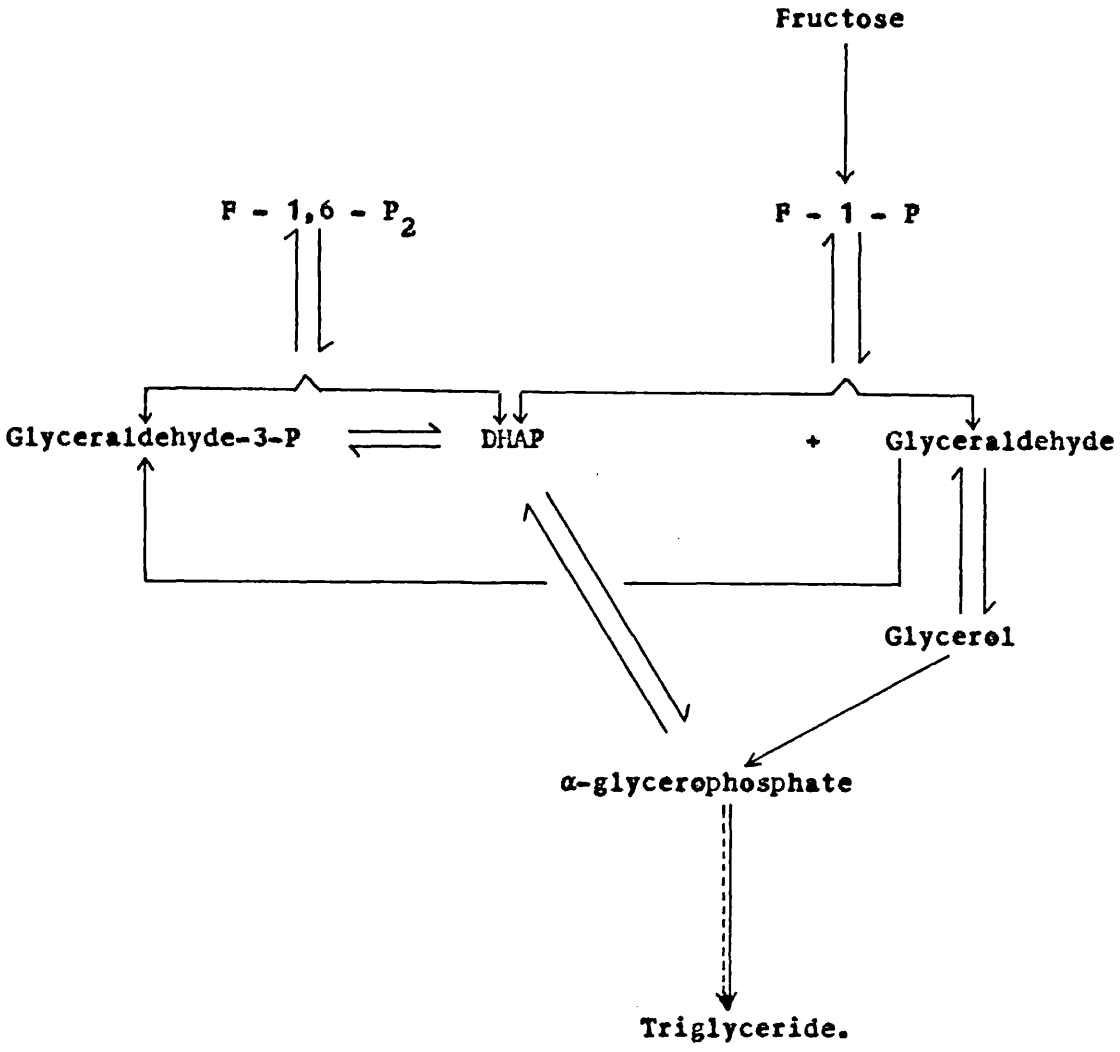


Fig. 20. The triose interconversions in fructose metabolism

This could result in increased conversion of dihydroxyacetone phosphate and glyceraldehyde to fructose 1,6-diphosphate (see Fig. 2). These ideas do not, of course, take into account possible differences in pool sizes between males and females.

When liver preparations from older (14-15 week) animals were incubated with ^{14}C-fructose and ATP using a higher initial concentration (0.2M) of ketose the male/female differences were hardly significant and in comparison with preparations from younger animals fructose utilization and fructose 1-phosphate formation were decreased. It is not known whether these results are due to inhibition caused by the use of higher concentrations of fructose or whether with the older animals the differences in metabolism between the sexes is less apparent. In this connection we have already discussed (p.70) our observation that the difference in rates of fructose phosphorylation between the sexes became less with increasing age of the animals.

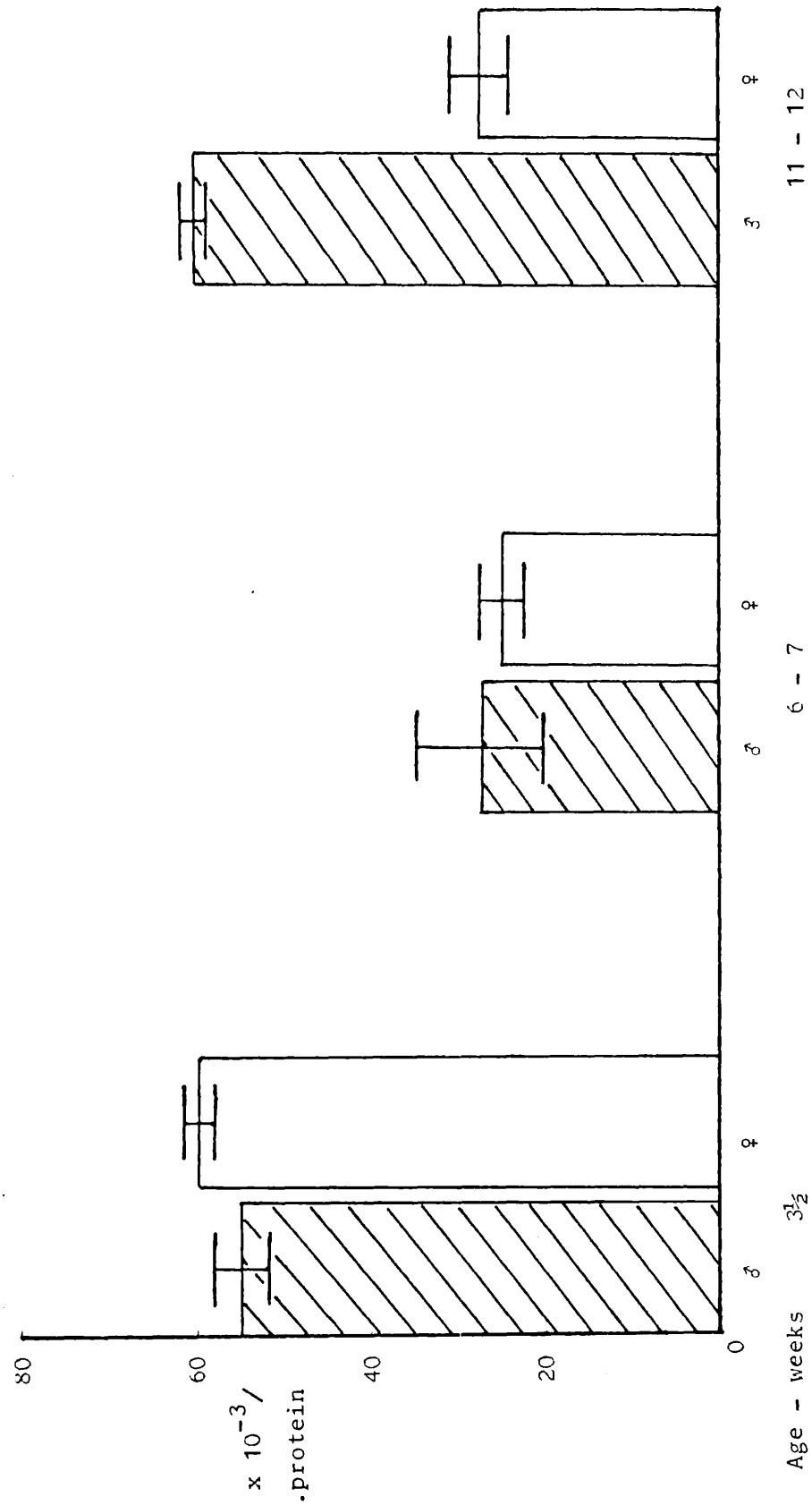


Fig. 21. Dihydroxyacetone phosphate formation from ^{14}C-fructose by male and female liver extracts from animals of different ages.

