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SEX-HORMONE DEPENDENT REACTION SEQUENCES IN THE INTERMEDIARY METABOLISM OF PRAOMYS (MASTOMYS) NATALENSIS

Thesis presented in fulfillment of the requirements for the degree of Master of Science at the University of the Witwatersrand

by

NEVILLE LIDDELL HOWARD

JOHANNESBURG.

February, 1969.

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DECLARATION

I, NEVILLE LIDDELL HOWARD, hereby declare that the work forming the basis of this thesis is my own and has not previously been submitted for any other degree at any university.

Signed: N L Howard.

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CHAPTER 1.

INTRODUCTION AND SUMMARY

It is over a century ago since it was first shown that fat can be synthesized from dietary carbohydrates, and today it is realized that dietary carbohydrates are more intimately and specifically involved in lipid metabolism than was considered possible twenty years ago. There seems little doubt now that dietary carbohydrates can lead to the accumulation of excess lipid in the liver and blood of man or animal.

Until recently there had not been any serious consideration of the possibility that the metabolic efficiency of males and females might differ. However, evidence has been accumulating which suggests that the incidence of coronary heart disease (a disorder of lipid metabolism) is much lower in premenopausal females than in males of the same age group. Similarly eunuchs fit better into the female pattern than into the pattern characteristic of sexually normal males.

These observations, which suggest a link between sex hormones and lipid metabolism, have led to renewed interest in the possibility of sex-dependent differences in the efficiency of conversion of carbohydrates into lipids.

Macdonald (1966a & 1966b) has shown that in humans, females are able to tolerate high-carbohydrate diets better than males. Indeed,

premenopausal females demonstrated a remarkable resistance towards the induction, by dietary carbohydrate, of the hypertriglyceridemia which occurs so readily in males and in post-menopausal females. This observation raises an interesting question: If the intake of dietary carbohydrate in males and females is essentially the same, and only the premenopausal females are able to resist the development of hypertriglyceridemia, is it possible that the female may have a physiological mechanism for the disposal of excess carbon and metabolic energy, which in the male would be used for lipid synthesis? How excess metabolic energy could be dissipated without generation of an uncomfortable quantity of heat is not known, but there may be sex-linked differences in the fundamental process associated with the production and utilization of metabolic energy.

Several other workers have detected sex-hormone-dependent metabolic differences in animals. Of these the most striking is the observation that males are capable of producing a hepatic protein which does not occur in females (Roy & Neuhaus, 1967). It seems reasonable to anticipate more sex-hormone-dependent differences and a search for these could lead to a better understanding of mechanisms which control lipid metabolism in the intact animal in vivo.

In this dissertation studies which were directed towards an investigation of some of the fundamental processes which link carbohydrate and lipid metabolism are described. We have studied:

1) The efficiency of oxidative phosphorylation in intact mitochondria of both sexes.

2) The acceptor control ratio of mitochondria from males and females.

3) The specific activities of some rate-determining lipogenic enzymes.

We were able to demonstrate that, in the oxidative phosphorylation P:O ratios and in the acceptor control ratios, there were no significant differences between male and female values while the mitochondria were respiring under controlled standard conditions. However, we did discern that, under these conditions, the female mitochondria produced larger quantities of adenosine triphosphate (ATP) and correspondingly used more oxygen than did the male mitochondria. This finding could indicate an inherent increased rate of substrate oxidation in the female, necessitating the dissipation of excess carbon, which in the male could be channelled towards fatty acid synthesis. To substantiate this hypothesis however, it would be necessary to demonstrate a more efficient system for the generation of reducing potential in the male.

The hexose-monophosphate shunt was believed to be the source of reducing potential for fatty acid synthesis. However, it has since been shown that this metabolic process supplies 50-60% of the required reducing equivalents necessary for the synthesis of fatty acids. Experimental results have shown that the activity of the malic enzyme was altered in

such a way as to suggest that malate may play a role in lipogenesis by furnishing reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) for reduction during utilization of acetyl-CoA for fatty acid synthesis (Wise & Ball, 1964).

We have demonstrated that, in the mouse, the activity of the hepatic malic enzyme is dependent on testosterone. In the liver of normal males the activity of this enzyme is considerably higher than in the liver of normal females. In castrated males the malic enzyme activity resembled that of the normal females. However, when castrated males were treated with daily injections of testosterone propionate the activity of the malic enzyme was again comparable with the level of activity found in normal males. Estrogen apparently had no effect upon the level of activity of this enzyme, for when castrated males were treated with daily injections of estradiol benzoate the activity of the malic enzyme was comparable with the level found in untreated castrated males.

These findings appear to suggest a mechanism for the generation of extra reducing equivalents in the male. However, this introduces an additional problem, i.e. the production of excess substrate, malate, in the male for the action of the malic enzyme.

We have demonstrated that the activity of the hepatic mitochondrial malate dehydrogenase, which catalyses the reduction of oxaloacetate to malate, is greater in the male than in the female. In addition the activity of this enzyme appears to be testosterone dependent,

for castration lowers its activity, and daily injections of testosterone propionate to ovariectomized females increases its activity. Since malate easily passes through the mitochondrial membrane, the source of substrate for the malic enzyme is assured.

Some workers have implicated the reaction catalyzed by isocitrate dehydrogenase as a source of extra-mitochondrial reducing potential. We investigated the activity of this enzyme on a comparable basis but were unable to show any sex-linked differences. However, the possibility that this enzyme does supply reducing equivalents to the system, as a whole, is not ruled out.

In our investigations of the activity of pyruvate kinase in liver cytoplasm we found, that in the female the activity of this enzyme was greater than in the male, and that this enzyme appeared to be estrogen dependent. In ovariectomized females, the pyruvate kinase activity resembled that of normal males. However, when ovariectomized females and castrated males were treated with daily injections of estradiol benzoate, the activity of this enzyme was elevated above that of normal males. In contrast, treatment with testosterone propionate neither elevated nor decreased the enzymic activity in ovariectomized and castrated animals.

It has been shown that pyruvate passes readily through the mitochondrial membrane, and the possibility exists that in the female pyruvate passes through the mitochondrial membrane at a greater rate than it does in the male. A possible course which can then follow is for the pyruvate

to be decarboxylated and then oxidized via the tricarboxylic acid (TCA) cycle resulting in the production of energy. This interpretation is substantiated, to a certain degree, by our previous findings, that during oxidative phosphorylation the respiring mitochondria from female animals produces more ATP than those from male animals.

These investigations are by no means complete, yet from a perusal of the results obtained it is clear that the stage is set for interesting developments in the study of the metabolism of lipids and carbohydrates in the mammalian system, on a comparative basis between male and female animals.

CHAPTER 2.

THEORETICAL

2.1. Nature and Theoretical Background of the Problem.

Obesity has always been a problem in affluent communities and has been attributed to overeating and a lack of adequate exercise. In recent years, it has been realized that obesity may also be due solely to genetically determined glandular disorders rather than overeating in all cases. However, it is now agreed that in otherwise healthy individuals obesity is due to excessive intake of fattening foods. Obesity is considered one of the most prevalent metabolic disorders of modern times. It predisposes to degenerative diseases, notably atherosclerosis and its complications, and to diabetes, arterial hypertension and diseases of the gall bladder (Duncan et al., 1964). In addition it increases the risks attending operative procedures and unfavourably affects the general mortality rate.

Simple exogenous obesity is the result of faulty eating and exercise habits and, as with other disturbances of discipline, its correction may be difficult and, if achieved, may yet be temporary. It has been stated that in 90 per cent of cases obesity has been due simply to overeating without any detectable cause. The remaining 10 per cent have their origin in a genetically determined predisposition to the disorder, or are secondary effects of other disorders which disrupt metabolism in such a way that obesity develops; e.g. von Gierke's disease; associated with diabetes; in Cushing's syndrome (hyperglycocorticoidism); in dis-

orders of the reproductive system.

According to Mayer (1964), obesities can be classified from the point of view of etiology or of pathogenesis. The etiological approach has been developed at length and in this genetic, traumatic, and environmental factors have been distinguished. Such a distinction, although useful necessitates a certain degree of over simplification. In order for obesity to develop, there has to be permissive interaction of genetic and environmental background.

Mayer (1964) found that a general distinction could be made between regulatory obesities, in which the primary impairment is of the central mechanism regulating food intake, and obesities in which the primary lesion is an inborn or acquired error in the metabolism of tissues, per se.

Regulatory obesities are exemplified by induced hypothalmic obesities. The experimental animals have an increased rate of lipogenesis and of cholesterogenesis and these rates are in proportion to the amount they are allowed to overeat. Their rate of absorption of glucose by the intestine increases. When such obese animals are reduced by fasting, their body composition returns to normal.

The situation in metabolic obesities, such as the hereditary obese hyperglycemic syndrome in mice (Mayer, 1964), which corresponds to diabetes mellitus in humans, or obesity due to grafting of Adrenocorticotropic hormone (ACTH)-secreting pituitary tumors, is in striking contrast

to that in regulatory obesities. In these metabolic disorders, the rates of lipogenesis during fasting are increased over the normal fasting rates, and reducing the animals to normal weight by underfeeding them does not bring body composition back to normal, but leaves them with a fat content still considerably greater than the normal fat content, at the expense of non-fat tissues (Mayer, 1960). The adipose tissue has many abnormal characteristics: a high coenzyme A activity (Thompson & Mayer, 1962), decreased uptake of glucose; increased lipogenesis from acetate (Christophe et al., 1961); absence of effect of the natural dietary fat, of epinephrine, and of fat mobilizing substance on fatty acid releases from adipose (Lochaya et al., 1961); presence of glycerokinase activity (Lochaya & Mayer, 1963).

Some of the recognised patterns in the etiology of obesity in man are listed below (Mayer, 1964).

Genetic: A multiplicity of genes have been studied; e.g. in congenital adipose macrosomia; in monstrous infantile obesity; associated with von Gierke's disease; in familial hypoglycemosis (a congenital lack of alpha cells in the pancreas).

Of hypothalmic origin: In dystrophia adiposogenitalis, with discrete or diffuse hypothalmic injury.

Of endocrine origin: With insulin-producing adenoma of the islets of Langerhans, with diffuse hyperplasia of the islets, and in association with diabetes; in Cushing's syndrome (hyperglycocorticoidism); in disorders

of the reproductive system; and hypogonadism postpuberal castration, menopause, and ovarian disorders.

Otherwise induced: By immobilization in adults and children; by social and cultural pressure.

Literal extension to man of the examples worked out in the mouse or in other experimental animals is not possible, although there are indications that certain non-ketotic types of diabetes associated with overweight in human subjects may be analogous to the hereditary obese hyperglycemic syndrome in mice. On the other hand, extension to man of the general concept of the existence of "regulatory" and metabolic" hyperphagias appears legitimate.

Finally, considerable evidence has been adduced that in man, too, the range of adaptation of normal regulation does not cover the complete range of possible energy expenditures, since in adult man at very low activity ranges, appetite does not decrease in proportion to caloric output, with resultant accumulation of fat (Mayer, 1964). In adolescents, obesity is more characteristically associated with inactivity than with intakes much in excess of normal (Johnson et al., 1956).

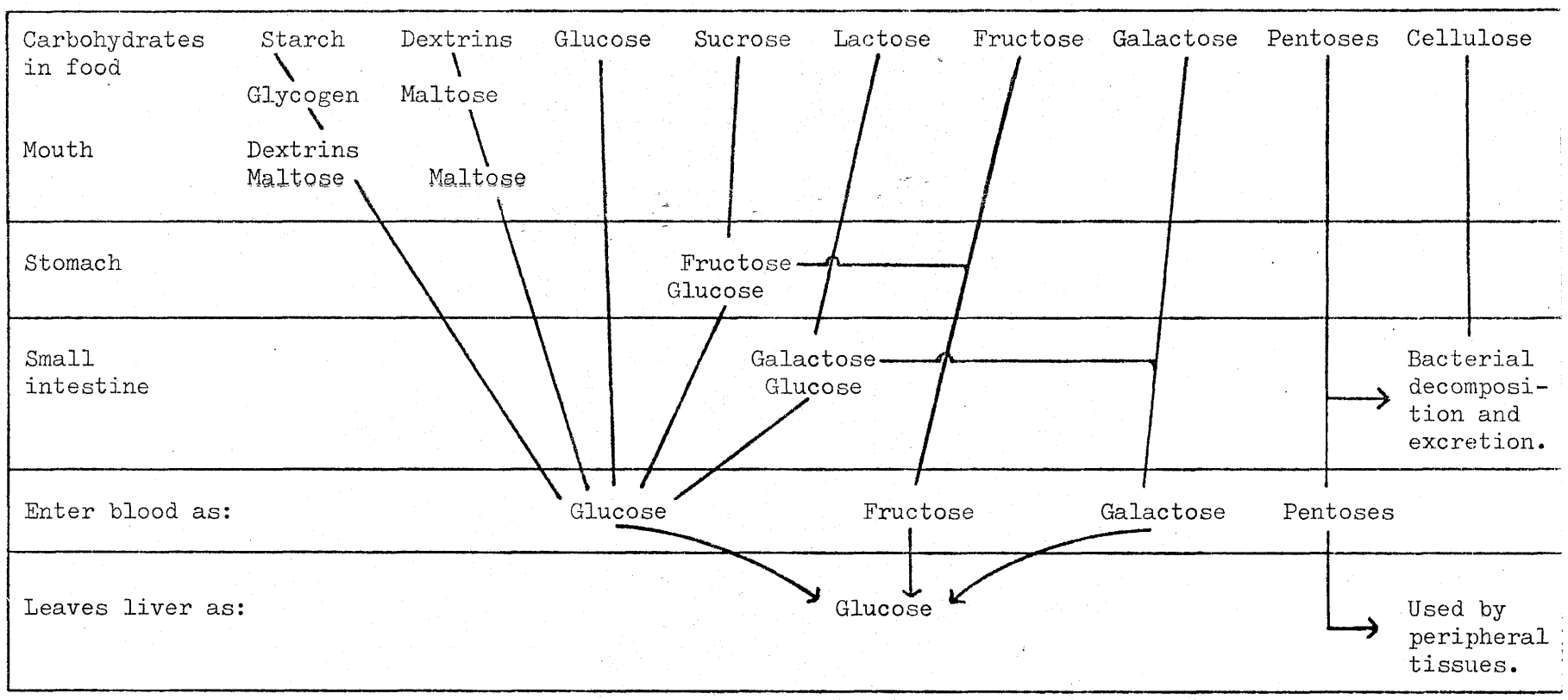
From the above conclusions it is seen that obesity can be attributed to some or other disorder in the metabolism (or excess intake) of either carbohydrates or lipids, or to a genetic aberration of these metabolites.

In this thesis, in the main, we are interested in the induced disorders of carbohydrate and lipid metabolism, i.e. the effects directly traceable to the excess intake of carbohydrates and/or lipids. For completeness, brief sections will deal with the digestion and absorption of these dietary constituents.

The monosaccharides, ingested or resulting from the digestion of carbohydrates, are almost completely absorbed in the small intestine (Table 2.1.). Small amounts may be absorbed from the stomach or the large bowel. Two types of absorption may occur in the small intestine; (a) a specific absorption of particular monosaccharides, and (b) a nonspecific absorption of all monosaccharides by diffusion resulting from osmotic forces across the mucous membrane. The absorption of glucose, fructose and galactose by both processes differs in two respects from that of those sugars absorbed by diffusion alone. Present evidence points against a phosphorylation process being involved in actual sugar uptake. A significant proportion of glucose may be degraded to lactic acid in the intestinal epithelium and may enter the blood in that form. Fructose is transferred unchanged to the extent of about 50%; some is transformed to glucose and another portion to lactic acid.

The position at present as regards digestion, absorption and transport of fat is as follows (Senior, 1964; Kinsell et al., 1967): the most important dietary lipid constituent is triglyceride, nearly all of which is normally absorbed when reasonable quantities are ingested.

Table 2.1. Products of carbohydrate digestion at various levels of the gastro-intestinal tract and subsequent fate (Soskin & Levine, 1952).



Division of the food triglyceride into a coarse emulsion occurs principally in the stomach, with ejection of the emulsion droplets into the duodenum.

The EXOGENOUS long chain and short chain TRIGLYCERIDES are completely or partially hydrolyzed in the small intestine through the action of pancreatic and intestinal lipases in the presence of bile salts. The short chain fatty acids released, are relatively more water-soluble and tend to be transported into the mesenteric portal blood without being activated and re-incorporated into triglycerides.

As the long chain triglyceride primary ester bonds are cleaved, free fatty acids and 2-monoglycerides are formed, within which there is a partial isomerization of 2- ester bonds to 1- or 3- ester bonds. Some free glycerol may be produced, which is quickly absorbed into the intestinal mucosa by passive transport, and mostly enters the mesenteric venous blood, although a small fraction of it may be phosphorylated by enzymes in the intestinal cell cytoplasm.

Within the intestinal mucosa the fatty acids are converted to water soluble, chemically reactive thiolester derivatives, using energy from ATP, catalyzed by enzymes principally located in membrane structures. The monoglycerides again are important as acceptors of fatty acyl chains on their free hydroxyl groups and this is the major pathway to diglyceride formation during fat absorption. In addition Glycerol-3-phosphate, derived from glucose metabolism, or to a minor extent from phosphorylation of free glycerol, may be esterified to phosphatidic acid from

which diglycerides may be formed alternatively. From diglycerides the major products are triglycerides which are discharged into the thoracic duct via lymphatic channels as chylomicrons, which represent the lowest density lipoprotein fraction synthesized by body tissues (Isselbacher, 1964). The triglyceride fatty acids typically resemble the ingested fat, whereas the fatty acids of cholesterol esters and phospholipids seem relatively unaffected by dietary fatty acid composition (Wood et al., 1964; Bierman, 1965). The entry of dietary chylomicrons into the blood-stream results in postprandial lipemia. As the actual chylomicron level at any time represents a balance between inflow and removal, its degree depends heavily on factors controlling absorption on one hand, and factors regulating utilization, such as the nutritional state of the individual, on the other. In normal subjects its peak may lie anywhere between 2 and 7 hours after a fat meal.

The major lipids of the plasma consist of cholesterol, cholesterol esters, triglycerides, phospholipids, and free fatty acids (FFA). The FFA's of the plasma are chiefly bound to albumin, while most of the other lipids are bound to specific globulins. Each of the specific lipoprotein complexes contain triglycerides, phospholipids, and cholesterol, although the proportions of the lipids may vary. In addition to the protein moiety the phospholipids are important in making the lipoprotein complex water soluble. The lipoprotein complexes with a greater relative lipid content have lower densities, and since proteins of different densities can be distinguished by their behaviour in the

ultra-centrifuge, this has provided the basis for one of the most commonly used schemes of classification. Since the rate of flotation of the lipoproteins is inversely proportional to their density, the different groups may also be subdivided on this basis, the unit of measure being the Svedberg flotation unit (SF) (Williams, 1962), see Table 2.2.

The fate of the chylomicrons after their discharge into the blood is still incompletely understood. A considerable portion of chylomicron triglyceride is hydrolyzed and taken up by extrahepatic tissues, particularly adipose tissue (Nestel et al., 1962a). Glucose and insulin appear to accelerate this process. The fatty acids are then re-esterified within the adipose tissue cell for storage. Adequate carbohydrate metabolism in adipose tissue is required to supply α-glycerol-phosphate for such re-esterification, since adipose tissue glycerokinase activity is very low and glycerol therefore cannot be used for esterification of fatty acids (Vaughan, 1961).

A significant role has been attributed to the liver in the initial removal of chylomicrons from plasma. A portion of the chylomicronous fat may be oxidized for energy metabolism, either directly or following hydrolysis. A significant proportion of plasma FFA's may be derived from chylomicrons (Nestel et al., 1962b).

Uptake of most of the chylomicron glycerides by adipose tissue is preceded by hydrolysis (Rodbell and Scow, 1965), which is mediated

by the enzyme(s) lipoprotein lipase located within the capillary wall.

ENDOGENOUS TRIGLYCERIDES are discharged by the liver as constituents of lipoproteins. The highest triglyceride content is found in lipoproteins of density between 0.96-1.006 (Sf20-400) (Kinsell et al., 1967). The triglyceride fatty acids may be obtained from the following sources;

(a) de novo synthesis from two-carbon fragments (Wakil & Bressler, 1962). The following sources have to be considered: dietary carbohydrates, proteins and short chain fatty acids (Uzawa et al., 1964).

(b) re-esterification of free fatty acids originating from adipose tissue (Havel, 1961).

(c) chylomicron triglycerides.

Since excess concentration in plasma of free fatty acids would be undesirable, the liver functions as an important link in the energy cycle by transforming such free fatty acids, regardless of source, to the transport form of triglycerides, which may then return to the adipose tissue bank.

There are no reliable data available regarding the exact proportion of dietary carbohydrate which is converted to triglycerides in the liver. With very high carbohydrate intake, VLDLP of Sf 20-400 are found in significant amounts in the plasma of most individuals, leading to elevation of total triglycerides in the fasting plasma (Ahrens, 1957a).

Table 2.2. Distribution of serum proteins and lipids in different lipoprotein fractions, correlated with densities and flotation rates.
Different fractions are shown as they become layered following centrifugation (Williams, 1962; Pilkington,1964).

Lipoprotein fraction and Electrophoretic mobility on Starch		Sf.	Density	Lipoprotein composition (per cent)			
				Protein	T.G.	Chol.	P.L.
1. VLDLP							
Chylomicron	α_2	10^3-10^5	0.96	1	87	4	8
	α_2	20 -400	0.96-1.006	7	56	17	20
2. LDLP	β_1	12 - 20	1.006-1.019	11	24	43	22
	β_1	0 - 12	1.019-1.063	21	9	47	23
3. HDLP	α_1		1.063-1.125	46	8	19	27
	α_1		1.125-1.21				
4. Albumin		Albumin residue contains the majority of FFA.					

VLDLP = very low density lipoproteins;

LDLP = low density lipoproteins;

HDLP = high density lipoproteins;

T.G. = triglycerides;

Chol. = cholesterol & -esters;

P.L. = phospholipid.

Their triglyceride content is intermediate between chylomicrons and low density lipoproteins (Gustafson et al., 1965), and can thus be easily differentiated.

There is some evidence to show that not only the amount but also the type of dietary carbohydrate influences the level and kind of lipids in serum. Macdonald and Braithwaite (1964) found higher plasma triglyceride levels in normal volunteers on high sucrose as compared to high starch diets, and of five preparations of carbohydrates used in a short term experiment (Macdonald, 1965a), only the feeding of sucrose resulted in elevation of the plasma triglyceride level. It has been suggested that fructose may be the agent responsible for these observations. Finally, no conclusive evidence has been presented as to whether endogenous and exogenous triglycerides differ as substrate for lipoprotein lipase.

The catabolisms of carbohydrate, protein and fat, respectively, pursue more or less independent, well documented courses, until they reach the stage of the alpha or beta keto acids. These keto acids (pyruvic, ketoglutaric, etc.,) then enter the tricarboxylic acid cycle directly or are broken down to a 2-carbon atom fragment ("Acetyl-CoA") which enters the cycle by condensing with oxaloacetate. (See figure 2.1.).

From this point on, the lower intermediates of all three foodstuffs are indistinguishable from one another; and from this "pool" of 2-carbon and 3-carbon intermediates, any of the three foodstuffs can

be built up again.

The position must now be clarified as regards the three major foodstuffs and their final common pathway. It is quite possible that an excess intake of any single division or combination of the three, could result in an excess of fat deposition.

Since obesity has been shown to be a contributory factor for numerous disorders of heart function and allied manifestations, and accounting for considerable morbidity and mortality, many clinical and biochemical results of obesity have been investigated.

Although the etiology of obesity is not adequately understood, four factors, often inseparable, seem to play prominent roles: (a) neurologic; (b) psychologic; (c) endocrine and metabolic, and (d) genetic. Many other factors particularly extrinsic also contribute.

Considerable data of diverse types have accumulated in recent years which indicate that obesity may be of prime importance in the etiology of arterial disease (Swell & Treadwell, 1963). The evidence for the participation of lipids in atherosclerosis is based on the following general observations: 1) a common factor in all experimental procedures for producing atherosclerosis in animals is the feeding of high lipid diets; 2) populations with high serum lipid levels have a higher incidence of heart disease than groups without elevated levels; 3) there is a greater incidence of atherosclerosis in individuals with abnormal lipid

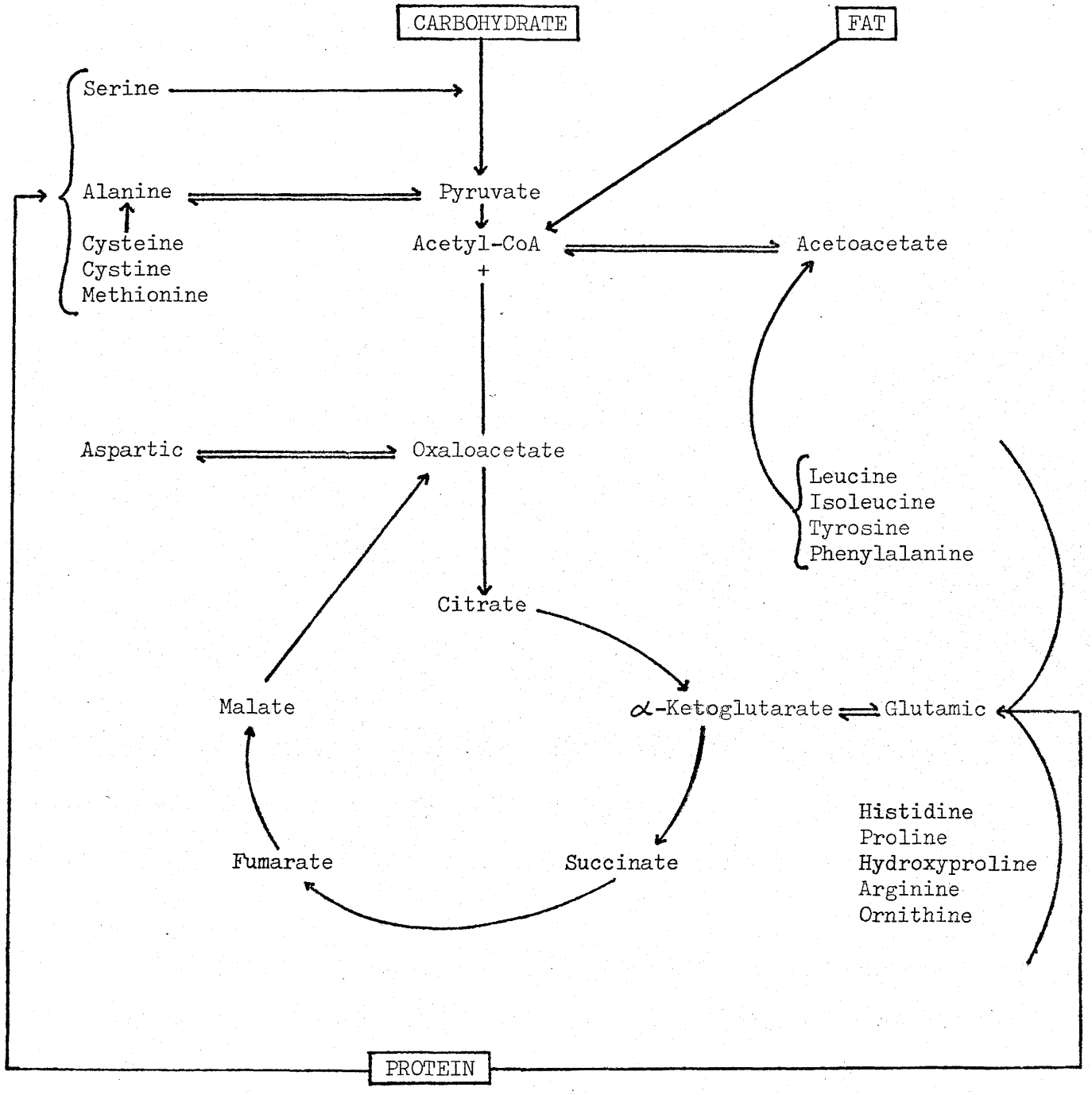


Fig. 2.1. Final common pathway of metabolism (Levine, 1964).

metabolism, such as diabetes, xanthomatosis, and nephrosis; 4) analysis of atheromatous lesions indicates the presence of large amounts of lipids. Currently there is some doubt as to which is the most meaningful indication of probable atherosclerosis, hypercholesterolemia or hypertriglyceridemia.

