ABSTRACT OF THESIS

Name of Candidate GRAHAME JOHN BULFIELD

Address INSTITUTE OF ANIMAL GENETICS, WEST MAINS ROAD, EDINBURGH.

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Title of Thesis GENETICAL AND BIOCHEMICAL STUDIES OF FATNESS IN MICE.

This investigation has attempted to find the primary biochemical lesion in obese and adipose mice by applying genetical criteria and elucidating the consequences of these lesions.

The physiology and biochemistry of the obese mouse syndrome has been reviewed, as has the theoretical and practical considerations involved in the concept of rate controlling enzymes, as well as the agents and mechanisms of the control of lipogenesis.

It has been found that the fatty acid profile of the triglyceride of the fat pad was very similar in obese, adipose, and normal animals. This indicates that there has been an increase in the synthesis of all the fatty acids in the genetically fat mice rather than any particular group of them.

The activity of several enzymes has been assayed in these animals. ATP citrate lyase, malic enzyme and pyruvate kinase have higher Vmaxs in obese, adipose, and their phenocopies, caused by auro-thio-glucose injections, than in their normal littermates, whereas there is little difference in the Vmax of isocitric dehydrogenase, lactic dehydrogenase, fumarase and malic dehydrogenase. By applying genetical criteria to these results, it is concluded that none of these enzymes can be the only or primary lesion in any of these animals.

However, another enzyme, 2 monoglyceride lipase, has been reported to have a lower activity in obese mice and possibly to exhibit other properties that would make it the site of the primary enzymic lesion in these animals on genetical criteria.

It was not possible to assay the activity of this enzyme in this investigation, but it was possible to examine one of the consequences of this lower activity, the low amount of free linoleic acid that may be available as a metabolic agent, in these animals.

The significance of free linoleic acid as an inhibitor of the activity and as a repressor of the synthesis of the lipogenic enzyme was discussed.

Use other side if necessary.

It was shown that linoleate was less able to reduce the activity of the lipogenic enzymes in obese than in normal animals when it was fed as a supplement to a fat free diet. It was considered that this effect might be due to the greater amount of linoleate being held in a glyceride form in obese animals, due to the lower activity of 2 monoglyceride lipase. Thus the low amount of free linoleate available in obese does not reduce the lipogenic enzymes as much as the large amounts in normal animals.

Linoleate is also a powerful inhibitor of the activity of pyruvate kinase. However, there was no difference in its ability to inhibit the activity of this enzyme taken from obese ATG or normal animals.

It was concluded, that, as the preliminary work on 2 monoglyceride lipase in obese, adipose and normal animals has shown that this enzyme might possibly be the primary lesion, in obese mice, then this enzyme's behaviour should be studied in great detail in all these animals to test the validity of these preliminary observations.

GENETICAL AND BIOCHEMICAL STUDIES OF FATNESS IN MICE

by

GRAHAME JOHN BULFIELD

B.Sc. (Leeds)
Dip.Anim.Gen. (Edinburgh)

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CHAPTER 1. GENERAL INTRODUCTION

Obesity in man is a complicated social and medical problem, whose nature is little understood. It is thought to be connected with an increase in the appetite although other factors have been implicated. Very little is known about the genetic basis of human obesity and one must presume that it is of a polygenic nature, as no single gene effects have been reported.

In animal production obesity is a wasteful form of food utilisation, a large proportion of fat in a carcass being undesirable. There has been some attempt to control the level of obesity in farm animals, especially in pigs, by selective breeding. As with human obesity no single gene effects have been reported in farm animals and again the obesity is presumably polygenically determined.

In mice, however, three distinct single genes causing obesity are known. The first of these, the "obese-hyperglycaemic" mutant, has been known since 1950 and has been the subject of much physiological and biochemical study which is reviewed in chapter 2 part (a). The second obese mutant, known as "adipose", was found in Edinburgh in 1959, and the third one, known as "diabetic", was found in the Jackson Laboratory in Bar Harbor in 1967.

Since the names "obese", "adipose" and "diabetic" used to refer to these genes also have common physiological and pathological meanings, the following terminology will be used throughout this thesis to distinguish these different meanings. When underlined, obese, adipose and diabetic will refer to animals homozygous for these mutant genes which display the observed syndrome. When not underlined, obese will refer to an excessively

fat animal, however caused, adipose will refer to fat tissue and diabetes to the condition of diabetes mellitus. The genes will be described by the symbols ob, ad and db.

Obese mice, illustrated in fig.1, have been particularly useful because of their gross obesity and their similarity to the forms of human obesity associated with maturity-onset diabetes. Adipose, illustrated in fig.2, resembles obese phenotypically, but prior to this investigation no biochemical work had been done on this animal. Diabetes seems to differ from the other two syndromes in showing an earlier onset of hyperglycaemia and perhaps less severe obesity. The differences, however, might be just due to the genetic background. Breeding tests which were carried out at Edinburgh prior to this investigation proved all these genes to be non-allelic. Further details of the breeding of obese and adipose animals will be found in chapter 3 part (a).

Obese and adipose thus provide two genetically different examples of a similar physiological condition. From the genetics of these two syndromes it is possible to establish criteria that can be used to test any biochemical abnormalities in these animals for the site of the primary lesion. The object of this investigation was to find the primary biochemical lesion in obese and adipose animals, and to elucidate the consequences of these lesions. This was done by using genetical criteria to compare the biochemistry, and specifically the enzymology, of obese and adipose animals with each other and with phenocopies produced in normal littermates and their normal littermates by auro-thio-glucose injections, (as will be

Fig. 1. Obese and normal litter mates.

OBESE
(72.7 gms)

NORMAL
(37. 3 gms)

Fig. 2. Adipose and normal litter mates.

ADIPOSE
(74.2 gms)

NORMAL
(45.6 gms)

described later). The known nature of genetic determination can provide criteria to pinpoint important stages of biochemical control. From current genetical theory, which holds that there are structural and regulatory genes, genetical criteria for the primary biochemical lesion can be established as follows. Structural genes code for one polypeptide and it is known that non-allelic genes, at separate loci, code for different polypeptides, although the polypeptide might be an enzyme or part of an enzyme. Mutational changes in a structural gene will produce an altered poly eptide and hence an altered enzyme activity. On the other hand regulatory genes are said to specify a substance which acts to alter the level of a polypeptide produced by another locus. Enzymic activity may therefore be altered in various ways and normal assays do not distinguish between these.

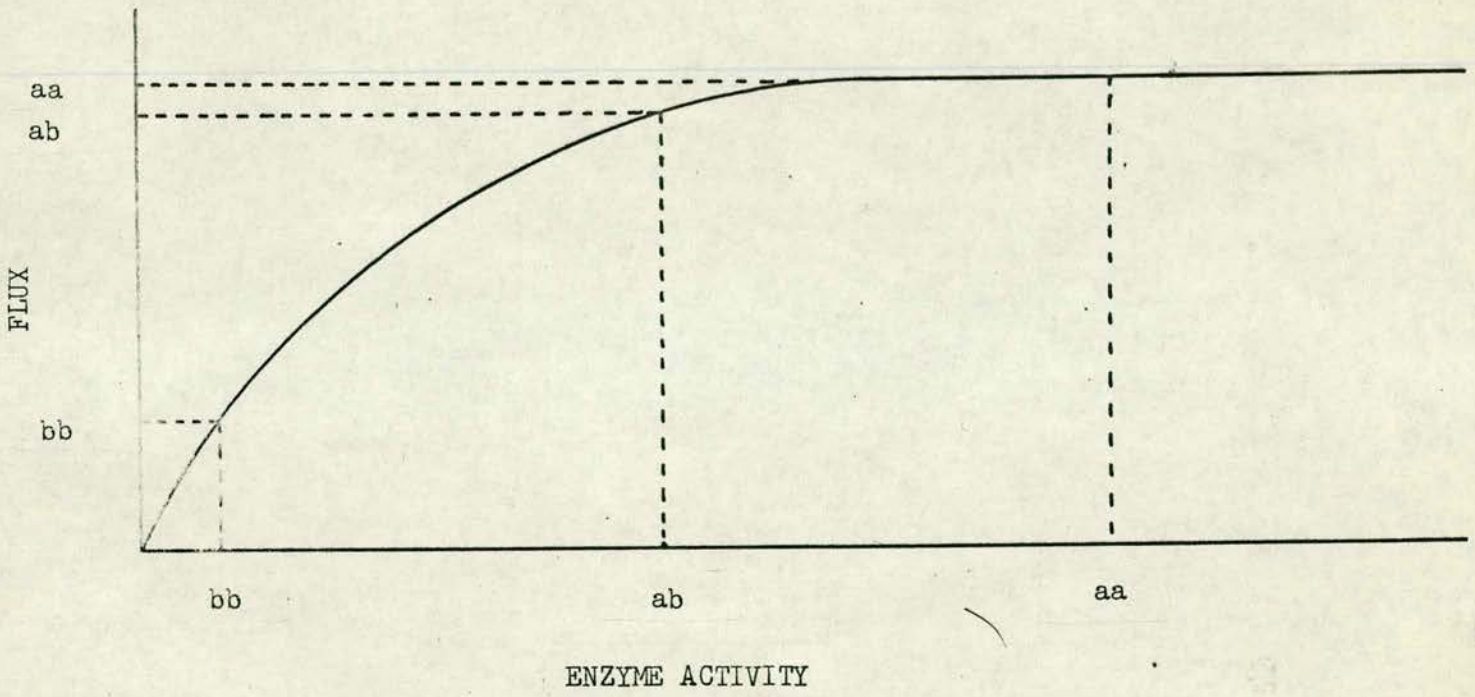
First, it may be observed that the genes ob and ad are non-allelic which leaves the following possibilities open. The genes ob and ad may code for different polypeptides which may either (1) catalyse different metabolic steps or (2) combine to form an enzyme complex catalysing one step, (compare haemoglobin). (3) If both ob and ad are regulatory genes these may alter the quantity of different enzymes. If (1) is correct we should find differences at some enzyme levels between obese and adipose mice, if (2) is true both obese and adipose should have similar alteration in the same enzyme. It may be noted that the expectation from (1) and (3) are similar when the specific activities of obese and adipose only are measured. In fact, evidence will be presented below which eliminates the

difficulty in distinguishing between (1) and (3). (4) The last possibility is that both ob and ad are involved in the metabolism of a general signal which acts in the control of one or several enzymes.

Secondly, the genes ob and ad are recessive. This means that both the heterozygote and homozygote wild type animals appear the same. If both genes are structural, then in each case, at the primary enzymic lesion one should be able to differentiate between these types and the apparently normal animals should fall into two classes, the heterozygote class having twice as many animals in it as the homozygote wild type class. As will be seen no such differentiation is possible for any of the enzymes studied in this investigation and so it is possible to eliminate these enzymes as the site of the primary biochemical lesion.

Thirdly, we have an expectation from the recessivity of the genes and the position of the enzymes in the biochemical pathway. If the product formation or flux (i.e. the rate of formation of product at steady state), is a positive function of the enzyme then the relationship between the flux and enzyme activity will be of the general shape indicated in fig.3. All enzymes that are in the "direct" pathway to lipogenesis fall into this class and so do those that contribute simple co-factors to this direct path. The reason for the non-linearity of the relationship in fig.3 is to be found in the fact that all such enzymes contribute positive terms in the net flux expression of the whole pathway. Zero activity clearly results in zero flux, (classical biochemical block), while very large activity tends to a

Fig. 3. The theoretical relationship between genetical alterations in the Vmax of an enzyme, which do not change the Michaelis constant, (Km), and the flux.

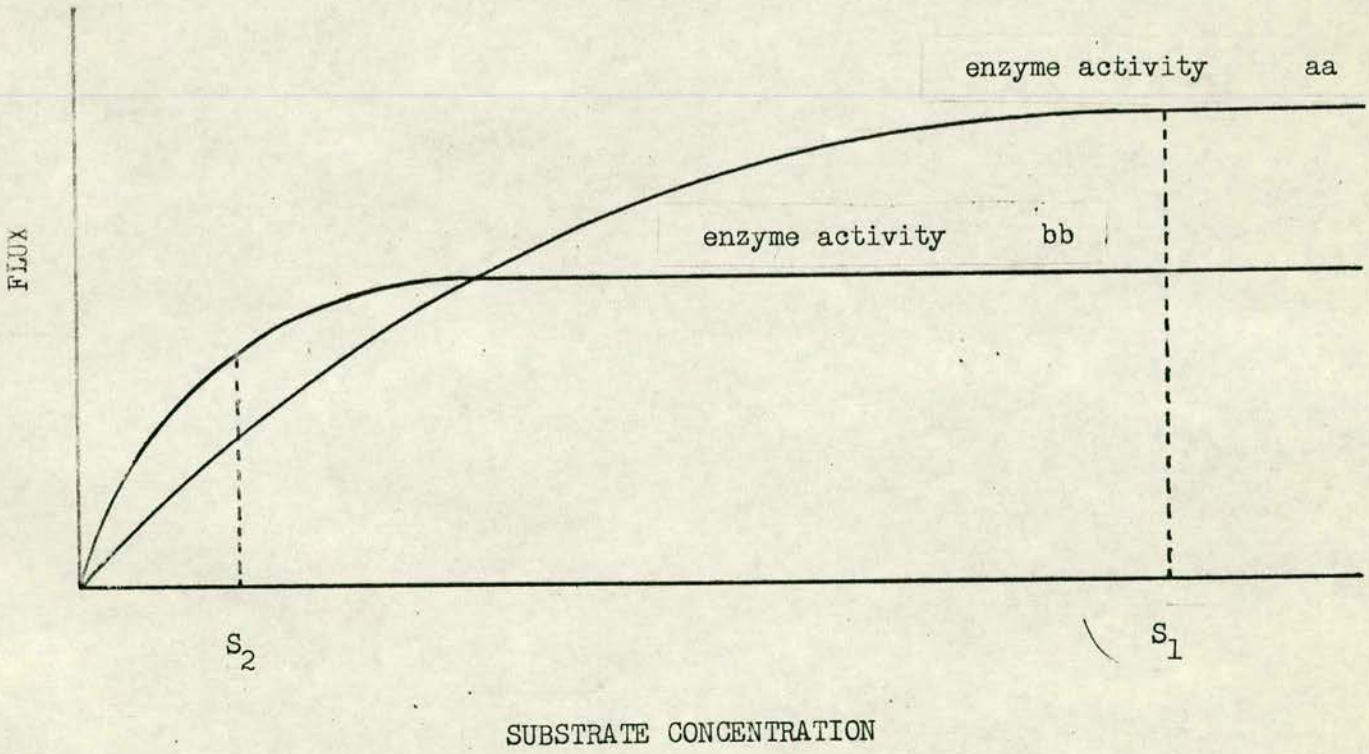


limiting value determined by other enzymes in the pathway, (Kacser, 1965; Kacser, 1968, personal communication).