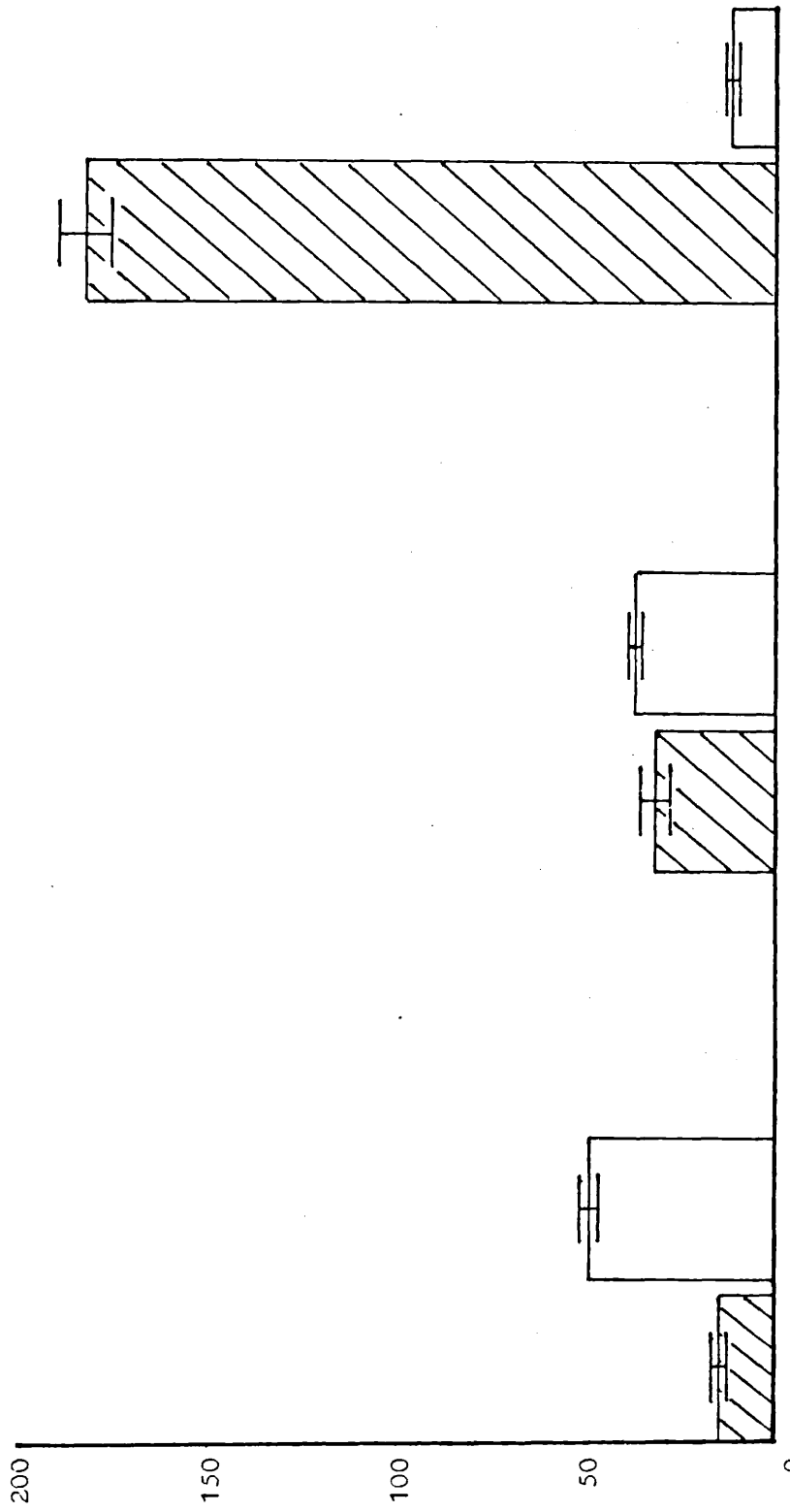


Fig. 22. α-Glycerophosphate formation from ^{14}C-fructose by male and female liver extracts from animals of different ages