Since atherosclerosis has been associated with obesity there have been many studies on the induction of hypercholesterolemia and general hyperlipemia by various types and amounts of dietary fats (Kinsell et al., 1952; Ahrens et al., 1957b; Bieberdorf & Wilson, 1965). It has recently been shown that dietary carbohydrates affect serum cholesterol levels as well (Grande et al., 1965; Lopez et al., 1966; and McGrandy et al., 1966). Furthermore it is well established that patients with overt coronary disease have higher levels of plasma lipids than do matched controls without discernible disease. The lipids involved in this difference are the low density lipoprotein fractions (Gofman et al., 1954). Since this fraction is rich in triglyceride it is not surprising that triglyceride levels are especially elevated in patients with coronary disease (Albrink & Man, 1959; Antonis & Bersohn, 1960; Carlson, 1960). Studies in different populations with widely differing disease rates have clearly shown that only those with relatively high lipid levels show a significant incidence of coronary disease. Populations with low levels of serum lipid such as the Bantu, and Yemenite Jews, all show a nearly

total absence of coronary heart disease (Bronte-Stewart, 1958). This is not a genetic characteristic. When such people migrate to affluent countries they develop the plasma lipid pattern and the coronary rates of their new environment. Elevated lipid levels are not secondary to coronary heart disease, but are significantly associated with the risk of developing it (Dawber et al., 1962). Increased levels of plasma lipids, specifically glyceride fraction, has also been demonstrated while test animals were under a regime of high fat and/or high carbohydrate diets (Ahrens et al., 1961).

The term hyperlipemia usually refers to an increase in plasma lipids (i.e. triglycerides, phospholipids, cholesterol and free fatty acids) above their normal level. Hyperlipemias are classed as shown in Fig. 2.2.

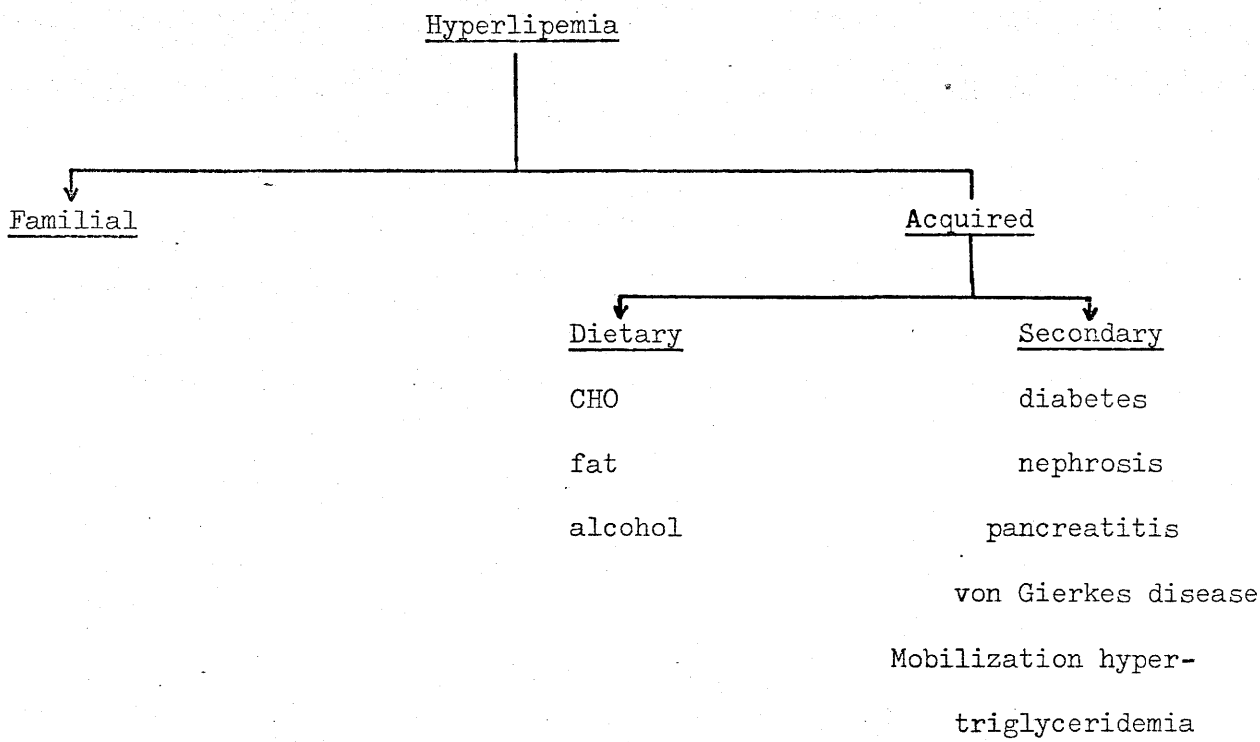


Fig. 2.2. Examples of Hyperlipemias.

The familial or hereditary hyperlipemia, differs from the acquired dietary type, in that the plasma lipid concentrations are increased above the normal level even though the individual is on a balanced normal diet. This genetic hyperlipemia is more severe than the acquired type (Frederickson et al., 1967), and may further be aggravated by feeding diets rich in carbohydrates or fats, or a mixture of both.

Frederickson & Lees (1965), have indicated that carbohydrate induced hyperlipemia differs from the lipid induced type, whereas, in the former there is an increase of endogenously synthesized plasma lipids, namely the very low density lipoprotein fraction, in the latter there is an increased concentration of chylomicrons i.e. lipids obtained from digestion of dietary fat.

The induction of experimental hyperlipemia by feeding of diets rich in carbohydrates or fats, or a mixture of both, is usually only transient in nature. Antonis and Bersohn (1961), have shown that on feeding a low fat, high carbohydrate "Bantu-type" diet, to European prisoners, resulted in a steady increase in triglyceride values, which reached a maximum in 5 weeks. After this period the values started to decrease reaching the normal basal level after 32 weeks.

Kinsell et al., (1967), have adopted a classification for the identification of hypertriglyceridemias, which is sufficiently non-specific to permit inclusion of most of the cases reported, and is (a)

exogenous hypertriglyceridemia and (b) endogenous hypertriglyceridemia.

Exogenous hypertriglyceridemia

Hyperchylomicronemia, type I of Fredrickson & Lees (1966), Fredrickson et al., (1967), fat-induced hyperlipemia, alimentary hypertriglyceridemia.

This disorder is characterized by intolerance to all types of dietary fat. On diets containing fat the serum shows various degrees of turbidity and a characteristic "creaming" of the particulate fat after standing overnight. Subsequent feeding of fat-free diets results in clearing of the lipemia.

It is now generally agreed that in these subjects hyperlipemia is caused by defective removal from the blood of dietary chylomicrons (Fredrickson & Lees, 1965), the half life of which is usually markedly prolonged, depending on the severity of the hyperlipemia. Whereas in normal persons a single fat load is cleared from the circulation within 8 hours or less. Fat loading in fat-induced hyperlipemia results in exaggerated and prolonged peak lipemia and clearing of a single fat load usually requires several days (Ahrens et al., 1961).

Estimation of postheparin lipoprotein lipase in the plasma of these subjects, using in vitro hydrolysis of triglyceride emulsions as a measurement, has uniformly yielded very low values. Further studies will be necessary to establish the physiologic role of the enzyme(s) more

firmly, particularly as regards its function in adipose tissue and liver (Kinsell et al., 1967).

Endogenous hypertriglyceridemias

Carbohydrate-induced hyperlipemia (Ahrens et al., 1961).

Mixed hyperlipemia (Kuo & Bassett, 1963).

Types III to V hyperlipoproteinemias (Fredrickson & Lees, 1965).

Non-alimentary hyperlipemia (Kinsell & Schlierf, 1965).

When one departs from hyperchylomicronemia, the classification of hyperlipemia becomes much less simple. However, it seems likely that a basic defect in all the syndromes discussed is associated with some type of abnormality of metabolism of endogenous very low density lipoprotein with regard to either formation or clearing of these particles. There is general agreement that the majority of these patients have gross or occult abnormality of carbohydrate metabolism.

In spite of evidence suggesting some basic common denominator in most endogenous hypertriglyceridemics, Kinsell et al., (1967), have divided these syndromes into three major groups:

1. Carbohydrate-induced hypertriglyceridemia.

Following reports of increases in serum triglyceride in normal subjects on high carbohydrate diets, Ahrens et al., (1961) proposed that essential hypertriglyceridemia could appear in two distinct varieties, "fat-induced" and "carbohydrate-induced" forms, when they had noted that

the majority of their hypertriglyceridemic patients had higher plasma triglyceride values if carbohydrate was isocalorically substituted for fat in a "formula diet". Ahrens et al., (1961) suggested that this phenomenon represented an exaggerated form of the normal biochemical process which occurs in all people on high carbohydrate diets and felt that the latter category represented the great majority of essential hypertriglyceridemics.

To qualify for membership in this group (similar to type IV hyperlipoproteinemia of Fredrickson & Lees, 1965), it is necessary that the individual have major and maintained elevation of plasma triglycerides on a high carbohydrate low fat diet and that the plasma triglycerides reach normal, or nearly normal, levels when a large portion of the carbohydrate is replaced by fat, regardless of the type of fat.

Data have now become available with regard to physical and chemical characteristics of the lipemic particles of carbohydrate-induced hyperlipemia (Ahrens et al., 1961; Bierman et al., 1965). The lipid particles which appear in a patient on a fat-free diet, are of endogenous origin, and differ in lipid composition from particles of alimentary origin (higher cholesterol/triglyceride ratio), and the paper electrophoresis of carbohydrate-induced hyperlipemics appears to be characterized by the appearance of excessive amounts of pre-β-lipoproteins which are probably hepatic in origin. Kuo & Bassett (1963) have compared the effect of starch with that of sucrose in five patients

with "carbohydrate-induced" hyperlipemia and observed significantly higher plasma triglyceride levels on the latter diet (Macdonald & Braithwaite, 1964).

2. Endogenous, carbohydrate and fat-inducible hypertriglyceridemia.

This category includes subjects who also have been described as having "mixed" hyperlipemia, "caloric" induced hyperlipemia and type V of Fredrickson's classification. These subjects exhibit hypertriglyceridemia on both high carbohydrate and high fat diets in which the total calorie content is not excessive. Several mechanisms appear to be operating in this category: On the one hand, chylomicrons may accumulate as a consequence of high fat diets. A hypercaloric intake with its increased demand for triglyceride transport and storage may exceed the ability of tissue to remove plasma triglycerides by deposition or by oxidation, so that an expanded endogenous pool may lead to deficient clearing of endogenous as well as exogenous triglycerides under the prescribed dietary conditions. In addition, the type of fat would also influence the extent or severity of the hyperlipemia (Kinsell et al., 1962). On the other hand, very low density lipoprotein (VLDLP, pre-β-lipoprotein) is present in increased amounts with both fat and carbohydrate induction. The VLDLP triglyceride fatty acids in these subjects on fat free diets correspond to the pattern as described for carbohydrate induced hyperlipemia. If however, hypertriglyceridemia is induced by high fat intake, the fatty acid composition of the pre-

β -lipoproteins (VLDLP) bears a close resemblance to that of the ingested fat (Kinsell et al., 1967).

3. Endogenous Hypertriglyceridemia with Essential Hypercholesterolemia.

Frederickson & Lees (1965) have described as their type III hyperlipoproteinemia, individuals who, in addition to clinical and laboratory evidence of essential hypercholesterolemia, show elevation of VLDLP and are markedly susceptible to induction by carbohydrates. In common with other endogenous hyperlipemics they manifest carbohydrate intolerance.

2.2. Dynamic aspects of Carbohydrate and Lipid Metabolism.

The conversion of glucose to fat is a process which occurs readily under conditions of optimal nutritional intake. With the exception of glycerol, fat (as fatty acids) cannot give rise to a net formation of glucose because of the irreversible nature of the oxidative decarboxylation of pyruvate to acetyl-CoA. Certain tissues, including the central nervous system and the erythrocytes, are much more dependent upon a continual supply of glucose than others. A minimal supply of glucose is possibly necessary in extrahepatic tissues to maintain the integrity of the citric acid cycle. In addition, glucose appears to be the main source of α -glycero-phosphate in tissues devoid of glycero-kinase. Certain mechanisms operate which safeguard essential supplies of glucose in times of shortage, allowing other substrates to spare its

general oxidation. Olson (1962) and Randle et al., (1963), have concluded that under conditions of carbohydrate shortage available fuels are oxidized in the following order of preference: (1) ketone bodies (and probably other short-chain fatty acids), (2) FFA, and (3) glucose.

As glucose is the fuel which is "burned last", it may be appreciated how adipose tissue is sensitive to a general deficiency in calorigenic substrates in the whole body through a mechanism based specifically on the availability of glucose.

On high carbohydrate diets FFA oxidation is spared; it is generally considered that this is due to the ability of tissues for esterification. As the animal passes from the fed to the fasting condition, the availability of glucose decreases, liver glycogen being drawn upon in an attempt to maintain the blood glucose level. Insulin activity in the blood decreases. As glucose utilization in adipose tissue diminishes, fat is mobilized as FFA and glycerol. The FFAs are esterified in other tissues, particularly the liver, according to the availability of glucose, and the remainder is oxidized.

Glycerol joins the carbohydrate pool after activation to α-glycerophosphate, mainly in the liver and kidney. During this transition phase from the fully fed to the fully fasting state, endogenous glucose production (from amino acids and glycerol) does not keep pace with its utilization and oxidation since the liver glycogen stores become depleted and blood glucose tends to fall. Thus fat is mobilized at an

ever increasing rate, but in several hours the FFA level stabilizes at the fasting level. At this point it must be presumed that in the whole animal the supply of glucose balances the obligatory demands for glucose utilization and oxidation. This is achieved by the increased oxidation of FFA and ketone bodies sparing the non-obligatory oxidation of glucose. This fine balance is disturbed in conditions which demand more glucose, or in which glucose utilization is impaired, and therefore lead to further mobilization of fat.

Under most conditions FFAs are mobilized in excess of oxidative requirements since a large proportion are esterified, even during fasting. The liver plays a regulatory role in removing excess FFA from the circulation. When carbohydrate supplies are adequate, most of the influx is esterified and ultimately retransported from the liver as VLDLP to be utilized by other tissues. However, when the capacity of the liver to esterify is not sufficient in the face of an increased influx of FFA, an alternative route, ketogenesis, is available which enables the liver to continue to retransport much of the influx of FFA in a form that is readily utilized by extrahepatic tissues under all nutritional conditions.

Most of these principles are depicted in Fig. 2.3. It will be noted that there is a carbohydrate cycle involving release of glycerol from adipose tissue and its conversion in the liver to glucose, followed by its transport back to adipose tissue to complete the cycle.

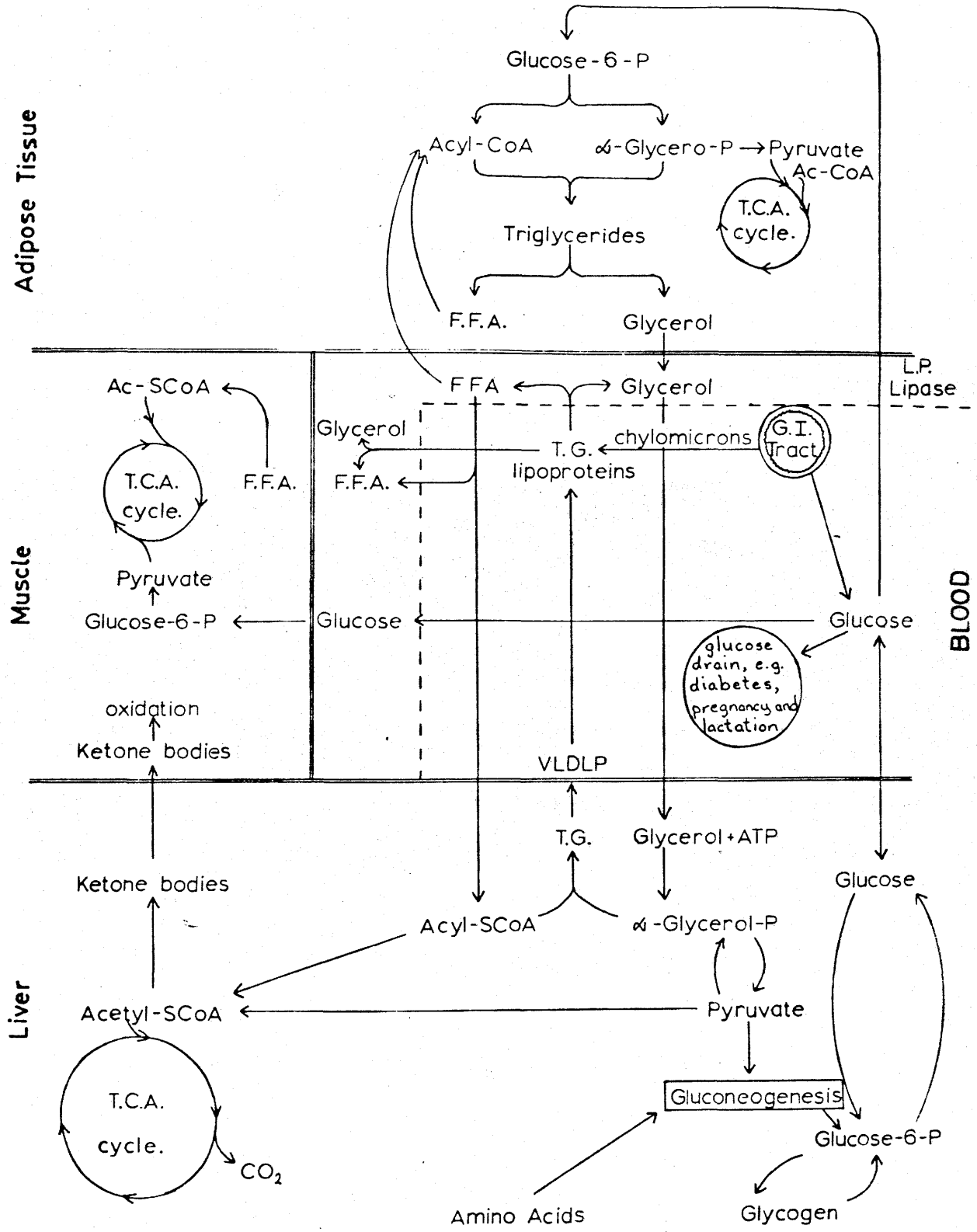


Fig. 2.3. Metabolic interrelationships between adipose tissue, the liver, and extrahepatic tissues.

The other cycle, a lipid cycle, involves release of FFA by adipose tissue, its transport to and esterification in the liver, and retransport as VLDLP back to adipose tissue. Disturbances in carbohydrate or lipid metabolism often involves these two interrelated cycles where they interact in adipose tissue and in the liver in the process of esterification (Harper, 1967).

2.3. Effects of Hormones.

Lipid metabolism is not only regulated by the nutritional state of the animal but also by hormones. This fact is admirably demonstrated in uncontrolled diabetes mellitus, where it has been found that insulin (or the lack of insulin) has a potent effect on adipose tissue, free fatty acids, elevation of lipoprotein Sf 12-400, in fact all factors which are directed towards gluconeogenesis and mobilization. In controlled diabetes there is a dramatic lowering of the blood glucose and FFA levels. However, it must be stressed that the effects of insulin (and other hormones) on carbohydrate and lipid metabolism need not necessarily be the direct result of the action of the hormone but also the secondary effect.

Glucose, from feeding diets rich in carbohydrates, stimulates the β-cells of the pancreas, causing them to release insulin. The insulin enables the glucose to enter the cardiac and skeletal muscle cells as well as adipose tissue and fibroblasts (Levine, 1965), where it can be used for glycogenesis, glucolysis and lipogenesis. Insulin has

been shown to stimulate the lipoprotein lipase enzyme(s) and in the liver the enzymes glucokinase (EC 2.7.1.2.) and glycogen synthetase (EC 2.4.1.11). Other hormones which are known to influence plasma lipid levels are shown in Table 2.3. (Williams, 1962; Rudman, 1963; and Steinberg, 1963).

Although some of these "lipolytic" hormones such as TSH, ACTH, CTH and epinephrine may stimulate the rate of formation of triglycerides, the effect that they exert on lipolysis is greater, thus leading to an accelerated triglyceride breakdown and FFA release. Epinephrine and norepinephrine stimulate lipolysis by enhancing the activity of the lipase enzyme(s).

Furthermore it has been found that insulin reduces the FFA of the plasma faster than it does the blood glucose. Glucagon reduces plasma FFA in normal persons faster than it does in diabetics. This is probably because the FFA level of the plasma is very much dependent on the rate of glucose utilization.

Hormones which increase the net release of fatty acids from adipose tissue cause the inflow of fatty acids to the liver to increase, and this in turn tends to cause a rise in the amount of β -lipoprotein triglyceride secreted by the liver. To the extent that this increases the total number of β -lipoprotein molecules in the plasma, there are increments in both the β -lipoprotein phospholipid and the cholesterol levels.

Table 2.3. Effect of Hormones on Plasma Lipids.

Hormone	Triglycerides	FFA
Growth hormone (GH).	I	I
Adrenocorticotropic (ACTH).		I
Pituitary fraction H. Peptides I & II.	I	I
Fat mobilizing substance (FMS).[a]	I	I
Lipid mobilizing hormone.	I	I
Thyroxin (TSH).	O	I
Glucagon.		D[b]
Epinephrine.	O	I
Norepinephrine.	O	I
Insulin.	D	D
Vasopressin.		I
Follicle-stimulating hormone (FSH).		I
α - and β - intermedin.		I
Estrogen.	D	O
Testosterone.	I	I

a) Peptide component isolated from the urine of fasting subjects.

b) In vivo, glucagon initially increases and later decreases FFA, because of hyperglycemia;
in vitro, glucagon increases FFA.

I = increase; D = decrease; O = little or no effect.

Hormones may also affect the β -lipoprotein lipids in other ways. Thus, in the hypothyroid individual cholesterol accumulates and an increased amount appears in the plasma β -lipoprotein fraction. Because of the lipoprotein structure, the β -lipoprotein triglycerides and phospholipids are also increased. Administration of thyroxin then causes the triglycerides and phospholipids to decrease with the cholesterol.

The sex hormones appear to have a direct effect on the synthesis of the lipoproteins. Estrogens increase the ratio of α -lipoproteins to β -lipoproteins and androgens decrease the ratio (Steinberg, 1963). Since the α -lipoproteins contain relatively more phospholipid and less cholesterol than the β -lipoproteins, the effects of the sex hormones on the plasma lipids are compatible with the effects on the relative quantities of α - and β -lipoproteins.

2.4. The Nature of the Problem at a Molecular Level.

Up to this stage we have looked at the nature of the problem from the "whole-body" aspect. We have seen that obesity is a social and metabolic problem, associated with many causes and effects; psychological, nutritional, insufficient physical activity, and endocrine imbalance.

The following sections will be devoted, in the main, to experimental hyperlipemia induced by diets rich in carbohydrates, to the nature of the problem at a molecular level, and to sex-linked differences.

Table 2.4. Adaptive enzymes of the liver.

	Enzyme	Activity in CHO FEEDING	Activity in Star-vation	Activator	Inhibitor
Enzymes of	Glucokinase	↑	↕	Insulin	
glycolysis	Glycogen synthetase	↑	↓	G-6-P, insulin	
and	Phosphofructokinase	↑	↓	AMP. insulin	Citrate, ATP
glycogenesis	Pyruvate kinase	↑	↓	Insulin	ATP
Enzymes of	Pyruvate carboxylase	↓	↑	Acetyl-CoA, glucocorticoids, glucagon	Insulin
gluconeo-	PEP-carboxykinase	↓	↑	Glucocorticoids	Insulin
genesis	Fructose-1,6-diphosphatase	↓	↑	Glucocorticoids	F-1,6-DiP. Insulin
	Glucose-6-phosphatase	↓	↑	Glucagon	Insulin
Enzymes of	Glucose-6-phos. dehydrogenase	↑	↓	Insulin	
the hexose	"Malic Enzyme"	↑	↓	Insulin	
monophos-	Citrate cleavage enzyme	↑	↓	Insulin	
phate shunt	6-phosphogluconate dehydrogenase	↑	↓		
and lipo-	phosphoglucomutase	↑	↓		
genesis	Malate dehydrogenase	↑	↓	Insulin	
	Acetyl-CoA carboxylase	↑	↓	Insulin	Fatty acyl-CoA.

CHO = Carbohydrate.
G-6-P. = Glucose-6-phosphate
ATP = Adenosine triphosphate
PEP- = Phosphoenol Pyruvate
F-1,6-DiP. = Fructose-1, 6-diphosphate.

2.4.1. Experimental Hyperlipemia.

There seems little doubt that dietary carbohydrate can lead to the accumulation of excess lipid in plasma and liver in both man and animals. In addition, there is evidence that the extent of the liver lipid accumulation is related to the type of dietary carbohydrate (Ahrens et al., 1961; Macdonald, 1962a; Carroll, 1964; Allen & Leaky, 1966).

This experimental hyperlipemia is believed to be the consequence of enhanced lipid synthesis, which results from the induction of certain rate-limiting enzymic steps in the Emden-Meyerhof pathway, pentose phosphate shunt, and the tricarboxylic acid cycle. An increase in the activities of these pathways will result in an increase of certain lipogenic precursors, e.g. acetyl Coenzyme A, $NADPH_2$ and adenosine triphosphate (ATP).*

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* The pyridine nucleotides, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), are notated in this dissertation as NAD and NADP for the oxidized forms, and as $NADH_2$ and $NADPH_2$ for the reduced forms. Further abbreviations and nomenclatures of enzymes, co-enzymes and co-factors are notated according to the International Union of Biochemistry as described in "Comprehensive Biochemistry" vol. 13, 2nd edition (1965). Editors: Florkin, M., and Stotz, E.H. Amsterdam, Elsevier.