Now, consider two homozygote genotypes aa and bb having different activities of the same enzyme, due to either changes in enzyme quantity or Vmax, the flux will be determined by the shape of the curve in fig.3. The heterozygote will be half way in activity between the two homozygotes. It can be seen that the flux of the heterozygote is nearer the higher homozygote than the lower one. However, in cases where the higher homozygote is further along the flat part of the curve, the heterozygote may be indistinguishable from the higher homozygote. From this example we can see that the lower homozygote will always appear recessive to the higher one (Kacser, 1968, personal communication). If, therefore, the enzyme allegedly responsible for the syndrome obese and adipose is being looked for, the recessivity of the ob and ad genes gives the expectation of a lower activity of the enzyme responsible. None of the enzymes investigated in this thesis show a lower activity in obese or adipose animals.

The situation is more complicated, however, when changes in the Michaelis constant (Km), as well as Vmax are caused by the genetical alteration. Situations can arise where a decrease in Vmax is accompanied by a decrease in Km such that at low concentrations of substrate one enzyme is "better", but a higher one is "worse", see fig.4. General predictions about dominance in such situations are not possible as the appearance of dominance will depend on the whole system. Possible allosteric interactions may further complicate the picture. This third criterion is therefore a much

Fig. 4. The relationship between the substrate concentration and the Vmax of an enzyme where the genetical alteration has altered the Michaelis constant (Km) as well as the Vmax.



weaker one and depends on the knowledge of changes (if any) in the Km of the enzyme. These values have not been determined in this investigation.

Thus, genetical theory gives us three criteria with which to examine potential primary enzymic lesions in the obese and adipose phenotypes. First the primary lesion is probably different in adipose to obese. Secondly, the primary biochemical lesion should exhibit segregation in apparently normal littermates of obese and adipose animals, and thirdly, if the lesion is a simple alteration of the Vmax of a lipogenic enzyme, then that enzyme should have a lower activity in obese or adipose animals.

If the primary genetic alteration, in both syndromes, is concerned with some general condition, such as hormone balance, it would be expected that rather wide effects on enzyme activities would be manifested and no specific identification with any one enzyme would be possible.

It is also possible to make phenocopies of obese and adipose mice by injecting normal animals with aurothioglucose. Animals treated in this way become extremely hyperphagic and eventually obese enough, in some cases, to be physically indistinguishable from obese and adipose animals.

A considerable body of information has been collected to show that these animals are physiologically quite different from obese and adipose ones, (this work is reviewed in detail in Chapter 2, part (a)). The obesity in these phenocopies, (referred to here as ATG animals), appears to be controlled by a different set of mechanisms to those causing the obesity in obese animals. Thus, primary changes in the biochemistry of obese (and for that matter adipose as well), should be at a different site

to those that are primary in ATG animals. Thus comparisons with ATG animals provide us with another method of checking potential biochemical sites for the primary lesion in obese animals.

In this thesis, while assessing data that might help to elucidate the primary lesion in obese and adipose mice, many enzymes are referred to. For easy reference each of these enzymes has been given a number which refers to its position on the metabolic chart, (fig.5), which may be folded out at the back of the thesis.

The general plan of this investigation was initiated by the reports of Lowenstein's group (Kornaker and Lowenstein, 1965; Spencer and Lowenstein, 1967), which implied that the primary effect in obese mice had been identified. Lowenstein's group found that the enzyme ATP citrate lyase (enzyme 18, in fig.5) has a two to four fold higher specific activity, (referred to here as Vmax), when assayed in liver supernatant from obese compared with that from normal littermates. Despite the recessivity of the ob gene these workers implied that this change in the activity of ATP citrate lyase, in obese mice, is the primary enzymic lesion which causes, eventually, all other biochemical and physiological manifestations in these animals.

Before repeating the work of Lowenstein's group or studying any of the other enzymes involved in synthesising the amount of fat in obese mice, it was thought necessary to examine the type of fat produced in these animals, as this might indicate the area in which the lesion is situated. At

the start of this investigation I had not found any reports on the type of fat deposited in these animals. The bulk of the fat in obese mice is deposited as an abdominal fat pad, in the form of triglyceride. The triglyceride is formed by the esterification of a whole range of fatty acids with glycerol. In triglyceride there are many types of fatty acid of varying chain length and saturation, and the amount of each one might be different in obese and adipose animals from that in their normal littermates. This would mean a different type of fat is produced in these animals and would also illustrate whether the primary biochemical lesion is in the synthesis of one, or one group, of fatty acids, or generally in the synthesis of all fatty acids. The type and amount of each type of fatty acid in the fat pad, can easily be assayed by gas/liquid chromatography.

The amount of the six main fatty acids from the triglyceride of the abdominal fat pad, was measured in obese and adipose mice and their normal littermates. No striking differences were found between any of the animals. Thus an alteration in the type of fat is not a significant indication of the site of action of these genes. This section of the investigation is dealt with in detail in Chapter 4, part (a).

From these results we can conclude that genes ob and ad affect the synthesis of all fatty acids and not just the synthesis of any one or class of them.

These findings resulted in the decision to investigate the general synthetic pathway for all fatty acids, starting with the reported rise in

the Vmax of ATP citrate lyase in obese animals that was mentioned earlier.

At the time this investigation was started the Vmax of ATP citrate lyase had been determined from the livers of obese and normal mice and found to be two to four fold higher in obese mice, (the theoretical validity of assumptions drawn from changes in Vmax, are discussed in Chapter 2, part (b)). ATP citrate lyase had been studied in obese mice because for several years the enzyme's biochemistry had been under intensive study by Lowenstein and his co-workers. They had previously assayed the Vmax of ATP citrate lyase in rats that had been maintained under a whole range of nutritional and hormonal conditions and the results of this work had led Lowenstein to postulate that the enzyme had a "rate controlling" function with respect to lipogenesis (a fuller discussion of this work will be found in Chapters 2, part (b) and 4, part (b)). As the obese mouse is a genetical example of hyperlipogenesis, it was logical, as will be described later, that Lowenstein's group should investigate the enzyme's Vmax in these animals. I have repeated the determination of the Vmax of this enzyme in liver supernatants from obese and extended the investigation to adipose mice and their normal littermates. The Vmax was two to four fold higher in both obese and adipose mice. Thus, if genetical theory is true, then the similarity in Vmax between obese and adipose mice together with their non-allelism indicates that the alteration in ATP citrate lyase activity in obese is possibly not the primary cause of the obesity. Work with litters from parents that were heterozygous for both the ob and ad genes tended to confirm this. (See chapter 4, part (b)).

The theoretical basis for judging an enzyme to be rate controlling with respect to the flux through the pathway it is involved in, is discussed in chapter 2 part (b). The details of the literature on the involvement of citrate and ATP citrate lyase, in lipogenesis, are dealt with in Chapter 4, part (b), as are the results of the experiments just mentioned.

Having concluded that the increase in the Vmax of ATP citrate lyase in obese and adipose mice is possibly not the primary biochemical lesion, I then looked for ways to show that this rise was not the only cause of the obesity in these animals.

If it could be shown that the Vmaxs of several other enzymes, catalysing steps in lipogenesis, had been altered in both obese and adipose mice, this would cast further doubt on the alteration in the Vmax of ATP citrate lyase as the only cause of the obesity. A co-ordinated alteration in the activity of several enzymes, in obese and adipose, would show that it is improbable that any simple alteration in any of these enzymes is the primary lesion.

For this reason I determined the Vmaxs of some enzymes that reports had identified as important rate controlling enzymes, as well as others identified as unimportant. The reasons for the enzymes chosen and the results of the assays on them are discussed in Chapter 4, part (c).

The results of this section of the investigation show that there is an alteration in the Vmax of two other enzymes, (malic enzyme, enzyme 21 in fig.5 and pyruvate kinase, enzyme 3 in fig.5), and little difference in

the Vmax of four others. There was again no marked difference between obese and adipose animals.

Recently several reports have confirmed and extended these results, so that there is now a range of known enzymic changes in obese and adipose mice. These are fully dealt with in Chapter 4, part (c).

At this stage the question arose whether the range of enzyme changes in obese and adipose animals, was peculiar to the special form of obesity found in these animals, or was general to other forms of obesity. If these same enzyme changes are present in other forms of obesity, such as in ATG animals, then it is certain that they are not the primary cause of the obesity in obese and adipose.

Thus the activity of these enzymes were assayed in ATG animals as well as obese and adipose. Despite being obese, ATG mice appear to be a completely different syndrome to obese and adipose, as they are extremely hyperphagic and have normal blood sugar levels, whereas obese and adipose are only mildly hyperphagic and generally hyperglycaemic.

The results of these experiments, dealt with in detail in chapter 4 part (d), show that the alterations in enzyme Vmax that occur in obese and adipose also occur in ATG mice. This confirms that none of the enzyme alterations so far studied, can be the only primary cause of these three types of obesity. Therefore in these three forms of obesity some controlling factor, other than the activity of any one of the enzymes so far studied, must

effect the co-ordinated rise in the Vmax of the lipogenic enzymes. Many factors, ranging from hormones to metal ions, have been reported to affect the activity of enzymes at different parts of lipogenesis; the literature on this subject is reviewed in Chapter 2, part (c). It is therefore essential to examine all these proposed mechanisms and see if they can be connected with the known physiological and biochemical changes in obese and adipose mice.

In chapters 2, part (c) and 4 part (e), work is reviewed that indicates the involvement of the fatty acids, especially the fatty acid linoleate, in the control of lipogenesis in the obese mouse. It is suggested that, as there is a lower activity of 2-monoglyceride lipase in obese mice, and that linoleate is held in an inert triglyceride form in these animals so that it is not able to 'repress' the lipogenic enzymes as well as it does in normal animals.

Experiments are described in Chapter 4, part (e) that show that linoleic acid is a more powerful inhibitor of lipogenic enzymes, in normal animals, than other fatty acids.

Other experiments were designed to find out if obese, ATG and normal animals, respond differently to various levels of dietary linoleate under different conditions. There was a tendency for obese mice to show less response in enzyme levels as a result of the action of dietary linoleate. It is considered in Chapters 4 part (e) and 5, that this affect of linoleate

might be made more meaningful by further intensive examination of the importance of 2-monoglyceride lipase. Using the genetic criteria, already discussed, it ought to be possible to show whether the change in the activity of the lipase is a primary biochemical lesion.

It is concluded, in Chapter 5, that the investigation in this thesis has made it possible to rule out any one change in the lipogenic enzymes as the primary lesion in the obese and adipose animals, and has shown the area in which research might gain an understanding of the similar effect that these genes have in mice.

CHAPTER 2. GENERAL REVIEW OF THE LITERATURE

Part (a): THE PHYSIOLOGY OF THE OBESE MOUSE

Before this investigation was started no biochemical or physiological work had been reported on adipose animals, apart from the work of Batt and Harrison, (1963), on the sterility of the males. However, a considerable amount of work has been published on the physiology and biochemistry of the obese mouse, which has been commonly referred to as the "obese hyperglycaemic" syndrome. Such a wide array of metabolic derangements have been reported in these animals that they are reviewed here and must form a necessary background to an examination and discussion of enzyme levels in these animals, since a change in any enzyme suspected to be the primary lesion must explain all the physiological changes mentioned below.

A considerable amount of work has been done on the endocrinology of the obese mouse. This has centered on the activity of insulin and the condition of the pancreas in these animals. The obese mouse (Mayer, 1960 review) exhibits pancreatic dysfunction with hyperplasia of the islets of Langerhans associated with increased insulin and glucagon secretion, (Shull and Mayer, 1956; Christophe et al, 1959). Obese animals are generally hyperglycaemic and moderately hyperphagic, the hyperglycaemia being generally increased by injections of growth hormone, and they have increased resistance to insulin injections, (Mayer, 1960; Strauffacher et al, 1967; Batt and Mailhe, 1967). The increased resistance to insulin in obese might be explained by the eight-fold greater destruction of insulin, IN VITRO, by fat pads from obese compared with those from normal littermates. The rate of insulin IN VIVO is at present being examined by

these workers (Westman, 1968). The size of the adrenal glands has been examined in obese. When taken from obese mice these are much larger than those from normal animals, but this is considered only to be a function of the higher weight of the obese animals, (Hellman, 1965).

The state of lipogenesis and lipolysis in obese animals has also been intensively examined. One of the first observations was that adipose tissue from obese contained more nitrogen than that from normal littermates suggesting an increase in cytoplasmic mass, (Mayer, 1965). The first attempt to measure lipogenesis in obese concluded that it was lower than in normal mice, as IN VITRO uptake of C_{14} glucose was depressed in these animals, (Chritophe et al, 1961), whereas recently it has been reported (Jansen et al, 1967) that C_{14} glucose given as a single dose IN VIVO was only converted into fatty acids in extra-hepatic tissue in obese at half the rate of normal littermates, but in the liver the conversion was greatly elevated in obese, whether the labelled glucose was added to the diet or injected. This work has been confirmed by Schreeve's group, (Shrigata and Schreeve, 1964; Schreeve et al, 1967), who found that when they sacrificed obese and normal animals, 90 minutes after intraperitoneal injections of trace amounts of carbohydrates labelled with tritium and carbon 14, (including glucose, lactate and glycerol) these were incorporated into liver fatty acids at five to eight-fold greater rates in the livers of obese mice compared with normal littermates. However, the incorporation into total carcass fatty acids was only 1.5 to 2 fold higher in obese animals. It can be seen, therefore, that although early workers found lower uptake of glucose in obese animals, this was

a particular case for IN VITRO conditions with adipose tissue, whereas IN VIVO liver tissue from these animals was far more active in incorporating glucose than that of normal animals. Both Schreeve's and Jansen's groups concluded that the metabolic derangements, especially the increased lipogenesis, of the liver could be more fundamental to the development of the obesity in obese animals, than the changes seen in the extra hepatic tissues, (Schreeve et al, 1967; Jansen et al, 1967).