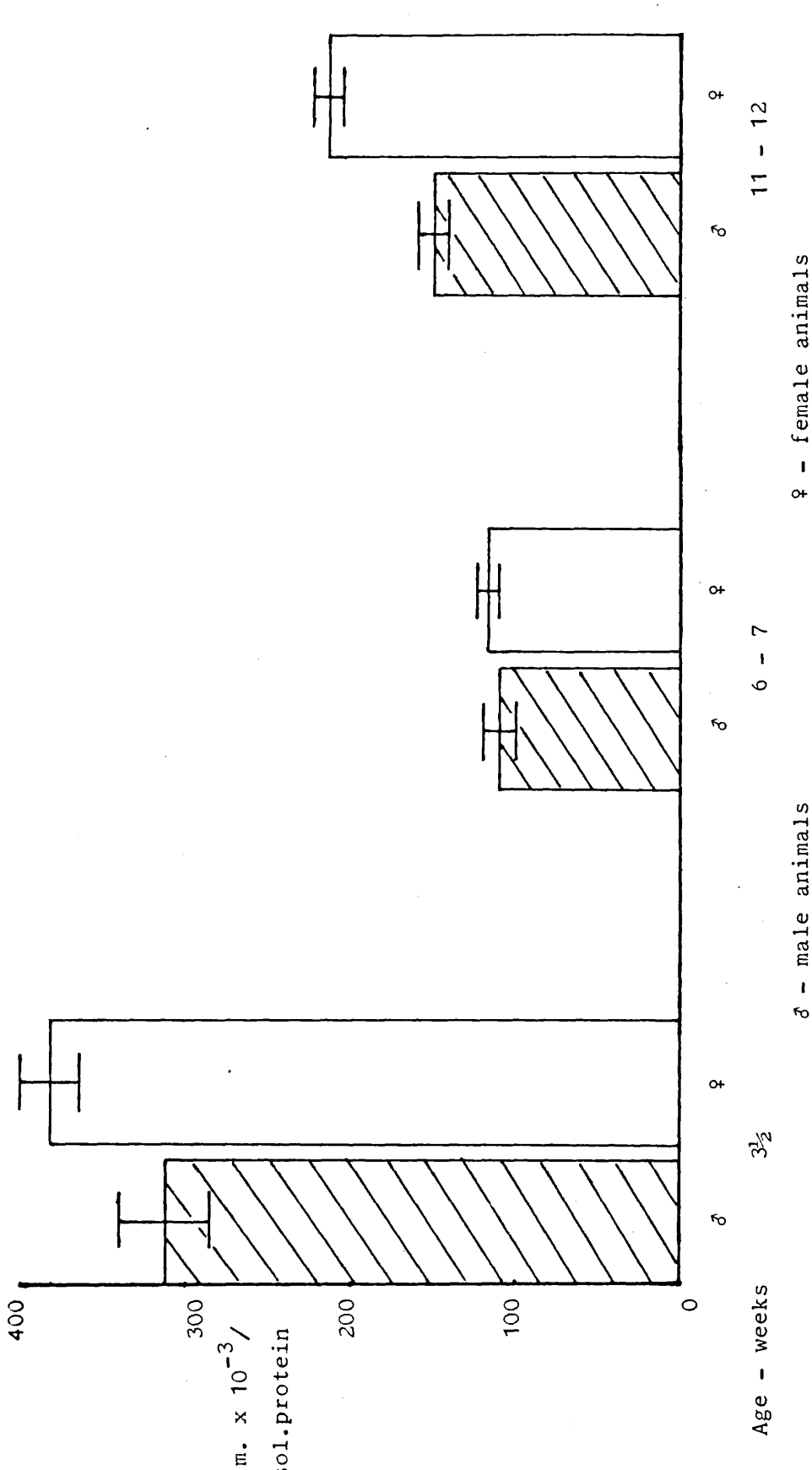


Fig. 23. Fructose 1,6-diphosphate formation from ^{14}C-fructose by male and female liver extracts from animals of different ages

In further work, some of the products of fructose metabolism by livers from rats of different ages and sex were studied using animals from the same litters. The results shown in Fig. 21-23 are difficult to interpret and again possible differences in pool sizes have not been taken into account. However, with young (3½ week) animals of both sexes there appears to be no significant difference in the amount of label appearing in the dihydroxyacetone phosphate (Fig. 21). This may mean that rates of formation and utilization of dihydroxyacetone phosphate are the same for both sexes or that these rates are different but so balanced as to produce the same amount of labelling in each case. With adult animals there is a differential and liver extracts from males appear to be more efficient in incorporating label than are those from females (cf. Table XIX). Conversion of fructose to α-glycerophosphate (Fig. 22) may proceed at a similar rate in young male and female rat livers. With adult animals however, the male tissue extracts incorporate 16 times as much label into α-glycerophosphate as do the female extracts (cf. Table XIX). It is possible from these results that triose formation from fructose is favoured in the case of male livers. This could be explained on the basis of higher levels of fructokinase, fructose 1-phosphate aldolase and glycerokinase in male as compared with female tissues, but it is possible that other enzymes in the fructose metabolic pathway shown in Fig. 2 do play an important role. The formation of fructose 1,6-diphosphate from fructose has already been referred to on P.97. The results shown in Table XIX and Fig.23 for the production of the diphosphate are similar, ie that slightly more is produced by female than by male liver tissue extracts from 11-13 week animals. Livers from 3½ week animals of both sexes incorporate significantly more label from fructose into fructose 1,6-diphosphate than do tissues from older rats.

Conclusion

The original intention was to discover sex differences in the metabolism of fructose in rats, which could possibly be related to those in man, and hence to sex differences in serum triglycerides and the incidence of heart disease.

It is apparent from the present study that there are important differences in fructose metabolism in male and female animals.

The activities of fructokinase, fructose 1-phosphate aldolase and glycerokinase (see P.97) are considerably greater in male than in female liver tissues of adult rats. Testosterone was observed to elevate fructokinase activity in both male and female animals whereas estrone decreased enzyme activity in the males but increasedit in females. It is fairly certain, therefore, that fructokinase activity in normal animals is under control of sex hormones as are probably the aldolase and glycerokinase. Castration and/or adrenalectomy of animals would provide further information regarding this theory as would experiments on liver enzymes obtained at different times in the estrous cycle. In the absence of further evidence control at the level of protein synthesis presumably occurs.

The higher levels of labelled dihydroxyacetone phosphate and α-glycerophosphate present in male rat liver tissues (after incubation with ^{14}C-fructose) compared with female tissues may also indicate sex differences in the pathway to triglycerides. It is obvious that future work should include an investigation of hormonal control of reactions leading from triose to fats.

In view of the differences in fructose metabolism and serum triglycerides in men and women and the closely allied effects of androgens and estrogens on fructose metabolism in rats, it is logical to suppose that sex hormones play related roles in both organisms. This, of course, must be confirmed by the appropriate experiments on enzyme levels in human liver tissues and the effect of exogenous sex hormones on these activities. The effect of changing hormone levels associated with the menstrual cycle and the onset of the menopause could also be investigated if suitable biopsy samples could be obtained.

In conclusion, there is every reason to suppose that the conversion

of fructose (and sucrose) to triglycerides is influenced by the products of the sex organs. The relationship between high serum lipid levels and coronary thrombosis is at present, however, mainly based on statistics. It is to be hoped that further biochemical investigations will show if there is a direct linkage between fructose and coronary thrombosis via serum lipids. With regard to preventive therapy, confirmation of such a linkage would be welcomed as the incidence of the disease might then be drastically reduced by careful control of sucrose in the diet.

E X P E R I M E N T A L

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

Glass distilled, deionised water was used for preparation of solutions.

Animals: Male and female Wistar-strain rats from 3 days of age to 17 weeks were used for *in vivo* studies. *In vitro* studies were conducted with animals aged 8-11 weeks. The animals were fed on a commercial stock diet *ad lib*, and killed as needed by cervical fracture.

General Methods

1) Paper chromatography: Whatman No. 1 and No. 3 papers were used throughout and these were developed by the descending elution technique. The chromatograms were developed for 1-48 hr.

Solvents

(i) ethyl acetate/acetic acid/formic acid/water (18:3:1:4; v/v, (345)).

(ii) methoxyethanol/methyl ethyl ketone/3N ammonia (7:2:3; v/v , (346)).

Locating reagents

(a) Carbohydrates were located using a silver nitrate solution in acetone, followed by ethanolic sodium hydroxide (0.5 - 2.0%) (347).

(b) Phosphates were detected by the formation of phosphomolybdate complexes which were subsequently reduced by irradiation with u.v. light (348)

2) Paper electrophoresis: Whatman No. 1 paper was used for the high voltage (4000V, 80mA) paper electrophoresis. The papers were developed for ¾ - 1½ hr.

Solvent

Ammonium formate buffer (pH 3.8) (349).

Locating reagents

The same reagents were used for detecting carbohydrates and phosphates as were described in the section on paper chromatography.

3) Radioactivity measurements: All radioactivity was measured on paper and counted to a 3% error with a Beckman LS100 scintillation

counter, using a toluene scintillant (5g PPO : 0.3g POPOP : 1 L toluene). The recovered radioactivity was 90-98% in all cases. Activity on paper chromatograms and electrophoretograms was located by the use of a Pye-Unicam 4 π Scanner prior to determination in the scintillation counter.

4) Protein estimation: The protein content of all enzyme preparations was measured spectrophotometrically by the method of Lowry et al (350) except that the Folin-Ciocalteu reagent was diluted with an equal quantity of water. Human serum albumin was used as a standard.

5) Spectroscopy: Absorbance of coloured solutions was measured using a Unicam S.P.500 spectrophotometer.

EXPERIMENTAL

1) Incorporation of ^{14}C-fructose and ^{14}C-glucose into liver triglycerides.