Many animals, including man, take their food as spaced meals, and therefore need to store much of the energy in their diet for use between meals. The process of lipogenesis is concerned with the conversion of glucose and intermediates such as pyruvate and acetyl-CoA to fat, which facilitates the anabolic phase of this cycle (Chaikoff et al., 1950). The nutritional state of the organism and tissues is the main factor controlling the rate of lipogenesis. During the late 1950's it was shown that the substitution of a stock diet containing no hexoses, for a diet containing a high percentage of glucose resulted in a 10 fold increase in the ability of the liver to convert glucose carbon into fatty acids (Hill et al., 1957), and in a twofold capacity to incorporate acetate carbon into fatty acids. In addition as little as 2.5% of fat in the diet causes a measurable depression of lipogenesis in the liver as measured by the incorporation of acetate carbon into fatty acids (Hill et al., 1958). Furthermore, the substitution of either 60% glucose or fructose for a standard diet containing no hexoses resulted in an increased activity of a number of enzymes (Fitch & Chaikoff, 1960), together with a simultaneous decrease in activity of the gluconeogenic enzymes. See Table 2.4 and Fig. 2.4.

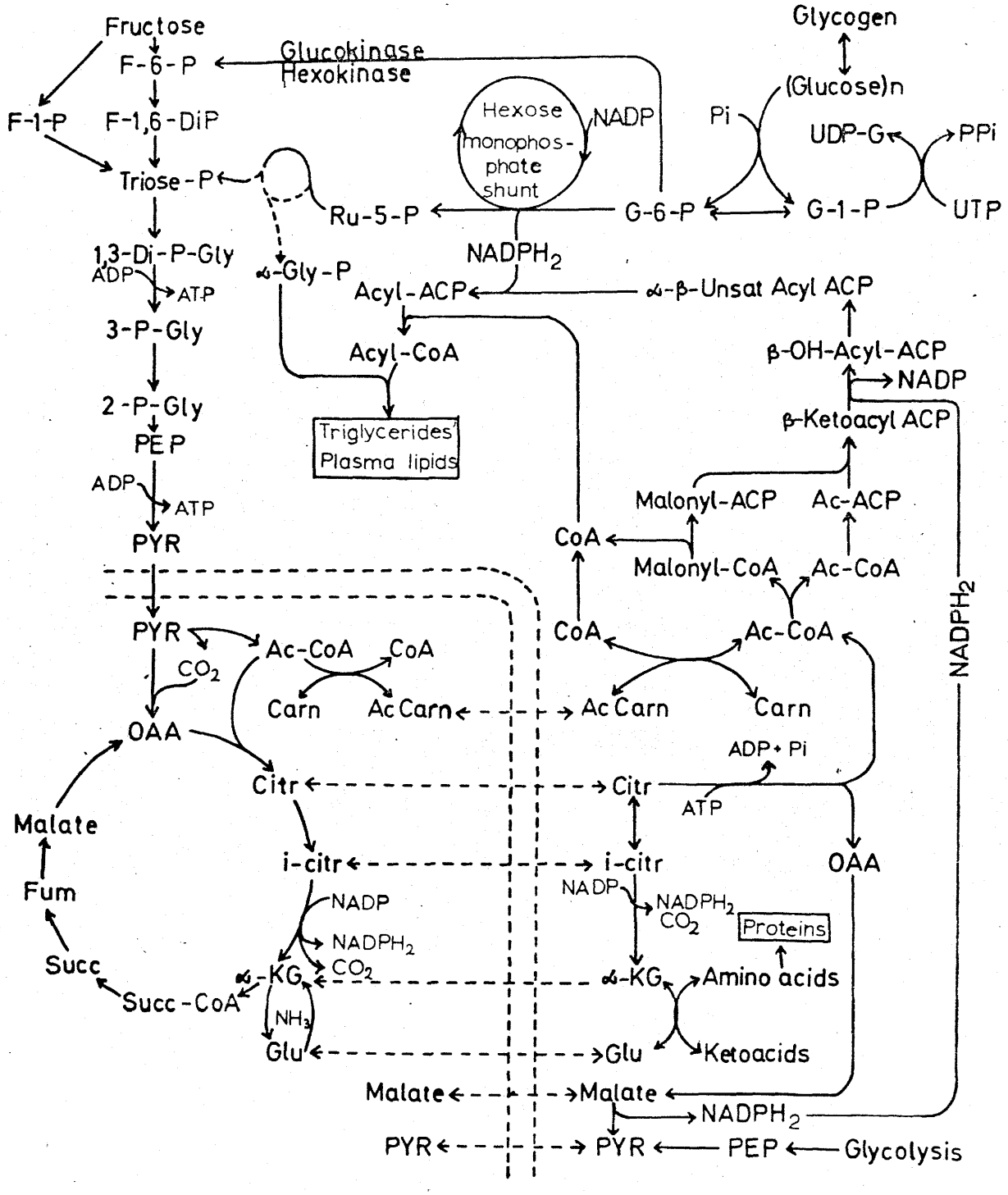


Fig. 2.4. Some pathways involved in the metabolism of carbohydrates and Lipids.

2.4.2. Nature of the Problem at a Molecular Level.

Masoro et al., (1950) demonstrated, in fasting rats, that the absence of lipogenesis was not due to lack of substrate (acetyl-CoA) and that the metabolic block existed in the rate of synthesis between acetyl-CoA and fatty acids. Because of the close association between the activities of the hexose monophosphate shunt and of the lipogenic pathway, it was considered that the block in lipogenesis was due to lack of $NADPH_2$ generation from the shunt pathway. However, subsequent work in which an $NADPH_2$ generating system was added to a liver homogenate from fasting rats failed to promote fatty acid synthesis (Sauer, 1960).

At the present time it is recognised that the rate-limiting reaction in the lipogenic pathway is at the acetyl-CoA carboxylase step, and more than one factor has been described which regulates the activity of this enzyme. Long-chain acyl-CoA molecules competitively inhibit acetyl-CoA carboxylase (EC 6.4.1.2.) (Bortz & Lynen, 1963), an example of metabolic negative feedback inhibition. This is the probable explanation of the depressed lipogenesis recorded under conditions of caloric deficiency, a high fat diet, or diabetes mellitus, all of which are associated with increased levels of plasma FFA.

The rate of incorporation of citrate into fatty acids is greater, under certain circumstances, than that of acetate (Spencer & Lowenstein, 1962). In addition, the activity of the extramitochondrial citrate

cleavage enzyme varies markedly with the nutritional state of the animal, closely paralleling the activity of the fatty acid synthesizing system. Utilization of pyruvate for lipogenesis by way of citrate could involve the decarboxylation of pyruvate to acetyl-CoA and subsequent condensation with oxaloacetate to form citrate within the mitochondria, followed by the diffusion of citrate into the extramitochondrial compartment, where it would undergo cleavage to acetyl-CoA catalyzed by the citrate cleavage enzyme (Fig. 2.5.). The acetyl-CoA would be available then for malonyl-CoA formation and synthesis to palmitate (Fig. 2.4.). However, studies by Flatt & Ball (1963) have shown that $NADPH_2$ generated in the conversion of hexose monophosphate to pentose phosphate supplies 50-60 per cent of the reducing equivalents used for fatty acid synthesis, and the possibility existed that pathways other than the hexose monophosphate shunt were used for its generation. Winegrad & Renold, (1958) showed that the synthesis of fatty acids from pyruvate occurs under circumstances where generation of $NADPH_2$ by the oxidation of hexose monophosphate seemed unlikely. This work suggested that the metabolism of pyruvate furnishes reduced coenzymes which could be used for fatty acid synthesis. Wise & Ball (1964) have shown that the activity of the malic enzyme (EC 1.1.1.40), first shown by Ochoa to catalyze the reaction

$$\begin{array}{l} CHOH - COOH \\ | \\ CH_2 - COOH \end{array} + NADP \rightleftharpoons CH_3 - \overset{O}{\overset{\|}{C}} - COOH + NADPH_2 + CO_2,$$

Malic acid pyruvic acid

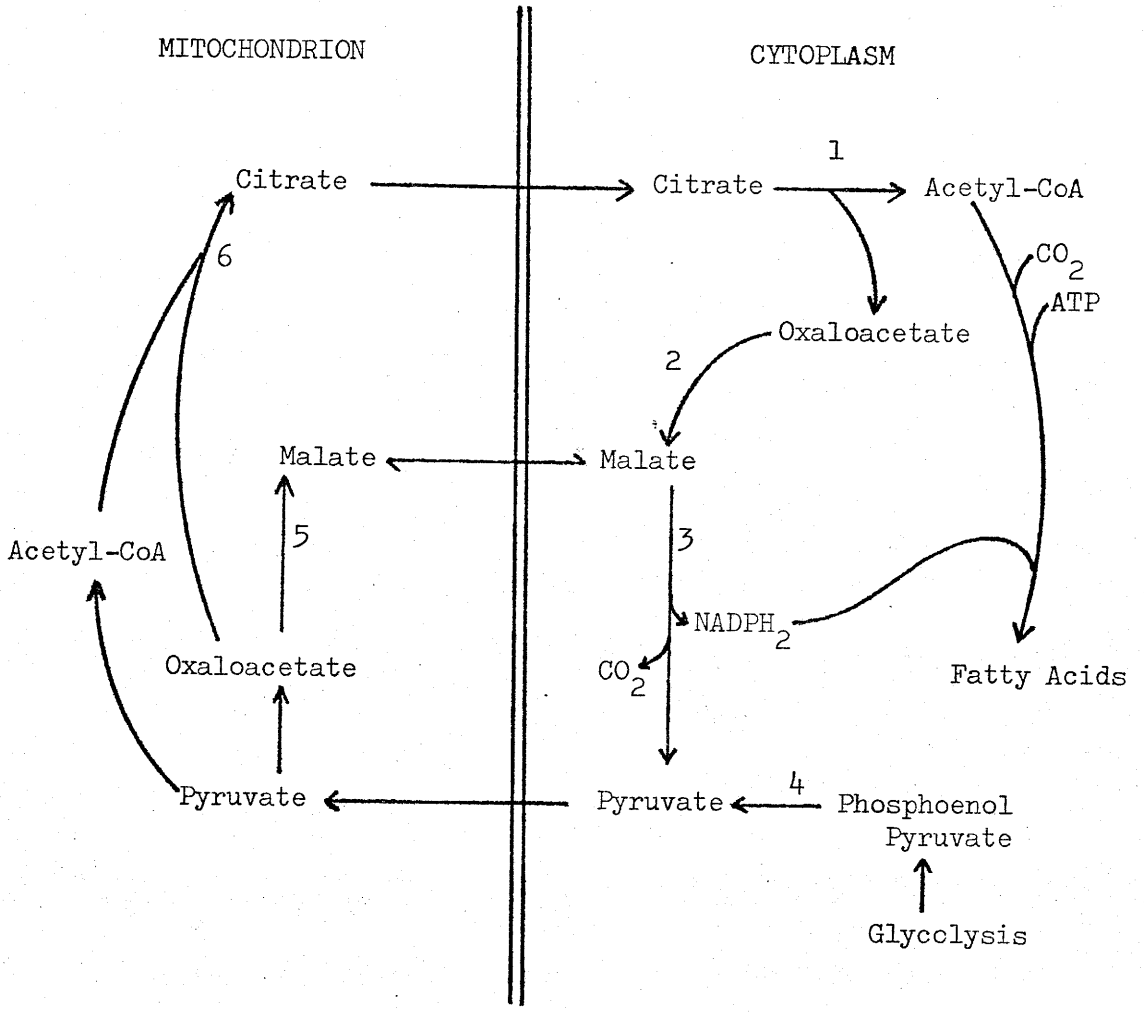


Fig. 2.5. Role of citrate cleavage, malic enzyme, pyruvate kinase, and malate dehydrogenase in lipogenesis.

1) EC 4.1.3.8. 2) EC 1.1.1.37. 3) EC 1.1.1.40.

4) EC 2.7.1.40 5) EC 1.1.1.37.(Mito.) 6) EC 4.1.3.7.

in both rat adipose tissue and liver was altered in such a way by experimental conditions, which are known to affect lipogenesis, as to suggest that malate may play a role in lipogenesis by furnishing $NADPH_2$ for the reduction of acetyl-CoA to fatty acids. The malic enzyme activity of liver rises when rats are placed on a high glucose or fructose diet, falls on fasting, and rebounds above normal on re-feeding (Fitch & Chaikoff, 1960).

A role for malic enzyme in lipogenesis has been postulated (Wise & Ball, 1964) which involves its interplay with malate dehydrogenase (EC 1.1.1.37) and pyruvate carboxylase (EC 6.4.1.1.) as shown by the following equations:

1 $\quad$ Pyruvate + CO_2 + ATP $\longrightarrow$ Oxaloacetate + ADP + Pi

2 $\quad$ Oxaloacetate + $NADH_2$ $\rightleftharpoons$ Malate + NAD

3 $\quad$ Malate + NADP $\rightleftharpoons$ Pyruvate + CO_2 + $NADPH_2$

---

4 $\quad$ Sum: $\quad$ NADP + $NADH_2$ + ATP $\longrightarrow$ $NADPH_2$ + NAD + ADP + Pi

This suggested role for malic enzyme has been postulated as well by Lardy et al., (1964) and by Young et al., (1964). The provision of $NADPH_2$ via the malic enzyme for lipogenesis could be only part of the role it could play in lipogenesis. Equally important may be the oxidation of $NADH_2$ which occurs when malic enzyme is coupled with the malate dehydrogenase reaction. The rate of oxidation of the reduced coenzymes formed during the conversion of glucose to acetyl-CoA could be a limiting step

in lipogenesis, since this process would grind to a halt unless these reduced coenzymes are reconverted to their oxidized form. Thus any process whereby $NADH_2$ can be converted to $NADPH_2$ would serve two purposes: It would regenerate NAD to permit the formation of more acetyl-CoA and simultaneously it would supply $NADPH_2$ for the reduction of acetyl-CoA to fatty acids. In addition, the participation of the pyruvate carboxylase reaction could further facilitate the oxidation of reduced coenzymes, since this reaction consumes ATP. (Fig. 2.5.).

It has recently been shown that hydrogen obtained from the isocitrate dehydrogenase (EC 1.1.1.42) reaction, which is NADP dependent, also serves as an active participant in fatty acid synthesis (Masoro, 1962). In the liver, glucokinase, which is responsible for the conversion of glucose to glucose-6-phosphate, is an inducible enzyme, and is not inhibited by high glucose-6-phosphate concentrations as hexokinase (EC 2.7.1.1.) is (Sols, 1965). This enzyme possibly acts as a functional barrier in the liver, for under gluconeogenic conditions the activity of this enzyme is decreased whereas under conditions of lipogenesis, viz. high carbohydrate load, it serves to stimulate glycolysis by catabolizing any excess glucose-6-phosphate that may inhibit the hexokinase enzyme. The activity of the glycogen synthetase enzyme (EC 2.4.1.11) has also been shown to be increased on feeding diets rich in carbohydrates (Steele, 1966). This will account for the increased glycogen synthesis during high carbohydrate loading.

Under increased carbohydrate loading there is necessarily an increase in glycolysis, resulting in an increased production of pyruvate. The pyruvate may enter the mitochondria where it may be decarboxylated to acetyl-CoA or carboxylated to oxaloacetate. These two products can condense to form citrate (Fig. 2.4. and 2.5.). On the other hand, a certain amount of the acetyl-CoA may be oxidized via the tricarboxylic acid cycle (TCA cycle) and the reducing equivalents are generated in the form of $NADH_2$ within the mitochondria by the dehydrogenations of the TCA cycle and of related reactions. The reducing equivalents are transferred to oxaloacetate and may leave the mitochondria either as malate (Krebs, 1967) or as citrate (Spence & Lowenstein, 1962) where the series of reactions shown in Fig. 2.5. can take place. This process gives an indication of how the reducing potential, originally present in carbohydrate can be utilized for synthetic processes in the cell.

The intra- and extra-mitochondrial transport of acetate is initiated and performed by carnitine (β -hydroxy- γ -trimethylammonium butyrate), and is considered to act as a carrier of both large and small acyl radicals across the mitochondrial membrane. Present in both compartments are two enzymes: acetyl transferase, which reacts with short-chain acyl-CoA molecules, and acyl transferase, reacting with long-chain acyl-CoA. Carnitine, by providing a method of transferring acetyl radicals from the mitochondria to the extra-mitochondrial fatty acid synthesizing system, provides a pathway for the conversion of pyruvate

to long-chain fatty acids which is an alternative to the pathway involving citrate and the citrate cleavage enzyme mentioned previously.

As shown in Fig. 2.6., the major enzymes involved in fatty acid synthesis are acetyl-CoA carboxylase (EC 6.4.1.2), which is an ATP dependent reaction and causes the carboxylation of the acetyl-CoA moiety to malonyl CoA , β -ketoacyl ACP reductase and α - β -unsaturated acyl ACP reductase enzymes. These latter two reductases are $NADPH_2$ dependent and have been collectively termed the "synthetase" enzyme. The free acetate, under the action of the thiokinase enzyme, is first activated to acetyl-CoA, which is further activated to acetyl-ACP (ACP=acyl Carrier Protein).

Summary: Substrate can be supplied directly from glycolysis and from utilization of the pathway as shown in Fig. 2.5. Reducing potential is supplied via: (1) the hexose monophosphate shunt pathway, (2) the direct action of the malic enzyme, (3) utilization of transportation of reducing equivalents from the mitochondria using the malate-, malate dehydrogenase's pathway and (4) use of the isocitrate-coupled-malate dehydrogenase system. Energy can be supplied directly from the glycolytic pathway.

The aforementioned findings are the major pathways undertaken on feeding diets rich in carbohydrates. During fasting and diabetes it has been found that the "complementary" enzymes are induced. The lipase enzyme activity is increased during fasting which results in higher concentrations of plasma free fatty acids from enhanced mobilization. The coenzyme A derivatives of these fatty acids act as competitive

a) Acetate thiokinase.

$$CH_3COOH + CoASH \xrightarrow{ATP} CH_3.COSCoA + H_2O$$

acetyl-CoA

b) Acetyl-CoA carboxylase.

$$CH_3.COSCoA + CO_2 \xrightarrow{ATP} HOOC.CH_2.COSCoA.$$

malonyl-CoA.

c) Condensing enzyme which condenses malonyl-ACP (ACP=acyl carrier protein) with acetyl-ACP to give β-ketoacyl-ACP.

d) β-Ketoacyl-ACP reductase.

$$CH_3.CO.CH_2.COS\text{-}ACP \xrightarrow{NADPH_2} CH_3.CHOH.CH_2.COS\text{-}ACP.$$

e) After removing water: α, β-unsaturated acyl-ACP reductase:

$$CH_3.CH{=}CH.COS\text{-}ACP \xrightarrow{NADPH_2} CH_3.CH_2.CH_2.COS\text{-}ACP.$$

f) Combination synthetase reaction, showing synthesis of palmityl-CoA.

$$CH_3COSCoA + 7HOOC.CH_2.COSCoA + 14NADPH_2 \longrightarrow$$

acetyl-CoA malonyl-CoA

$$CH_3(CH_2)14.COSCoA + 7CO_2 + 7CoASH + 14NADP.$$

Fig. 2.6. Reactions involved in part of the extra-mitochondrial synthesis of fatty acids.

inhibitors of acetyl-CoA carboxylase, the rate-limiting enzyme of fatty acid synthesis. This explains, in part, the decrease in lipogenesis demonstrated, even although the concentration of acetyl-CoA is fairly high, due of course to the oxidation of fatty acids. Wieland (1965) has also demonstrated the inhibition of the citrate synthetase enzyme by long chain fatty acyl-CoA derivatives.

The metabolic pathways involved in gluconeogenesis are modifications and adaptations of the Embden-Meyerhof pathway and the TCA cycle. These pathways are well documented and need not be reviewed here. However, for gluconeogenesis to take place there would have to be a diminishing of lipogenesis, glycolysis, and an increase in lipolysis. The increase in lipolysis on starvation has been well demonstrated and this increase in fatty acyl-CoA derivatives, as has been described, necessarily inhibits the rate-limiting step in lipogenesis. It remains therefore for the rate of glycolysis to be decreased. Randle (1965) has indicated how this may take place. Five abnormalities of glucose metabolism have been identified in the muscles of alloxan-diabetic and fasting rats. One of these (impaired glucose transport) is due solely to lack of insulin and it may be corrected by addition of insulin to the diabetic tissue. The remaining four abnormalities are: a diminished ability of insulin at physiological concentrations to stimulate membrane transport of glucose and other sugars; a reduced rate of phosphorylation of glucose by hexokinase; a reduced

rate of glycolysis due to inhibition of phosphofructokinase; and a diminished rate of oxidation of pyruvate due to inhibition of pyruvate dehydrogenase. Each of these abnormalities can be induced in vitro by the addition of fatty acids to the incubation medium.

The overall oxidation of glucose is diminished by diabetes and by fatty acids and ketone bodies, partly because of impaired uptake of glucose, partly because of impaired glycolysis, and partly because the oxidation of pyruvate is impaired. The basic mechanism for this inhibition lies in the relationship of Coenzyme A and acetyl-CoA to the oxidation of pyruvate and of fatty acids and to the formation of citrate (Fig. 2.7.). The oxidation of pyruvate to acetyl-CoA, catalyzed by pyruvate dehydrogenase is inhibited by a rise in the intracellular concentration of acetyl-CoA relative to that of CoA. Such an increase is induced by fatty acids and this change is believed to account for the inhibition of pyruvate oxidation which they induce. A rise in the ratio of acetyl-CoA/CoA may also lead, through the citrate synthetase reaction, to a rise in the concentration of citrate. The concentration of citrate in muscle is increased by fatty acids and citrate is an inhibitor of phosphofructokinase and hence glycolysis. Finally the inhibition of hexokinase is believed to be secondary to phosphofructokinase inhibition which leads to accumulation of glucose-6-phosphate, which is a known inhibitor of hexokinase (Fig. 2.7.).

Thus a number of enzymes are inhibited under gluconeogenic

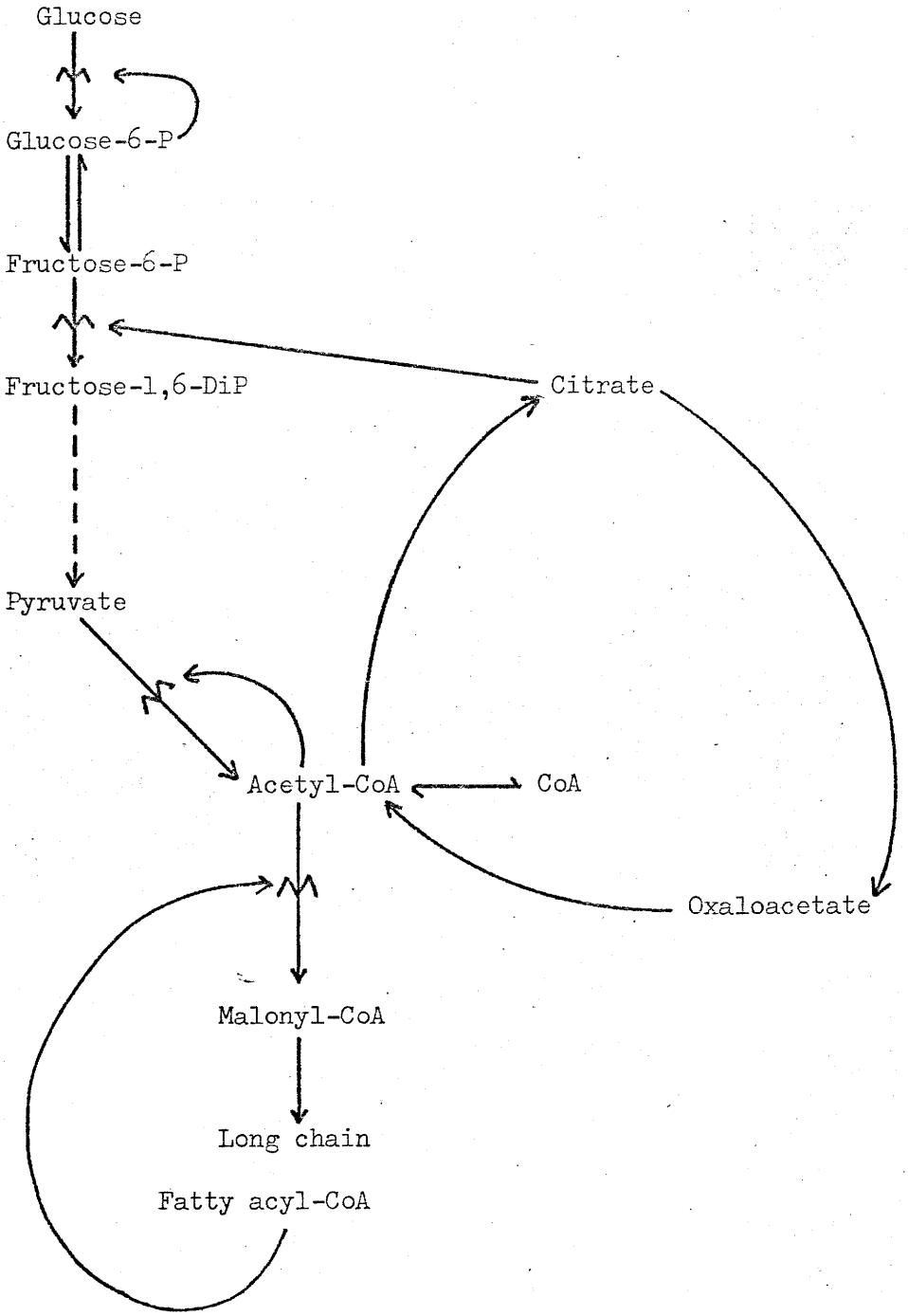


Fig. 2.7. Feedback control mechanisms in glycolysis and lipogenesis.

conditions. However, the main gluconeogenic enzymes e.g. mitochondrial pyruvate carboxylase (EC 6.4.1.1.) and the cytoplasmic phosphoenolpyruvate carboxylase (EC 4.1.1.32), are increased, leading generally to an increased production of glucose.

Yet another control point, of glucose metabolism, is the dependence of glycolysis and related metabolic pathways on the state of respiration in the cell. Both in aerobic and anaerobic glycolysis the concentration of ATP present has a controlling influence on the activity of phosphofructokinase. A high concentration of ATP offers a feedback inhibition of phosphofructokinase which is aggrevated by accumulation of citrate. The oxidation of citrate through the TCA cycle is controlled at the level of its first irreversible step, the NAD dependent isocitrate dehydrogenase, by the allosteric dependence of this enzyme on ADP as activator (Sols, 1965).

In the intact mitochondria the rate of respiration and the steady state equilibrium of the electron carriers are exquisitely poised and are a function of concentration of respiratory substrate, ADP, ATP, inorganic phosphate, and oxygen.

Chance and Williams (1956) have placed great emphasis on the concentration of ADP as the most critical element in the determination of respiratory rate, in either isolated mitochondria or in the intact cell. These workers have also tabulated the metabolic states of mitochondria, i.e. states 1 to 5. The respiratory control index (or

better, acceptor control ratio, ACR) has been defined as the respiration in state 3 (excess ADP) divided by the respiration in state 4 (ADP-less), and gives an indication of the "coupling" of the mitochondria, i.e. the extent to which respiration is coupled with phosphorylation (Lehninger, 1964). Tightly coupled mitochondria having a high acceptor control ratio.