The incorporation of intermediates of carbohydrate metabolism into lipids has also been studied in obese mice. Mayer et al (1953), postulated that the genetic lesion in obese was a partial block in the oxidation of acetate, causing more pyruvate to be converted to fatty acids. Hughes and Talbot (1958) also found a decrease in the conversion of injected labelled acetate into 14 CO_2 in obese animals. However, Parson and Crispell (1955) reported that equal amounts of 14 CO_2 were produced from the fat pads of obese and normal mice after an injection of labelled acetate. In contrast, an increased incorporation of C_{14} acetate into fatty acids in both liver and extra-hepatic tissues has been found in obese mice IN VIVO (Bates et al, 1955b) and IN VITRO (Mayer et al, 1955; Christophe et al, 1961b) in the presence of glucose, however, the IN VITRO lipogenesis was lower in obese than in normal animals. It appears, therefore, that obese mice do show an increased incorporation into fatty acids of C_{14} acetate although this might be masked by the presence of glucose. Mayer (1965) interpreted these findings to suggest that adipose tissue from obese mice metabolises an excess amount of

substrate carbon to fatty acids and that fatty acid synthesis in this tissue is less dependent on the simultaneous occurrence of accelerated glucose metabolism than is true for normal adipose tissue from rats and mice. Although in any case, the rate-limiting step in normal animals does not appear to be from glucose to acetate, as Flatt and Ball (1963), with rat liver slices IN VITRO, have shown that the rate of fatty acid synthesis is 60% greater when both glucose and acetate are present, than with just glucose alone. Therefore, Mayer's interpretation of the higher acetate incorporation in obese animals might not be correct and therefore will not help to pin point the primary lesion in these animals.

Therefore, in obese mice, there is a large rise in overall lipogenesis as shown by increased incorporation of glucose and acetate (and other carbohydrate intermediates) into fatty acids, especially in liver tissue. As well as this extensive increase in the parameters of lipogenesis in obese mice, these animals also exhibit decreased lipolysis. This has been shown by the incubation of adipose tissue from obese mice and normal animals, IN VITRO, with a range of substances and measuring the release of free fatty acids, as a measure of lipolysis. Obese adipose tissue failed to give a rise in the release of free fatty acids in response to adrenaline, fat mobilising substance, or a 16 hour fast, whereas normal littermates gave the normal rise in the release of free fatty acids in response to all these treatments, (Marshall and Engell, 1960; Lebocuf et al, 1961; Yen and Allan, 1967).

Therefore, not only is there decreased lipolysis in obese animals, but there is also increased resistance to factors which increase lipolysis in normal mice.

It is with this background of extensive metabolic derangements in obese animals, both in lipogenesis and lipolysis, that the work described later on individual enzymes, must be viewed. Any postulated primary enzymic lesion must explain all these wide metabolic effects.

CHAPTER 2. GENERAL REVIEW OF THE LITERATURE

Part (b) : ENZYMES AND RATE CONTROL

Over the last fifty years, the sequence of reactions and the enzymes that catalyse them have been identified for the major pathways of metabolism. So much information has been collected on these pathways that they are often shown in the form of a map (see fig.5).

Since it has been discovered that genetic control of metabolism is mediated through enzymes there has been great interest in enzyme function. For not only is it known that many gross inborn errors of metabolism are due to the absence of an enzyme, but also that finer control of metabolism might be exercised by alteration of the rate of synthesis, or the efficiency, of an enzyme, or by its interaction with other metabolites.

The dramatic effect, that the removal of some enzymes has on metabolism has led to the belief that the flow or flux of material through a biochemical pathway may be determined by the actvity of a "key" or "pace making" enzyme. Umbarger (1961), using enteric bacteria, has even gone so far as to say that the first step in the pathway might be the rate determining one. This belief in the existence of "key" enzymes, has given rise to an intensive study of individual enzymes to see to what extent alterations in their activity, in catalysing their specific reactions, can control the flux through the pathway. However, we can say an enzyme is a "pace maker" only after its importance in controlling the flux of the pathway has been compared with that of all the other enzymes in the pathway.

If the change in activity of an enzyme does significantly affect the flux through the pathway, then it is important to know which other metabolites interact with the enzyme, altering either its efficiency or its synthesis. The activity of an enzyme might also be affected by a physical alteration in its structure by gene substitution.

Unfortunately, to get this apparently simple information to describe an enzyme's rate controlling ability, is not easy. There are serious methodological problems. Although it is easy to extract an enzyme from something like liver or adipose tissue, saturate it with substrate and co-factors and measure its maximum activity (known here as Vmax) in isolation IN VITRO, we do not know whether the substrates and co-factors are at saturation level IN VIVO. If they are not, the Vmax cannot tell us much about the enzyme's activity in the cell, because there is a specific non-linear relationship for each enzyme between enzyme activity and substrate concentration. Unless this relationship, specified by the Michaelis constant, (Km), is known, the enzyme activity at substrate levels below saturation, cannot be predicted from the Vmax. Another difficulty in inferring IN VIVO activity from IN VITRO determination, is that saturation of the enzyme with the substrate will remove any competitive inhibitors that may alter enzyme activity in the cell.

Even if we do know the substrate and co-factor pool size in the cell we do not know the importance of cellular localisation on enzyme activity. Besides gross cellular isolation, for example inside or outside the mitochondria, the substrates or co-factors might have to be in the close physical proximity of the enzyme to be able to react or the reactants might

have to be located at a specific site on the membrane surface. Moreover, the situation in the cell is probably even further complicated as we undoubtedly do not know all the co-factors that influence the activity of any one enzyme IN VIVO.

In order to interpret enzyme activity determinations we must know the size of the IN VIVO substrate pools, and the relevant Km values. In very few cases is one able to measure substrate pool sizes in mammalian systems and there has never been any systematic attempt to measure all the pool sizes in one pathway.

Thus there are great difficulties in looking at the isolated Vmax of one enzyme IN VITRO and trying to relate it to the enzymes activity IN VIVO. The problem is even greater because one must remember that the substrate and product of one reaction are only two metabolites in an integrated chain of reactions, which govern the flux of the system. Other metabolites in the pathway might alter this one enzyme's activity and indeed the substrate and product of the enzyme itself might affect the activity IN VIVO of other enzymes, changing the flux and possibly altering the activity of the original enzyme. Thus the behaviour of an enzyme might be quite different in the system outside it. In fact the complications and permutations that can be envisaged are enormous for a system with even a short list of co-factors and substrates.

However, if it is possible to get a rough estimation of IN VIVO enzyme activity then, to make meaningful statements about the rate controlling ability of the enzyme in its pathway, we must examine the

effect of a change in enzyme activity on the flux. This means we have to find some way of measuring the flux.

In mammalian systems this is often done by defining a beginning and an end to the pathway. Then to masure, for example, the incorporation of the labelled initial substrate into labelled final product, mainly IN VITRO tissue slices, which it is hoped represents the IN VIVO situation more closely than tissue-free supernatants. This is then taken as the flux through the pathway and used as a basis of comparison with enzyme activity. This measurement of the flux is unsatisfactory because the arbitrary classification of one metabolite as the "initial" substrate of the pathway ignores the fact that this substrate itself is an intermediate in a larger chain of events and thus also bound by the rules of the system. Saturating tissue slices IN VITRO might again be very different to the IN VIVO situation because the concentration of the initial substrate IN VIVO is not known.

Having attempted as closely as possible to find the IN VIVO enzyme activity and the IN VIVO flux, we can investigate the effect of changes in the activity of each enzyme in the pathway on the flux. We need some parameter which will measure this phenomenon. Kacser and Burns (1968) have developed one in the form of a Sensitivity Coefficient of the flux with respect to the enzyme. This simply relates the percentage change in flux to the percent change in activity of the enzyme, for small values of the change.

i.e.

sensitivity coefficient or C $= \frac{dF}{F} / \frac{dE}{E}$

= % change in the flux divided by

% change in enzyme activity.

Thus a coefficient with the value 1.0 would be identified with absolute rate control. Unfortunately, once again this relationship is non-linear. So for each enzyme we will have to establish the relationship between % change in flux and % change in enzyme activity for each level in enzyme activity, before we can use the parameter in a predictive capacity.

To calculate a sensitivity coefficient for an enzyme we need to be able to alter the enzyme activity IN VIVO without altering anything else in the system. In micro-organisms it is often easy to find mutations that alter the activity of one enzyme. In fact it is possible to obtain large numbers of mutations that confer different activities on each enzyme in a pathway. Thus we can estimate a sensitivity coefficient fairly accurately in these organisms because we can alter an enzyme's activity and keep everything else constant. In mammals, so far, one rarely has mutations that affect the activity only of the enzyme under examination and never does one have mutations affecting the activity of each of a whole sequence of enzymes in the same pathway. The only way usually available to alter enzyme activity is to change the nutritional or hormonal environment of the animal. This causes a whole range of complex alterations in the system and therefore is

most unsatisfactory. Although, if these complications are always respected and if the information collected to calculate the sensitivity coefficient, adheres as closely as possible to the IN VIVO situation, then it might be possible to make a crude investigation of the rate controlling ability of an enzyme.

Furthermore, to be able to say whether an enzyme is a "key" or "pace maker" one we need to know the sensitivity coefficients of every enzyme in the pathway. If one has a sensitivity coefficient a lot higher than the others, then it might be called a "key" enzyme, but only under the conditions in which the investigations were made.

There is a second sense in which the term "rate control" is used, which is not necessarily relevant to the problem discussed above. This refers to the physiological and biochemical mechanisms which are said to operate in maintaining the flux at some steady value or to adjust it to some optimum in the face of environmental variation. Even if it can be shown that an enzyme is a "key" one, (i.e. it has a high coefficient), it does not follow that the organism itself 'uses' a mechanism to control the activity of the enzyme. "Control", i.e. effective alteration of flux, might in fact be mediated by a number of "non-key" enzymes simultaneously. The assumption that "key" enzymes and actual control are always synonomous is false.

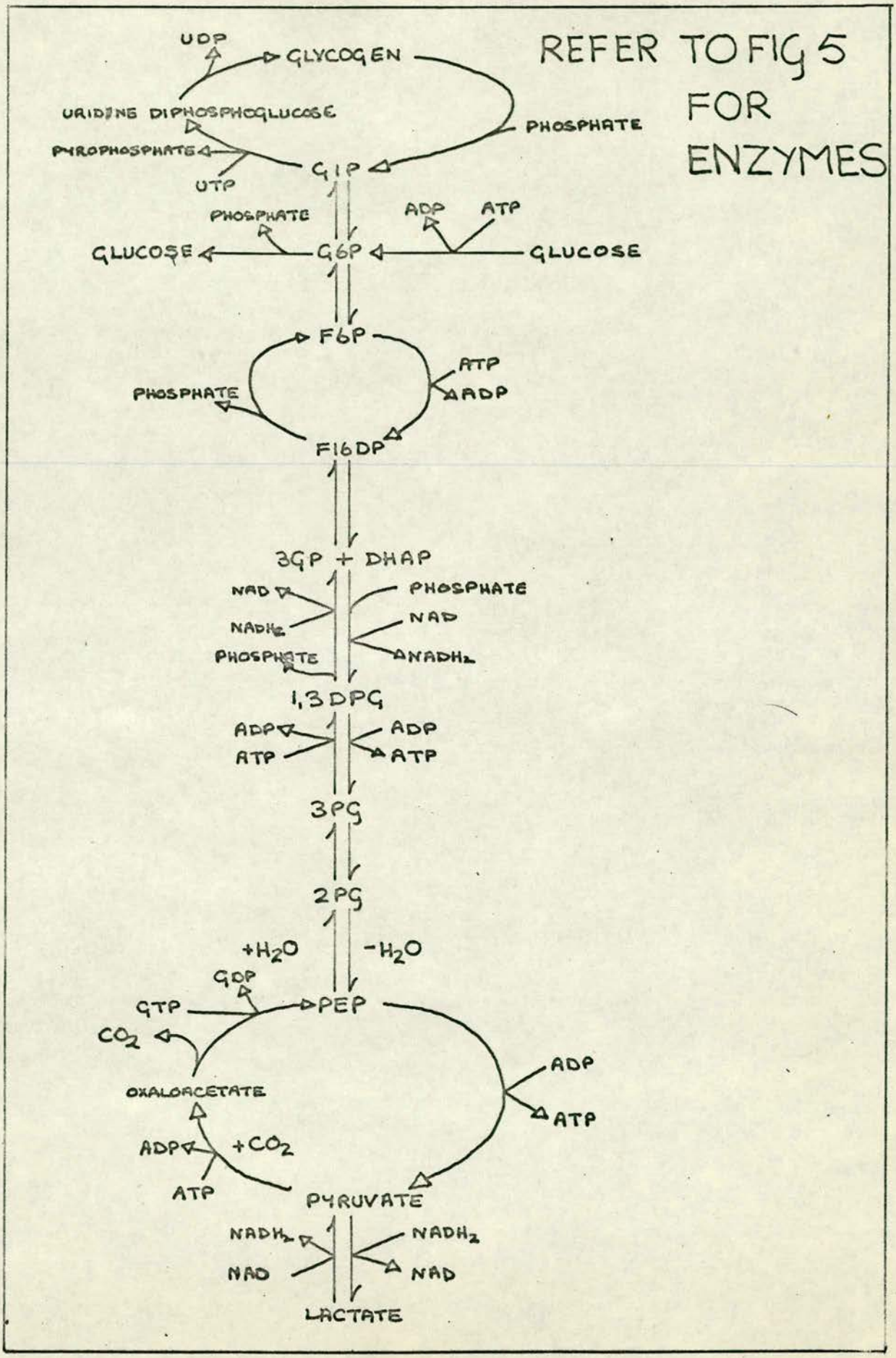
Conversely, if it can be shown that under some circumstances enzyme activities are altered, (for example starvation, hormone treatment) this does not entitle us to conclude that such enzymes are "pace-makers", nor, for that matter, that the flux has in fact changed.

The apparently paradoxical situation might be found that a particular genetic alteration affects a "key" enzyme and hence the flux and phenotype, while control, in the physiological sense, is exercised by a different mechanism. Considerable confusion exists in the literature on this point.

There is much literature reporting investigations examining the way individual enzymes might control the flux through a pathway. Quite often these findings have been synthesised into a complete explanation of control of large areas of metabolism. They include some work on the enzymic control of the parts of metabolism that I am interested in - glycolysis, gluconeogenesis and lipogenesis.