Liver slices from male and female rats were incubated in Krebs-Ringer bicarbonate solutions (10ml.) (351) containing ^{14}C-(U)-fructose (0.023 µmoles; 2.89mCi/mMole and ^{14}C-(U)-glucose (0.18 x 10^{-3}µmoles; 289mCi/mMole), respectively. Incubation time varied from 1-2hr. after which the slices were washed twice with ice-cold Krebs-Ringer solution. The washings were added to the original incubation medium which was evaporated to dryness and redissolved in a known volume of water (1ml) to determine the residual radioactivity. The procedure for the extraction of lipids as described in Appendix 2, was carried out. After saponification and acid treatment the aqueous solution containing glycerol was evaporated to dryness. The glycerol was extracted twice with ethanol, reduced to dryness both times and finally taken up in a known volume of ethanol.

Similarly, the petroleum layer containing the fatty acids was evaporated to dryness and the fatty acids finally dissolved in a known volume of petroleum ether. The radioactivities of the glycerol and triglyceride fatty acid fractions were measured.

2) Preparation of soluble liver protein fractions

(a) Livers were homogenized in 50 mM-sodium cacodylate buffer (1:3 (w/v); pH 6.5) using a Potter-Elvehjem homogenizer with a bore volume of 60ml. and 0.48mm. clearance. The homogenates were either filtered through 4 layers of muslin or centrifuged at 5000g for 10 min. to remove ruptured membrane protein before centrifugation at 100,000g for 1 hr. The resulting supernatant solutions were used to perform all fructokinase assays.

(b) In the case of fructose 1-phosphate aldolase studies, livers were homogenized in 75mM-glycyl-glycine buffer (1:3 (w/v); pH 7.5), and the procedure described above repeated.

3) Fructokinase assay

Two methods for determining fructokinase activity were used:

(a) In several of the assays in which the effect of testosterone and estrone on male and female rat liver slices was investigated, fructokinase activity was determined on the basis of fructose disappearance as described by Hers (257,258). Fructose in this case was estimated by the colourimetric method developed by Roe et al (352) and later modified by Hers (353). Enzyme solution (0.5ml.) was added to a well shaken, preheated (37° for 20 min.) mixture of 40mM-fructose (0.3ml.), 50mM-adenosine triphosphate (0.3ml.), (50mM-magnesium acetate (0.3ml), 4.0M-potassium acetate (0.9ml.) to which 0.5M-sodium fluoride (0.3ml.) had been added prior to the addition of the enzyme. The 37° temperature at pH 6.5 was maintained for 15 min. and then deproteinization was effected by the successive addition of zinc sulphate and barium hydroxide solutions.

(b) When measuring fructokinase activity in:-

(i) the livers of male and female rats injected with testosterone and estrone;

(ii) the livers of rats over a range of ages;

(iii) enzyme preparations incubated with large concentrations of ^{14}C-fructose;

(iv) liver slices incubated with testosterone, and

(v) liver slices incubated with testosterone and cycloheximide,

a modified version of the procedure described in (a) was used. The reaction mixtures were incubated in capillary tubes at 37°. The composition of the digests was as follows: 40mM-^{14}C-fructose (2.89mCi/mMole) (5µl); 50 mM-adenosine triphosphate (5µl), (50mM-magnesium acetate (5µl)), 4.0M-potassium acetate (15µl), 0.5M-sodium fluoride (5µl) and enzyme extract (5-20µl). After incubation the reaction was stopped by placing the tubes in an acetone-dry ice mixture. Each tube was individually thawed and the contents quickly spotted on chromatography paper which was then developed as described in General Methods (1). The chromatograms were scanned for radioactivity using a Pye-Unicam 4π scanner and the areas of paper containing the labelled products (identified by the use of standards) were then cut from the chromatograms and the radioactivity measured as described in General Methods (3).

Enzyme activity was expressed in terms of μmoles fructose utilized/mg. soluble protein or/g.wet wt. liver at 37°.

4) Preparation of ^{14}C-fructose 1-phosphate

^{14}C-Fructose 1-phosphate was prepared using the soluble liver protein fraction (described in Experimental (2)) as source of fructokinase. 15.2mM-^{14}C-fructose (6.08μmoles; 65.8mCi/mMole) plus 38.5mM fructose (15.4μmoles) was incubated with enzyme (160μl), 50mM-adenosine triphosphate (40μl), 50mM magnesium acetate (40μl), 4.0M-potassium acetate (120μl) and 0.5M sodium fluoride (40μl) for 15 min. at pH 6.5 and the resulting fructose 1-phosphate isolated by preparative paper chromatography. The conversion was 90% and a total of 4.56μmoles of ^{14}C-fructose 1-phosphate (65.8mCi/mMole) and 11.5μmoles fructose 1-phosphate was obtained.

5) Fructose 1-phosphate aldolase assay

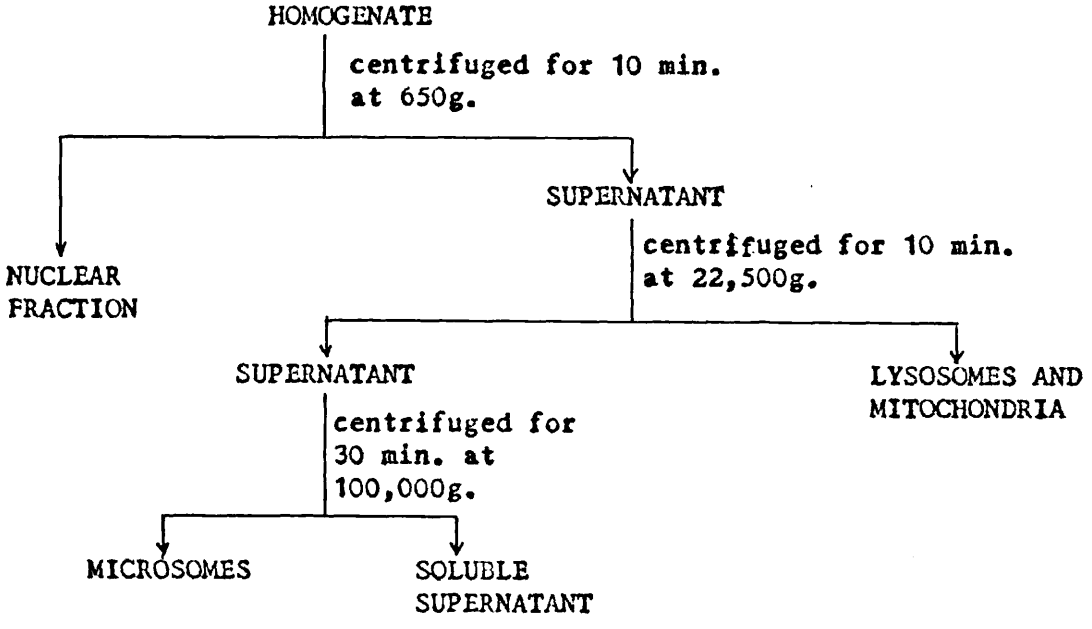
Fructose 1-phosphate aldolase was assayed by the addition of the enzyme preparation (20μl) (prepared as described in Experimental (2b)) to 10.9mM ^{14}C-fructose 1-phosphate (5μl) and 0.5M sodium fluoride (5μl) (cf 354). Incubations were carried out in capillary tubes at 37°. Reactions were stopped by immersion in acetone-dry ice mixtures and the contents of the tubes were subsequently spotted on paper chromatograms which were treated as described in General Methods (1). The residual fructose 1-phosphate and products were detected with a Pye Unicam 4π Scanner and accurate measurements of ^{14}C-content then made by scintillation counting of the appropriate strips removed from the chromatograms.