However, respiration has also been shown (Lehninger, 1964) to be inhibited by high concentrations of ATP which causes a reversal of electron transport from cytochrome c to NAD. The reduced NAD has been shown to reduce acetoacetate to β-hydroxybutyrate, increasing the ratio of β-hydroxybutyrate/acetoacetate, which serves as a good indication of the $NADH_2$/NAD and ATP/ADP + Pi ratios of the mitochondrion and may act as a chemical messenger between the mitochondrion of the liver, where ketone bodies are produced, and the peripheral tissue where the ketone bodies are used.

The rate of oxidation of pyruvate or fatty acids via the TCA cycle is determined not only by the supply of these fuels in the cytoplasm, by the availability and concentration of ADP, Pi and ATP, as we have seen above, and by the enzymatic capacity of the cycle and of the respiratory chain, but also by the concentration and proportions of the tricarboxylic and dicarboxylic acids that comprise the intermediates of the cycle. The rate of each step of the TCA cycle depends not only on the concentrations of its substrate but also on the relative concentra-

tion of other intermediates, since some of these intermediates are capable of specific inhibition of other steps. The major intermediate in the formation and removal of TCA intermediates is oxaloacetate and the related enzymatic reactions which it may undergo.

Finally the rate of glycolysis and the activity of the hexose monophosphate shunt will depend on the removal of reduced nicotinamide dinucleotides. The reducing potential of $NADPH_2$ may be used for synthetic pathways or transferred via a specific transhydrogenase enzyme to NAD which can then, by means of shuttle systems, be oxidized via the mitochondrial cytochrome chain. The best known example of these shuttle systems is that of α-glycerophosphate dehydrogenase (Fig. 2.8.), other examples are: lactate dehydrogenase; malate dehydrogenase (NAD-linked); β-hydroxybutyrate/acetoacetate shuttle.

There is thus evidence that there is a close interrelationship between glucolysis and lipogenesis, gluconeogenesis and lipolysis, with respiration. Experimental hypertriglyceridemia, carbohydrate induced (or fat induced), has evidence of being intimately linked to and controlled by the molecular process outlined.

2.4.3. Carbohydrate Equality in Induction of Hypertriglyceridemia.

There is evidence to show that both the amount and type of dietary carbohydrate influences the amount and kind of lipid in the serum, liver and adipose tissue of experimental animals (Macdonald,

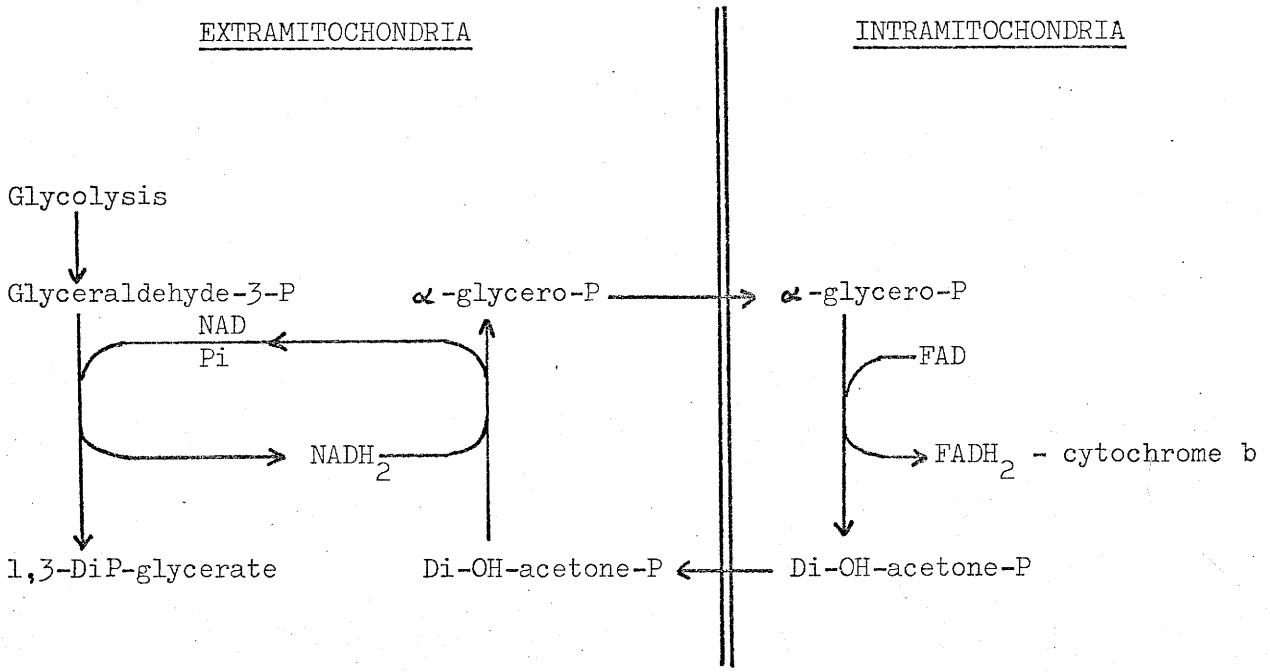


Fig. 2.8. The α-glycerophosphate dehydrogenase shuttle system.

1962a & b). Anderson et al., (1963) have suggested that high intakes of dietary sucrose may be responsible for the high incidence of ischemic heart disease, and many features of the malnutritional syndrome, kwashiorkor, are probably the result of a relative excess of carbohydrate in a protein-deficient diet in children. The effects of varying the dietary carbohydrates are most significant in the serum lipids, which decreased during the starch containing diet, whereas sucrose diet caused the serum lipid level to rise to values higher than those found on normal free-choice diets (Macdonald & Braithwaite, 1964). The increase in serum lipids on the sucrose diet is due to the rise in the triglyceride fraction, whereas on the starch diet the sterol ester and phospholipid fractions fall, the triglyceride level remaining unchanged. Thus it seems that dietary sucrose may travel along different metabolic pathways to dietary starch.

Furthermore, other experimental workers have demonstrated that in patients with carbohydrate inducible hypertriglyceridemia, a high sucrose diet was more efficient than either glucose or starch diets in stimulating hypertriglyceridemia. These results have been confirmed in both laboratory animals and in normal human male subjects (Kuo & Bassett, 1965; Beveridge et al., 1964; Antar & Ohlson, 1965; Kuo et al., 1967). Furthermore a decrease of triglyceride concentrations was observed by Macdonald (1965b) when maltose or dextrose were substituted for sucrose.

Kaufman et al., (1967) have demonstrated that where the carbo-

hydrate equivalents were administered as fructose, the serum glycerides were elevated to a greater extent than when the carbohydrate was given as sucrose, glucose or starch. An increasing order of potency of the carbohydrate inducible hypertriglyceridemias has evolved; viz. starch (complex polysaccharides), maltose, glucose, sucrose and lastly fructose causing the highest serum lipid levels.

Prior to these findings, workers had endeavoured to elucidate the mechanisms and molecular pathways, on a differential basis, for the absorption and metabolism of the individual carbohydrates (Kiyasu & Chaikoff, 1957; Ginsberg & Hers, 1960; Fitch & Chaikoff, 1960; Dahlqvist, 1964; and Ockerman & Lundborg, 1965). The conclusion drawn was that, although some fructose is converted in the intestinal lumen to glucose and absorbed and metabolized as such, some of the fructose is absorbed intact and follows a different metabolic pathway to that of glucose. In the liver the major pathway for fructose metabolism is: phosphorylation of fructose by the enzyme fructokinase to fructose-1-phosphate. Fructokinase, unlike glucokinase, is not affected by insulin. Fructose-1-phosphate is then split into two triose units by a fructose-specific aldolase, and free glyceraldehyde and dihydroxy-acetone phosphate are formed. Triokinase catalyses the phosphorylation of glyceraldehyde to glyceraldehyde- 3- phosphate. The two triose phosphates may then be degraded via the Embden-Meyerhof pathway, producing active acetate, the main precursor of lipid synthesis. From

the position of entry of the triose phosphates into the natural glycolytic pathway, they come under the influence of the normal control points such as pyruvate kinase, pyruvate carboxylase and the citrate synthetase. However, the initial metabolism of fructose, as shown above, eludes the major glycolytic control points at the phosphofructokinase and hexokinase steps. This could be the reason for the greater levels of serum glycerides observed on feeding diets rich in fructose or sucrose. The metabolic pathways of fructose degradation are shown schematically in Fig. 2.9.

Further confirmatory evidence for these findings was elegantly demonstrated by Macdonald (1967) when he administered C^{14} - labelled carbohydrates to baboons and humans, and found that the label from the C^{14}-fructose and C^{14}-sucrose was incorporated at a greater rate into triglycerides than was either that of glucose or dextrose.

Thus it has been concluded that the fructose moiety of sucrose was responsible for the increase in the triglyceride values in the plasma and that it was preferentially converted to triglycerides by the liver.

2.4.4. Sex-linked Metabolic Differences.

In 1958, Bersohn & Oelofse reported that the incidence of coronary heart diseases was higher in males than in females. This work, together with similar findings (Albrink & Mann, 1959), first presented evidence that there are sex-linked differences in certain vascular dis-

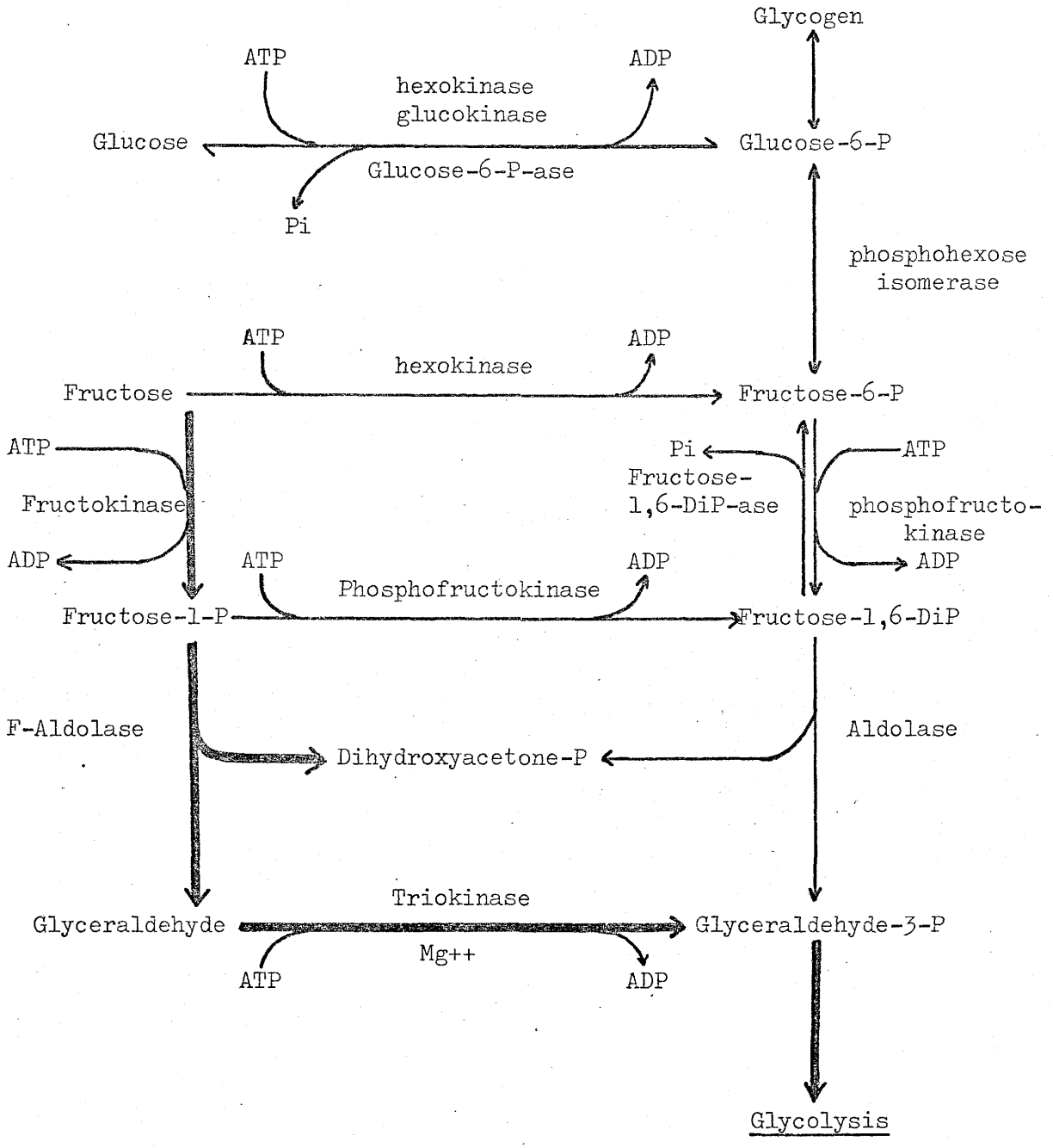


Fig. 2.9. Metabolism of Fructose.

eases. Furthermore the evidence that has been presented in this field has shown a correlation between the incidence of coronary heart diseases and the high concentration of lipids in both liver and plasma.

Morton & Horner (1961) reported that male rats, on a diet containing 73% sucrose, had three times as much liver triglyceride as controls, whereas the female on the sucrose diet showed no increase in liver triglycerides. Later work, in humans, confirmed the findings of Morton and Horner for the effect of dietary sucrose (Macdonald & Roberts, 1965).

Plasma lipid fractions have been investigated, on a comparative basis, as well. Beveridge et al. (1964), demonstrated that on a fat free control diet the serum triglyceride level rose in males and showed no change in females. When young females were given a low fat diet with either corn-starch or sucrose as the carbohydrate, it was found that while on the starch diet no alteration occurred in the level of serum triglycerides, whereas on the sucrose diet the triglycerides fell (Macdonald, 1965a). This is in contrast to similar experiments in males where sucrose was associated with a marked rise in serum triglycerides (Macdonald & Braithwaite, 1964). In yet another series of comparable experiments in postmenopausal females it was discovered that, while they were on a sucrose diet their serum triglycerides showed an increase similar to that found in the males (Macdonald, 1966a). In addition post-menopausal females also had a significant rise in triglyceride level while on a starch diet.

This influence of sex on dietary carbohydrate-lipid interrelationships is in line with the incidence of ischemic heart disease, namely a high incidence in males and postmenopausal females and a low incidence in premenopausal females. It is not possible at this stage to state where or how the influence of the sex hormones operate. Findings do however suggest that the sex hormones are influencing the metabolism of dietary sucrose.

The feeding of diets rich in carbohydrates to a cross section of human subjects results in different observable effects in males and females. It would seem that in the males, and in postmenopausal females, the excess carbon, reducing potential and energy is utilized for the production of excess lipids, which are deposited and result in an increase in total body weight. However, in the premenopausal females this fact is not observed. The prevalent questions are: 1) what happens to this excess carbon intake; and 2) have the sex hormones, male and/or female, any effect upon the lipogenic enzyme pathways? Could there be an uncoupling of oxidative phosphorylation resulting in the dissipation of excess chemical energy as heat? Up to date there is no convincing proof for this hypothesis. If there is no uncoupling of oxidative phosphorylation then the excess energy would be trapped as ATP.

Ilse et al., (1968a) have duplicated the findings of the above authors in the laboratory mouse, Praomys (Mastomys) natalensis, and have demonstrated a sex difference in the blood triglyceride levels with only

the male triglyceride levels responding to high carbohydrate feeding. In addition, Ilse and co-workers (1968b) have demonstrated that the ATP-hydrolyzing activity of the female liver mitochondria is greater than that of the males, and that this hydrolyzing activity is testosterone dependent. This experimental finding provides a substantial method for the dissipation of excess energy, in that the trans-membrane ionic "pump" could be more efficiently used by the female system. Levin (1968) has demonstrated that the transvaginal potential difference is reduced by ovariectomy and increased following injection of estradiol to ovariectomized rats. In addition laboratory rats, and to some extent human females, have a higher basal metabolic temperature than their male counterparts (Car & Krantz, 1942).

Although the sex hormones have been shown to influence metabolic processes in the gonadal organs, recent experimental evidence has indicated that they may very well affect the metabolism of the liver (Roy & Neuhaus, 1967). These workers have demonstrated that testosterone, injected intraperitoneally, induced the de novo synthesis of an a_{2u} protein in the liver of females and castrated males. Furthermore, Fuji & Villee (1968) have shown that a single injection of testosterone stimulates the synthesis of ribonucleic acid (RNA) not only in the prostate but also in the liver of the immature rats.

Experimental evidence shows that RNA and protein synthesis, in the female gonads, is stimulated by estrogen (Emmens & Martin, 1965).

Estrogen has been found to increase the activity of certain enzymes such as phosphofructokinase (Singhal et al., 1967a), phosphohexose isomerase (Singhal et al., 1967b), and uterine hexokinase (Valadares et al., 1968), which may entail an acceleration of the synthesis of certain RNA and protein species. Estrogen has been shown to stimulate the activities of other enzymes, some of which are involved in carbohydrate metabolism; viz. the transhydrogenase enzyme (Hagerman & Villee, 1959), the glycogen synthetase enzyme, while suppressing glycogenolysis by inhibiting phosphorylase activity (Bo et al., 1967), 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase (Barker & Warren, 1966), and the acid proteolytic enzymes such as acid phosphatase and acid ribonuclease (Goodall, 1965).

In addition both estrogen and testosterone have been found to either stimulate or suppress other enzyme systems. Sasaki & Ketkar (1968) demonstrated that the activities of UDP-glucuronic acid dehydrogenase and L-glutamine-D-fructose-6-phosphate transamidase in rats were increased twofold by the administration of estrogen, whereas their activities were slightly decreased by testosterone. Furthermore van Pilsum & Ungar (1968) compared the activities of the kidney enzyme, transamidase, in female and male rats. The

activity of this enzyme was lower in the female and ovariectomy caused an increase in its activity. The enzyme levels in intact males was lowered by daily injections of estradiol, whereas intact females given larger doses of testosterone showed an increased activity of this enzyme.

Finally to conclude this section; the *in vitro* experiments of Merola *et al.*, (1968) provided evidence for the inhibition of cholesterol synthesis by estrogen, at the decarboxylation of mevalonic acid, while the *in vivo* experiments of Doeg (1968) showed that testosterone stimulated lipid synthesis in the "target organs" and liver mitochondria of rats.

2.5. Aim of Present study.

In a study of the etiology of atherosclerosis numerous workers have demonstrated the role of diets as a possible cause of this disorder. Recent experimental work has placed the emphasis on the lipogenic effect of dietary carbohydrates. In a study of the effect of dietary carbohydrates on lipogenesis the nature of the carbohydrate must be clearly defined, since simple carbohydrates e.g. sucrose and fructose, have a greater hypertriglyceridemic effect than that of complex carbohydrates, e.g. starch.

Furthermore, it has been unequivocally demonstrated that males and postmenopausal females are more susceptible to the induction of hypertriglyceridemia by dietary sucrose than are young premenopausal females.

In addition the sex hormones have been shown to play either an inhibitory or inducing role upon the activities of certain key lipogenic and gluconeogenic enzymes. However, no conclusive explanation for this phenomenon is at hand.

The problem is then essentially as follows: As the males (and postmenopausal females) show a tendency towards hypertriglyceridemia, the cellular mechanisms involved in lipogenesis, in the male, should show a greater tendency towards the production of triglycerides, with a concurrent decreasing effect discernible in the female organism. If the females show a greater metabolic efficiency than do the males, then the female system should demonstrate mechanisms whereby the excess energy derived from carbohydrate loading could be dissipated. To a certain extent this has been shown by the work of Ilse et al., (1968b) in that the female system has a far more active ATP-hydrolyzing activity than that of the male system.

In this project we intend investigating on a comparative basis:

1) The efficiency of cellular mechanisms for energy production by liver of male and female mice, Praomys (Mastomys) natalensis.

1.1. Mitochondrial phosphorylation.

1.2. Efficiency of coupling of respiration and phosphorylation.

2) Mechanisms for energy utilization. Do they differ in male and female? This will include the assay of the activity and levels of key enzymes of the lipogenic sequence.

CHAPTER 3.

EXPERIMENTAL PROCEDURE

3.1. Materials.

Glucose-6-phosphate dehydrogenase, phospho-enolpyruvic acid, oxaloacetic acid, hexokinase, ADP, ATP and reduced and oxidized pyridine nucleotides were obtained from Messrs. C.F. Boehringer & Soehne (Germany). The disodium salt of ethylene diaminetetracetic acid (EDTA), succinic acid, mono- and di-saccharides, cytochrome c, lactic dehydrogenase, tri-ethanolamine, DL-malic acid, DL-isocitric acid, hydrazine sulphate, sodium deoxycholate and methylene blue were obtained from Messrs. British Drug Houses Ltd. (England).

Trihydroxymethylamino-methane (Tris.) was obtained from Messrs. Light & Co. Ltd. (Colnbrook, England). Crystalline bovine albumin was obtained from the Armour Pharmaceutical Co. Ltd. (England). Biogel P-300 was supplied by Bio-RAD Laboratories (Richmond). DEAE cellulose powder De50 was obtained from Whatman, 2-mercapto-ethanol from Sigma, and the sephadex G-200 from Pharmacia (Sweden). All additional reagents and chemicals used were of analar reagent grade, unless otherwise stated.

Specialized equipment used: MSE 18 highspeed refrigerated centrifuge; Unicam SP 500 (Cam) spectrophotometer; Beckman, model DB, spectrophotometer with attached recorder; Warburg apparatus, manufactured by B. Braun (Melsungen); and the LKB fraction collector (rotating type) with attached distributor, counter, uvicord and recorder.

3.2. Laboratory Animals.

A highly inbred strain, R, of multi-mammate mice, Praomys (Mastomys) natalensis, was used throughout these experiments. These animals were obtained from the National Cancer Research Unit, housed in the confines of the South African Institute for Medical Research. The male and female mice were separated while still weanlings, age 4 to 5 weeks, and kept in groups of five, under controlled standard conditions.

The test animals were allowed to feed "ad libitum" on mice cubes obtained from "Epol" until they were approximately 9-11 weeks old. After this period they were separated into various groups, viz. test control animals, males and females; females for ovariectomy; males for castration. The ovariectomized and castrated animals were later treated with either estrogen, testosterone or progesterone. The constitution of the "Epol" diet was; 18% protein, 14% fat and 68% starch, together with added minerals and vitamins.

3.3. Ovariectomies and Castrations.

3.3.1. Materials.

Trifluoromazine "Siquil" (Siquil- Squibb), 20 mg./ml. This acts as a tranquilizer and was used for preoperative sedation. The stock solution was appropriately diluted with an aqueous solution containing 10% ethanol and 20% propylene glycol (v/v), and administered intramuscularly at a dosage of 12.5 µg./50 g. body weight, about 30-40

minutes prior to anaesthetization.

Sodium-Pentobarbitone (Petersons (Pty) Ltd.). This anaesthetic was used in conjunction with "Siquil". The stock solution was appropriately diluted with an aqueous solution containing 10% ethanol and 20% propylene glycol (v/v) and was administered at a dosage of 37.5 µg./50 g. body weight.

Megimide Bemegride (Megimide-Nicholas (Pty) Ltd.). This was used in order to decrease the anaesthetic effect of the sodium-pentobarbitone. The stock solution was diluted with normal physiological saline and administered intraperitoneally as required. Usually 1-4 µg./50 g. body weight was sufficient.

The working solutions of "Siquil", sodium-pentobarbitone, and megimide were sterilized by passing through a micropore filter (GSWP GS 0.22 µ plain white 13 mm.) with the aid of a Swinney millipore adaptor and a Luerlok syringe. The sterilized solutions were stored in 2 ml. quantities in sterile screw-capped bottles at 4°C. These solutions were stable for 7 days.

Hibicol. This solution was prepared by mixing hibitane (1 vol.), absolute alcohol (10 vols.), and de-ionised water (1.5 vols.). For the duration of the operations all the instruments were stored in hibicol.

Catgut. Sterile absorbable surgical sutures, USP plain 4-0. (Davis and Geck Division of the American Cyanamid Division).

Silk suture. Non capillary, braided. Arbrasilk size 3/0 BPC-USP. (Armour Pharmaceutical Co. Ltd.).

Suture needles. Eye curved TCP. (Glaxo-Allenburys Cat. 18066, size 6).

Tuberculin syringes. Luer-lok of 1 ml. capacity.

Hypodermic needles, 26G 3/8" for intramuscular injections, and 23G 1½" for intraperitoneal injections (B-D Yale Beeton, Rickinson and Co., Rutherford N.J.).

"Nair" depilatory reagent. (Petersons (Pty) Ltd.).

Mercurochrome. Standard pharmaceutical grade, 1% aqueous.

Sterilization of all equipment and instruments was done by autoclaving them at 20 lbs./sq. in. for 20 minutes.

Hormonal Preparations were dissolved in Arachis oil.

Estradiol benzoate. (2mg./ml., British Drug Houses, London). The estradiol treatment was commenced two to three weeks after successful operative procedure had been performed. The hormone was administered at a dosage of 5 µg. of estradiol benzoate/50 g. body weight on alternate days for two weeks.

Testosterone propionate, (25 mg./ml., British Drug Houses, London). Two courses of testosterone treatment were undertaken: 1) The hormone treatment was commenced two to three weeks after successful operative procedure had been performed, and was administered to both ovariectomized and castrated animals at dosage rates of 25 µg., 250 µg. and 1.5 mg. of testosterone propionate/50 g. body weight on alternate days for 10-14 days; 2) The hormone treatment was commenced immediately after the successful operations at the same dosage as in the first

course. In this case only castrated males were thus treated.

Progesterone, (25 mg./ml., British Drug Houses, London). The progesterone was administered to ovariectomized and castrated animals at a dosage of 1.5 mg. progesterone/day/50 g. body weight. In the case of the castrated males the course began immediately after the operations and lasted for 10 days, whereas the ovariectomized females were started two weeks after the operations and also continued for a duration of 10 days.

After the various hormones were appropriately diluted with arachis oil they were sterilized and injected into sterile containers and sealed.

3.3.2. Operational Procedure.

After weighing, the mice were injected with the tranquilizer in the muscle of the hind limb. Subsequent to the tranquilizer taking effect they were injected intraperitoneally with sodium-pentobarbitone. As soon as the mice were under the influence of the anaesthetic their backs were cleaned of all hair by means of an electric shaver and the depilatory reagent.

The mice were then placed on their stomachs on a covered operating board and their limbs secured by means of padded clips. Their backs were swabbed with mercurochrome and a medial dorsal longitudinal incision, approximately half an inch long, was made from $^{1}/_{8}$" above the

position of the crest of the iliac bones of the pelvis to the level of the lower poles of the kidneys. The connective tissue holding the skin to the muscle was carefully cut away with a fine pair of scissors, and the skin, with the incision, was moved laterally and held in position with a pair of artery clamps.