Glycolysis and gluconeogenesis are the over-all reversal of the same metabolic process: pyruvate or lactate to or from glucose or glycogen. Krebs (1964) has summarised the two sets of reactions involved (Fig.6). He says that it is generally accepted that gluconeogenesis (lactate to glucose) involves some reactions of glycolysis in reverse and some additional reactions which overcome the energy barriers, preventing the direct reversal of glycolysis, (i.e. the reactions catalysed by enzymes 4,5,6 and 7 in figs. 5 and 6). These reactions that are specific to gluconeogenesis Krebs calls "pacemaker" reactions, because the Vmaxs of their enzymes are low, whereas the Vmaxs for the enzymes catalysing the other reactions are over 100 times the activity expected from the known overall rate of gluconeogenesis. Krebs says that two of these enzymes possess special characteristics that may be

Fig.6. Reactions of Glycolysis and Gluconeogenesis (From Krebs, 1964).



expected of pacemakers in that they are inhibited by excess of their own substrate, the inhibition being removed by precursors of gluconeogenesis such as lactate.

Thus Krebs describes enzymes as rate controlling either (a) if they catalyse alternative routes to overcome thermodynamic barriers, (b) if they have low Vmaxs or (c) if they suffer substrate inhibition. The evidence, presented by Krebs, may well be consistent with these enzymes having some rate controlling function, but it does not show that the other enzymes involved in glycolysis have not. This is necessary before any enzyme can be described as a pacemaker enzyme. First, the thermodynamic barriers, postulated by Krebs, just represent a free energy difference. In fact one of the reactions (from fructose 1.6-diphosphate to fructose 6-phosphate) the free energy difference (Kreb's thermodynamic barrier) appears from fig.6 (taken from Kreb's paper) to be in the opposite direction to that needed to postulate the reaction as a thermodynamic barrier for gluconeogenesis. Unfortunately, as Krebs does not discuss the equilibrium constants for these reactions in his paper, it is difficult to make a conclusion on his pinpointing of the pacemaker reactions for gluconeogenesis. For example, the step from 1,3 diphosphoglycerate to 3 phosphoglycerate involves ADP to ATP. Does this reaction constitute a thermodynamic barrier? If so, why, by Kreb's criteria, do not two enzymes, one for glycolysis and one for gluconeogenesis, catalyse the step? But we cannot say whether there is a "thermodynamic barrier" as we do not know the equilibrium constant of this reaction.

Secondly, although the Vmax of Kreb's pacemaker enzymes are 100 fold lower than the others, and just enought to account for the apparent flux, we have seen that because of the non-linear relationship between activity and substrate concentration, the ratio of the Vmaxs is not guide to the relative importance of the two sets of enzymes IN VIVO.

Thirdly, the flow data presented from the incorporation work, to show that inhibition of certain enzymes by their substrates is removed by gluconeogenic precursors, such as lactate, are limited in their value as the precursors are provided in saturated amounts to the IN VITRO tissue slices and this may not represent the IN VIVO situation. Also, we must again eliminate such an effect at all the other enzyme steps, IN VIVO, before we can say that this shows that these enzymes are pacemaking.

These complications make it impossible, at this stage, to estimate sensitivity coefficients for each enzyme in gluconeogenesis and thus get some quantitative relation between the enzymes as to their value in controlling the rate of flux through the pathway. Therefore we cannot, on this basis, yet classify the gluconeogenic enzymes into pacemaking and non-pacemaking ones. The concept that Krebs has worked out for the reactions in glycolysis and gluconeogenesis has been expanded into a far more sweeping control theory by Weber and his co-workers.

Weber (1966,67) has classified the enzymes that catalyse the reactions in fig.6 into three groups - (i) "bifunctional" enzymes - those that are involved both in glycolysis and gluconeogenesis (enzymes, 8,9,10, 11,12); (ii) key glycolytic enzymes - those that are involved in glycolysis alone (enzymes 1,2,3) and (iii) key gluconeogenic enzymes - those that are involved in gluconeogensis alone (enzymes 4,5,6,7).

Thus Weber has suggested three classes of enzymes, which he calls Functional Genic Units (FGU's). He postulates that "these FGU's are "produced" on the same unit of the genome" (Weber et al, 1967). The "bifunctional" FGU contains enzymes with high Vmaxs that do not alter under differing nutritional and hormonal conditions and thus, says Weber, are not pacemaking reactions; the key glycolytic and key glyconeogenic enzymes alter their Vmaxs in a reciprocal way to the hormonal or nutritional environment. The "simultaneous but antagonistic action", says Weber, "on key gluconeogenic and glycolytic enzymes, may well determine the overall metabolic flow for gluconeogenesis or glycolysis", (Weber et al 1966). Thus Weber postulates an all-embracing theory for rate control of glycolysis and glucogenesis that ""rate limiting" enzymes are "one-way" reactions on both sides of metabolic pools of reversable reactions" (Weber et al, 1966).

This may be so, but the experiments reported by Weber's group far from show this. Firstly, they give no data on the IN VIVO flux of glycolysis or gluconeogenesis under the nutritional and hormonal regemes that are imposed in these experiments. Without this information it is impossible to correlate the enzyme's Vmax with flux. Weber et al make the implicit assumption that the enzyme's Vmax represents the flux through the enzyme step, but there can only be one flux through the whole pathway which must be the same for each step. Secondly, the evidence that the glycolytic and gluconeogenic enzymes are on three "Functional Genic Units" is based on evidence showing that the FGU's or enzymes show reciprocal response or non-

response to hormones and nutritional conditions, and that this response can be blocked by actinomycin D. Although actinomycin D is supposed to prevent DNA-like RNA synthesis this is not direct evidence that the genes coding for these enzymes are "produced on the same unit of the genome". Before the concept of Functional Genic Units can be proved, linkage data will be needed from genetical studies. No such data are at the moment available.

It appears, therefore, that the evidence for the rate control of glycolysis and gluconeogensis does not adhere at all closely to the criteria for rate control investigations that I have previously outlined.

In lipogenesis, "rate control" has two separate claims, each for a different enzyme. These are ATP citrate lyase (EC 4.1.3.8., enzyme 18) and acetyl CoA carboxylase (EC 6.1.4.2., enzyme 22).

Kornaker and Lowenstein (1964a, 1965a) consider that ATP citrate lyase catalyses the first step in the extra mitochondrial synthesis of fatty acids. Moreover, they say "differences in the enzyme activity of the first step may be responsible for different rates of operation of the whole pathway". They then show that in different hormonal, physiological and nutritional conditions, the Vmax of ATP citrate lyase is correlated with lipogenesis, either seen as incorporation of labelled citrate into fatty acids or manifested as obesity. The implication is that the Vmax of ATP citrate lyase controls the flux of material from citrate to fatty acids.

Unfortunately, once again, there is not adequate data to estimate a sensitivity coefficient for this enzyme with respect to lipogenesis. Furthermore, it is known from this investigation, and others that many

enzymes have correlated responses in lipogenesis. Another enzyme has been claimed to control the rate of lipogenesis by Lynen et al (1964), who considers that there are only two steps in the extramitochondrial synthesis of fatty acids. These reactions are those catalysed by the enzymes acetyl CoA carboxylase (enzyme 22) and fatty acid synthetase (enzyme 23) of which acetyl CoA carboxylase catalyses the first step. Of course, Lowenstein and his co-workers disagree with this and consider the reaction catalysed by ATP citrate lyase to be the first step in lipogenesis.

Despite this Lynen and his co-workers have intensively studied the acetyl CoA carboxylase reaction. They have measured the Vmaxs of the two enzymes (enzymes 22 and 23) and have found that the Vmax of fatty acid synthetase complex is always 20 to 100 fold in excess of the Vmax of acetyl CoA carboxylase and of the incorporation of labelled acetate into fatty acids. Once again these results may not reflect the situation IN VIVO.

Lynen et al do show that if purified acetyl CoA carboxylase is added to liver extracts from starved rats there is a proportional rise in the produce of the reaction - fatty acid. This would imply a sensitivity coefficient of 1.0. Unfortunately the flux is measured at substrate (in this case labelled acetate) saturation and also there are no data on how the flux would respond to additions of other enzyme, fatty acid synthetase (i.e. this enzyme might have a sensitivity coefficient of 1.0 as well).

Therefore, none of the studies so far reported have fulfilled the criteria necessary to enable us to obtain safe evidence of the role of enzymes involved in glycolysis, gluconeogenesis or lipogenesis. Thus it is difficult in the present state of knowledge to compare the ability of these enzymes to control the rate of flux through the pathway. This makes it difficult to pinpoint any enzyme as a pacemaker or key enzyme at this stage.

As well as the theoretical anomalies of the work discussed above for each of the studies there are practical difficulties.

A corollary of the work of Weber's group, is that on starvation the glucocorticoids derepress the "key" gluconeogenic enzymes thus enabling blood sugar levels to be maintained. However, in adrenalectomised rats although the "key" gluconeogenic enzymes stay repressed, as Weber predicted, blood sugar levels are maintained. Therefore a rise in the activity of the "key" gluconeogenic enzymes is not necessary to maintain blood sugar levels, (Lardy et al 1967). Weber's group have also suggested that the "key" glycolytic enzymes are derepressed by insulin and repressed in diabetes. However, glycerol feeding to alloxan diabetic rats results in an increase to normal level of pyruvate kinase (enzyme 3) and phosphofructokinase (enzyme 2), although glucokinase remains low. This suggests that the "key" glycolytic enzymes do not always respond as a "Functional Genic Unit" and that glucokinase alone (enzyme 1) might be primarily affected by insulin levels.

The basic relationship found by Lowenstein's group was the

correlation between the Vmax of ATP citrate lyase (enzyme 18) and the incorporation of labelled citrate into fatty acid in different nutritional and hormonal conditions. After three days starvation both citrate incorporation and the Vmax of ATP citrate lyase had decreased. However, Srere and Foster (1967) have shown that fatty acid synthesis has decreased to zero after 24 hours starvation, whereas the Vmax of ATP citrate lyase start to drop until 48 hours of starvation. This throws doubt on the possibility that the Vmax of ATP citrate lyase controls fatty acid synthesis.

In a parallel way with acetyl CoA carboxylase (enzyme 22) for which a similar relationship to that found by Lowenstein's group for ATP citrate lyase with fatty acid synthesis, had been found by Lynen et al, Korchak and Masoro (1962) found that after 24 hours starvation fatty acid synthesis had fallen to 1-5% of normal, whereas the Vmax of acetyl CoA carboxylase had only fallen 50%, and Gibson and Hubbard found no drop at all in Vmax under similar conditions. Once again it does not appear that the fall in lipogenesis could be controlled by a drop in the Vmax of the enzyme.

From the evidence I have reviewed above, it appears that at practical and theoretical levels, the singling out of any enzyme or enzyme group as the pacemakers in glycolysis, gluconeogenesis or lipogenesis is not yet justified. However, in the work reported below, from this investigation, it was possible to show, by the three genetic criteria mentioned in the introduction that the enzyme originally reported to be rate controlling, in <u>obese</u> animals, was not the primary and probably not the only cause of

the obesity in these animals. Until it is possible, by genetic criteria, to pinpoint the primary enzymic lesion we do not have to estimate the coefficient described above. It is important, however, to bear the considerations, mentioned in this chapter, in mind when working with any enzymes that have been reported to be "rate controlling".

CHAPTER 2. GENERAL REVIEW OF THE LITERATURE

Part (c) : THE CONTROL OF LIPOGENESIS.

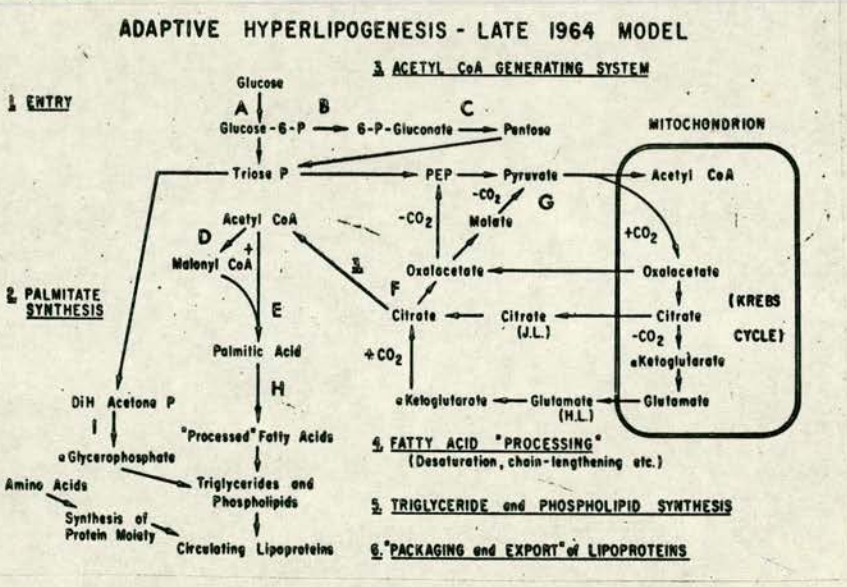
There are several areas and levels of metabolism at which control of lipogenesis is said to be exerted. Many classes and individual controlling agents have been postulated to affect the overall or a highly specific part of the system. Thus the metabolic system in which control may be exerted may be divided into several subjects or areas, (Tepperman and Tepperman, 1965): (1) food intake, (2) glucose entry to the cell, (3) acetyl CoA generating system, (4) palmitate synthesis, (5) fatty acid changes - chain lengthening and desaturation, (6) triglyceride and phospholipid synthesis, (7) transport of lipoproteins, (8) lipolysis and gluconeogenesis, (fig.7).

Control that will affect obesity can be exerted in any of these areas or a combination of them.

We have already seen that obese mice are only mildly hyperphagic (Mayer 1960) so it is probable that this gene does not primarily affect the food intake level.

However, several classes of effector are known that both inhibit the activity and repress the synthesis of enzymes involved in all areas of lipogenesis. Besides chemical effects on enzymes there are other cellular properties that must be taken into account, such as, compartmentation and localisation of enzyme substrates and their cofactors, the control of permeability and integrity of membranes, and so on. The classes of effector and their effect on lipogenesis are as follows:

Fig.7. Adaptive hyperlipogenesis, (from Tepperman and Tepperman, 1965).