The enzyme activity was expressed as c.p.m. products/mg. sol. protein at 37°.

6) Subcellular fractionation of rat livers

The method employed was essentially that of Schneider and Hogeboom (337). The livers (7.0-10.5g) were removed and washed with ice-cold 0.25M-sucrose-50mM-sodium cacodylate solution (pH 6.5), blotted on filter paper, and then cut into pieces and homogenised in 5 vol. (w/v) of fresh ice-cold 0.25M-sucrose-50mM-sodium cacodylate

solution by ten passes in a Potter-Elvehjem homogenizer. The homogenate was filtered through 4 layers of muslin and then fractionated with a M.S.E. Superspeed 50 centrifuge by differential centrifugation at 0-4° as follows:



Each sediment was re-suspended in the same sucrose-buffer medium (5ml) and recentrifuged at the appropriate speed. This resuspension was repeated and the washings were added to the corresponding supernatant solutions. All the fractions were frozen and thawed 3 times using acetone and dry ice, and the ruptured membranes then removed by centrifugation at 100,000g for 1 hr.

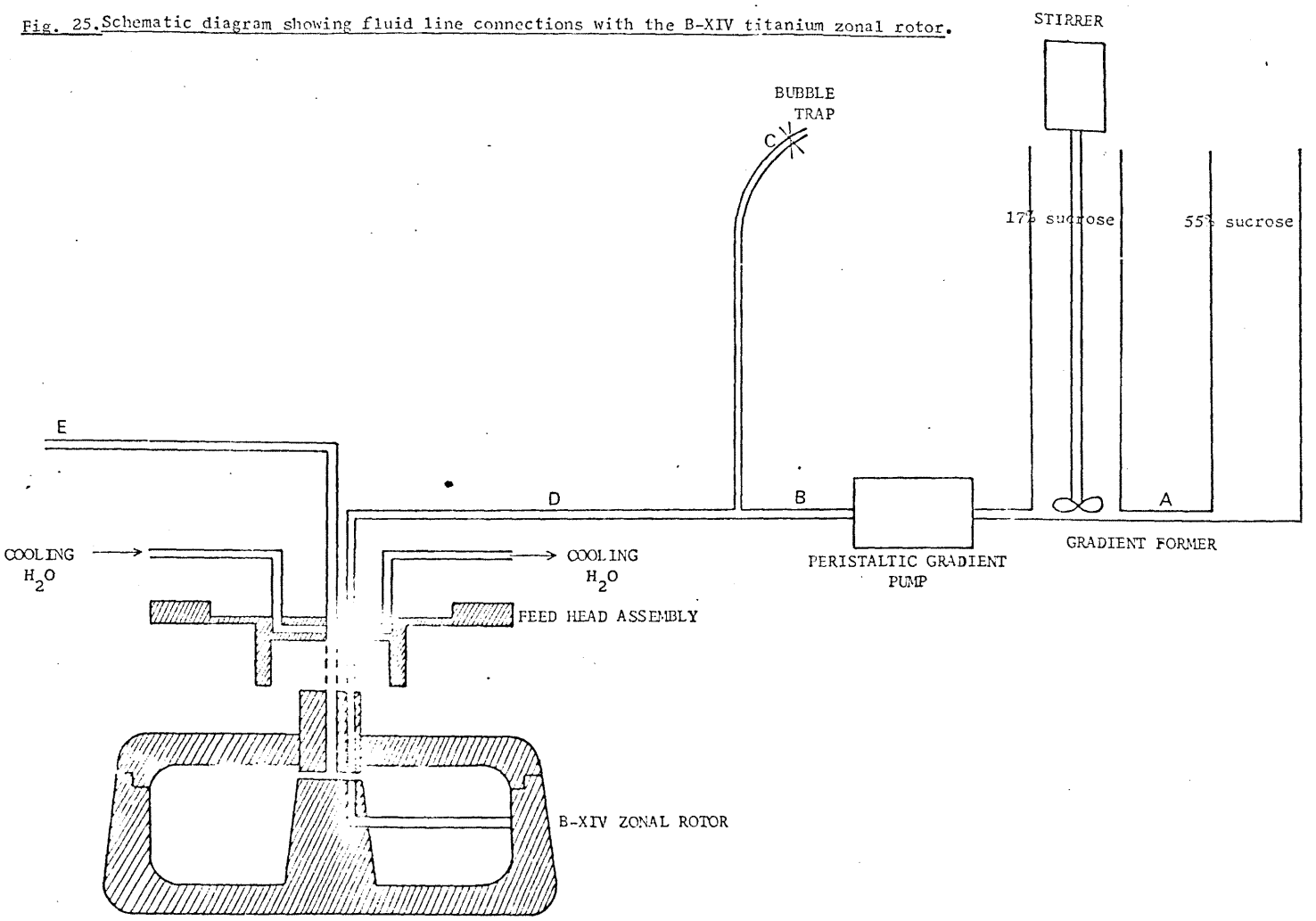
Fructokinase assays were performed on all the soluble supernatant solutions.

7) Zonal centrifugation technique (cf K. Clarke, Ph.D. Thesis, University of London, 1971)

Subcellular fractions of rat liver were obtained by the method described in Experimental (6). Zonal centrifugation using a M.S.E. Superspeed 65 centrifuge fitted with a B-XIV titanium zonal rotor was used to separate the Lysosomal & Mitochondrial fractions. Fig. 25 shows all the connected fluid lines which were kept as short as possible.

(i) Loading the rotor: 17% (w/w, 1.0719g cm^{-3}) sucrose (325ml) and 55% (w/w, 1.2663g Cm^{-3}) sucrose (275ml) solutions (355) were placed in their respective compartments of the gradient former,

Fig. 25. Schematic diagram showing fluid line connections with the B-XIV titanium zonal rotor.



which was kept at 0°C by surrounding it with ice. With fluid lines A and D clamped, 17% sucrose solution was pumped through the vertical line C in order to check that there were no air bubbles in the system. Line C was clamped and lines A,B,D and E opened. The stirrer was set in motion to ensure thorough mixing of the light and heavy sucrose solutions and a linear with volume sucrose-density gradient was pumped into the zonal rotor at a flow rate of 15ml./min. whilst the rotor was rotating at 2,500 r.p.m. This was immediately followed by 60% (w/w, 1.2955g cm^{-3}) sucrose solution (50ml.; the 'cushion' fluid), pre-cooled to 0°, which resulted in the 17% sucrose emerging through line E and indicated that the rotor was full. The temperature for all subsequent operations was kept at 0-4°. Lines E and B were then clamped and 0.25M sucrose-50mM sodium cacodylate (10ml.; 1.0349g cm^{-3}, pH 6.5, 0°) containing the sample was applied with a 20ml. syringe at a flow rate of 2.5ml./min. to the rotor through line E, with line C open. The flow rate was found by measuring the exudate from line C. Air was not allowed to enter line C, so that the gradient would not be disturbed by air bubbles. Line E was clamped and the 20ml. syringe replaced by a 50ml. syringe, containing light mineral oil; with E open, the light oil was pumped into the rotor at a flow rate of 2.5ml./min. and this ensured the sample zone was moved from the core of the rotor into a region of higher gravitational field. On completion, lines B,C,D and E were clamped, the rotor feed head assembly removed and the rotor capped.

After centrifuging the rotor was decelerated to 2,500 r.p.m. before unloading.