In the female, the ovaries (which are located in a dorsal-lateral position) were usually visible through the thin muscle wall of the back and an incision could be made just above them. These incisions were of minimum length so as to allow convenient extrusion of the ovary with a fine pair of forceps. After hemostasis was ensured by ligation of the upper horn of the uterus with catgut, the ovary, together with the surrounding fat pods, oviduct, and a small portion of the uterus, was excised. The uterus was then placed back in the abdominal cavity and the muscle was stitched together with catgut. Care was taken not to sew any stray hairs into the wound as these might have caused an infection. The procedure was then repeated in the removal of the second ovary. Finally the skin was stitched with silk suture and swabbed with mercurochrome. If, after the operation, there was slackening of respiration, megimide was administered to the animal intraperitoneally.

With the males the testes were removed via the scrotal sac or from a dorsal lateral position. The latter method was employed only when the testes were retracted into the abdominal cavity and could not

be massaged down. Here, as in the case of the female, a dorsal longitudinal incision was made through the skin. The connective tissue was cut away and the skin moved laterally, and a small incision was made in the cutaneous maximus muscle of the back. After probing carefully in a ventral lateral direction with a blunt pair of forceps, the epididymal fat could be grasped and drawn out through the lateral incision. During this process the testes would also be drawn out together with the epididymus. The spermatic vessels, the deferential vessels and the ductus deferens were then tied off with a single catgut ligature. The testicle, together with the epididymus, was then excised. The vas deferens was placed back in the abdominal cavity and the muscles were stitched together with silk and swabbed with mercurochrome.

When the testes were not retracted they were removed directly from the scrotal sac. In this case after anaesthetizing the animal, the hair was removed from the scrotal sac with the aid of the depilatory agent alone. The mice were then placed on their backs on the operating board and their limbs secured. Mercurochrome was swabbed on the scrotal sac and a lateral incision was made. The fine membranes covering the testes were removed and the testes drawn out of the sac. The gubernaculum was then cut away from each testis and the spermatic vessel, the deferential vessel and the ductus deferens of each testis was ligated and excised. The remains of the vas deferens belonging to each testis was replaced in the empty scrotal sac. This was then stitched together

with the silk suture and swabbed with mercurochrome.

After the operation the animals were covered with a towell and kept in a warm place till they had recovered. Since the animals tended to become cannibalistic they were isolated throughout convalescence and during the subsequent experiments. These animals were injected with various hormonal preparations as described. Control animals were injected only with arachis oil (60 µl./day/50 g. body weight).

3.4. Oxidative Phosphorylation in Liver Mitochondria.

3.4.1. Preparation of mitochondria.

Two buffered Tris-HCI solutions, pH 7.4, were required. Solution A contained 0.25M Sucrose, 0.5mM EDTA and 10mM Tris. Solution B contained 0.25M Sucrose and 10mM Tris. All preparative steps were carried out at 4°C, according to a modified method of Myers and Slater (1957).

After the animals were sacrificed, weighed, and their livers removed, the blood vessels and fat attached to the liver were also removed. The liver was weighed (ca. 1.2 g.), dissected into small pieces and evenly homogenized in sucrose buffer A (1 g. liver/10 ml. of buffer) for 20 seconds, in a Potter-Elvehjem homogenizer. The crude homogenate was centrifuged at 1,000 x g. for 5 minutes in the MSE

centrifuge, in order to sediment the cell debris. The deposit was discarded and the supernatant, S_1, was recentrifuged at 18,500 x g. for 10 minutes in order to sediment the mitochondrial particles. The supernatant, S_2, was poured off and used in later experiments for the determination of certain enzyme activities. The mitochondrial residue was resuspended in sucrose buffer A and recentrifuged at 19,000 x g. for 10 minutes. The supernatant so obtained, S_3, was discarded and the "fluffy" lipid layer carefully removed from the mitochondrial pellet by washing with buffered sucrose B. The mitochondrial pellet was finally resuspended in buffered sucrose B to give a protein concentration of approximately 10 mg./ml. In order to obtain an even distribution of mitochondria, the mitochondrial suspension was gently homogenized, at low speed in a Potter-Elvehjem homogenizer, for 4 seconds.

3.4.2. Manometry.

Principle. The manometric method for the estimation of gas exchange, as originally devised by Warburg in the 1920's (Umbreit et al., 1964), was used in the experimental estimation of the oxygen uptake by mouse liver mitochondria. The apparatus consisted essentially of three parts: 1) A detachable reaction flask equipped with two stoppered side-arms and a centre-well. The stopper of one side-arm served as a gas-valve during equilibration; 2) a simple U-tube capillary manometer containing Brodie's fluid (10,000 mm. equivalent to 760 mm. Hg); 3) a constant temperature water-bath and mechanical shaker unit. In use the

reaction flask was attached to the manometer by means of a standard tapered ground glass joint and kept in place by an elastic band. The manometer-flask assembly was fitted, by means of a slotted socket, to the water-bath apparatus such that the flask was fully immersed in the water. Between readings the assembly was mechanically shaken in order to promote a rapid exchange of gases between the reaction fluid and the gaseous phase.

A thermobarometer, containing a volume of water equal to the volume of the reaction mixture in the test flasks, was used in order to correct any variations in atmospheric pressure. The above description is that of the Warburg constant volume respirometer and was used throughout these experiments.

To measure the oxygen consumption occurring in the reaction flask, the apparatus containing all the required reagents was set-up. With all stopcocks and valves opened the system was equilibrated. Stopcocks and valves were closed and the fluid in the closed arm of the manometer was adjusted to the 150 mm. mark. Equilibration was continued so as to obtain the initial reading on the open arm, e.g. 216.5 mm. The reaction was started and the levels of the fluid in the manometer arms were regularly adjusted, between shaking, so that the closed arm fluid registered on the 150 mm. mark. After a certain optimum time, x minutes, the final reading on the open arm was taken, e.g. 157 mm. For both the initial and x minute readings the fluid in the closed arm of the mano-

meter was adjusted to 150 mm. However, during the interval the reading on the open arm decreased from 216.5 to 157 mm. (59.5 mm.) as a result of oxygen consumption in the flask. Since the gas volume of the flask (Vg), the volume of the fluid in the flask (Vf), the temperature of operation, the gas being exchanged, and the density of the fluid in the manometer are all known, it is possible to calculate the amount of gas used during the experiment. The essence of the method was to hold the gas and fluid volumes constant and to measure the decrease or increase in pressure when one gas altered in amount. The flask constant, k, was the calculated value for a particular experimental assembly, such that the amount of gas utilized or given off could be calculated and expressed as microlitres (μl.) of gas at 0°C and 760 mm. pressure. The derivation of the flask constant resulted in the following formula (Umbreit et al., 1964):

$$\text{Gas taken up, } (\mu l) = x = h \left[\frac{Vg \frac{273}{T} + Vf\alpha}{Po} \right]$$

$$= hk$$

Vg = Volume of gas phase in flask including connecting tubes down to the reference point (150 mm. on closed arm of manometer).

Vf = Volume of fluid in vessel.

Po = Standard pressure, which is 760 mm. Hg or 10,000 mm. Brodie's fluid.

T = Absolute temperature of water-bath.

α = Solubility in reaction liquid of oxygen (expressed as ml. gas/ml. liquid at S.T.P.).

H = The observed change in the manometer (open side) reading in mm.

In the derivation of the above formula the initial pressure, P, and the vapour pressure, R, are cancelled out.

Method.

The reaction mixture was placed in the main compartment of the flask, and contained: 40 /um. phosphate; 6 /um. of ADP; 20 /um. glucose; cytochrome c (4.8%); albumin (5%); each delivered in 0.1 ml. of solution. These were made up to a volume of 3 ml. with 0.25 M sucrose-Tris buffer, pH 7.4, which contained 20 /um. succinate, 15 /um. potassium chloride and 5 /um. magnesium chloride. Hexokinase, 0.02 ml./reaction mixture, was added to promote the continued formation of ADP and to trap as glucose-6-phosphate via the following reaction:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

The first side-arm contained sufficient mitochondrial preparation to sustain respiration under the prescribed conditions, and the most suitable quantity was found to be 0.3 ml. The second side-arm contained sufficient perchloric acid to stop all reactions and to precipitate the protein. The centre well contained concentrated potassium hydroxide, on folded filter paper, for the absorption of carbon dioxide. These conditions gave adequate respiration, equivalent to state 3 respiration of Chance and Williams (1956).

With each test-experiment a control-experiment was performed.

Conditions were equivalent, however, in this case, no substrate, succinate, was added. This control was measured in order to determine the endogenous substrate respiration and the ATP formed due to the myokinase activity normally present in mitochondrial preparations. The ATP produced by myokinase or adenylate kinase (EC 2.7.4.3) activity in the mitochondria (Dixon & Webb, 1964) was significant and had to be taken into account in order to obtain optimum results. The reaction was essentially:

$$2\ ADP \rightleftharpoons ATP + AMP$$

After equilibration, at the standard temperature of 30°C, and setting of the apparatus for oxygen consumption, the mitochondrial preparations were tipped into the main compartment of the reaction flask, from the first side-arm. The rate of oxygen utilized was measured, at regular intervals for 30 minutes, by the decrease in pressure shown on the manometer (Table 3.4.1).

On the completion of the 30 minute reaction period the flasks were rapidly removed from the water-bath. The perchloric acid was immediately added to the reaction mixture, mixed well and rapidly cooled to 4°C. The reaction mixture was then neutralized with 5 M potassium carbonate.

Finally, a second control-experiment was performed with each mitochondrial preparation, viz. the zero-time control. This control was performed in order to determine the amount of ATP present in the

reaction mixture at zero-time. All the required reagents, together with the perchloric acid and the mitochondrial preparation, were added to a conical test tube and incubated at 30°C for 30 minutes with periodic shaking. Subsequently the reaction mixture was neutralized with 5 M potassium carbonate.

After removing the precipitated protein the concentration of ATP in the clear supernatant solution from each experiment was assayed.

3.4.3. Estimation of ATP.

The next step in the investigation of phosphorous:oxygen (P:O) ratios in oxidative phosphorylation of liver mitochondria was the assay of the ATP produced during respiration, as described in section 3.4.2. For efficient phosphorylation to take place a system which constantly regenerates ADP must be present. This was facilitated by the addition of hexokinase and glucose, as noted previously, to the reaction mixture. The ATP formed was trapped as glucose-6-phosphate (G-6-P), and the amount of phosphate (phosphorous) bound during the reaction period was estimated by assaying the amount of G-6-P present in the reaction mixture. Since the endogenous formation of ATP, by the activity of myokinase, had been determined, the net formation of ATP could be calculated.

The technique used for the assay of ATP was that of Lamprecht & Trautsehold (1965). The principle of the method is that ATP, in the presence of excess glucose, hexokinase and magnesium, phosphorylates the

glucose stoichiometrically (Gamble & Najjar, 1955), to G-6-P (Equation 1). Glucose-6-phosphate dehydrogenase (G-6-P-DH) catalyses the oxidation of G-6-P, in the presence of excess NADP, to 6-phosphogluconolactone (equation 2).

1. $\text{Glucose} + \text{ATP} \xrightarrow[Mg^{++}]{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$

2. $\text{G-6-P} + \text{NADP} \rightleftharpoons \text{6-phosphogluconolactone} + \text{NADPH}_2$

The equilibrium for equation 2 lies far to the right (Lamprecht, et al., 1965).

Method: The quantities added to the cuvette were arranged so as to give an optimum optical density change and complete reaction within 8 minutes. The assay was read against a control cuvette containing triethanolamine (TEA) buffer, pH 7.5, at a wavelength of 340 mμ. and a light path of 1 cm.

Reaction Mixture	Volume (ml.)
0.05M TEA buffer, pH 7.5	1.9
Deproteinized supernatant	1.0
0.05M Magnesium chloride	0.05
7mM NADP	0.05
G-6-P-DH (ca 140 units/mg.)	0.02

The solutions were added to the silica cuvettes in the order shown. Before the addition of G-6-P-DH the initial optical density reading, E_1, was taken. The G-6-P-DH was added and on completion of the reaction the final optical density reading, E_2, was recorded. The optical density change, $E_2 - E_1$, gave the absorbancy due to the $NADPH_2$ formed from the G-6-P present in the deproteinized supernatant which was directly pro-

portional to the concentration of ATP formed during oxidative phosphorylation.

Table 3.4.1. Randomly selected example of the readings obtained for the manometric determination of oxygen uptake by mouse liver mitochondria, and the subsequent calculations for P:O ratio.

Time	Thermobarometer		Test 1.		Control 1.	
	Reading mm.	change mm.	Reading mm.	Corrected reading mm.	Reading mm.	Corrected reading mm.
10.57 a.m.	150	0	217	217	164	164
10.58	151	-1	217.5	216.5	164.5	163.5
10.59	151.5	-1.5	218	216.5	165	163.5
11.00	Tip in mitochondria					
11.05	151.5	-1.5	208.5	207	164	162.5
11.10	152	-2	197.5	195.5	164	162
11.15	152	-2	188	186	163	161
11.20	152.5	-2.5	178	175.5	163	160.5
11.25	153	-3	168.5	165.5	163	160
11.30	154	-4	161	157	163.5	159.5
Actual change in mm. from 11.00 - 11.30.			59.5		4.0	
Flask constants, k.			1.4		1.64	
/ul. O_2 uptake.			83.3		6.56	
/uG. atoms O_2.			0.753		0.06	
ATP produced.			0.845		0.150	

Temperature = $30^{o}C$

Net oxygen uptake = (test-control) in /uG. atoms O_2.

= 0.693 /uG. atoms O_2.

Net ATP produced = (Test-control) /um. of ATP.

= 0.695 /um. of ATP.

P:O ratio = $\frac{\text{ATP produced}}{O_2 \text{ uptake}} = \frac{0.695}{0.693} = 1.005$

3.4.4. Calculations:

1. Oxygen consumption:

Let h = pressure change in mm.

Then amount of oxygen used = h x k = X μl.

X x 22.414 = micromoles of oxygen/30 minutes

X x 22.414 x 2 = μG atoms of oxygen/30 minutes

i.e. h x k x 22.414 x 2 = μG atoms of oxygen used/30 minutes.

The net oxygen consumed is equal to the overall oxygen consumption during the test experiment, minus the oxygen consumption during the control experiment.

2. Phosphorous, as ATP, produced during respiration.

The amount of $NADPH_2$ formed is directly equivalent to the amount of G-6-P in the reaction mixture. This is equivalent to the amount of ATP produced during respiration, and it is simply a matter of arithmetical calculation in order to convert the optical density change, $E_2 - E_1$, to micromoles of $NADPH_2$ formed. Hence micromoles of ATP produced per 30 minutes respiration can be calculated.

The net ATP produced is equal to the overall ATP produced during the test experiment, minus the sum of ATP produced due to myokinase activity, and that produced in the zero-time control.

3. Phosphorous:oxygen ratio (P:O).

This ratio is given by:

$$\frac{\mu M \text{ of Pi produced/mg. mitochondrial protein/unit time}}{\mu G \text{ atoms of O used/mg. mitochondrial protein/unit time.}}$$

For a summary of these results see Table 3.4.1.

3.4.5. The determination of the acceptor control ratio of mouse liver mitochondria.

Chance and Williams (1956) have systematized the general conditions affecting the respiratory rate of intact mitochondria and the oxidation-reduction state of the components of the respiratory chain. State 1 is the condition in which both ADP and respiratory substrate are lacking, and state 2 is the condition in which respiratory substrate only is lacking. State 3 is the condition in which all required components are present and the respiratory chain itself is the rate-limiting factor; that is, it is the "active" state of respiration. State 4 is the condition in which only ADP is lacking; this is the so-called controlled or resting or ADP-less state. State 5 is the condition in which only oxygen is lacking.

Great emphasis has been placed on the concentration of ADP as the most critical element in the determination of respiratory rate in either isolated mitochondria or in the intact cell (Chance & Williams, 1956). The rate of respiration in the presence of ADP divided by the rate in its absence is defined as the acceptor control ratio.

In the techniques used the procedure followed in the determination of A.C.R. values was adapted to the experimental apparatus available.

Method:

The procedure followed was similar to that described in the section 3.4.3., except that the ADP-glucose solution was added to the

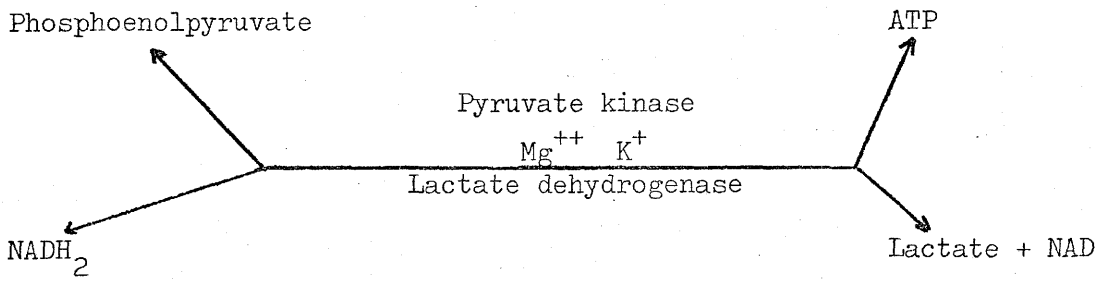
second side-arm instead of the perchloric acid, and not to the main compartment. The apparatus was equilibrated at 30°C and set up for oxygen consumption. The mitochondrial preparation was tipped into the main compartment from side-arm 1, and the oxygen taken up was measured. This gave respiration in state 4; i.e. no ADP was present. After 10 minutes the ADP-glucose solution was tipped in, resulting in state 3 respiration. The oxygen consumption was noted for a further 20 minutes.

The ratio given by the respiration in state 3, to the respiration given in state 4, was calculated. This gave the acceptor control ratio, and was a measure of the "intactness" of the mitochondria.

3.5. The assay of some Hepatic Enzymes.

3.5.1. Assay of ATP:Pyruvate phosphotransferase (Pyruvate kinase EC 2.7.1.40).

Principle: Pyruvate kinase catalyses the transfer of a high energy phosphate ester from phosphoenolpyruvate to ADP. Like all kinases, pyruvate kinase exhibits an absolute requirement for magnesium ions. In addition, this enzyme functions only in the presence of a relatively high concentration of potassium ions (Bücher & Pfleiderer, 1955). The compound optical assay in which the pyruvate kinase reaction is coupled with that of lactic dehydrogenase was first developed by Negelein (1944).



As the pyruvate kinase assay is a compound assay, the coupling enzyme, lactate dehydrogenase, was added in excess to the assay mixture.

Method: The supernatant, S_2, obtained during the preparation of liver mitochondria (section 3.4.1.), was recentrifuged at 26,000 x g for 45 minutes. This supernatant, S_4, was used as the source of all the cytoplasmic enzymes studied. The buffer used was 0.5M triethanolamine buffer pH 7.4 containing 8mM magnesium sulphate and 75mM potassium chloride.

Reaction Mixture	Volume (ml.)
0.05M TEA-HCl buffer	2.8
0.01M $NADH_2$	0.05
0.024M ADP	0.05
Lactate dehydrogenase (1150 I.U./ml.)	0.001
Supernatant, S_4	0.05
0.005M Phosphoenolpyruvate (PEP)	0.05

The solutions were added to the silica cuvettes in the order shown. Before adding the PEP the initial decrease in optical density per minute was recorded, reading against a blank cuvette containing TEA-HCl buffer. The reaction was started by adding the required volume

of PEP and the velocity of the reaction was measured for a further 3 minutes, by the gradual decrease in optical density due to the oxidation of $NADH_2$ in the reaction cuvette. The reaction was followed in the Beckman spectrophotometer, wave-length 340 mμ at 30°C.

Calculation: The initial decrease in optical density per minute was subtracted from the final decrease in optical density per minute, after addition of PEP, and from this value the specific activity of the pyruvate kinase was determined. The units of activity were expressed as International Units (I.U.).

I.U. = micromoles of pyruvate formed/mg. S_4 protein/minute.

3.5.2. Assay of L-malate : NADP oxido-reductase (decarboxylating) EC 1.1.1.40 (Malic enzyme).

Principle: The specific activity determinations were based on the reaction below, first shown by Ochoa (1955) to be catalyzed by the malic enzyme.

$$\text{L-malate} + \text{NADP} \xrightleftharpoons{Mn^{++}} \text{Pyruvate} + CO_2 + NADPH_2$$

The early rate of reduction of NADP, in the presence of enzyme, manganese ions, and excess malate, is, within certain limits, proportional to the enzyme concentration.

Method: The reaction was followed in a Beckman or Unicam spectrophotometer, wavelength 340 mμ, in silica cuvettes (d=1 cm.), at 30°C.

Reaction mixture	Volume (ml.)
De-ionized water	2.5
0.25M Tris- HCl, pH 7.4	0.3
0.05M Manganese chloride	0.05
0.003M NADP	0.05
Supernatant S_4	0.05
0.062M Potassium malate	0.05

The solutions were added to the cuvettes in the order shown. Before adding the malate, the activity caused by endogenous substrates and reductive systems present in the assay mixture was measured, E_1 per minute, readings taken against a blank containing 0.25M Tris-HCl buffer. The substrate, malate, was added and the increase in optical density caused by the reduction of NADP was measured, E_2/minute. E_2-E_1/minute was a measure of the activity of the malic enzyme present in the supernatant, S_4, enzyme preparation.

Calculation: The specific activity of the enzyme was calculated and expressed in International Units (I.U.).

I.U. = micromoles of pyruvate formed/mg. of S_4 protein/minute.

3.5.3. Assay of L-malate:NAD oxido-reductase (EC 1.1.1.37.)

Mitochondrial malate dehydrogenase (M-MDH).

Cytoplasmic malate dehydrogenase (C-MDH.).

Principle: Malate dehydrogenase catalyses the oxidation of L-malate by NAD (Hohorst, 1965).

$$\text{L-malate} + \text{NAD} \rightleftharpoons \text{oxaloacetate} + \text{NADH}_2$$

The equilibrium for the reaction lies far to the left (Hohorst, et al., 1959). To obtain a quantitative oxidation of malate the reaction products had to be removed, in order to drive the reaction to the right. Thus, this analytical procedure was carried out in an alkaline medium, in order to bind the protons, and the oxaloacetate formed was trapped as the hydrazone. The basic equation for the spectrophotometric assay of MDH was:

$$\text{Malate} + \text{NAD} + \text{hydrazine} \overset{\text{pH } 9.6}{\rightleftharpoons} \text{oxaloacetate-hydrazone} + \text{NADH}_2 + \text{H}_2\text{O}$$

Method: A relatively high concentration of NAD was necessary to obtain a quantitative reaction. The course of the reaction was followed in the Beckman spectrophotometer, wavelength 340 mμ, in silica cuvettes (d = 1 cm.), at 30°C.

The mitochondrial preparation from section 3.4.1. was further blended with sodium deoxycholate (10 mg. Na-deoxycholate per 1 ml. of mitochondrial preparation) in a Potter-Elvehjem homogenizer. This procedure solubilised the malate dehydrogenase and facilitated the assay of this enzyme's activity.

Reaction Mixture	Volume (ml.)
1M Hydrazine - 0.2M glycine buffer, pH 9.6	2.8
0.03M NAD	0.05
Supernatant, S_4, or mitochondrial preparation	0.1
To start: 0.2M Potassium malate	0.05

Readings were taken against a control cuvette containing all the solutions except the malate.

Calculation: The initial rate of reaction was measured and extrapolated to time. From this the specific activity of the enzyme was calculated and expressed in International Units.

I.U. = micromoles of oxaloacetate formed/mg. protein/minute.

3.5.4. Alternative assay method for cytoplasmic malate dehydrogenase (EC 1.1.1.37)

Principle: The activity of the cytoplasmic MDH has been found to be greater than the activity of the mitochondrial MDH(Delbrück et al., 1959a; & 1959b; Dixon & Webb, 1964). It has also been found that the equilibrium of the catalysed reaction favours the reduction of oxaloacetate (Hohorst et al., 1959). For these reasons an additional assay technique was utilized (Bücher, 1959) in order to determine the specific activity

of the cytoplasmic malate dehydrogenase in the favoured direction.

$$\text{Oxaloacetate} + NADH_2 \xrightleftharpoons{\text{pH } 7.6} \text{Malate} + NAD$$

Method: The course of the reaction was followed in the Beckman spectrophotometer, wavelength 340 mμ, in silica cuvettes (d = 1 cm.) at 30°C.

Reaction Mixture	Volume (ml.)
0.1M TEA-phosphate buffer, pH 7.6.	1.5
0.01M NADH	0.05
0.15M EDTA	0.1
Supernatant, S_4, (diluted 1:10)	0.025
De-ionized water	1.125
To start: 0.002M Oxaloacetate	0.1

The solutions were added to the cuvettes in the order shown. Before adding the substrate the reaction mixture was allowed to equilibrate for 3 minutes at 30°C.

Calculation: On starting the reaction the initial rate of reaction was recorded and extrapolated to time. The specific activity of the enzyme was calculated and expressed in International Units.

I.U. = micromoles of malate formed/mg. protein/minute.

3.5.5. Assay of Isocitrate:NADP oxidoreductase (decarboxylating) (EC 1.1.1.42.)

Principle: The specific activity determinations for this enzyme, Isocitrate dehydrogenase, were based on the overall reversible reaction

in which isocitrate is oxidatively decarboxylated to α-ketoglutarate (Siebert et al., 1957; Plaut, 1962).

$$\text{Isocitrate} + \text{NADP} \xrightleftharpoons{Mn^{++}} \alpha\text{-ketoglutarate} + CO_2 + NADPH_2$$

Method: The reaction was followed on the Beckman spectrophotometer, wavelength 340 mμ, in silica cuvettes (d = 1 cm.), at 30°C.

Reaction Mixture	Volume (ml.)
0.11M Tris-HCl buffer containing 0.001M EDTA, pH 7.4	2.1
De-ionized water	0.5
0.02M Manganese sulphate	0.2
0.08M Potassium isocitrate	0.05
Supernatant, S_4.	0.05
To start: 1.5 mM NADP	0.1

Readings were recorded against a control cuvette containing all reagents except NADP. The solutions were added to the cuvettes in the order shown. Before adding the NADP the reaction mixture was allowed to equilibrate for 3 minutes at 30°C.

Calculation: The initial rate of reaction was recorded and extrapolated to time. The specific activity of the enzyme was calculated and expressed in International Units.

I.U. = micromoles of α-ketoglutarate formed/mg. protein/minute.

3.6. Determination of Protein Concentrations.

3.6.1. Kjeldahl.

The amount of protein present in each mitochondrial sample was determined by the modified micro-Kjeldahl method of Hiller (1948). The reaction mixture was placed into 3 test tubes as follows:

Reaction Mixture	Blank	Standard	Test
De-ionized water	0.2	---	---
Albumin (10 mg./ml.)	---	0.2	---
Mitochondrial preparation	---	---	0.2
Kjeldahl acid[a)]	1.0	1.0	1.0

Each test tube was then heated on a digestion rack until the solutions were clear, normally for 3 hours. The ammonia, from the clear samples, was then steam distilled after adding 7.5 ml. of Kjeldahl alkali[b)] per ml. of acid, into 10 ml. of 0.01N HCl containing 2 drops of Kjeldahl indicator.[c)] After distilling for 9 minutes the erlenmeyer flask, containing the acid and indicator, was lowered and allowed to steam bubble for 2 minutes. The ammonia released into the HCl was back titrated with 0.01N NaOH, and on subtracting this titre volume from the blank, the concentration of protein present was calculated.

a) Kjeldahl acid: Mix 60g. K_2SO_4, 2g. $CuSO_4$, 1.1g. Na_2SeO_3, and 1.373g. HgO with 200 ml. of concentrated sulphuric acid.

b) Kjeldahl alkali: 240g. of NaOH pellets were dissolved in 560 ml. of de-ionized water containing 2.5g. of $Na_2S_2O_3$.

c) Kjeldahl indicator: Mix 42g. of methyl red in 85 ml. of alcohol, add 10 mg. methylene blue in 15 ml. of de-ionized water.