1. HORMONES

(a) Thyroid. Thyroxine increases the activity of malic enzyme (EC 1.1.1.40, enzyme 21) seven fold (Tepperman and Tepperman, 1964), but decreases fatty acid synthesis from acetate by diminishing the glycogen content of liver S_{10} preparations and thus leading to a failure to regenerate ATP, (Myant and Illiffe, 1964). Moreover lipolysis was enhanced in hyperthyroid conditions and reduced in hypothyroid conditions by an alteration in the mechanism of lipase activation, (Fisher and Ball, 1967). So thyroid hormones can both decrease lipogenesis and increase lipolysis.

(b) Growth hormone. Growth hormone given to rats, IN VIVO, depleted their fat depots (Young, 1945), inhibited lipogenesis and elevated the plasma free fatty acids (FFA) levels within two to six hours, (Roben, 1959; Knobil and Hotchkiss, 1964). It has been suggested, (Swislocki and Szego, 1965) that growth hormone does not deplete fat depots directly but by increasing the oxidation of fatty acids in the liver it causes the release of free fatty acids by the depot fats.

Although growth hormone mobilises fatty acids IN VIVO it was needed in high concentrations to cause lipolysis IN VITRO and it was suggested that action by contaminants could not be ruled out (ACTH, FSH, etc.) (Korner, 1967). However, very small amounts of growth hormone could mobilise fatty acids from epididymal fat pads IN VITRO provided the synthetic steroid dexamethasone was present, (Fain Kovacey and Scow, 1965).

(c) Glucocorticoids. Experiments were discussed in part (b) of this chapter where injection of glucocorticoids increased the activity of the "key" gluconeogenic enzymes - pyruvate carboxylase (EC 6.4.1.1)(enzyme 4 in fig. 5), PEP carboxykinase (EC 2.7.1.40)(enzyme 5 in fig.5) fructose 1,6 diphosphatase (EC 3.1.3.11)(enzyme 6 in fig.5); triamcinolone was more active than cortisone and hydrocortisone; the glucocorticoids had no effect on the activity of any of the "key" glycolytic enzymes, (enzymes 1,2,3 in fig.5), (Weber et al, 1963, 1965; Weber, 1965). Thus, as actinomycin D blocks the action of the glucocorticoids, Weber et al suggested that they effect the synthesis of these enzymes and hence a controlling part in gluconeogenesis. The theoretical anomalies of this work are discussed in part (b). Furthermore in adrenalectomized rats there is the same rise in PEP carboxykinase as there is in normal rats and high doses of actinomycin D, while completely blocking the synthesis of PEP carboxykinase, do not affect the ability of hydrocortisone to maintain blood sugar levels in fasted mice, (Lardy et al 1967). So, although the adrenocorticoids increase the activity of gluconeogenic enzymes, it is doubtful whether this is necessary to maintain blood glucose levels.

(d) Epinephrine. Epinephrine stimulates the mobilisation of free fatty acids and glycerol and glycogenolysis in adipose tissue (Mayer, 1963). It also causes ACTH to be released from the pituitary which in turn stimulates the release of the adrenocorticoids.

(e) Insulin. Alloxan diabetic rats have blood glucose levels over 300 mg/ 100 mls. of blood. It has been found that the IN VITRO synthesis of total

fatty acids and of mono-unsaturated fatty acids from acetate-1-C_{14} and stearate-1-C_{14} is considerably reduced in liver slices of such animals compared with normal ones. The total fatty acid synthesis was repaired by the addition of insulin IN VITRO whereas mono-unsaturated fatty acid synthesis was only repaired by injecting insulin IN VIVO, (Benjamin and Gellhorn, 1964). Alloxan diabetes is a chronic diabetes that gradually reduces fatty acid synthesis IN VITRO to a fairly low level in about 72 hours and lipogenesis is restored about 6-8 hours after insulin injection. Acute diabetes can be brought about by injecting animals with anti-insulin serum (AIS) ~~injections~~. The same pattern was produced in normal liver homogenates by adding 1 to 5 μ equivalent of palmitate or 125 mμ moles of palmityl CoA per ml of incubation media. Both these inhibitions were reversed by adding carnitine which reduces the level of Coenzyme A.

Insulin stimulates the synthesis of lipids, increasing the rate of labelled glucose incorporation into fatty acids in epidydimal fat pads and stimulates the synthesis of triglyceride from fatty acids and α-glycero phosphate, (Korner, 1967), the latter part probably being an indirect result of its action on carbohydrate metabolism. The action of adrenaline or ACTH in releasing fatty acids in isolated fat pads, or isolated fat cells is inhibited by the presence of insulin in the incubation medium, (Rodbell, 1964).

Studies with starvation and realimentation, in rats, with acute or chronic insulin insufficiency, or with large injections of insulin, have shown that insulin increases the Vmaxs of glucokinase (EC 2.7.1.2) (enzyme 1 in fig.5) (Niemeyer et al, 1967; Sols et al, 1964). In fact

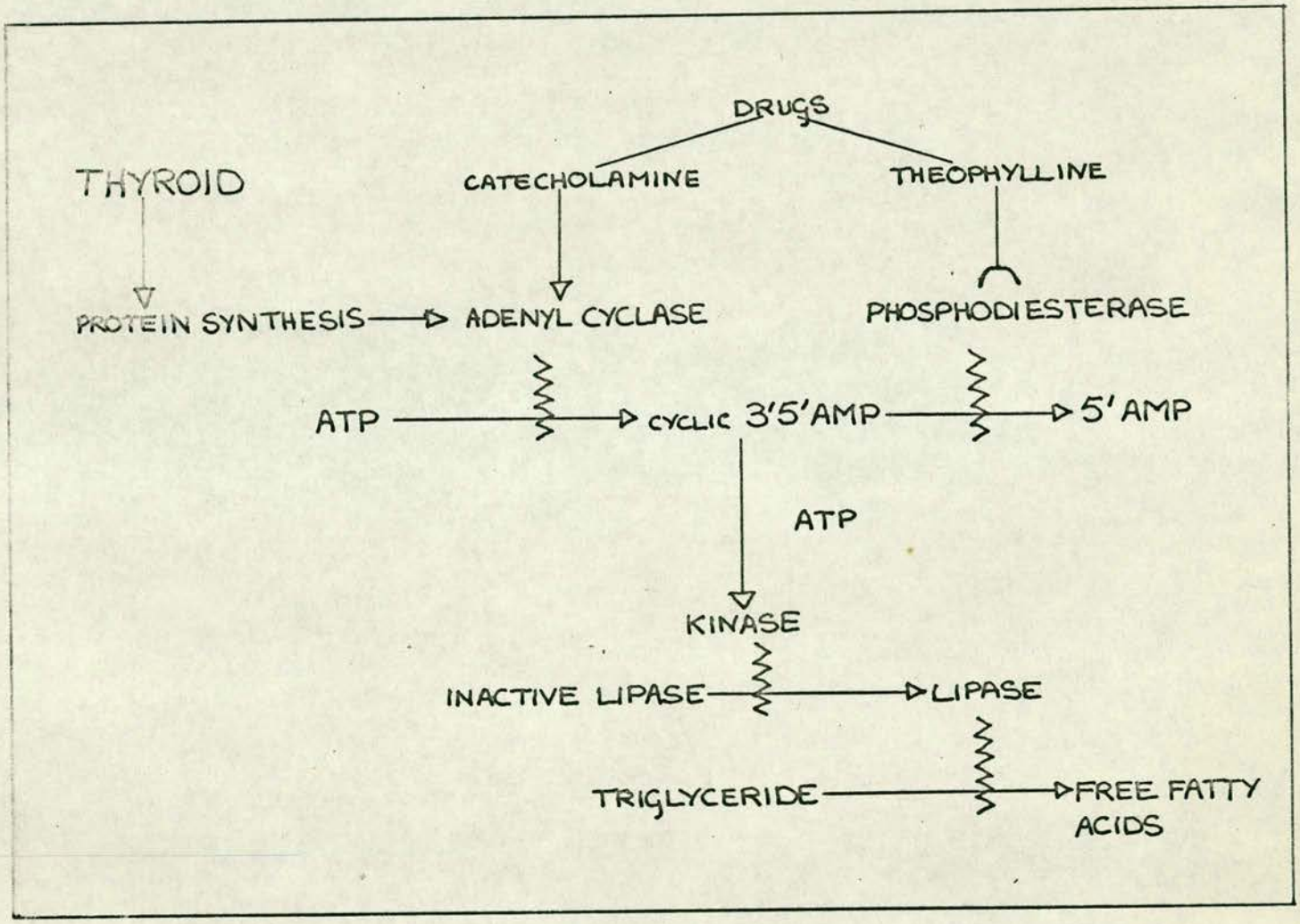
insulin appears to "derepress" all of the "key" gluconeogenic enzymes, (enzymes 4,5,6,7 in fig.5), while not affecting the "bifunctional" enzymes (enzymes 8, 9, 10,11,12 in fig.5), (Weber, 1965a,b,c,d,e). The theoretical objections of this work have already been discussed in part (b) of this chapter. A further point to note is that glycerol feeding to alloxan diabetic rats results in an increase to normal level of pyruvate kinase while glucokinase remains low, (Takeda et al, 1967). This suggests that glucokinase, alone of the "key" glycolytic enzymes may be regulated by insulin and that pyruvate kinase may be secondarily regulated by the rate of metabolic flow through the glycolytic pathway. In the same way, the Vmaxs of ATP citrate lyase (enzyme 18, in fig.5) and acetyl CoA carboxylase (enzyme 22 in fig.5) are increased by glycerol feeding (Greenspan and Lowenstein, 1967), and they too appear to be indirectly affected by insulin.

Thus, although insulin does have wide effects on glycolysis and lipogenesis, its primary source of action might be only at the glucokinase level.

(f) Mechanisms of hormone action. There have been three theories proposed for the mechanisms of hormone action. They are as follows.

(i) There is an hypothesis that hormones are released from the endocrine glands in response to a stimulus, and act on the adenyl cyclase system, (see fig.8) in the target cell, so that more ATP is converted to 3'5' AMP. The 3'5' AMP affects a secondary target system to produce the recognised physiological change. This hypothesis has been constructed, in general terms, from the fact that 3'5' AMP mimics the action of a trophic hormone from the pituitary, (Sutherland Øye and Butcher, 1965), adrenaline and glucagon in liver slices by stimulating the catabolism of glycogen, (Sutherland and Ball,

Fig.8. An example of how a hormone, (in this case from the Thyroid), might affect the activity of an enzyme, (in this case lipase), through the adenyl cyclase system, (after Krishna et al, 1968, see also Davies 1968).



1958), ACTH on rat adrenal cortex, lutein zing hormone in the corpus luteum and vasopressin on isolated toad bladders, (Butcher, Ho Meng and Sutherland, 1965). 3'5' AMP is also implicated in the lipolytic response to epididymal fat pad to adrenaline, (Butcher et al, 1965) and mimics the action of thyroid hormones in causing the mobilisation of FFA, see fig. 8 (Fisher and Ball, 1967; Krishna et al, 1968). It has also been shown that insulin decreased adenyl cyclase/activity and hence the amount of 3'5' AMP (Jungas, 1968) and this is contrary to the action of adrenaline suggesting an integrated mechanism for the control of lipid synthesis, (Korner, 1967). At several levels of this system further inhibitions and activations can take place. For instance, 3'5' AMP can be inactivated to 5' AMP by a phosphodiesterase and this inhibition can itself be inhibited by the methyl xanthines, (Davies, 1968). This provides an integrated system that probably plays and important part in the control of carbohydrate and lipid metabolism, (Korner, 1967).

(ii) Another mechanism for hormone action, which has been proposed, is at the level of RNA synthesis. Weber (1965a,b,c,d,e) reported that the "key" glycolytic enzymes (enzymes 1,2,3 in fig.5) increase in activity and the "key" gluconeogenic enzymes (enzymes 4,5,6,7 in fig.5) decrease in activity, due to insulin injections in alloxan diabetic rats, and the "key" gluconeogenic enzymes increase in activity due to glucocorticoid injection, could all be completely blocked by a prior injection of actinomycin D. He concluded that the hormones must act at the DNA-like RNA synthesis level and that the "key" glycolytic and "key" gluconeogenic enzymes, two co-ordinated "functional genic units", and that the activity of the enzymes

represented the functional activity of their pathways and that the two pathways worked in a reciprocal manner.

However, it has been shown that actinomycin inhibition of RNA synthesis still permits growth hormone stimulation of protein synthesis in rat liver ribosomes, (Korner, 1964), nor does actinomycin prevent the ability of insulin to stimulate glucose uptake by the diaphragm, (Eboue-Bouis et al, 1963; Wool and Mayer, 1964), or insulin stimulation of fatty acid synthesis in mammary gland slices, (Mayne and Barry, 1965), although it does prevent the synthesis of the lipogenic enzymes, (Gellhorn and Benjamin, 1964). Also actinomycin D does not impair the ability of hydrocortisone to maintain blood glucose levels in adrenolectomized rats, whereas the synthesis of PEP carboxykinase (enzyme 5 in fig.5) one of the "key" gluconeogenic enzymes, is impaired, (Kipnis and Kalkhoff, 1965).

Moreover, insulin possibly only directly controls the synthesis of glucokinase in diabetic animals, (Sharma et al, 1963; Kornaker and Lowenstein, 1964b, 1965a,b). Also, the <u>obese</u> animals are hyperinsulinaemic and yet have both high gluconeogenic enzymes and glycolytic enzymes, (Seidman et al, 1967). Thus in respect to insulin stimulation of synthesis, the "key" glycolytic enzymes do not always work as a co-ordinated unit, nor are their Vmaxs always the reciprocal of that of the gluconeogenic enzymes.

The mechanism of hormone action on protein synthesis therefore appears less simple than was at first imagined. We do not know which of the steps in protein synthesis are rate limiting, or which are stimulated or suppressed by hormone action (Korner, 1967). Nor is it clear which enzymes have their

synthesis directly effected by hormone action and which have their synthesis altered indirectly.

(iii) One of the earliest theories of insulin action came from the observation that it could facilitate the entry of glucose into muscle and other cells, having its primary effect at the level of the cell membrane (Levine and Goldstein, 1965), although its actual physical effect on the membrane surface is still not known, (Korner, 1967).

Although all the hormones mentioned have some kind of effect on lipid synthesis, the ones most implicated seem to be insulin and glucocorticoids, with the possibility that the adenyl cyclase system might provide the control framework for the action of these hormones.