(ii) Unloading the rotor:

The feed assembly was replaced and the fluid lines B,D and E opened. Sucrose solution (60% w/w) was pumped into the rotor, through line D, at a flow rate of 15ml./min. The gradient containing the separated sample material was displaced through the rotor core and line E and fractions collected (5ml.) which were kept surrounded by ice.

The lysosomal fractions and the mitochondrial fractions were separately pooled, diluted to 250ml. with buffer, and centrifuged at 12,000 r.p.m. for 1 hour. The subcellular particles were resuspended in 50mM-sodium cacodylate buffer (5 ml.), frozen and thawed three times using acetone and dry ice and the ruptured membranes then removed by centrifugation at 100,000g for 1 hr. The supernatant solutions were assayed for fructokinase activity.

8) Relative fructokinase and hexokinase activity in liver tissue preparations.

One male rat (244g) and one female rat (218g) were killed and the livers immediately excised, dried and weighed, and each liver independently homogenized. The soluble protein preparations were prepared as described in Experimental 2(a) and both enzyme preparations were divided into two fractions. A fructokinase assay (assay (b)) was performed on one fraction; the second fraction was acidified to pH 6.0, centrifuged at 20,000g for 20 min. and readjusted to pH 6.5. Fructokinase activity was then determined using assay (b) after the addition of 0.5M 2-acetamido 2-deoxy-D-glucose (5µl) (259).

9) Hormone injection studies

Male and female rats weighing between 150-280g were injected subcutaneously with the following hormones in a saline/propylene glycol mixture (1ml.) (see Experimental (10)):

(i) testosterone (3, 12 and 24mg./Kg. body wt.);

(ii) estrone (24mg./Kg. body wt.).

Control animals were injected with saline/propylene glycol (1ml.). 6 rats were used with each hormone.

The animals were killed at various times (1-72hr.) after injection; the livers were immediately excised and soluble protein fractions prepared independently from each liver as described in Experimental (2).

Fructokinase and fructose 1-phosphate aldolase activities were determined.

10) Preparation of steroid suspensions

The steroid hormones are relatively insoluble in aqueous media, hence, all treatments were made with a fine suspension of hormones.

Testosterone and estrone were first dissolved in propylene glycol at 50° before the addition of saline solution (0.9%) or Krebs-Ringer bicarbonate solution. In this procedure the final organic phase in the medium did not exceed 0.5% (356).

11) Studies with liver slices

Liver slices were prepared as described by Deutsch (357). The slices (200-300mg. wet wt.) were placed in stoppered glass tubes containing Krebs-Ringer bicarbonate solution (10ml) with and without added steroid hormone. Control media also contained propylene glycol. The tubes were incubated in a water bath at 37° with shaking.

After incubation, the slices were removed from the medium, washed thoroughly in ice-cold Krebs-Ringer bicarbonate solution and dried on filter-paper. The slices from each tube were homogenized in 50mM-sodium cacodylate buffer (pH 6.5; 3ml), in a Potter-Elvehjem homogenizer (vol. 7ml, fitted with a teflon pestle with a clearance of 0.23mm.) and centrifuged at 100,000g for 1 hr. (0-4°). Enzyme assays were carried out on the supernatant fraction.

12) Effect of cycloheximide on fructokinase activity in rat liver slices.

Liver slices were incubated in Krebs-Ringer bicarbonate solution (as described in Experimental (11)) with testosterone (1mg./10ml.sol.) and testosterone (1mg./10ml.sol.) plus cycloheximide (final concentration in medium 4mM) (358). Control media contained propylene glycol. Soluble protein preparations were made and enzyme assays carried out as described in Experimental (11).

Appendix 1

Partial purification of Fructokinase

A purification of fructokinase was carried out with the intention of studying possible direct *in vitro* effects of sex hormones on the enzyme.

A 20-fold purification of fructokinase from male rat liver was obtained by a modification of the method of Hers (257).

The rat was killed by cervical fracture, the liver excised and macerated in a M.S.E. Homogenizer (3 min. max. r.p.m.) with ice-cold 50mM sodium cacodylate buffer (1:3, w/v; pH 6.5). The homogenate maintained at 0^{o} was acidified to pH 4.5 with N-HCl and then immediately adjusted to pH 5.5 with 0.5N NaOH. The denatured protein was centrifuged off (9000g, 30 min.) and the clear supernatant adjusted to pH 7.5 and fractionated with ammonium sulphate. The precipitate appearing at 0.4-0.6 saturation was centrifuged off (9,000g, 20 min.) and dissolved in 50mM sodium cacodylate buffer and then dialyzed against cacodylate buffer (2L) for 24 hr. at 4^{o}.

The enzyme activity was assayed using ^{14}C-fructose (see P.112) and the results expressed as fructose 1-phosphate c.p.m. x 10^{-3}/ min./mg.sol. protein.

	Activity
Crude enzyme	3.0
0.4-0.6 ammonium sulphate fraction	66.0

The purified enzyme was used to investigate the action of sex hormones *in vitro*, however, severe difficulties were experienced with hormone solubilities and the results obtained were considered to be dubious, and hence are not reported in this thesis.

Appendix 2

Gas-liquid chromatographic separation of fatty acids

In an early stage of this investigation attempts were made to examine the incorporation of label from ^{14}C-fructose into individual glyceride fatty acids. This study was later abandoned, however, in view of the difficulty of interpreting the very complex series of reactions involved. However, a preliminary examination of the glyceride fatty acid composition of a number of rat tissues was made and this is reported here.

Rat tissues (liver, heart, arterial) and serum were added to Bloor's solvent (ethanol:diethyl ether; 3:1 v/v) (344) and the mixtures brought to the boil and filtered. The protein precipitates were washed twice with Bloor's solvent and the combined extracts evaporated to dryness. The residues after three extractions with hot petroleum ether (B.P.40-60^{o}), and filtration, were then reduced in volume and applied to Silica gel thin layer chromatography plates. These were developed with a petroleum ether (40-60^{o}): diethyl ether : glacial acetic acid (82:18:1; v/v) solvent, then dried and stained with iodine vapour to identify the glyceride fraction. The iodine was allowed to sublime off and the glycerides scraped off the plate, dissolved in petroleum ether, centrifuged and finally saponified with ethanolic KOH (11% w/v). The mixtures were acidified with N-HCl and the two layers allowed to separate. The petroleum layers containing the fatty acids were isolated and reduced in volume, then placed in flasks and treated with excess boron trifluoride-methanol complex (14% BF_3 in methanol). The flasks were gassed with nitrogen, sealed and incubated at 60^{o} for 30 min. The BF_3-methanol mixtures were removed by washing with water and the organic layers then separated, dried with anhydrous sodium sulphate and evaporated to dryness. The methylated acids were taken up in a known volume of petroleum ether (B.P.40-60^{o}) and analysed immediately by gas-liquid chromatography using a Perkin Elmer F11 Gas Chromatograph fitted with a hydrogen flame ionization detector with nitrogen as a carrier gas. The temperature used was in the range 175-190^{o} and the column (6ft x ¼ in.) contained

20% Diethyl glycol succinate (DEGS) on a HMDS Chromosorb W.80-100 mesh support.

The major bound fatty acids in the rat liver (see Fig. 24) were found to be myristic, palmitic, stearic, oleic and linoleic. There appeared to be qualitative differences between various tissues and it is particularly interesting to note differences between different lobes of the liver. This latter observation should be borne in mind when examining the metabolism of liver cells.