3.6.2. Biuret.

The amount of protein present in each supernatant sample was determined by the modified methods of Gornall et al., (1949), and Cleland et al., (1953).

Reaction Mixture	Volume (ml.) in 20 ml. test tubes		
	Blank	Standard	Test
De-ionized water	1.0	0.8	0.8
Albumin standard (10 mg./ml.)	---	0.2	---
Supernatant, S_4.	---	---	0.2
Biuret reagent [a)]	4.0	4.0	4.0

After the addition of the reaction mixture to the test tubes as shown each test tube was well shaken. The reaction mixtures were allowed to stand for 30 minutes at room temperature and the optical density, at wavelength 540 mμ, of tests and standard were recorded on the Unicam spectrophotometer, against the blank reference cell. A standard curve showed no variation over a period of one year.

a) Biuret reagent: 1.5g. of $CuSO_4.5H_2O$ and 6g. of sodium-potassium tartrate were dissolved in 500 ml. of de-ionized water. 300 ml. of 10% NaOH solution was added and well stirred. After the solution had cooled it was transferred quantitatively to a 1 litre volumetric flask and made up to the mark with de-ionized water.

3.7. Purification Procedure for Mouse Liver Malic Enzyme (EC 1.1.1.40).

All purification steps were carried out at 0-5°C and all solutions contained 10^{-5}M NADP and 10^{-5}M EDTA. The latter steps in the procedure were modified from the method of Hsu & Lardy (1967), and Soldin (1968).

A typical protocol from among successful preparations is presented in Table 3.7.1. Successive purification procedures were carried out on the supernatant fractions, S_4, obtained either from 10 male livers or from 10 female livers.

Step 1. To the supernatant fractions, S_4, mercaptoethanol was added to a concentration of 2mM (Fraction I). The protein concentration, the activity of the malic enzyme, and the volume, were measured.

Step 2. Ammonium Sulphate Fractionation: Solid ammonium sulphate was added to Fraction I, with stirring, to give 45% saturation. Stirring was continued for 15 minutes and the precipitate was removed by centrifugation at 15,000 x g. for 20 minutes (Fraction II). The supernatant Fraction II was brought to 70% saturation by the addition of solid ammonium sulphate, stirred for 15 minutes, and again centrifuged as before. The 45 to 70% saturated ammonium sulphate precipitate was dissolved in a small amount of 0.05M Tris-HCl-2mM mercaptoethanol at pH 7.7, and dialysed against 1.5 litre portions of the same buffer overnight with one change of buffer (Fraction III).

Step 3. DEAE-cellulose Chromatography: A column (1 x 10 cm.) was prepared from washed DEAE-cellulose and equilibrated with 1 litre of 0.005M Tris-HCl buffer at pH 7.7. Fractions were collected on the LKB fraction collector. Protein peaks were detected by recording light absorption at 280 mμ. Fraction III was added to the column and eluted with 0.005M Tris-HCl buffer at pH 7.7 containing 0.05M KCl. In eluting

Fraction III the protein peak which contained the enzyme activity in each case was collected (Fraction IV). Again the protein concentrations, the activities of the enzymes, and the volumes, of the fractions were measured.

Step 4. Ammonium Sulphate Fractionation: Fraction IV was made to 70% saturation with ammonium sulphate, with stirring. The precipitate was centrifuged at 15,000 x g. for 15 minutes and then resuspended in a minimum quantity of 0.05M Tris-HCl buffer at pH 7.4 (Fraction V).

Step 5. Sephadex G-200 Chromatography: A column (1 x 20 cm.) was prepared from washed Sephadex G-200 and equilibrated with a litre of 0.05M Tris-HCl buffer at pH 7.4. The fractions were collected on the LKB fraction collector. Protein peaks were detected as described. Fraction V was added to the column and eluted with the 0.05M Tris-HCl buffer at pH 7.4. The protein peak which contained the enzyme activity was collected (Fraction VI).

Table 3.7.1. Purification of Mouse Liver Malic Enzyme.

Fraction	Volume (ml.)	Total activity (I.U.)	Total protein (mg.)	Specific activity (I.U.)	Yield (%)	Purification factor
I	119	53.11	1,890	0.0281	100	1.0
II	116	46.33	1,130	0.041	87	1.5
III	17	36.3	234	0.155	68	5.5
IV	49	25.4	79	0.320	48	11.4
V	1.9	21.3	43	0.490	40	17.4
VI	5.2	14.1	18	0.785	27	28.0

Portions of FractionVI were used in the starch gel electrophoresis in order to determine the mobilities of the enzyme on a comparative basis between male and female.

3.8. Starch Gel Electrophoresis of Mouse Liver Malic Enzyme.

The results of earlier experiments indicated the possibility that isoenzymes of malic enzyme could exist in male and female mice. Electrophoretic analysis of the malic enzyme was made on crude liver homogenate supernatants and on 28-fold purified enzyme preparations. The procedures followed for the preparation of the enzyme fractions have been outlined.

Materials: The standard Vokam power supply, Shandon type 2541, together with the standard Shandon electrophoresis tank and starch gel moulds (21 x 3.5 x 0.6 cm.) were used throughout this experiment. In the staining mixture 2(p-Iodophenyl)-3-(p-Nitrophenyl)-5-phenyltetrazolium chloride (INT) obtained from Nutritional Biochemicals Corporation, and N-methyl-phenazonium metho-sulphate (PMS) obtained from Light & Co. were used.

Procedure: Horizontal starch gel electrophoresis was carried out in the cold for 5-6 hours at a constant current of 15 milli-amps and voltage varying from 160 V, at the start, to 300 V at the end of the experiment. The discontinuous buffer system of Poulik's (1957) was used, viz. gel buffer 0.076M Tris-citrate at pH 8.65, and bridge buffer 0.3M borate at pH 8.0.

The procedure followed was that of Henderson (1966) and Roller (1968). After electrophoresis, the malic enzyme was located in the

starch by incubation of the starch strips at 37°C for 20 minutes in a staining mixture containing the following components:

Reaction Mixture	Volume (ml.)
0.2M Tris-HCl buffer at pH 8.0	110
1M DL-malate at pH 7.0	16
0.25M $MnCl_2$	0.4
6mM NADP	2
INT (10 mg./ml.)	10
PMS (1.6 mg./ml.)	16

Finally, a permanent record, photograph, of the stained starch gel's was made and the distances travelled by the malic enzymes from male and female livers were compared.

CHAPTER 4.

RESULTS

4.1. Mitochondrial Oxidative Phosphorylation.

The results obtained for the oxidation of succinate and the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP), by mouse liver mitochondria, are listed in Tables 4.1.1. and 4.1.2. The acceptor control ratio (ACR) results are tabulated in Table 4.1.3.

The results depicted in Table 4.1.1. show that the Phosphorous: Oxygen (P:O) ratios in both male and female liver mitochondria are essentially equivalent (male mean : 1.042 ± 0.042; female mean : 1.014 ± 0.055). However, it was found that, during respiration, the mitochondria prepared from female livers consumed greater quantities of oxygen (mean : 0.715 ± 0.15 microgram atoms of oxygen per mg. protein per 30 minutes) than did those prepared from male livers (mean : 0.548 ± 0.12 microgram atoms of oxygen per mg. protein per 30 minutes). Correspondingly, the females produced larger amounts of ATP (mean : 0.723 ± 0.15 micromoles of ATP per mg. protein per 30 minutes). These differences for oxygen consumption and ATP production, between the sexes, were highly significant ($P < 0.001$).

A comparison of the values obtained for the acceptor control ratios indicates that there were no significant differences ($P > 0.1$) in the state of "coupling" of the prepared mitochondria between the sexes.

Table 4.1.1. Phosphorous:Oxygen ratio as determined during oxidative phosphorylation of mouse liver mitochondria.

Type of animal	n[a]	ATP mean[b]	Oxygen mean[c]	P:O	S.D.(±)
Male	22	0.559	0.548	1.042	0.042
Female	22	0.723	0.715	1.014	0.055

Table 4.1.2. The significance of the difference in ATP production and oxygen consumption between males and females.

Investigation	n[a]	MALES		FEMALES		Significance[d]
		mean	S.D.(±)	mean	S.D.(±)	
ATP[b]	22	0.559	0.12	0.723	0.15	$P < 0.001$
Oxygen[c]	22	0.548	0.12	0.715	0.15	

Table 4.1.3. Acceptor control ratios (ACR)

Type of animal	n[a]	ACR	S.D.(±)
Male	27	2.4	0.45
Female	23	2.8	0.50

(a) Number of animals used.

(b) Micromoles of ATP produced per mg. protein per 30 minutes.

(c) Microgram atoms of oxygen consumed per mg. protein per 30 minutes.

(d) Refer to Fisher (1958), "Statistical Methods for Research Workers".

4.2. The activity of the hepatic pyruvate kinase enzyme (EC 2.7.1.40)

The supernatant fractions (S_4) from livers of normal, ovariectomized, castrated, and hormone treated mice were assayed for pyruvate kinase activity. The results are tabulated in Table 4.2.1.

From a study of the results shown in Table 4.2.1. it would appear that the activity of the pyruvate kinase enzyme is dependent on estrogen. In the liver of normal males, the activity of this enzyme is considerably lower than that in the liver of normal females. In ovariectomized females and castrated males, the pyruvate kinase activity resembled that of normal males. Daily treatment of ovariectomized and castrated mice with testosterone propionate resulted in pyruvate kinase activities within the normal male range. However, when ovariectomized females and castrated males were treated with daily injections of estradiol benzoate, the activity of the pyruvate kinase enzyme was elevated towards the level of activity found in normal females.

These findings are summarized in Fig. 4.2.1.

Table 4.2.1. Pyruvate Kinase activities.

Type of animal		Treatment	n[c)]	Mean[a)]	S.D.(±)
MALE:	normal	Nil	20	0.0236	0.0046
	castrated	Nil	7	0.028	0.0032
	castrated	Estradiol benzoate.[b)] 1.5 mg./day/50g. i.m.	4	0.034	0.005
	castrated	Testosterone propionate 1.5 mg./day/50g. i.m.[b)]	8	0.021	0.006
FEMALE:	normal	Nil	15	0.0469	0.0072
	ovariectomized	Nil	6	0.029	0.0045
	ovariectomized	Estradiol benzoate. 1.5 mg./day/50g. i.m.[b)]	4	0.036	0.004
	ovariectomized	Testosterone propionate 1.5 mg./day/50g. i.m.[b)]	7	0.024	0.001

a) International Units. Micromoles of pyruvate produced/mg. protein/minute.

b) In arachis oil, 60µl, per injection, intramuscularly. Courses of injections were commenced on the day of the operation and continued for ten successive days.

c) Number of animals used.

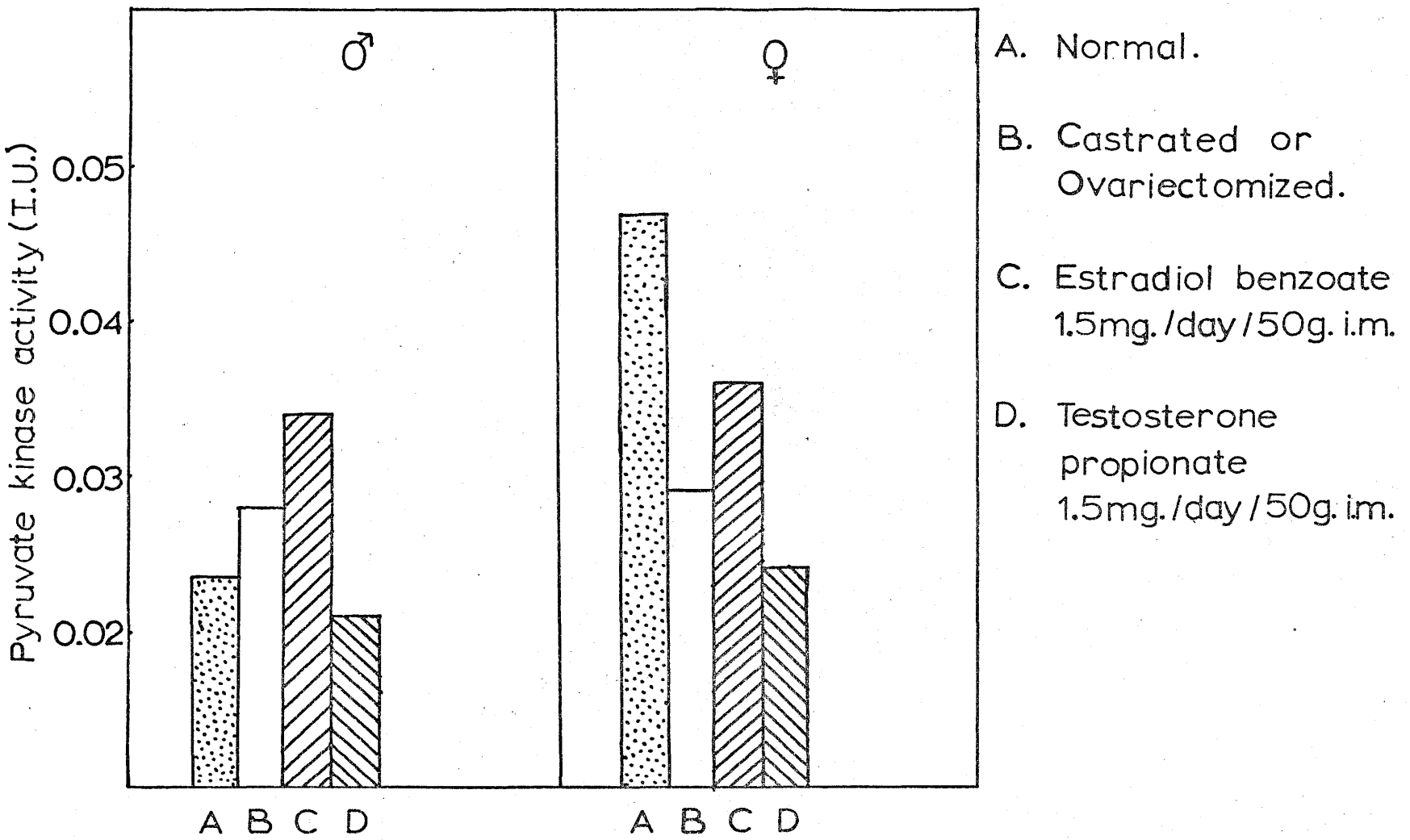


Fig. 4.2.1. The pyruvate kinase activity in normal, castrated, ovariectomized, and hormone treated mice, Praomys (Mastomys) natalensis

4.3. The Activity of the Hepatic Malic Enzyme (EC 1.1.1.40)

The supernatant fractions (S_4) from livers of normal, ovariectomized, castrated, and hormone treated mice were assayed for malic enzyme activity. The results obtained are tabulated in Table 4.3.1. and Figs. 4.3.1. and 4.3.2.

A study of Table 4.3.1. and the relevant figures demonstrate that the activity of the hepatic malic enzyme is dependent on testosterone. In the liver of normal males, the activity of this enzyme is considerably higher than that in the liver of normal females.

Castration of males resulted in the progressive decrease in the activity of the malic enzyme until it resembled that of normal females. Daily treatment of castrated males with estradiol benzoate appeared to have little effect upon the level of this enzyme, since the activity remained comparable to that of castrated males and normal females. However, when castrated males were treated with daily injections of testosterone propionate, the activity of the malic enzyme was again comparable with the level of activity found in normal males.

The level of the enzyme activity in ovariectomized females, and in those operated animals given daily injections of estradiol benzoate, remained within the normal female range. Daily treatment of ovariectomized females with testosterone propionate resulted in a slight increase in enzyme activity, however, this increase was not as dramatic as in the case of the castrated males. Thus the hepatic malic enzyme appears to be dependent upon testosterone.

Table 4.3.1. Malic Enzyme activities.

Type of animal	Treatment	n[c)]	Mean[a)]	S.D.(±)
MALES: normal	Nil	23	0.053	0.0027
castrated	Assayed 1 day after operation	5	0.0469	0.0006
castrated	Assayed 4 days after operation	5	0.0385	0.0015
castrated	Assayed 6 days after operation	5	0.0353	0.0013
castrated	Assayed 8 days after operation	5	0.0297	0.001
castrated	Assayed 14 days after operation	3	0.0272	0.0012
castrated	Assayed 19 days after operation	4	0.0254	0.0007
castrated	Assayed 27 days after operation	4	0.0234	0.0008
castrated	Estradiol benzoate* [b)]	8	0.0251	0.0036
castrated	Testosterone propionate**	8	0.0340	0.0014
castrated	Testosterone propionate***	6	0.0366	0.0024
castrated	Testosterone propionate****	6	0.0464	0.0120
FEMALES: normal	Nil	20	0.0224	0.0053
ovariectomized	Assayed 15 days after operation	5	0.0231	0.0024
ovariectomized	Estradiol benzoate*	4	0.0235	0.0031
ovariectomized	Testosterone propionate ****	6	0.0297	0.0015

a) International Units; micromoles of pyruvate produced/mg. protein/minute.

b) In arachis oil, 60µl. per injection, intramuscularly. Courses of injections were commenced on the day of the operation and continued for ten successive days.

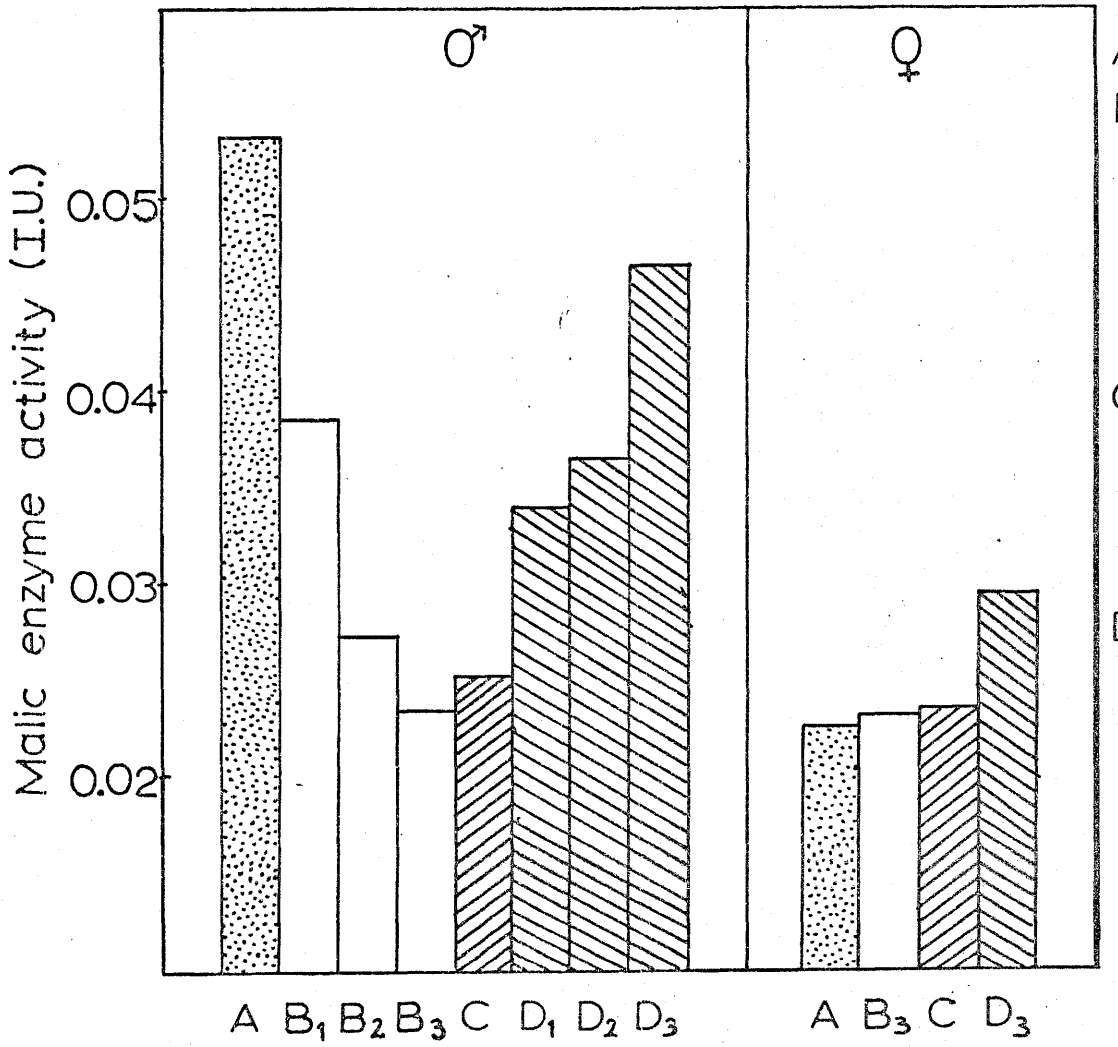
* 1.5 mg. estradiol benzoate/day/50g. body weight.

** 0.025 mg. testosterone propionate/day/50g. body weight.

*** 0.250 mg. testosterone propionate/day/50g. body weight.

**** 1.5 mg. testosterone propionate/day/50g. body weight.

c) Number of animals used.



A. Normal.
B. Castrated or Ovariectomized.
1. 4 days after operation
2. 14 days after operation
3. 27 days after operation.
C. Castrated or Ovariectomized plus Estradiol benzoate
1.5mg./day/50g.
D Castrated or Ovariectomized plus Testosterone propionate
1. 0.025mg./day/50g.
2. 0.25mg./day/50g.
3. 1.5mg./day/50g.

Fig.4.3.1. Malic enzyme activity in normal, castrated, ovariectomized, and hormone treated mice, Praomys (Mastomys) natalensis.

Fig. 4.3.2. A, B & C represent malic enzyme activities with increasing doses of testosterone to castrated males. D represents the decrease in malic enzyme activities on castration.

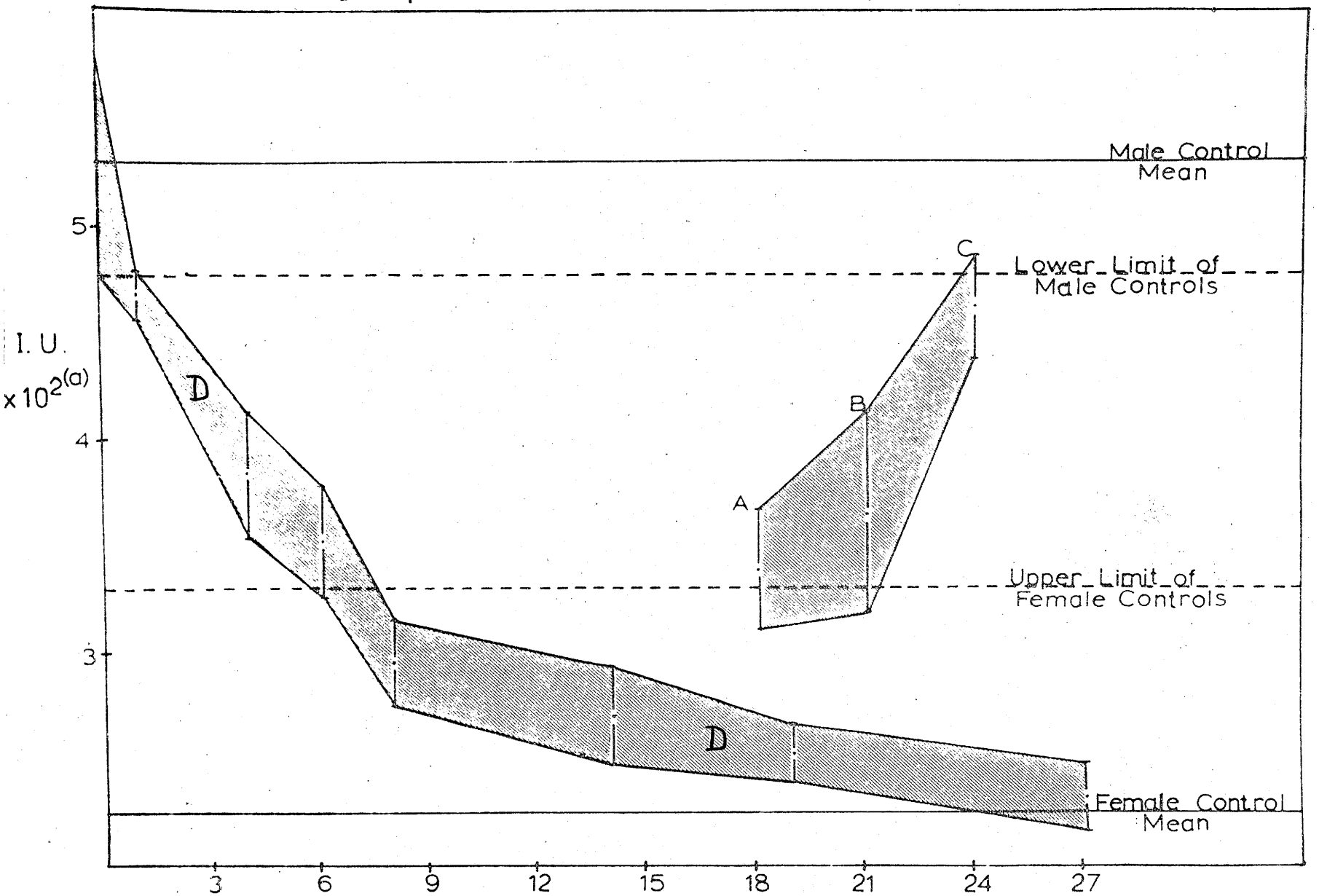


Fig. 4.3.2.

4.4. The Activity of the Mitochondrial Malate Dehydrogenase (EC 1.1.1.37).

The mitochondrial fractions from livers of normal, castrated, ovariectomized, and hormone treated mice were assayed for malate dehydrogenase activity. The results obtained are tabulated in Table 4.4.1. and Fig. 4.4.1.

A study of the results demonstrates that the activity of the hepatic mitochondrial malate dehydrogenase is dependent on testosterone. In the liver of normal males, the activity of this enzyme is considerably higher than that in the liver of normal females.

In castrated males and ovariectomized females, the malate dehydrogenase activity resembled that of normal females. However, when operated mice, both male and female, were treated with daily injections of testosterone propionate, the activity of the malate dehydrogenase was again comparable with the level of activity found in normal males. When castrated males were treated with daily injections of progesterone, the activity of the malate dehydrogenase was intermediate to that found in normal males and females.

Table 4.4.1. Mitochondrial Malate Dehydrogenase Activities.

Type of animal		Treatment	n[c)]	Mean[a)]	S.D.(±)
MALE:	normal	Nil	8	0.1258	0.016
	castrated	Nil	4	0.0850	0.009
	castrated	Testosterone propionate*	5	0.124	0.01
	castrated	Progesterone**	4	0.105	0.01
FEMALE:	normal	Nil	8	0.09	0.012
	ovariectomized	Nil	4	0.088	0.01
	ovariectomized	Testosterone propionate*	4	0.121	0.006

a) International units; micromoles of oxaloacetate produced per mg. protein per minute.

b) In arachis oil, 60 µl per injection, intramuscularly.