Thus although the hormones might provide a general framework for the control of lipid synthesis there are many more agents that have more specific affects.

(2) TRICARBOXYLIC ACIDS (TCA)

The activity of highly purified acetyl CoA carboxylase (enzyme 22 in fig.5) was stimulated by prior incubation with citrate, (Brady and Gurin, 1952; Vagelos et al, 1963; Lynen et al, 1964) possibly due to trimerization, (Srere, 1965). However, C_{14} acetate incorporation into fatty acids was not stimulated by either citrate or isocitrate, (Myant and Iliffe, 1964), and the amount of citrate required to stimulate the enzyme is higher than could reasonably be expected under physiological conditions, (Lowenstein, 1964; Srere, 1965). Neither does citrate, in physiological concentrations, show the reported inhibition of phosphofructokinase, (enzyme 2 in fig.5)

or the reported stimulation of iso citric dehydrogenase, (enzyme 15 in fig.5), (review Srere, 1965).

(3) ADENOSINE NUCLEOTIDES. Besides being involved in the adenyl cyclase system, AMP inhibits fructose 1,6 diphosphatase (enzyme 6 in fig.5)(Krebs et al, 1963) and PEP carboxykinase (enzyme 5 in fig.5). ATP is a competitive inhibitor, with the substrate, for glucose 6 phosphate dehydrogenase, (Avigard, 1966) and inhibits pyruvate kinase, (Weber et al, 1967).

(4) METAL IONS, also affect specific enzymes. Mg^{++} and K^{+} activate pyruvate kinase and Ca^{++} and Na^{+} inhibits it, (Bygrave, 1967), Mg^{++} activates acetyl CoA carboxylase, (Greenspan and Lowenstein, 1967) and K^{+} activates and Na^{+} inhibits acetyl CoA synthetase, (von Korff, 1953). Although there are several more examples in the literature of metal ion action, their actual significance, under physiological conditions, is not known.

(5) DRUGS. Clinically, several drugs have been used recently to reduce weight in obese patients with maturity onset diabetes. These include sulphonyl urea and phenoformin, (Abraham and Arky, 1967; Wilansky and Hahn, 1967), although their action is in no way understood and there is not any indication of how they could help to investigate the control of obesity in the fat mutant mice.

(6) INHIBITORS OF LIPOLYSIS. Several agents have been reported to inhibit the release of free fatty acids from adipose tissue and thereby inhibiting

lipolysis. They include, nicotinic acid, prostaglandin E_1, glucose and 2-deoxy-d-glucose. The sites of action of these agents are often different and they have sometimes different affects IN VIVO from IN VITRO, but their significance in the control of lipid metabolism is not yet known, (review Carlson, 1965).

(7) FREE FATTY ACIDS AND THEIR ACYL CoA ESTERS. Of all the possible lipogenic controlling agents, so far discussed, only some lesion in the postulated adenyl cyclase system of hormonal control would seem able to effect the sweeping changes that have occurred in the obese and adipose mice, although there is no direct evidence that this system is involved. However, from work on obese mice there is a suggestion that the primary lesion in these animals might involve a lipogenic control system not yet discussed. This is the control of lipogenesis and the synthesis and activity of lipogenic enzymes by free fatty acids and their acyl - CoA esters. The most extensive work in this area has been on acetyl CoA carboxylase (enzyme 22 in fig.5). The activity of this enzyme, which had been purified many times is strongly inhibited IN VITRO by long chain acyl - CoA derivatives of fatty acids (for example palmityl CoA), in competition with the acetyl CoA substrate for the active enzyme sites, whereas free fatty acids have no effect, (Robinson et al, 1963; Borts and Lynen, 1963). Other workers, however, find no competition between acetyl CoA and palmityl CoA and have found that the free acids have been found to inhibit this enzyme. (Wieland and Weiss, 1963; Tubbs and Garland, 1964).

Other work has shown that the shorter chain fatty acid octanoate inhibits the activity of the glycolytic enzymes, (enzymes 1,2,3 in fig.5), and the pentose phosphate shunt enzymes (29,30 in fig.5), whereas it does not affect the Vmax of the "bifunctional" or gluconeogenic enzymes, (4,5,6, 7,8,9,10,11,12 in fig.5), (Weber et al, 1967a). Glucokinase was protected from octanoate inhibition by glucose and phosphofructokinase by fructose 6 phosphate but pyruvate kinase was not protected by its substrate, phospho enol pyruvate.

Thus the fatty acids, both in their free form and acyl-CoA esters inhibit the activity of a range of enzymes IN VITRO, (Lea et al 1967; Weber et al, 1967b).

Fatty acids are also known to affect lipogenesis and the synthesis of the lipogenic enzymes IN VIVO. The enzymes catalysing the synthesis of long chain fatty acids from acetate, (enzymes 22, 23 in fig.5) rise when assayed in liver or adipose tissue homogenates from rats on a fat-free high carbohydrate diet, (Chaikoff, 1953; Lyon et al, 1952; Tepperman and Tepperman, 1958, 1961). Similarly different parameters of lipogenesis: the activity of fatty acid synthetase (enzyme 23 in fig.5)(measured both spectrophotometrically and radioactively) or the incorporation of radioactive acetate or pyruvate: rose when measured in liver supernatant extracted from young mice, kept on a fat free diet. If the fat free diet given to these animals, is supplemented with 2% methyl palmitate or oleate, or 2% coconut oil and 1% cholesterol, the lipogenic parameters still remain four

fold higher than in control animals. However, addition of 2% linoleate or 2% corn oil, (which has a 50% linoleate content) brings these parameters down to normal levels within four days, (Allman and Gibson, 1967; Allman et al, 1965; Bartley et al, 1967). This work specifically points to linoleic as the important fatty acid in controlling lipogenesis.

Linoleic acid (with arachidonic and linolenic) has been known for a long time to be an essential fatty acid in the diet of rats, (Baldwin, 1947); its absence causes a deficiency disease characterised by a scaly condition of the tail (caudal necrosis). Linoleate is also essential in the diet for complete clearing of a condition known as "fatty liver" and for normal liver metabolism, (Mitchell, 1946; Mohrhauer and Holman, 1963; Sinclair and Collins, 1968).

Recently work on obese animals has suggested that they might have altered linoleic acid metabolism. It is shown in chapter 4, part (a) (table 1) from the work of Stein et al (1967) on the fatty acid profile of the triglyceride of the fat pad, of obese and normal mice, that, on starvation, there was a significant rise in the proportion of oleic acid and no change in the proportion of linolic acid in normal mice, whereas in obese there was no change in the proportion of oleic acid but a significant rise in linoleic acid, on starvation. Stein et al also found that oleic and linoleic acids make up to 88%of the fatty acids at the 2' position of the triglyceride from the fat pad of obese animals. This led them to assay the activity of 2-monoglyceride lipase and they found it to be five times higher in adipose tissue from normal mice than from

obese mice. The only agent that effects lipogenesis that is known to have an altered metabolism in obese animals is linoleic acid. The low activity of monoglyceride lipase in obese would lower the breakdown of monoglyceride, with its high linoleate content, to free fatty acids. Stein et al (1967) suggest that this monoglyceride is directly acylated at the 1' and 3' positions, back to triglyceride, thus lowering the amount of free linoleate that would be available to be used as a controlling agent in lipogenesis. This might account for the general rise in lipogenesis in the obese animals. These implications of Stein et al's work introduce chapter 4 part (c) and lead on to experiments that I have done to try to discover whether some lesion in control of lipogenesis by linoleate can be found in the obese and adipose syndromes.

Although it can be seen from this section that many agents can influence the rate of lipogenesis under different conditions, only the effect of linoleate has been studied in this thesis. If it is shown that linoleate is not the controlling agent in the obese or adipose syndrome then a further application will have to be made of the other factors mentioned in this section. In this light, the adenyl cyclase system for the control of hormone action appears to show most of the features of a crucial control mechanism and would warrant further study in these animals.

CHAPTER 3. METHODS

Part (a) : BREEDING

The obese mouse first appeared in a stock at the Jackson Laboratory, Bar Harbor, (Ingall et al 1950), and the adipose mouse in this laboratory in Edinburgh, (Falconer and Isaacson, 1959). Both adipose and obese animals are homozygous for recessive genes (ob/ob, ad/ad), that are fully penetrant and non allelic to each other. Obese and adipose mice are normally sterile, (Bunner and Gates, 1954; Lane and Dickie, 1954; Batt and Harrison, 1963). Recently another obese animal has appeared in a mouse stock at the Jackson Laboratory and has been called diabetes. Diabetes is also homozygous for a recessive gene (db/db), (Coleman and Hummel, 1967), and is non allelic to both adipose and obese, (Falconer, 1968).

The sterility of the obese and adipose animals complicates the breeding of the mouse stocks. In order to maintain a constant supply of the fat animals for experiments, littermates of obese (or adipose) had to be routinely crossed, as follows.

A normal littermate of obese (or adipose) has a 2/3rds chance of being heterozygous. On mating these littermates, there is a 4/9th chance that both parents will be heterozygous and their litter will contain obese animals. Unfortunately this breeding programme uses a lot of space, especially as the only way to tell whether both members of a mating are heterozygous, is to wait until it is possible to identify any obese (or adipose) mice in the litter. The obese mice are about five weeks old before they can be recognised whereas adipose cannot be recognised until they are

about seven weeks old, although this difference is probably an effect of the genetic background.

Of course, because of the nature of the breeding plan, mating littermates of adipose and obese animals, inbreeding depression occurred in both strains. To remedy this at various times both strains were crossed to the J.U inbred strain; the strain containing obese mice was crossed twice and the strain containing adipose mice was crossed once. This outcrossing again complicated the breeding plan.

At one stage in this investigation it was decided that it would be useful to try and get both adipose and obese mice in the same litter, and to look for the doubly homozygous animal adipose/obese. This would enable an investigation of adipose and obese in the same genetic background and thus it could be seen whether there was any difference between them.

This was done by taking known heterozygous ob/+ animals and ad/+ animals and crossing them. The F 1's were intercrossed. This gave a 1/16 chance that any one F 1 mating would be of doubly heterozygous animals. It was hoped to recognise these matings as the litters should have a high proportion of fat animals, i.e. 9 thin to 7 fat, or 9 thin to 6 fat, or 10 thin to 6 fat, depending on the phenotype and viability of the double mutant, compared with 3 thin to 1 fat from litters of F 1 matings where the animals are heterozygous for only one of these genes.

Out of fifty such F 1 matings, four had a very large number of fat animals in their litters. These matings were allowed to breed for three

litters and then the matings were split and both members of each mating were crossed, first to a known heterozygote ob/+ and then to a known heterozygote ad/+, to test whether they were indeed doubly heterozygous. One of the members of one mating died, but it was possible to prove that both members of the other three matings were in fact doubly heterozygous, (ad/+, ob/+).

CHAPTER 3. METHODS

Part (b): BIOCHEMISTRY

1. Extraction of Fat. A method of freezing the mice in liquid nitrogen and then grinding in a pestle and mortar was tried. This was inefficient, because, no matter how cold one tried to get the mortar some of the fat from the mouse stuck to the side. This is objectionable if one wants to get an estimate of the amount of fat per carcass. A Waring Blender was therefore used. The mice were killed and then immediately chopped up with some solvent in the Waring Blender. The chopped mouse was then washed with solvent into the filter paper cup of a large Soxhlet apparatus and refluxed gently with solvent.

Several types of solvent were tried for different lengths of time. Petroleum ether, diethyl ether and acetone, were experimented with, both singly and in combination. The most efficient solvent was found to be diethyl ether for a 12 hour period.

After the extraction the diethyl ether was gently distilled from the fat, the later stages taking place by blowing nitrogen across the fat. The nitrogen atmosphere was used to prevent the fat from hydrolysing and to try to keep the fat as close as possible to its in vivo condition.

The fat was then weighed and expressed as a percentage of the total carcass.

2. Fatty Acid Profile. The triglyceride was separated from the other lipid components by the method of Goodman et al (1962). A column of 10 mm internal diameter was used packed with 5 grams of silicic acid in 21%

benzene in hexane. The column was always kept moist. A sample of lipid, weighing about 100mgm was placed on top of the column and extracted with the following solvents. First, 85 mls of 21% benzene in hexane; second, 25 mls of benzene; and, third, 125 mls of chloroform. The triglyceride fraction comes down with the second and third solvents.

The triglyceride was concentrated under nitrogen and was then hydrolysed and the free fatty acids methylated for Gas Liquid chromatography by the following method, kindly supplied by Mr. I. McCartney. First the triglyceride was refluxed for 3 hours with 20% methanolic KOH and then it was extracted three times with 20 mls. of petroleum ether, (60°C to 80°C fraction). The petroleum ether was discarded. The aqueous fraction was acidified with concentrated HCl until the pH was brought down to 1.0 and then it was extracted again three times with 20 mls. of petroleum ether and the petroleum ether fractions were retained. These fractions were pooled and extracted three times with 20 mls of distilled water, the water being discarded. The petroleum ether fractions were then dried with anhydrous sodium sulphate overnight, under nitrogen, in the fridge. The petroleum ether was then blown off and the fatty acids concentrated under nitrogen. They were then methylated by boron trifluoride/methanol complex (from B.D.H.) for 2 minutes in a boiling water bath. The methylating reagent was destroyed with distilled water and the resulting mixture was extracted three times with 20 mls. of petroleum ether and the pooled petroleum ether fractions were again dried overnight in the fridge with sodium sulphate in a nitrogen atmosphere. Then the methylated fatty acids were

concentrated under nitrogen and a little taken up in a capillary and introduced into a Gas-Liquid chromatogram. The column used was of Apiezon M at a temperature of 200°C under a pressure of 10 lbs per square inch.

The fatty acids were easily identified by comparing their retention volumes with standard retention volumes and the amount of each fatty acid was calculated from the recorder chart by triangulation and expressed as a percentage of the total fatty acids present.

3. Extraction of Enzymes. Livers were extracted to provide a cell free supernatant for enzyme assays by the method of Kornaker and Lowenstein (1965). This was the method these workers used when they reported the alteration in ATP citrate lyase activity in obese animals. The first aim was to repeat this work and it was thought advisable to use their method, and it was proved satisfactory, it has been used for all the enzymes that have been investigated in this thesis.