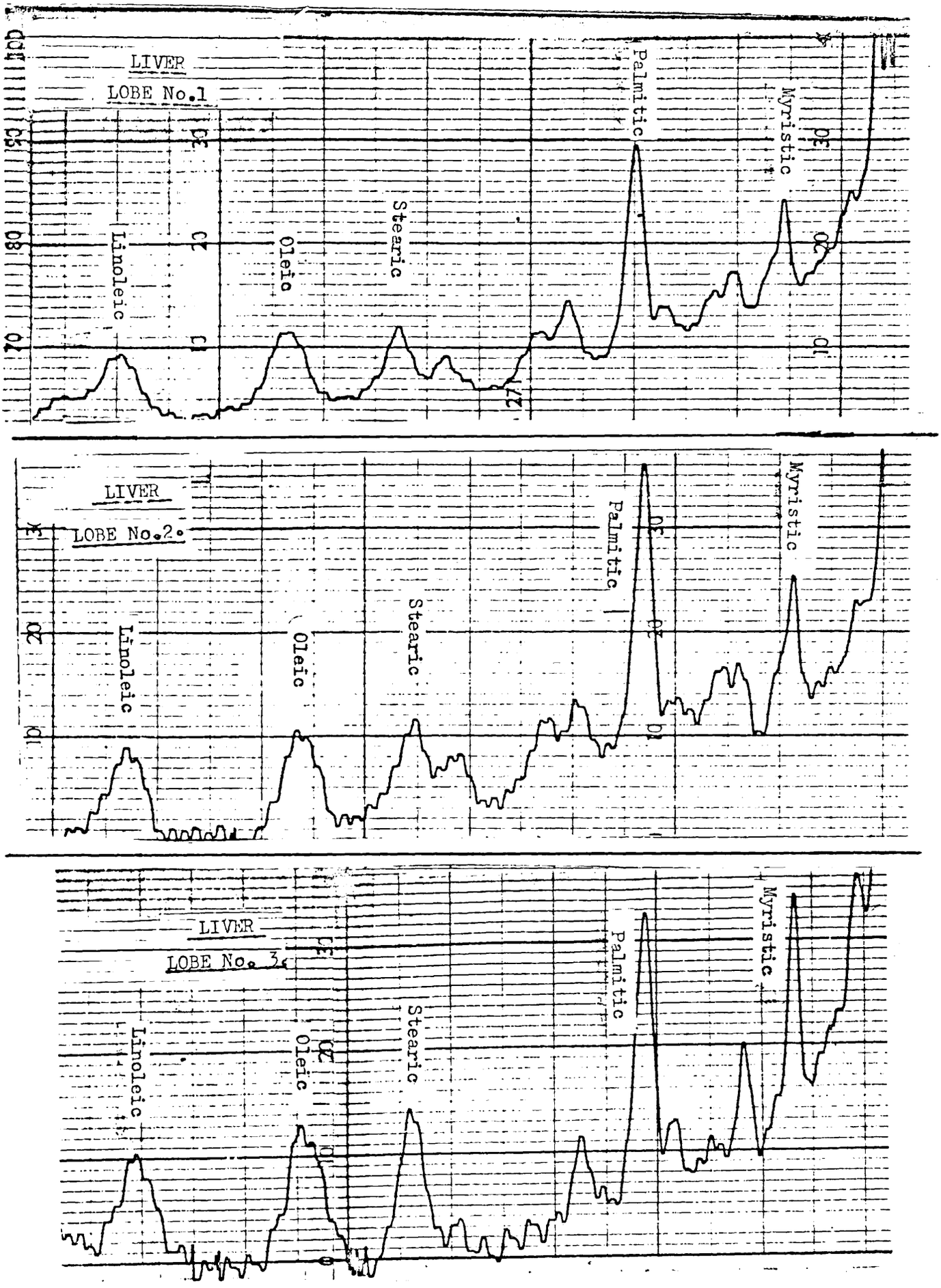


Fig.24. Fatty acids from liver glycerides.
Conditions - Temp. 190°; Air, 19lb/in^2; H_2, 14lb/in^2, Flame 3lb/in^2.
Sensitivity 1/10th; Chart speed, 600 mm/h.

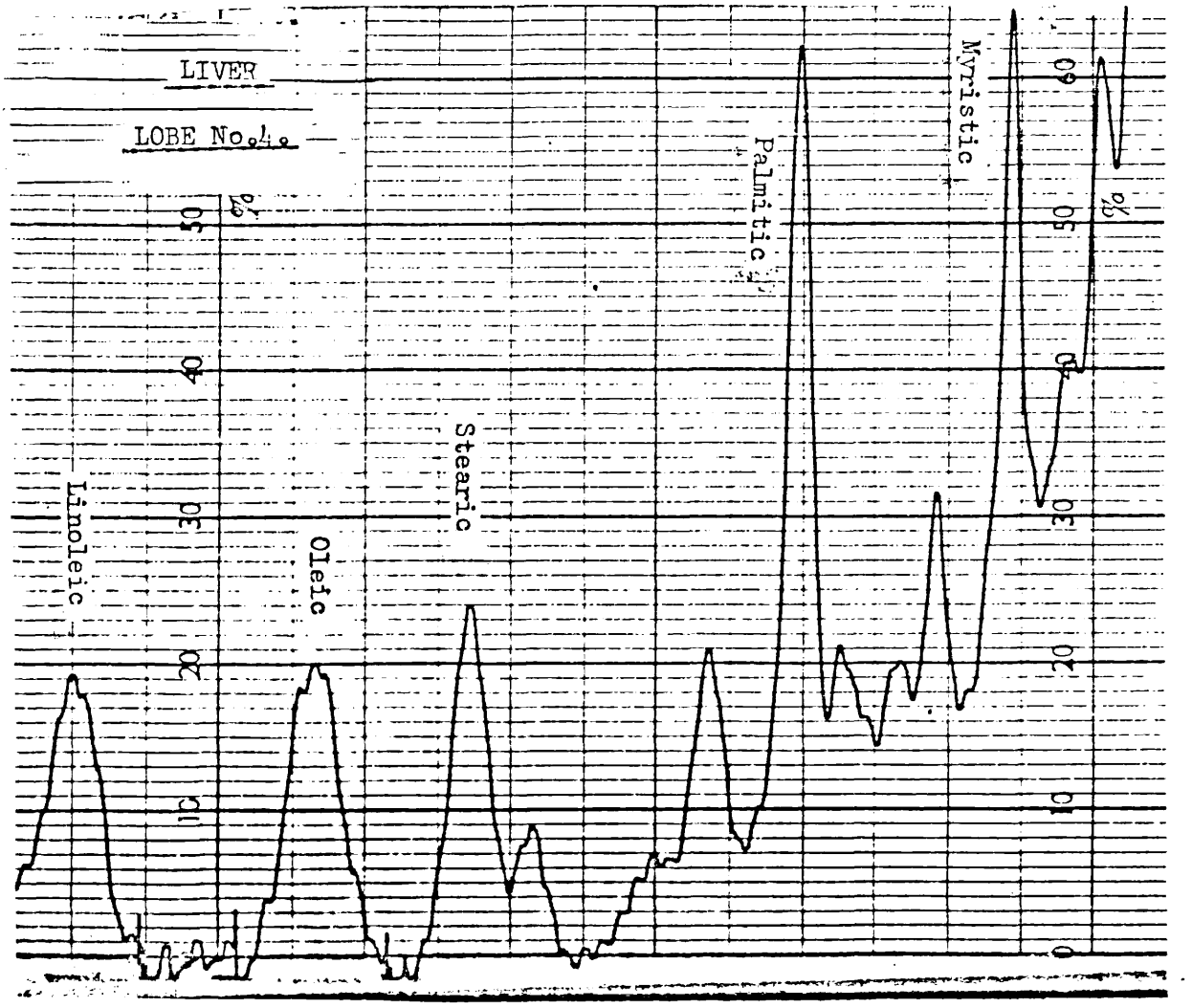


Fig.24-cont'd. Fatty acids from liver glycerides.
Conditions - Temp, 190°; Air, 19lb/in^2; H_2, 14lb/in^2, Flame 3lb/in^2.
Sensitivity 1/20th; Chart speed, 600 mm/h.