Courses of injection were commenced on the day of the operation and continued for ten successive days.

* 1.5 mg. testosterone propionate/day/50g. body weight.

** 1.5 mg. progesterone/day/50g. body weight.

c) Number of animals used.

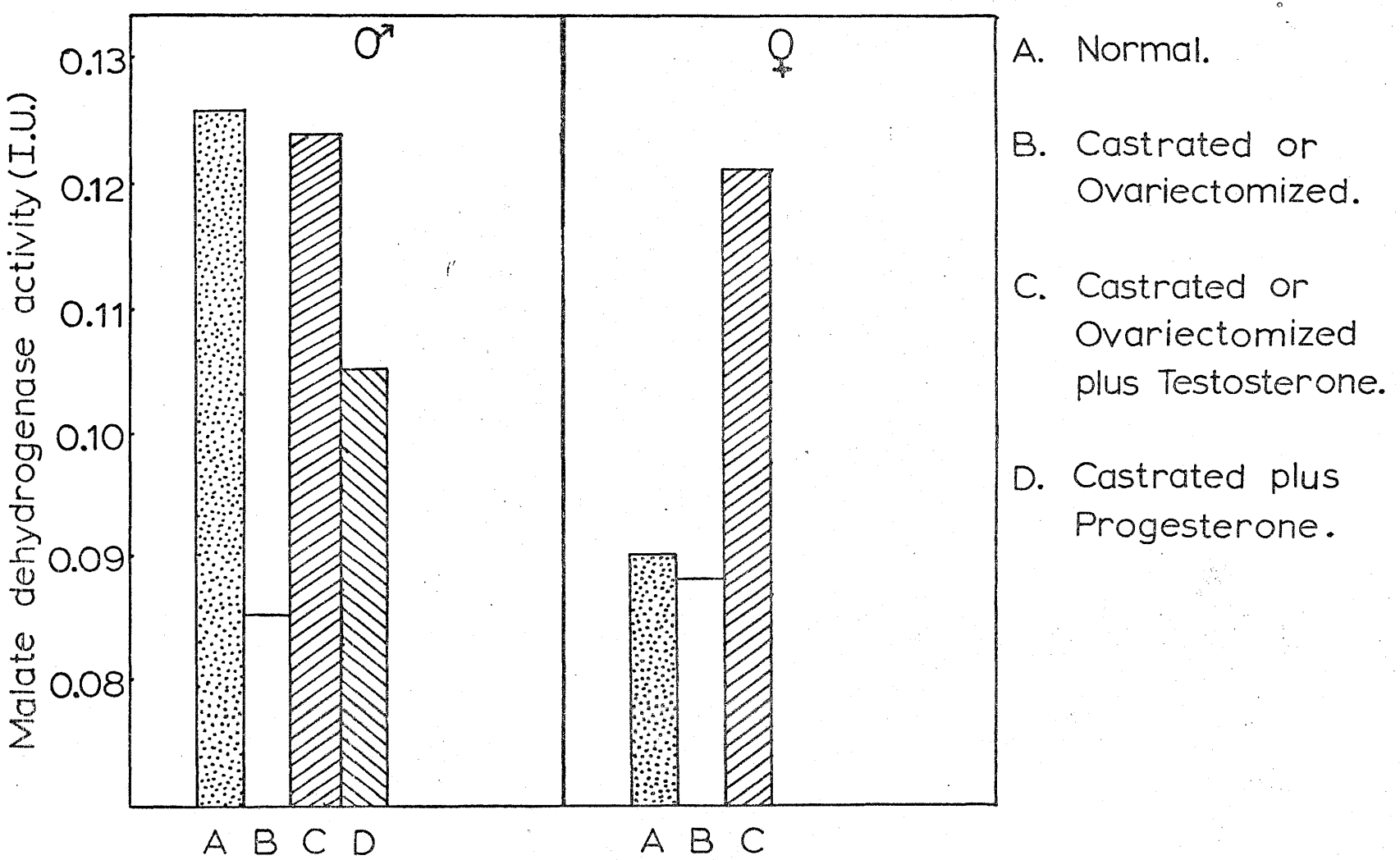


Fig.4.4.1. Mitochondrial malate dehydrogenase activity in normal, castrated, ovariectomized, and hormone treated mice, Praomys (Mastomys) natalensis.

4.5. The Activity of Liver Cytoplasm Malate Dehydrogenase (EC 1.1.1.37).

In the liver cytoplasm the natural direction of the reaction catalysed by this enzyme favours the formation of L-malate. However, the mitochondrial malate dehydrogenase activity was measured via the formation of oxaloacetate and for comparison purposes the cytoplasmic enzyme was measured in both directions. The two rates of reactions differed markedly, however, the conclusions drawn were the same. The supernatant fractions (S_4) from livers of normal, castrated, ovariectomized, and hormone treated mice were assayed for cytoplasmic malate dehydrogenase activity, and the results obtained, for both methods of assay, are tabulated in Tables 4.5.1. and 4.5.2. respectively.

We demonstrated in the mouse, Praomys (Mastomys) natalensis, that the activities of the liver cytoplasmic malate dehydrogenase in normal males and normal females are essentially equivalent. In castrated males and ovariectomized females the activities of this enzyme resembled that in normal mice. Daily injections of the hormone preparations appeared to have no effect upon the activity of this enzyme. Our conclusion, for this enzyme, is that there are no apparent sex-linkages in its activity under the experimental conditions used.

Table 4.5.1. Cytoplasmic Malate Dehydrogenase Activities
(Formation of oxaloacetate)

Type of animal		Treatment	n[c)]	Mean[a)]	S.D. (±)
MALE:	normal	Nil	8	0.0731	0.007
	castrated	Nil	4	0.0670	0.0042
	castrated	Testosterone propionate*	5	0.0774	0.0025
	castrated	Progesterone**	4	0.0776	0.0057
FEMALE:	normal	Nil	8	0.0772	0.0036
	ovariectomized	Nil	4	0.0714	0.0055
	ovariectomized	Testosterone propionate*	4	0.0752	0.005

Table 4.5.2. Cytoplasmic Malate Dehydrogenase Activities.
(Formation of L-Malate).

Type of animal		Treatment	n[c)]	Mean[a)]	S.D.(±)
MALE:	normal	Nil	8	3.29	0.29
	castrated	Nil	6	3.27	0.22
	castrated	Testosterone propionate*	5	3.72	0.27
	castrated	Progesterone**	4	3.33	0.16
FEMALE:	normal	Nil	8	3.51	0.21
	ovariectomized	Nil	4	3.70	0.28
	ovariectomized	Testosterone propionate*	4	3.69	0.70

a) International units; micromoles of oxaloacetate or L-malate produced per mg. protein per minute.

b) In arachis oil, 60 µl per injection, intramuscularly.

Courses of injections were commenced on the day of the operation and continued for ten successive days.

* 1.5 mg. testosterone propionate/day/50g. body weight.

** 1.5 mg. progesterone/day/50g. body weight.

c) Number of animals used.

4.6. The Activity of Liver Cytoplasmic Isocitrate Dehydrogenase. (EC 1.1.1.42)

The supernatant fractions (S_4) from livers of normal, castrated, ovariectomized, and hormone treated mice were assayed for isocitrate dehydrogenase activity. The results are presented in Table 4.6.1.

From a study of the results presented, it would appear that there are no clear cut sex-linked differences in the activity of this enzyme.

Table 4.6.1. Isocitrate Dehydrogenase Activities.

Type of animal		Treatment	n[c]	Mean[a]	S.D. (±)
MALE:	normal	Nil	8	0.244	0.03
	castrated	Nil	4	0.268	0.023
	castrated	Testosterone propionate*[b]	5	0.248	0.005
	castrated	Progesterone**[b]	4	0.273	0.027
FEMALE:	normal	Nil	8	0.269	0.020
	ovariectomized	Nil	4	0.297	0.009
	ovariectomized	Testosterone propionate*[b]	4	0.240	0.026

a) International Units; micromoles of α-ketoglutarate produced per mg. protein per minute.

b) In arachis oil, 60 µl per injection, intramuscularly. Courses of injections were commenced on the day of the operation and continued for ten successive days.

* 1.5 mg. testosterone propionate/day/50g. body weight.

** 1.5 mg. progesterone/day/50g. body weight.

c) Number of animals used.

4.7. Purification and Electrophoresis of Mouse Liver Malic Enzyme.

The results obtained for the activities of the malic enzyme demonstrated that the activity of this enzyme is testosterone dependent. However, treatment of ovariectomized females with injections of testosterone did not elevate the activity of this enzyme to the range of the normal males. It was tentatively proposed that isoenzymes of malic enzyme might exist, viz. a male form, M, and a female form, F. The enzyme from the male livers consisting of both the M and F forms with only the M form being testosterone dependent, whereas the enzyme from the female livers containing only the F form. In order to test this hypothesis a purification procedure, for malic enzyme, was attempted and the resultant purified enzymes, together with crude enzyme samples, were subjected to electrophoresis so as to compare the electrophoretic mobilities of the so-named F and M forms.

The actual electrophoresis, Fig. 4.7.1, demonstrated that, under the prevailing conditions, there was no electrophoretic difference in the mobilities of the enzymes obtained from the males or from the females. Fig. 4.7.2. is an example of the eluting patterns obtained during column chromatography.

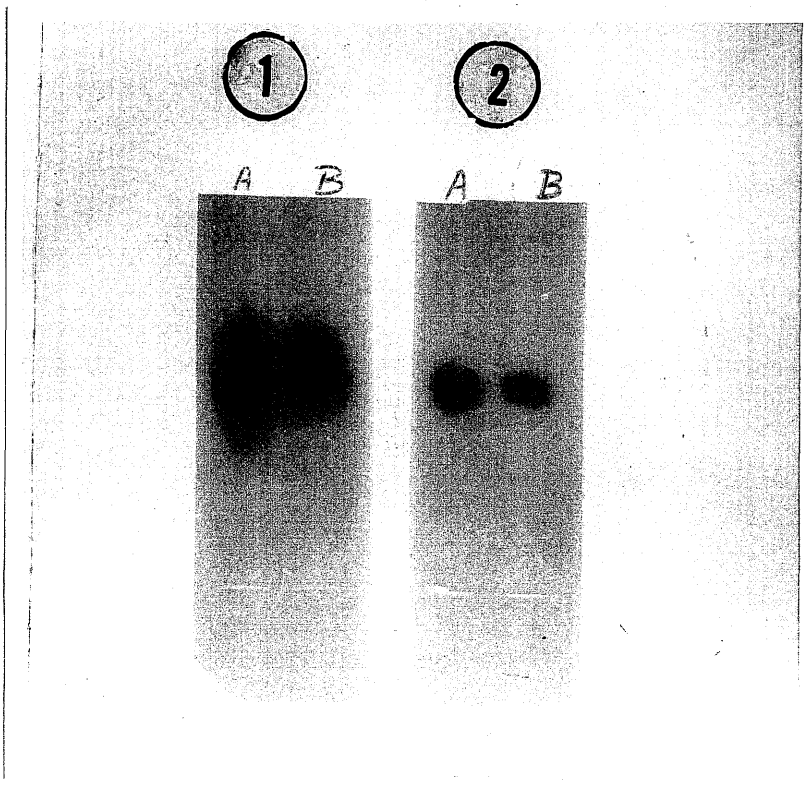
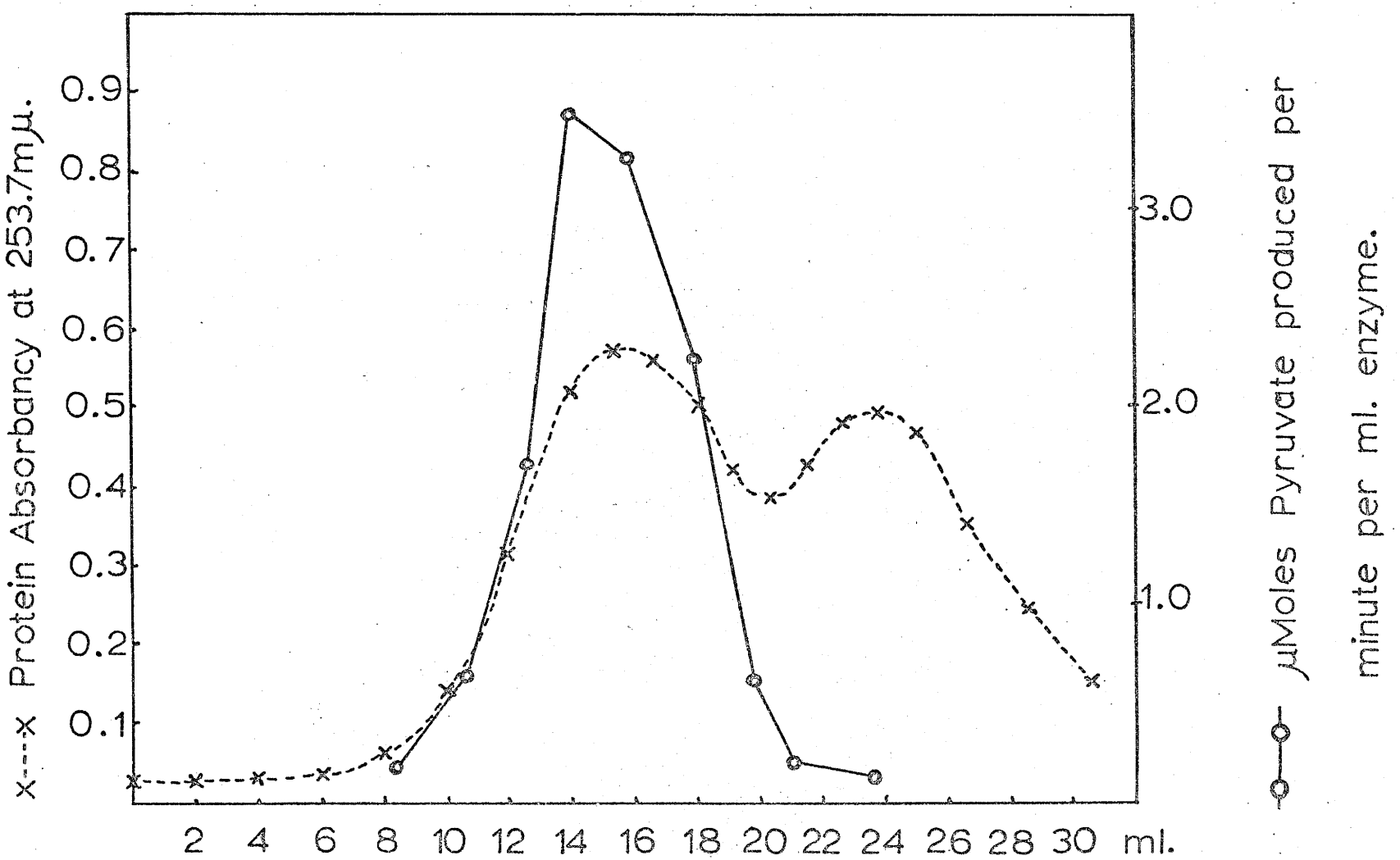


Fig. 4.7.1. A Photograph of starch gel showing mouse liver malic enzyme preparations, resolved by electrophoresis. (1) Showing the migration of the purified enzymes, and (2) the migration of the enzymes from crude extract preparations. In both cases (A) is the enzyme prepared from female livers, and (B) is the enzyme prepared from male livers.

Fig.4.7.2. Example of an eluting pattern in column chromatographic purification of Malic Enzyme; Sephadex G·200.



CHAPTER 5.

DISCUSSION.

In any biochemical studies it is more attractive to make use of human subjects, since, in most cases, many pertinent findings can be related to the understanding and sometimes even curing of diseases known to afflict the human race. However, this form of study can only be performed on living subjects in which the treatment prescribed can have no possible detrimental effects.

In the past decade, this type of study has been applied in an attempt to elucidate the role of carbohydrate and lipid metabolism in the incidence of coronary heart disease (Bersohn & Oelofse, 1958; Antonis & Bersohn, 1960; Carlson, 1960; Albrink et al., 1961; Stamler, 1963.) It was concluded that dietary carbohydrates can lead to the accumulation of excess lipid in the liver and plasma of both man and animal (Antonis & Bersohn, 1961; Ahrens et al., 1961); Macdonald, 1966a).

During the course of these studies evidence was established that the incidence of coronary heart disease is much lower in pre-menopausal females than in males of the same age group. In addition Macdonald (1966b; 1967) has shown that in humans, the females are able to tolerate high-carbohydrate diets better than the males. The findings of these workers set the stage for further experimental studies

into the determination, if any, of sex-linked differences in lipid metabolism.

Due to the nature of the experimental investigations undertaken and the laboratory facilities on hand the multimammate mouse, Praomys (Mastomys)natalensis, was used.

Ilse et al. (1968a) demonstrated in this mouse, on diets rich in carbohydrates, that the serum lipid pattern response was similar to that found by Macdonald in human volunteers. They found that the females resisted the induction, by dietary carbohydrates, of hypertriglyceridemia which occurred readily in the males. Thus the problems which are associated with lipid metabolism in humans appear, to some extent, also in the Mastomys.

It is possible that these problems, which are: (1) the greater tolerance of females to diets rich in carbohydrates, and (2) the apparent increased ability of the male system to synthesize fatty acids, are regulated at molecular control points within the cells. It also seems reasonable, at this stage, to suggest that some molecular control points can be influenced by the level of the relevant sex-hormones.

In a study of control points within a cell it seems reasonable to study, among other aspects, also the respiratory systems within the mitochondria. The respiratory chain is the "power chain" of the

mitochondrion, the site of conversion of respiratory energy into phosphate bond energy, into mechano-chemical energy, and into osmotic energy. The general nature of the respiratory chain has been considered as a sequential multienzyme system which accepts electrons from the various NAD - and NADP - linked dehydrogenases and flavoprotein (FP) dehydrogenases of the tricarboxylic acid (TCA) cycle and the fatty acid oxidation cycle.

The aerobic regeneration of ATP from ADP and phosphate by energy coupling in the respiratory chain is the final step in cellular respiration, for which the TCA and the fatty acid oxidation cycles are essentially preparatory processes. The major fate of the oxidation-reduction energy of electron transport is its conversion to the chemical energy of ATP.

Experimental findings to date appear fundamental to the concept of the respiratory assembly. This is an organized macromolecular assembly made up of one molecule of each component in a geometry favourable to their interaction (Lehninger, 1964). These findings are schematically shown in Fig. 5.1.

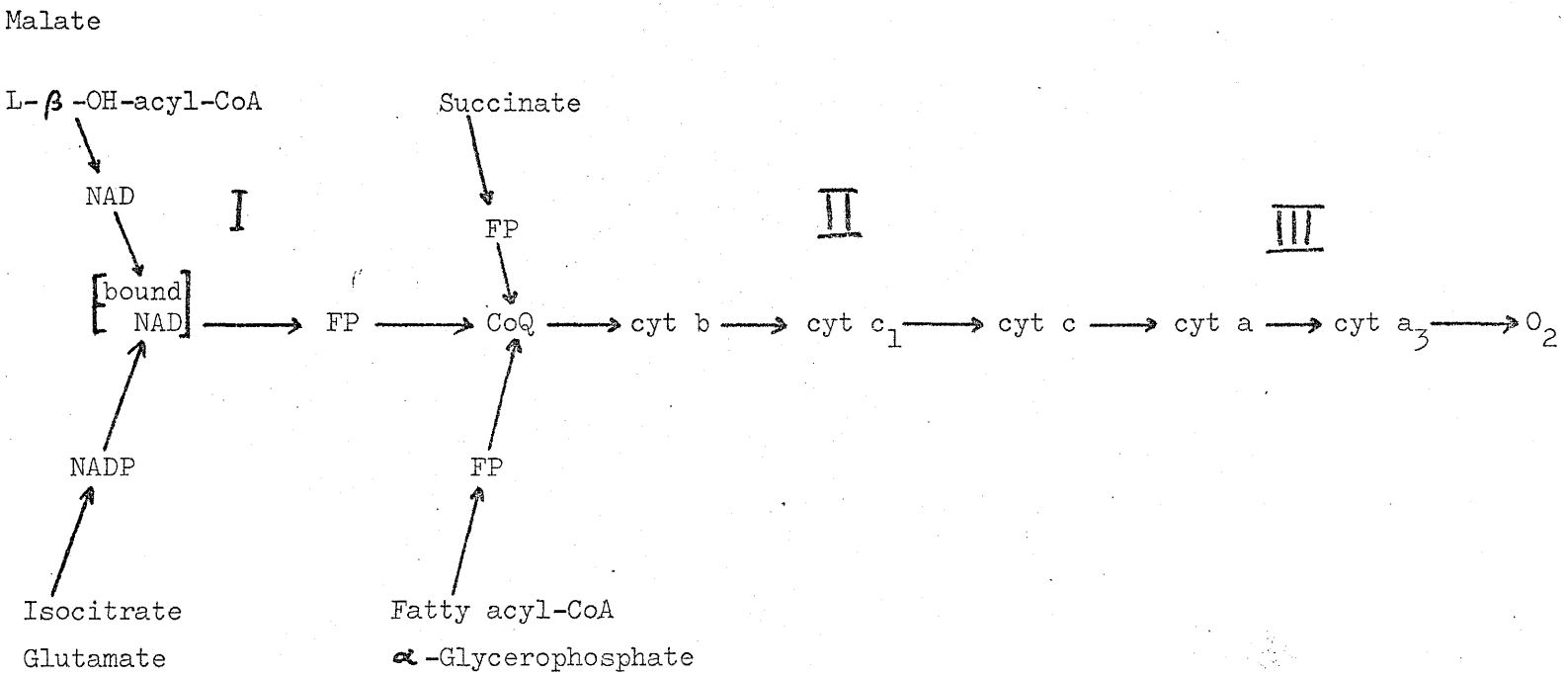


Fig. 5.1. Schematic diagram of the respiratory chain. The scheme shows the sites of phosphorylation in Roman numerals. There is still significant uncertainty concerning the place of ubiquinone, or coenzyme Q, in the chain, as well as the sequence and identity of the cytochromes (Lehninger, 1964).

Several workers have studied the organization of electron transport as applied to the types of chains required for the oxidation of various substrates (Kimura & Singer, 1959; Chance & Hess, 1962). They have concluded that there is a common span in the cytochrome region having substrate-specific bifurcations and entry points. In addition it is evident that competition for entry into the chain could cause disparities in the rate of oxidation of different intermediates of the oxidative cycles, which may be one basis of integration and control of cyclic oxidations. For example, Krebs et al., (1961) have shown that succinate oxidation may "monopolize" the respiratory chain to the exclusion of other substrates.

Belitzer (1939) was first to systematically compare the stoichiometry of coupled uptake of phosphate and oxygen and observed that the P:O ratio (that is, the ratio of molecules of Pi esterified per atom of oxygen utilized) during respiration is at least 2. Further studies in numerous other laboratories (for review see Lehninger, 1964) have shown that the oxidations of the TCA cycle yielded an average P:O ratio of 3.0, and the oxidation of succinate to fumarate a ratio of 2.0.

Studies on mitochondria isolated by the sucrose method showed that oxidative phosphorylation is dependent on reasonably intact structure. Chance and Williams (1956) have demonstrated that the

acceptor control ratio is an index of the state of "coupling" of the mitochondria.

As has been previously noted the process of mitochondrial oxidative phosphorylation can be "uncoupled", that is, oxidation can take place without the formation of ATP. This has been demonstrated *in vitro* by the action of dinitrophenol on respiring mitochondria. In "uncoupled" oxidation the energy is probably dissipated as heat, for in the whole animal, severe hyperthermia follows administration of dinitrophenol (Slater, 1966).

Passmore & Draper (1964) have found that obese subjects do not develop ketosis as readily as non-obese subjects while on a regime favouring the production of ketone bodies. Similarly, the rate of free fatty acid mobilization on starvation seems to be less in obese subjects than in non-obese subjects. Furthermore, it has been suggested that, in normal individuals, there could be a mechanism for the disposal of excess metabolic energy without the undue development of heat or adipose tissue (Passmore & Draper, 1964). The problem of the greater tolerance of females to diets rich in carbohydrates may perhaps be considered as the presence, in the female, of a more efficient mechanism for the dissipation of excess energy. We find that the crux of the problem is the *method* of energy dissipation.

The "uncoupling" of oxidative phosphorylation could be a

possible mechanism for the removal of excess energy. However, this does give rise to the additional problem of how the female system copes with this excess heat given off. Carr & Krantz (1942) have indicated that female rats, and to some extent human females, have a higher basal metabolic temperature than their male counterparts. This finding of thirty-six years ago could indicate the presence of a metabolic process in the female which can produce heat, from ingested foodstuffs, instead of storing the energy in some form.

The results obtained for the P:O ratios in the mice are depicted in Table 4.1.1., and showed that these ratios in both male and female liver mitochondria were essentially the same. Further investigations into determining the state of "uncoupling" of the mitochondria, that is, the acceptor control ratios, demonstrated that there was no appreciable difference in the ratios obtained from either male or female liver mitochondria. In addition the results recorded (Tables 4.1.1. & 4.1.3) showed that the intactness of the mitochondria was reasonable and that the state of "uncoupling", taking into consideration the experimental conditions, was fair. It was found that the P:O ratios oscillated about a mean of 2.6.

Although these results were below the theoretically possible values [oxidation of succinate yields P:O ratios of 2, and Chance & Williams (1956) have demonstrated acceptor control ratios of 46] which

could be obtained, they do indicate fairly substantially that the state of intactness of the mitochondria was the same in both male and female livers. Similarly the state of "uncoupling" was the same in both sexes.

Umbreit et al. (1964) and Chance and colleagues have demonstrated that far more accurate results are obtained by using the oxygen electrograph than when using the Warburg constant pressure respirometer. Since the former instrument completes the experiments within seconds, the results show extreme accuracy.

While performing these experiments it was found that, while no differences were discernible in the ratios, there were appreciable differences in the absolute quantities of oxygen consumed and ATP produced (Table 4.1.2.).

The feeding of high carbohydrate diets to male and female Praomys (Mastomys) natalensis resulted in the males only becoming hypertriglyceridemic (Ilse et al., 1968a). Furthermore, similar feeding experiments by these authors demonstrated that the male ratio of total reduced to total oxidized pyridine nucleotides had an overall increase, whereas the female ratios remained comparable to the normal female range.

Combining the studies of Ilse et al. (1968a) and the studies discussed in this dissertation to this point, it can be seen that the

problem of the dissipation of excess energy by the female has not been solved. The results discussed do however show that, under standard conditions, the female liver mitochondria produce relatively more ATP than do the mitochondria from male livers.