The enzymes were, therefore, extracted by homogenising just less than one gram of rapidly cooled liver tissue with three volumes of cold (about 5°C) 0.25 molar sucrose solution, freshly prepared. The homogeniser used was an electric Tri-R, with Teflon pestle and a toughened glass mortar of length 4 inches and internal diameter half an inch. The pestle was plunged twice into the extraction media in the mortar, the whole process taking about 8 seconds. The extract was then put into 3 ml centrifuge tubes and spun at 60,000g_{av} for one hour in an MSE automatic superspeed 50 centrifuge at 0°C. The supernatant was withdrawn by Pasteur pipette and used as the basic material for all the enzyme assays.

All the apparatus used was pre-cooled in fresh ice before touching the extract and all operations were carried out at 0-5°C.

4. Estimation of Protein. The activity of all the enzymes were expressed per mgm of protein. The protein of the tissue extract supernatant was measured by a modification of the method of Lowry et al (1951), kindly supplied by Dr. M. Birnstiel. The estimation was carried out as follows:

Two stock solutions were made called Lowry A and Lowry B:-

Lowry A -	Na_2CO_3...	30 grams
	NaOH...	6 grams
	Na K tartrate	0.3 grams
	distilled water to	1,500 mls.
Lowry B -	$CuSO_4.4H_2O$...	2.5 grams.
	distilled water to	500 mls.

From these two stock solutions Lowry C was made fresh daily from 50 parts of Lowry A to 1 part of Lowry B.

A phenol reagent was made fresh daily from one part of Folin Ciocalteau's reagent, (from B.D.H.), to one part of distilled water. A protein stock solution from crystallised and lyophillised bovine serum albumin (from Sigma) to 500 µgrams per ml, were made fresh daily and used as comparisons with the experimental solutions to be assayed.

The enzyme extract to be assayed for protein concentration, was diluted 100 fold with distilled water. This dilution was found, in practice, to bring the extract within the range of this assay.

The 0.4 mls of the protein solution, (either standard or experimental) were added to 2 mls of Lowry C and well mixed by inversion. This mixture was left for 20 minutes at room temperature and then 0.2 mls of the phenol reagent were jetted in (to mix quickly) and again well mixed by inversion, and left to stand at room temperature for 30 minutes. The assay mixture was then read at 750 mμ in Beckman DB or Hilger Watt recording spectrophotometer, against a water blank that had been treated in the same way as the assay mixture. By comparing the absorbance reading of the assay solution with that of the five standard protein solutions a fairly accurate estimate could be made of the protein concentration. This estimation of protein concentration was repeatable at about 98% level.

5. Enzyme assays. In all the assays mentioned below the object was to measure the specific activity (Vmax) of the enzyme in μmoles of substrate removed or product formed per hour per mgm. of protein Thus any alteration to a published enzyme assay that increased the activity was considered justified. The assays were made as reliable and repeatable as possible under the conditions. All the assays were performed on either a Beckman DB recording spectrophotometer or on a Hilger Watt spectrophotometer with a Honeywell chart recorder attachment, at 37°C, and the rate of the reaction was measured over the first two minutes. For each animal each assay was done three to five times at different protein concentrations to ensure proportionality between enzyme activity and protein concentration. The chemicals were obtained from either Sigma or B.D.H. The assays used are listed below...

(a) ATP citrate lyase (EC 4.1.3.8 enzyme 18 in fig.5). The assay used for this enzyme is based on that of Srere (1959 a & b) and Kornaker and Lowenstein (1965). In the assay the production of oxaloacetate is measured by its reaction with NADH in the presence of malic dehydrogenase. The disappearance of NADH is measured at 340 mμ on the spectrophotometer. The assay mixture contained, 150 μmoles of Tris buffer pH 7.5 30 μmoles of potassium citrate, 10 μmoles of $MgCl_2$, 30μ moles of mercaptoethanol, 500 O.D. units of malic dehydrogenase, 0.5 mgm of coenzyme A, 0.2 mgm of NADH and 5 μmoles of ATP. The enzyme solution and distilled water were added to make the final volume up to 3 mls. The reaction was started by adding the ATP last and progress was measured at 340 mμ against a blank cell containing all the components except NADH and Coenzyme A.

The basic assay was developed by Srere (1959a & b) for the partly purified enzyme. However, when the purified malic dehydrogenase, (pig heart muscle from Sigma) was added to the crude enzyme extract being used, a lower and more variable Vmax was obtained than if this malic dehydrogenase was left out of the reaction mixture altogether. There was native malic dehydrogenase in the enzyme extract which is known to have a specific activity about 300 fold higher than ATP citrate lyase and using this alone gave a higher Vmax of ATP citrate lyase than if used in combination with the commercial type. So the commerical malic dehydrogenase was left out of the reaction mixture. Although this makes the assay more repeatable, both/one with enzyme extract and from week to week, it does not affect the ratio of the Vmaxs of extracts taken from obese or adipose animals to that of their normal littermates.

To keep this assay reliable it is also necessary to use freshly prepared sucrose extraction media and freshly prepared NADH, as both these substances, on storage, appear to accumulate material that in some way distorts this assay.

(b) Malic enzyme (EC 1.1.1.40 enzyme 21 in fig.5). The specific assay for the activity of malic enzyme was based on the method of Ochoa (1955c). The following assay mixture gave the best conditions to measure the Vmax of this enzyme: 1.5 mls. of 2.25 m.Molar Tris buffer of pH 7.5, 3 μmoles of $MnCl_2$, 0.3μmoles of NADPH and 50 μmoles of monosodium hydrogen L-malate. The enzyme solution and water were added to make the final volume up to 3 mls. The reaction was started by adding the L- malate and progress was measured at 340 mμ against a blank cell containing all the components except NADPH and L- malate.

(c) Malic Dehydrogenase (EC 1.1.1.37 enzyme 20 in fig.5). The Vmax malic dehydrogenase (MDH) was assayed with a method based on that of Ochoa (1955b) using the following reaction mixture: 150 μmoles of Tris buffer pH 7.5, 0.2 mgm of NADH and 10 μ moles of cisoxaloacetic acid. The enzyme solution and water were added to make the final volume up to 3 mls. The reaction was started by adding the cis oxaloacetic acid and progress was measured in the spectrophotometer at 340 mμ against a blank cell containing all the components except NADH and cis oxaloacetic acid.

(d) Pyruvate Kinase (EC 2.7.1.40 enzyme 3 in fig.5). There are several assays for the Vmax of partly purified pyruvate kinase reported in the literature, (for example Bucher and Pfeiderer (1955)) but only one method

is given that is applicable to a crude extract. This is the method of Weber et al (1965) and this was used as a basis for the assay employed here.

After a considerable number of trials the most reliable assay giving the highest Vmax for this enzyme was found to contain the following reaction mixture: 150 µmoles of Tris buffer pH 7.5, 100 µmoles of $MgCl_2$, 300 µmoles of KCl, 100 µmoles of potassium phospho enol pyruvate, 5 µmoles of ADP and 0.2 mgm of NADH. The enzyme solution and water were added to make the final volume up to 3 mls. The reaction was started by adding K PEP and progress was measured at 340 mµ in the spectrophotometer against a blank cell containing all the components except ADP and NADH.

The assay mixture by Weber et al (1965) contained lactic dehydrogenase but this was omitted from the assay mixture used here for two reasons. First, it was impossible to obtain commercial LDH from any supplier that did not have pyruvate kinase or pyruvate kinase-like activity and second, the normal enzyme extract contained 50 times as much LDH as pyruvate kinase, so further addition in the reaction mixture was unnecessary.

It must also be noted that the Vmax of the enzyme is higher if potassium ions are included in the reaction mixture in large amounts, especially if K PEP is used instead of Na PEP, unfortunately K PEP has to be used in rather large amounts and is very expensive and is also difficult to obtain. However, it has been shown that fructose 1.6 diphosphate added to the reaction mixture lowers the amount of K PEP needed in the assay mixture by at least ten fold, (Weber et al, 1967; Brock, 1968 personal

communication). Unfortunately as this effect could not be stabilised and repeatable results could not be obtained, the larger amounts of K PEP were still used.

(e) Lactic Dehydrogenase (EC 1.1.1. 27/28 enzyme 31 in fig.5). The Vmax was measured with an assay based on the method of Kornberg (1955) using the following reaction mixtures: 150 µmoles of Tris buffer pH 7.5, 0.2 mgm of NADH and 50 µmoles of sodium pyruvate. The enzyme solution and water were added to make the final volume 3 mls. The reaction was started by adding the sodium pyruvate and progress was measured on the spectro-:photometer at 340 mµ against a blank cell containing all the components except NADH and sodium pyruvate.

(f) Isocitric Dehydrogenase (EC 1.1.1.41, enzyme 15 in fig.5). The Vmax of isocitric dehydrogenase was measured with an assay based on the method of Ochoa (1955a) using the following reaction mixture: 100 µmoles of Tris buffer pH 7.4, 0.3 µmoles of NADPH, 1.0 µmoles of ethylamine diamine tetraacetic acid (EDTA), 4 µmoles $MnSO_4$, 10 µmoles of sodium D.L. isocitrate. The enzyme solution and water were added to make the final volume up to 3 mls. The reaction was started by adding the sodium D.L isocitrate and progress was measured on the spectrophotometer at 340 mµ against a blank cell containing all the components except the sodium D.L. isocitrate and NADPH.

(g) Fumarase (EC 4.2.1.2 enzyme 16 in fig.5). The Vmax of fumarase was measured with an assay based on the method of Racker (1950), using the following assay mix: 1.5 mls of 0.5 Molar malic acid, 1.4 mls. of 0.1

Molar sodium phosphate buffer pH 7.5 and enzyme solution and distilled water to make the total volume up to 3 mls. The reaction was started by adding the enzyme and progress was measured on the spectrophotometer at 240 mμ against a blank cell containing all the reagents except malic acid.

6. Preparation of Sodium Octanoate, Oleate and Linoleate... Sodium octanoate (caprylate) and oleate were bought from B.D.H.. They were also prepared by direct hydrolysis of the acids with NaOH and stored in 0.05 M solution of Tris buffer pH 7.5, sodium linoleate was also prepared in this way. Sodium octanoate or oleate behaved in the same way, as inhibitors, whether they were bought or prepared.

7. Preparation of the special diets ... A fat free diet was bought from Nutritional Biochemical (Chicago), (after Wooley and Sebrell, 1945). This diet was also of a high carbohydrate content containing about 60% Sucrose. Pure methyl linoleate and methyl oleate were obtained from Sigma as was the 75% crude linoleic acid.

8. Auro-thio-glucose injections ... To destroy the ventro-medial nuclei of the hypothalamus and make phenocopies of obese and adipose mice, auro-thio-glucose was used, (after Marshall and Mayer, 1954). This was injected intra-peritoneally at 1 mgm per gram body weight. This is a 50% lethal dose. Dead animals were seen in two to three days and the survivors became obese about two weeks to four weeks after injections.

CHAPTER 3. METHODS

Part (c): STATISTICAL

The statistical tests used were taken from Snedecor (1962) and were calculated on the Olivetti Programma Computer with programmes written by Professor D.S. Falconer and used in this Institute. The exception was the "t" test that was used. In most of my experiments pairs of obese (or adipose) and their littermates were used and to analyse this data there is a special paired "t" test which is more appropriate than the one usually used. Dr. R.C. Roberts kindly wrote the programme for this test which was also used on the Olivetti Programme.

CHAPTER 4. GENERAL PLAN OF THE INVESTIGATION

Part (a) : THE FATTY ACID CONTENT OF THE TRIGLYCERIDE.

It is important to find out if there is any difference in the type of fatty acids being synthesised in the fat pad of the obese or adipose mice before getting a possible indication of the amount of fatty acids being synthesised through looking at proposed rate controlling enzymes. Thus the percentages of the different fatty acids in the triglycerides of the abdominal fat pad were measured, using gas-liquid chromatography, in both obese and adipose and their littermates.

Table 1 shows a comparison of the percentages of the amount of the six most common fatty acids present in the triglyceride of the fat pad of obese and normal mice, calculated from my results and from the results of Haessler and Crawford 1965 and Stein et al 1967. The comparisons in Table 1 show that there are no large differences in percent of fatty acid between obese or adipose and normal animals. For none of the data in Table 1 is it possible to calculate standard errors, so it is not possible to say whether some of the small differences are statistically significant or not. In fact the reasonable similarity of data from different sources suggests that there is little strain difference in fatty acid profile. Stein et al did, however, point to the fact that, on starvation, there was a significant rise in the proportion of acid and no change in the proportion of linoleic acid in normal animals, whereas in obese mice, on starvation,

TABLE 1

Composition of Fat

Amounts of six fatty acids, expressed as a percentage of the total fatty acids, in the triglycerides of obese (ob), adipose (ad), and normal littermates (+)

		Source of data (references given below)									
		1		2		3		4		1	
Fatty acid		ob	+	ob	+	ob	+	ob	+	ad	+
Myristic	(14.0)	1	1	2	2	1	2	1	1	1	1
Palmitic	(16.0)	20	24	27	24	20	26	20	20	21	20
Palmitoleic	(16.1)	6	4	8	5	11	6	8	4	6	4
Stearic	(18.0)	3	3	3	2	2	5	1	5	3	4
Oleic	(18.1)	40	38	43	39	39	37	39	45	37	40
Linoleic	(18.2)	32	28	17	29	25	22	30	22	30	31

Sources of data.

1. Present work
2. Haessler and Crawford (1965)
3. Stein et al (1967) : fed mice
4. Stein et al (1967) : starved mice.

there was a significant rise in the proportion of linoleic acid but no change in the proportion of oleic acid. The significance of this observation is dealt with in chapter 4 part (e). Table 1 also shows that the fatty acid profile for adipose animals does not differ significantly from their littermates or from obese animals. There is no other work with which to compare the results for adipose animals and their littermates.

All these results indicate that it is not the type of fatty acid present in the triglyceride of the fat pad that is the significant difference between obese or adipose mice and their normal littermates.