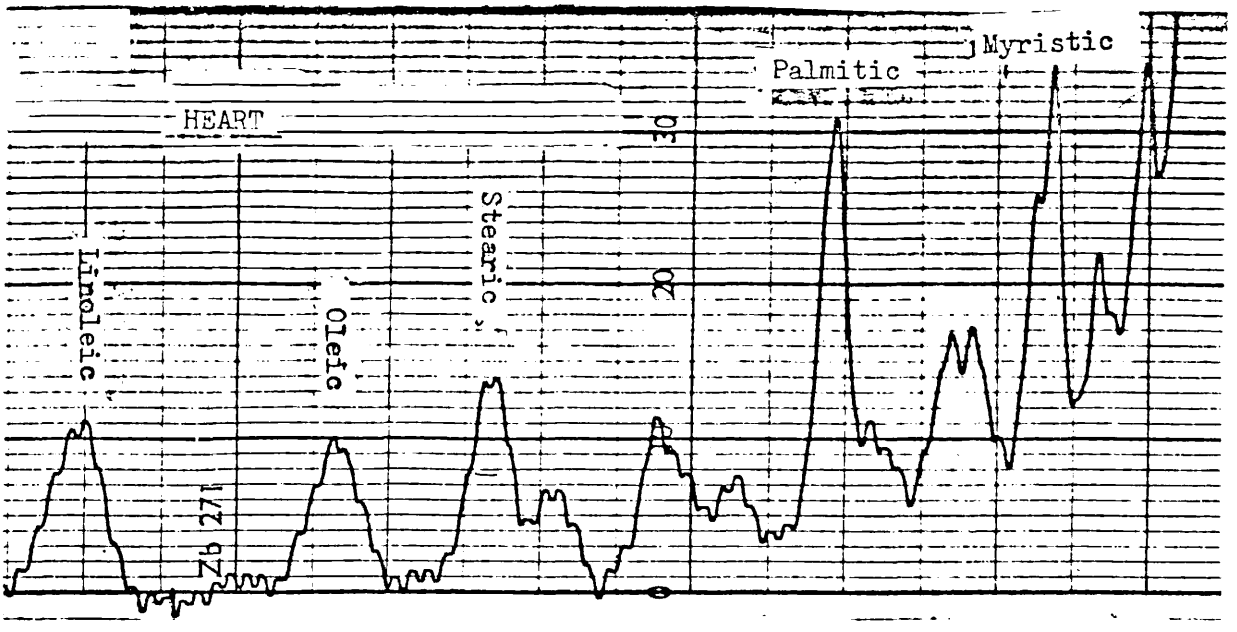


Fig.24-cont'd. Fatty acids from heart glycerides.
Conditions - Temp, 190°; Air, 19lb/in^2; H_2, 14lb/in^2, Flame 3lb/in^2
Sensitivity 1/10th; Chart speed, 600 mm/h.

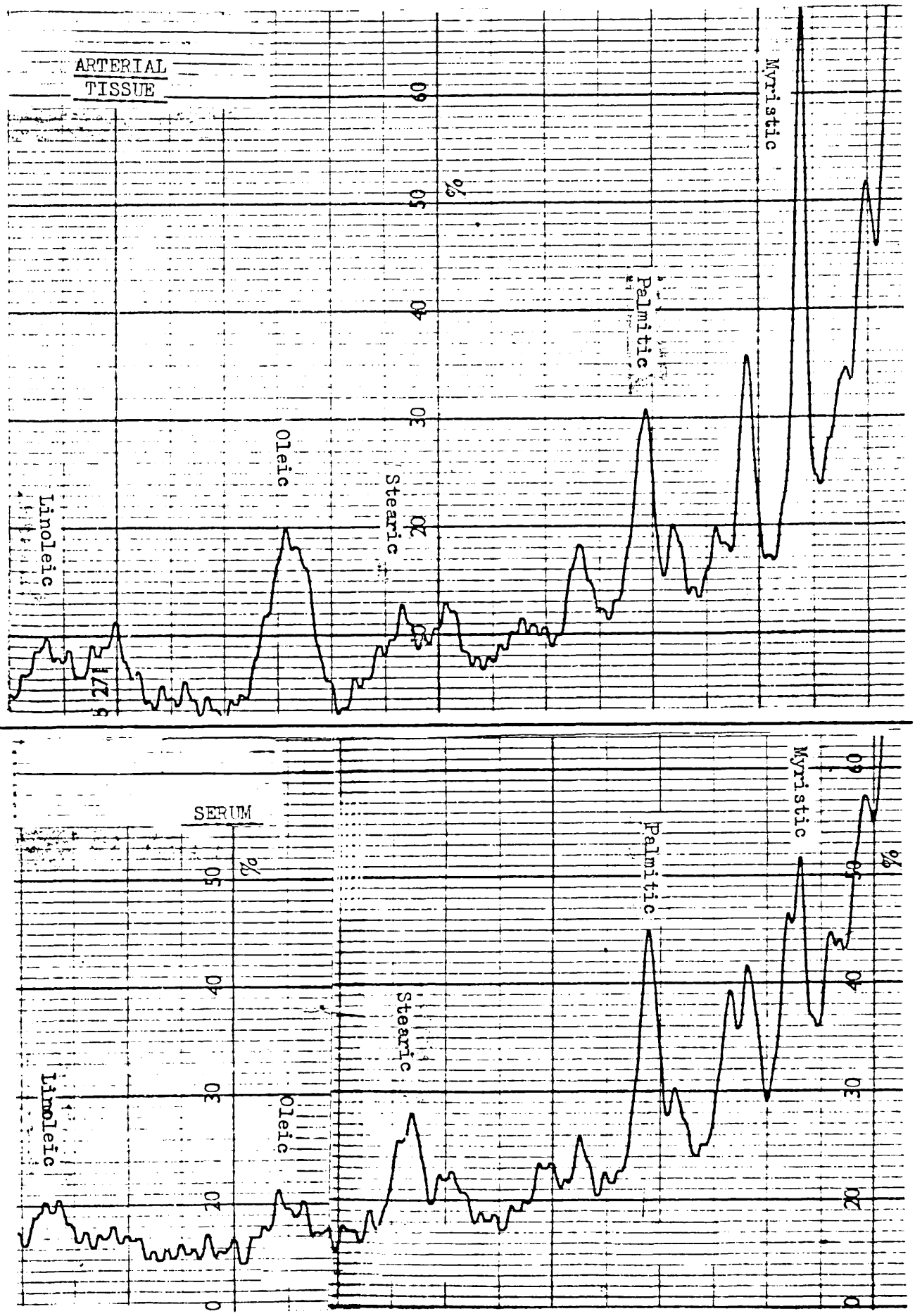


Fig.24-cont'd. Fatty acids from arterial tissue and serum glycerides.
Conditions - Temp, 190°; Air, 10lb/in^2; H_2, 14lb/in^2, Flame 5lb/in^2.
Sensitivity 1/10th; Chart speed, 600 mm/h.

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