It is known that steroid hormones do affect the translocation of cations (Landon et al., 1966; and Dransfeld et al., 1967). However, it is not known with any certainty what mitochondrial substances or enzyme activities participate in the translocation of cations across the mitochondrial membrane. There seems to be agreement that the process is energy-linked and that it may be mediated by utilization of the free energy of breakdown of high energy intermediates formed as a consequence of respiratory chain electron transport (Brierley et al., 1963). Alternatively, the cation transport could be driven by energy derived from hydrolysis of ATP, that is, by the action of ATPase. Racker (1965, 1967 & 1968) has shown that the ATPase of mitochondria couples energy production with the actual phosphorylation. Ilse et al., (1968b) have demonstrated that there is an apparent sex-linked difference in the ATP-hydrolyzing activity of liver mitochondria of Praomys (Mastomys) natalensis. These authors demonstrated that the ATP-hydrolyzing activity in female liver mitochondria was higher than the activity in male livers. Castration increased the ATP-hydrolyzing activity to that found in normal females. Intramuscular injections of large doses of testosterone to castrated males and ovariectomized females lowered the

ATP-hydrolyzing activity to that found in normal males.

The higher ATP formation that was demonstrated during oxidative phosphorylation, in the females, could possibly be linked to the higher ATP-hydrolyzing activity mentioned by Ilse and co-workers. These findings by Ilse and co-workers could, to some extent, explain the utilization of excess energy, by the female, in a more efficient cation transport across mitochondrial membrane structures. If energy is to be used for a sex hormone dependent increase in some or other ionic translocations, the original ionic status could be restored by an increased "passive" back flow. However, in the male, this excess energy could be used for the synthesis of triglycerides.

If one is to continue along the lines proposed above a secondary problem should be solved. This problem is essentially the presence of a possible pathway in the female, for the conductance of excess potential energy, supplied via glycolysis, to the oxidation sites of the tricarboxylic acid cycle within the mitochondria.

Under the regime of high-carbohydrate and low-fat diets there is an increase in glycolysis and an increase in lipogenesis if the calorie intake is sufficiently high (see Chapter 2). Under these experimental conditions the induction of hypertriglyceridemia was probably due to enhanced lipogenesis. This results from the induction of certain key enzymes such as glucose-6-phosphate dehydrogenase, 6-phospho-

gluconate dehydrogenase, phosphoglucomutase, glycerophosphate dehydrogenase, lacate dehydrogenase, and NAD - and NADP dependent malate dehydrogenase (Fitch & Chaikoff, 1960) as well as glycogen synthetase (Steele, 1966). The induction of these enzymes would cause an increase in the activity of certain rate-limiting steps in the Embden-Meyerhoff pathway, the pentose phosphate shunt and the tricarboxylic acid cycle. The enhancement of these pathways would in turn result in high levels of ATP, acetyl-CoA, and reducing potential which are essential for the synthesis of fatty acids and triglycerides. The feeding of diets rich in carbohydrate would also cause an increase in the activity of ATP citrate lyase (citrate cleavage enzyme) (Kornacker & Lowenstein, 1965; Kornacker & Ball, 1965), and an enhancement of the malic enzyme (Tepperman & Tepperman, 1964), both of which have been postulated to be involved in lipogenesis.

Krebs & Eggleston (1965) and Yudkin & Krauss (1967) have ably demonstrated that the activity of pyruvate kinase (EC 2.7.1.40) is greatly influenced by the prevailing dietary conditions. Under conditions of active lipogenesis (high carbohydrate - low fat diets) the activity of this enzyme is greatly increased.

During glycolysis the hexose sugar units are degraded to 3 - carbon units and finally yield the high-energy intermediate phosphoenolpyruvate (PEP). At this stage the enzyme pyruvate kinase acts

upon the PEP to form pyruvate and ATP. Reference to Fig. 2.5. clarifies the role undertaken by pyruvate kinase in the production of pyruvate. Brierley & Green (1965) have demonstrated that small molecules can diffuse extremely rapidly into part of the mitochondrial water. Indeed, Amoore (1958) found that about two-thirds of the mitochondrial water is accessible to pyruvate.

Once the pyruvate has diffused into the mitochondria there are two major reactions which it can undergo; (1) the pyruvate can be decarboxylated to acetyl-CoA, and 2) it can be carboxylated to oxaloacetate (Fig. 2.5). If the former route is favoured then a portion of the pyruvate present will probably enter the tricarboxylic acid cycle and be oxidized with concurrent production of ATP. However, in the latter case, besides entering the tricarboxylic acid cycle, the oxaloacetate could be oxidized by the NAD-linked malate dehydrogenase to malate.

If it were presumed that the rate of glycolysis in both male and female was similar, this would provide almost equivalent amounts of substrate for mitochondrial oxidations, and any difference in the fate of the carbon compounds would have to be sought in oxidation or lipogenesis. Our results indicate that the female mitochondria have a higher rate of substrate oxidation than the male mitochondria. This is borne out by the greater rate of ATP production by the female, under the experimental conditions described. In making these proposals

however, one must not neglect the outlook that this finding may have in some part been due to a greater permeability of the female mitochondrial membrane to succinate. However, in the system studied the evidence does not point to such a process taking place.

As depicted in Table 4.2.1. and Fig. 4.2.1. it would appear that the activity of the pyruvate kinase enzyme is greater in the female and that its activity is in some way connected to the endogenous estrogen levels.

Soon after a meal most of the tricarboxylic acid cycle and glycolytic intermediates in liver will come from glycerol and, to a lesser extent, from lactate. The lactate having its origin in muscle and the glycerol in adipose tissue. In the tissue of normal animals, glycerophosphate for triglyceride synthesis is not made simply by utilization of dihydroxyacetone phosphate arising from glycolysis. Instead, glycolysis continues through to pyruvate and the levels of triose phosphates and of pyruvate are probably supplemented through action of the non-oxidative phase of the pentose phosphate pathway. Pyruvate could then undergo mitochondrial carboxylation to oxaloacetate which is then converted to phosphoenolpyruvate (PEP) as in the common gluconeogenic pathway (Chakrabarty & Leveille, 1968). In adipose tissue glycolysis is separated from glycerophosphate formation which provides a means of regulating the relative proportions of glycerol and free fatty

acids (FFA) on a 1:1 basis after triglyceride turnover in normal adipose tissue. Chlouverakis (1968) found that the ratio of 1:1 for glycerol and FFA coming from adipose tissue skews up on overloading with carbohydrate. Chakrabarty & Leveille (1968) found that in obese animals the mechanism for glycerophosphate synthesis changes and that L-α-glycerophosphate is then drawn off directly from the Embden-Meyerhof pathway so that on overfeeding, more glycerol gets out and moves to the liver.

In liver such as we have studied, the major means of regulating the extent of the glycerophosphate pool, is by keeping glycerol out or by rapidly degrading it through the Embden-Meyerhof pathway. The females may use their elevated pyruvate kinase activity to push carbon rapidly down the Embden-Meyerhof pathway, converting it to pyruvate, then to acetate which is oxidized intra-mitochondrially at a rate perhaps greater than in the male (Fig. 5.2.).

The increased rate in the activity of the pyruvate kinase, in the females, could supply greater quantities of substrate carbon for mitochondrial oxidation. However, this does not explain the lowered rate of pyruvate kinase activity in the male. A possibility to be considered, and a subject for future study, is that the production of glycerophosphate could be increased in the male. This mechanism could channel off a certain percentage of the carbohydrate during glycolysis.

The α-glycero-phosphate is required for the synthesis of triglyceride and since it has been shown that the male actively synthesises more triglyceride under prescribed conditions, than does the female, this may be a useful hypothesis.

Tanaka et al. (1967) have described two types of pyruvate kinases present in the liver. Types L and M which differ in their chemical and physical properties. Type M (which is also found in muscle, brain, heart, adipose tissues and kidney) is not affected by hormones nor by the feeding of high carbohydrate diets. The type L enzyme is affected by insulin administration and feeding high carbohydrate diets. Under these conditions the activity of the L form is increased. This finding may have some bearing on the problem of the sex-linked differences in the enzyme activities in that there may be one or more pyruvate kinase isozymes of which only one is affected by sex hormones. However, the exact nature of the problem cannot be explained at this stage. It could be that the different types of enzymes, L and M, are influenced by the levels of the sex-hormones to a greater or lesser extent in either sex, in addition to the influences described by Tanaka et al., (1967). Possibly it is only the L form which is so affected, however, this can only be shown by further experimental studies.

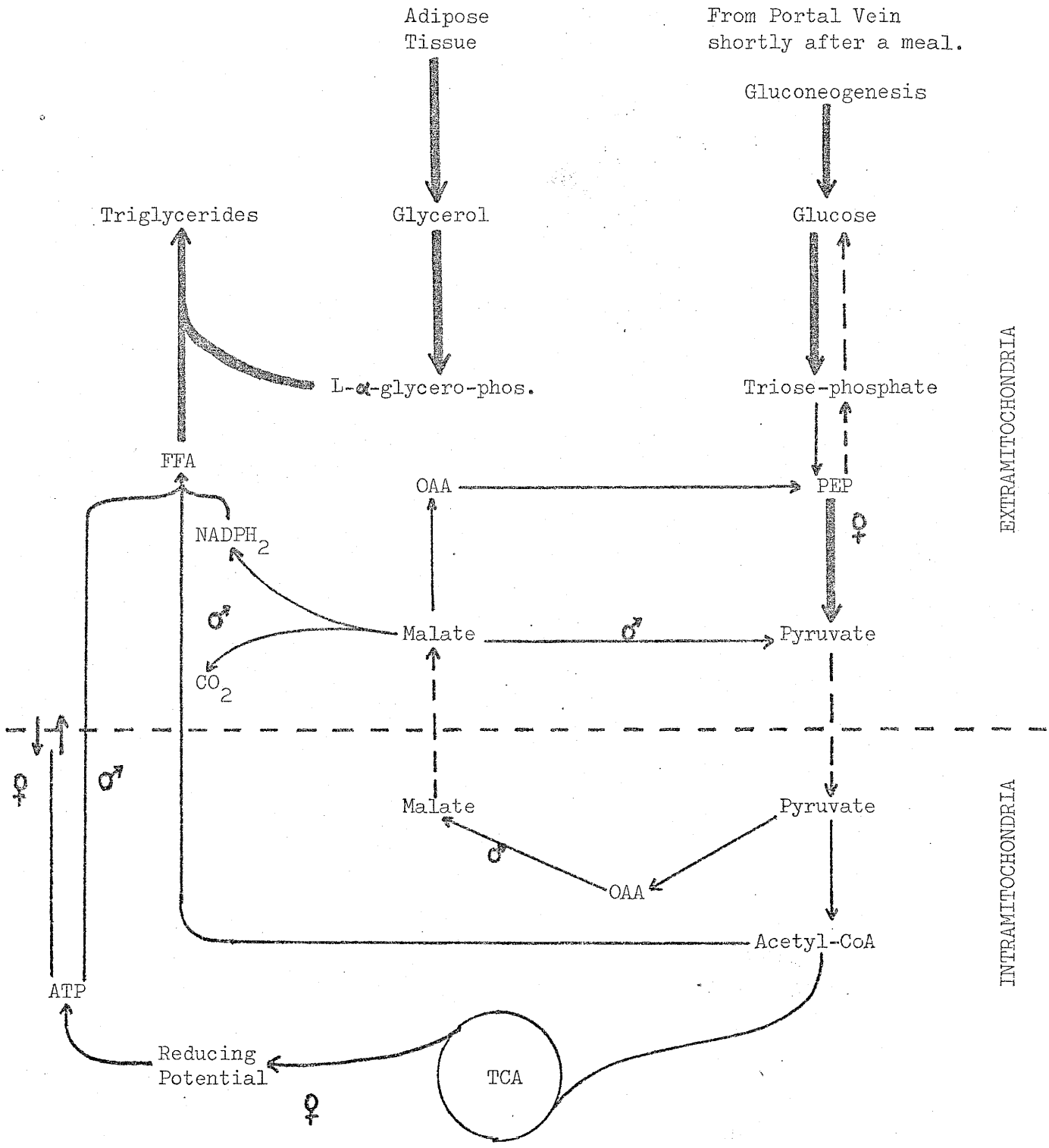


Fig. 5.2. Male and female factors influencing carbohydrate utilization.

Stimulated by carbohydrate loading.

♂ & ♀ Elevated through male or female factors.

The living cell employs $NADPH_2$ in preference to $NADH_2$ in many reductive synthetic processes. One case in point is the conversion of acetyl-CoA to fatty acids where $NADPH_2$ appears to be the reductant of choice (Bressler & Wakil, 1963; and Lynen, 1961). Studies by Flatt & Ball (1963) have shown that $NADPH_2$ generated in the conversion of hexose monophosphate to pentose phosphate supplies 50-60 per cent of the reducing equivalents used for fatty acid synthesis. The work of Winegrad & Renold (1958), and Jeanrenaud & Renold (1960) showed that synthesis of fatty acid from pyruvate can occur under circumstances where generation of $NADPH_2$ by the oxidation of hexose monophosphate seemed unlikely. This work suggested that the metabolism of pyruvate can furnish the reduced coenzymes necessary for fatty acid synthesis. The results of Wise & Ball (1964) have shown that the activity of the malic enzyme (EC 1.1.1.40), in both rat adipose tissue and liver was altered in such a way, by experimental conditions, as to suggest that malate may play a role in lipogenesis by furnishing $NADPH_2$ for the reduction of acetyl-CoA to fatty acids. The lipogenic tissues, adipose tissue and liver, are richer in malic enzyme than non-lipogenic tissues, muscle, brain and heart. Alterations in dietary regime alter the activity of the liver malic enzyme, so much so that the activity of this enzyme rises when rats are placed on a high glucose or fructose diet (Fitch & Chaikoff, 1960).

A role for malic enzyme in lipogenesis has been postulated by Wise & Ball (1964), which involves its interplay with malate dehydrogenase and pyruvate carboxylase as shown by equations 1, 2, 3 and 4 in Chapter 2, page 41.

The overall result is essentially an ATP - requiring trans-hydrogenation between NADP and $NADH_2$ to yield $NADPH_2$. Although pyruvate carboxylase is present in liver, its activity is low in comparison to those of malic enzyme and malate dehydrogenase. Thus additional pathways for the contribution of oxaloacetate must be found if this hypothesis is to be valid.

The provision of $NADPH_2$ via the malic enzyme for lipogenesis may be only part of the role it could play in lipogenesis. Equally important is the oxidation of $NADH_2$. As discussed by Flatt & Ball (1963), the rate of oxidation of the reduced coenzymes formed during the conversion of glucose to acetyl-CoA could be a limiting step in lipogenesis. Thus any process whereby $NADH_2$ can be converted to $NADPH_2$ would serve two purposes: It would regenerate NAD to permit the formation of more acetyl-CoA and simultaneously supply $NADPH_2$ for the reduction of acetyl-CoA to fatty acids. It is interesting to note that Goodridge & Ball (1966) have shown that in the pigeon the liver is geared to lipogenesis to a much greater extent than the adipose tissue. The activity of the malic enzyme is correspondingly increased, thus

meeting the increased requirement for $NADPH_2$.

As shown in Table 4.3.1. and Fig.4.3.1. and 4.3.2, the activity of the malic enzyme is dependent on testosterone. The activity of this enzyme in the male livers is considerably higher than in the female livers. Figure 4.3.2. graphically demonstrates the progressive decrease, with time after castration, of the activity of the malic enzyme. In addition on treatment with increasing amounts of testosterone, the activity of this enzyme was shown to become progressively higher until it was comparable to the normal male range.

Under the dietary conditions which stimulate lipogenesis, it has been shown that the rate of lipogenesis, i.e. fatty acid and triglyceride synthesis, is stimulated to a greater extent in the males and in post-menopausal females, than in the premenopausal females. In addition the malic enzyme has been implicated as a source of reducing potential for fatty acid synthesis.

The malic enzyme activities found, could indicate that in the male animal, more substrate and reducing potential are utilized via a system coupled to this enzyme, in order to supply sufficient reducing potential required for the indicated increase in fatty acid and triglyceride synthesis. However, this hypothesis poses additional problems. The first being the source of additional substrate for the action of the

malic enzyme. If the system proposed by Wise & Ball (1964) is correct, then under these conditions a comparison of the activities of the constituent enzymes should show comparable differences between the sexes. The results obtained for the cytoplasmic NAD-linked malate dehydrogenase are depicted in Table 4.5.1. and Fig. 4.5.1. It is clearly shown that there is no appreciable difference in the activity of this enzyme between males and females. These results could indicate that this coupled enzymic system, which incorporates the enzymes pyruvate carboxylase, NAD-malate dehydrogenase, and the malic enzyme, is not favoured by the male for the production of the required extra reducing potential. However, this system could still be used to supply equivalent amounts of $NADPH_2$ by both sexes. The activity of the pyruvate carboxylase enzyme was not measured, and is a possible project for future investigation. However, Wise & Ball (1964) did find that the specific activity of this enzyme was not comparable to the specific activities of the other two enzymes in this system. Thus additional pathways for the contribution of oxaloacetate or malate must be found.

At this stage Ilse (unpublished) suggested that the citrate cleavage enzyme (EC 4.1.1.38) could possibly supply the extra substrate for the action of malate dehydrogenase. The citrate cleavage enzyme catalyses the following reaction (Kornacker & Ball, 1965):

Citrate + CoA + ATP $\longrightarrow$ Acetyl-CoA + Oxaloacetate + ADP + Pi

The operation of this pathway in liver is supported by evidence that both the rate of citrate incorporation into fatty acids and the activity of the citrate cleavage enzyme vary with nutritional and hormonal states in which the role of lipogenesis is altered. However in this laboratory the investigation of the citrate cleavage enzyme activity in Praomys (Mastomys) natalensis gave inconclusive comparative results. Recently the role of the citrate cleavage enzyme in lipogenesis was challenged (Foster & Srere, 1968), so that an additional source of substrate for the malic enzyme would have to be sought.

Krebs (1967) has shown that malate readily diffuses across the mitochondrial membrane. In addition it is known that pyruvate forms an important linkage between intra- and extra-mitochondrial substrates. Pyruvate in the mitochondria can then be carboxylated via the mitochondrial pyruvate carboxylase to oxaloacetate which can then be reduced to malate. The malate can then re-diffuse into the cytoplasm and be oxidized to pyruvate by the malic enzyme forming the required reducing potential. It is suggested that this could be a possible mechanism which the male may utilize for the generation of the required reducing potential. The results shown in Table 4.4.1. and Fig. 4.4.1. demonstrate that the mitochondrial NAD-linked malate dehydrogenase activity is greater in the male, and that the activity of this enzyme is also affected by levels of testosterone. These

results indicate that the system outlined (Fig. 5.3) could be used for the maintenance of the increased malic enzyme activity in the male. This postulate is essentially the same as that of Wise & Ball (1964) except that part of the system lies within the mitochondria and not solely in the cytoplasm.

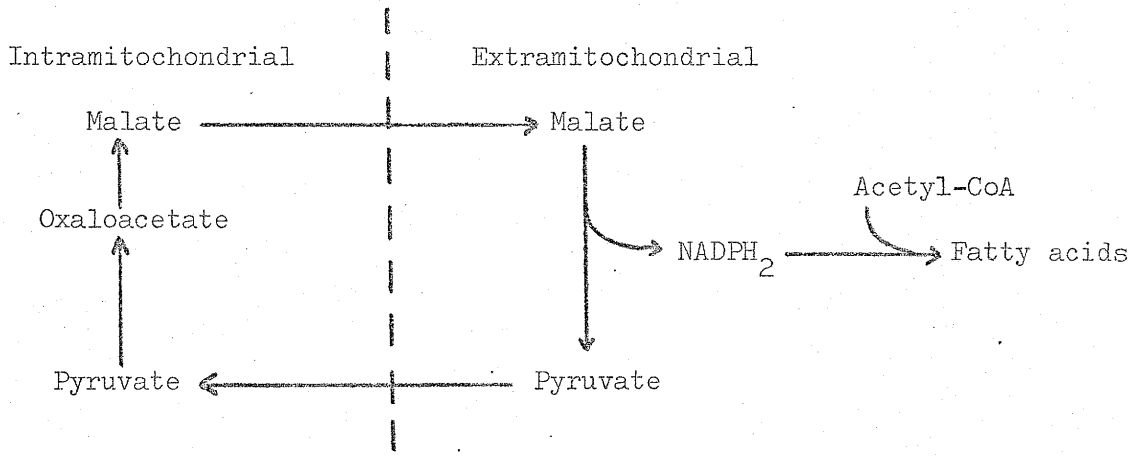


Fig. 5.3. See text.

The cytoplasmic isocitrate dehydrogenase (NADP-linked) can also supply a percentage of the required reducing potential for fatty acid synthesis. Keeping in mind the results demonstrated for the malic enzyme, the activity of the isocitrate dehydrogenase was investigated. The activities obtained for this enzyme are shown in Table 4.6.1. It was not possible to show any sex-linked differences in the activity of this enzyme, isocitrate dehydrogenase. This could indicate that, although this enzyme still supplies a certain amount of reducing potential in both sexes, it is not enhanced in the male in order to cope with increased fatty acid synthesis.

Henderson (1967) showed that malic enzyme exists in two isozymic forms in mouse tissue. The isozymes of malic enzyme differ in electrophoretic mobility and tissue and subcellular location. The results depicted in Table 4.3.1. demonstrate that testosterone affects the level of the malic enzyme activity in the male. However, testosterone did not appear to have a great effect on the enzyme activity from female livers. On this evidence it was decided to attempt an investigation of the electrophoretic mobilities of the malic enzyme isolated from male and female livers. To this end the enzymes were purified 28-fold and both crude homogenates and the slightly purified enzymes were subjected to electrophoresis on starch gels. No discernible differences in electrophoretic mobilities were found (Fig. 4.7.1.). It appears that the malic enzymes isolated from male and female livers are similar in many physical properties, and that under the experimental conditions chosen no isozymes of malic enzyme were demonstrated. Thus the hypothesis of F and M forms of the malic enzyme existing in female and male livers was not proven. The fact that the activity of the malic enzyme from female livers was lower than that from male livers and that its activity, in ovariectomized females, did not respond to testosterone treatment could perhaps be explained in a more fundamental manner. Perhaps the treatment by testosterone propionate to ovariectomized females was of too short a duration for the effect to become noticeable in the enzymes specific activity.

Further experimental work with these conditions in mind should be attempted in order to verify this. However, the fact does remain that, in normal animals, there are significant differences in the specific activities of the malic enzyme, and that these differences correspond to the finding of greater triglyceride synthesis in the male than in the female.

It has been shown that both estrogen and testosterone activate certain lipogenic enzymes. Pyruvate kinase activity appears to be increased by high levels of estrogen. However, malic enzyme and mitochondrial malate dehydrogenase activities appear to be dependent upon testosterone. In addition it has been shown that neither estrogen nor testosterone have any effect upon the activities of isocitrate dehydrogenase or cytoplasmic malate dehydrogenase.

The activities of numerous other enzymes have been shown to be affected by the sex-hormones. It has been demonstrated that these hormones either activate or inhibit enzymes isolated from the gonads, liver, kidneys, heart, and other extra-genital tissues of laboratory animals.

In fact de Asua et al., (1968) demonstrated that the activity of pyruvate kinase, isolated from rat uterus, was increased by estradiol administration. In the uterus of rats estrogen stimulates the activity of phosphofructokinase (Singhal et al., 1967a), glycogen

synthetase (Bo et al., 1967), phosphohexose isomerase (Singhal et al., 1967b), and hexokinase (Valadares et al., 1968). The activity of alkaline phosphatase in fibroblasts of mouse subcutaneous connective tissue has been found to be estrogen induced (Schaefer & Fischer, 1968). However, estrogen has been shown to inhibit glycogenolysis by inhibiting phosphorylase activity (Bo et al., 1967). Further in vitro studies by Merola et al., (1968) demonstrated that estrogen inhibits cholesterol synthesis at the decarboxylation of mevalonic acid.

Furthermore, testosterone has been shown to influence certain enzyme systems. Salivary amylase activity is 50% higher in male mice than it is in female mice, and it appears to be testosterone dependent (McGeachin et al., 1965). Lipid synthesis in target organs and liver mitochondria was shown by Doeg (1968) to be stimulated by testosterone.

Finally, a number of experimental workers have demonstrated that estrogen and testosterone can have opposing effects upon the same enzyme systems (Sasaki & Ketkar, 1968; Wenk, 1968; van Pilsum & Ungar, 1968).

Thus it seems clear that estrogen and testosterone can affect the specific activities of a number of enzyme systems. A perusal of the results presented in this dissertation, together with those of other experimental workers, indicates that testosterone can enhance

the rate of fatty acid synthesis whereas estrogen appears to increase the degradation of any excess carbon via the tricarboxylic acid cycle.

However, the mechanism of action of the hormones on these systems has not been finally elucidated. The hormone may act at the gene level and activate or inhibit the synthesis of these enzymes. This postulate, though not proven, is substantiated by the findings in Singhal's laboratory (1967a & b). These authors found that estrogen stimulated the synthesis of RNA which was related to the increased activity of the enzymes isolated from the uterus. In addition, Fuji & Villee (1968) demonstrated that testosterone stimulated the synthesis of RNA in the prostate and liver of immature male rats. These findings could indicate that there is a stimulation, by these hormones, of protein synthesis. However, it is possible that there are some other controlling influences present, since it was found (Table 4.2.1., 4.3.1. & 4.4.1.) that the effects of these hormones were not directly transferable to the opposite sex (Sasaki & Ketkar, 1968).

On the other hand, the presence of the hormone(s) might cause a change in the molecular configuration of the enzyme proteins which are involved in the reactions. To take this hypothesis one step further, the two hormones could have a complementary influence on the activities of the enzymes. Estrogen could change the configuration of pyruvate kinase such that its activity is maximal, whereas testo-

sterone could have a "blocking" effect upon the activity of this enzyme. In a similar manner testosterone could exert maximal activity upon malic enzyme and mitochondrial malate dehydrogenase, while estrogen either has no effect or tends to decrease these enzyme reactions. However, the results shown do not conclusively support this hypothesis, since solely by the removal of the "activating" hormone the enzyme activity could be decreased.

Alternatively the hormone(s) could react with a hypothetical carrier molecule to form an active complex, or its presence could change the molecular configuration of the carrier in some way so that its role in the reaction could be enhanced.

The gradual decrease observed in the activity of the malic enzyme of castrated males, that were left for various periods of time after castration, was probably due to the endogenous levels of testosterone gradually decreasing. This is substantiated by the finding that the activity of malic enzyme increased with increasing doses of testosterone propionate administered to castrated males. However, one would expect that most of the endogenous testosterone would be degraded in one or two days. The slow decrease in enzyme activity could be due to the formation of a testosterone- protein complex. The availibility of testosterone for degradation would then depend upon the dissociation of the complex which would probably occur only

when the endogenous levels of testosterone are extremely low. The same process probably occurs with estrogen.

In conclusion therefore, a number of identifiable sex-linked differences in energy responses have been tabulated. A reasonable explanation has been proposed as to the probable functions of these differences. Together with the work of Ilse et al., (1968a & b) a start has been made on the understanding of the problem at a molecular level, viz. the greater tolerance by premenopausal females to dietary carbohydrates than that of males of the same age group and of post-menopausal females.

These results provide some insight into some of the effects which sex hormones may have on metabolism in general. In addition the stage is set for important future work in the field of lipid and carbohydrate metabolism, which may eventually contribute to the elucidation of the sex differences in the incidence of coronary thrombosis, and allied disorders, in the male and female.

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