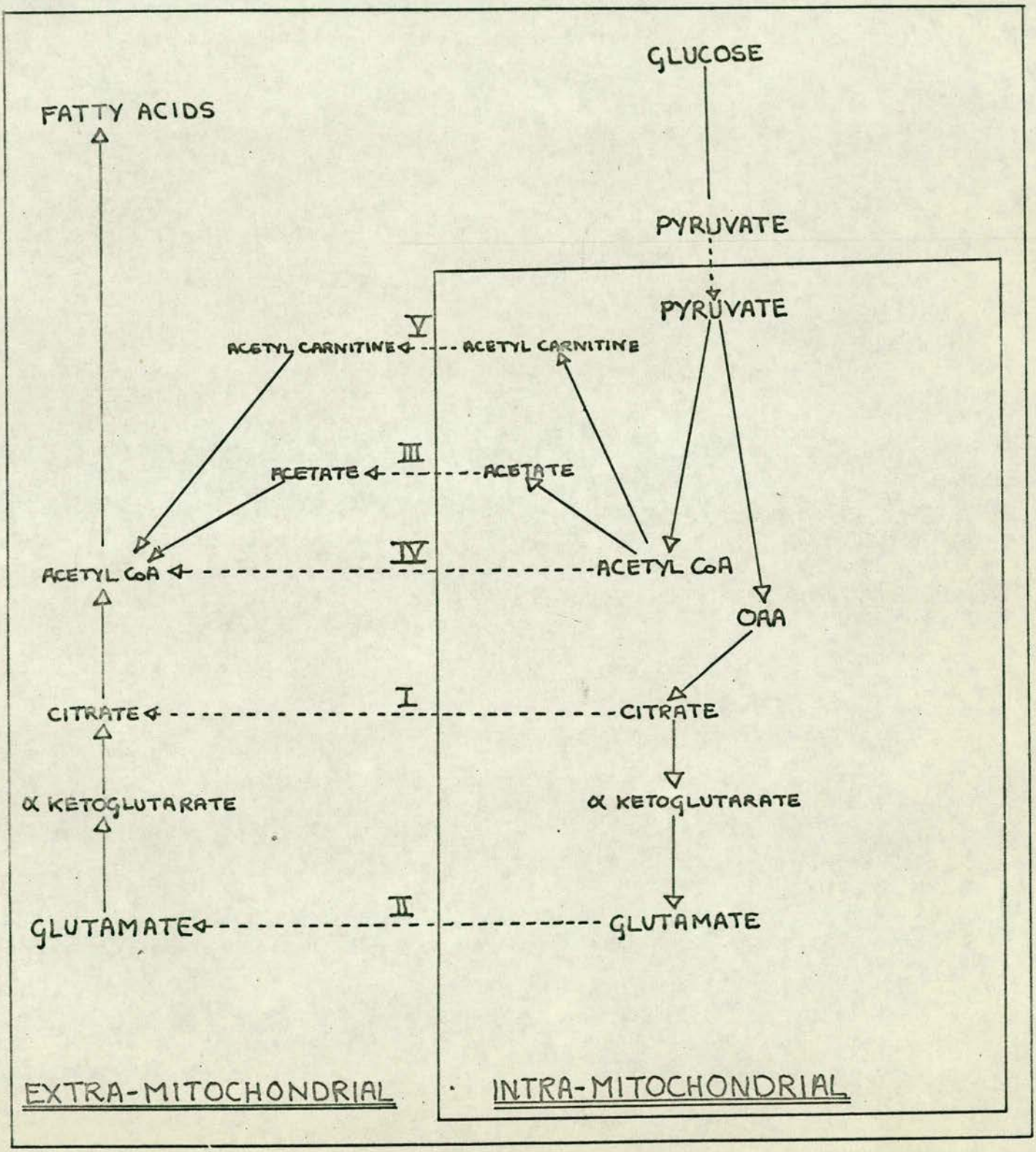
Part (b) : CITRATE AND THE ACTIVITY OF ATP CITRATE LYASE IN METABOLISM AND OBESITY.

The impetus for this investigation came from the report of Lowenstein's group, (Kornacker and Lowenstein, 1964; Spencer and Lowenstein, 1966), that the Vmax of ATP citrate lyase, (EC 4.1. 3.8, enzyme 18 in fig.5), was two to four fold higher when measured in liver supernatant extracted from obese animals compared with that from normal ones.

This discovery was not important on its own, but Lowenstein's group combined it with a lot of theoretical and experimental information on the function of this enzyme in lipogenesis with which they implied that the enzyme was "rate-limiting" and indeed that it might be the site of the primary biochemical lesion in these mice. The position and possible importance of ATP citrate lyase, in this part of metabolism, rests on the fact that some of the steps catalysing the conversion of carbohydrate to fat are situated in the mitochondria and some are outside it. I will first briefly survey the literature on the function of ATP citrate lyase before discussing my repetition of the work of Lowenstein's group with obese mice.

The main steps in the synthesis of fatty acids from carbohydrates are illustrated in fig.9, whereas fig.5 shows the detailed steps and the enzymes involved. It can be seen that carbohydrate is converted to pyruvate outside the mitochondria. The pyruvate diffuses through the mitochondrial wall and is converted to acetyl coenzyme A. Then, under conditions of

Fig. 9. The five possible routes from Pyruvate to Acetyl coenzyme A across the mitochondrial wall.



excess energy, (Green and Wakil, 1960; Wakil, 1962), much of the acetyl coenzyme A may be synthesised, into fatty acids. Many studies have shown that the synthesis of fatty from acetyl coenzyme A takes place outside the mitochondria, (Popjak and Tietz, 1955; Brady et al, 1956; Langdon, 1957; Matthes et al, 1960; Masaro et al, 1962; Dils and Popjak, 1962; Abraham et al, 1963; Harlan and Wakil, 1963; Spencer, Gorman and Lowenstein, 1964). Therefore the acetyl group of the intra-mitochondrial acetyl coenzyme A has to be transferred across the mitochondrial wall. Fig. 9 shows the five ways that this transfer could be effected. Most workers consider that the transfer of the acetyl group takes place as citrate, either by direct diffusion, (transfer I in fig.9), (Spencer and Lowenstein, 1962; Bhadure and Srere, 1963; Lowenstein, 1964; Lardy et al, 1964; Lardy et al, 1965; Greville, 1965; Daikuhara et al, 1968), or indirectly as glutamate, via α-keto glutarate (transfer II) (D'Adamo and Haft, 1962 and 1965; Madson et al, 1964; Hardwick, 1965); rather than as acetate (transfer III), acetyl coenzyme A (transfer IV) on acetyl carnitine (transfer V).

However, there is rather contradictory evidence for the direct diffusion of citrate across the mitochondrial wall, for it has been shown that, although mitochondria do contain 70% of the cell citrate, this concentration is not reduced by repeated washing of the isolated mitochondria, (Schneider et al, 1965; Srere, 1965). Although doubt has arisen over the direct diffusion of citrate, Lowenstein's group have approached the problem of the transfer of the acetyl group across the mitochondrial wall, from a

different direction. They compared the incorporation of labelled acetate (i.e. from transfer III) and labelled citrate (i.e. from transfer I) into fatty acids in liver slices and came to the conclusion, that of the two, only the rate of citrate incorporation was high enough to account for the rates of fatty acid synthesis that are known to occur in the liver. Their investigation of the importance of citrate or acetate as the precursor of acetyl coenzyme A, and therefore of fatty acid synthesis, led Lowenstein's group into a series of investigations of the activity of the enzymes that can supply acetyl coenzyme A, for fatty acid synthesis, from citrate, (ATP citrate lyase, EC 4.1.3.8, enzyme 18 in fig.5) and from acetate, (acetyl CoA synthetase, EC 6.2.1.1, enzyme 19 in fig.5), in relation to the rate of lipogenesis.

The activity of ATP citrate lyase responds in very close parallel to the rate of lipogenesis under a variety of condition of nutrition and hormone imbalance, whereas acetyl CoA synthetase does not. This has been interpreted to imply that ATP citrate lyase activity is causally related to fatty acid synthesis, (Kornaker and Lowenstein, 1963, 1965 a & b; Brown and McLean, 1965; Howanita and Levy, 1965; Brown et al, 1966; Kornaker and Ball, 1965), and rate controlling with respect to it (see Chapter 3 part (b)) (Kornaker and Lowenstein, 1963).

Thus with the work on the activity of these two enzymes in different conditions, it was logical that Lowenstein's group should examine their activity in a case of extreme hyperlipogenesis and obesity - the <u>obese</u> mouse.

ATP citrate lyase has a two to four fold higher specific activity, in the supernatant of a sucrose extract of livers from obese mice than in that of their normal littermates, whereas the activity of acetyl coA synthetase is only about 20% higher (Kornaker and Lowenstein, 1964; Spencer and Lowenstein, 1966). The implication is that this is a causal alteration in a rate controlling enzyme in lipogenesis and possibly the site of the primary genetic lesion in obese animals. This implication can be checked against the genetic criteria outlined in the Introduction, (chapter 1). First, the primary genetic alterations are probably different in adipose to obese. Secondly, the primary biochemical lesion should exhibit segregation in apparently normal littermates of obese animals and thirdly, if the primary lesion is simply an alteration in enzyme activity, then the enzyme should have a lower activity in obese animals because the ob gene is recessive.

The third criterion is known not to be satisfied in the obese syndrome as these animals have a higher Vmax for ATP citrate lyase than their normal littermates, and the second criterion also appears not to be satisfied as there has been no report of different classes of Vmax within littermates of obese animals.

It is possible to investigate this second criterion more fully and to see whether the first criterion (i.e. is the Vmax of this enzyme different in obese compared with adipose animals) is fulfilled.

Accordingly the Vmax of ATP citrate lyase was determined in pairs of obese or adipose animals and their normal littermates. The body weight and

carcass fat of these four types of animals was also determined.

Also a breeding programme was carried out to see whether the double mutant obese/adipose(ob ob/ad ad) was any different, either physically or in terms of the Vmax of ATP citrate lyase, from either adipose or obese. This programme at the same time produced obese and adipose animals in the same litter and therefore most of the difference in genetic background was removed that was previously present between these animals.

RESULTS

Table 2 shows the live weight, amount of fat and percentage fat of obese and adipose animals and their normal littermates. Table 3 shows the Vmax of ATP citrate lyase for the same four types of animal, and fig.10 shows the frequency distribution of Vmaxs of the same enzyme for animals whose parents were heterozygous for both the ob and ad gene. Table 3 shows that the Vmax of ATP citrate lyase is 2.04 fold higher in obese animals than in normal littermates and that adipose animals is 1.95 fold higher than from normal littermates. There is very little difference in the Vmax of the enzyme between adipose and obese animals although there is some difference between their littermates, probably due to differences in genetic background. In fact, a difference of genetic background is evident in the carcass composition of the two strains (Table 2). Both the percentage and the total amount of fat is higher in the littermates of adipose than in those of obese. This suggests that the slight difference in Vmax between the obese and adipose animals is correlated with the amount of fat in the carcass as determined by the genetic background.

TABLE 2

The live weight and the weight of fat expressed both in grams and as a percentage of live weight of obese animals, adipose animals and their normal littermates.

	number of pairs of observations	Mean of obese	Mean of normals	Mean difference ± standard error	Obese as a percentage of normal	t	P
Live weight	19	60.7	32.4	28.3 ± 2.6	187	10.9	<0.001
weight of fat	10	14.2	3.0	11.2 ± 2.7	-	4.2	<0.01
percentage of fat	10	33.4	12.0	21.4 ± 2.1	-	10.0	<0.001
		Mean of adipose	Mean of normals		Adipose as a percentage of normal		
Live weight	13	49.8	34.6	15.2 ± 2.1	144	7.4	<0.001
weight of fat	10	17.0	6.6	10.4 ± 1.5	-	7.1	<0.001
percentage of fat	10	36.8	20.2	16.6 ± 2.3	-	7.4	<0.001

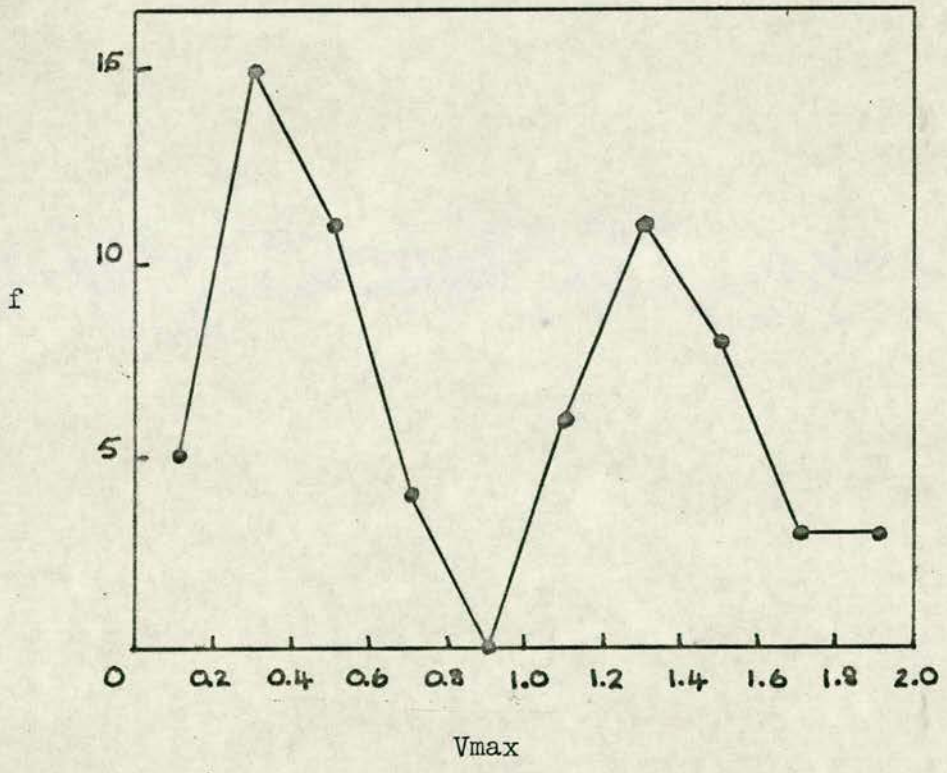
TABLE 3

The Vmax of ATP citrate lyase (as μ moles/hour/mgm protein) of obese animals, adipose animals and their normal littermates.

	number of pairs of observations	mean	mean differences ±	standard error	mutant as a % of normal	t	P
obese normal	23	1.19 0.45	0.74 ±	0.102	264	7.3	<0.001
adipose normal	13	1.25 0.64	0.61 ±	0.125	195	4.9	<0.001

Fig.10. Frequency distribution of the Vmax of ATP citrate lyase, expressed a μ moles/hour/mgm protein, of animals from litters whose parents were known to be heterozygous for both the ad and ob genes, (i.e. ob+, ad+).

total animals	66
animals classed "fat"	35
animals classed "thin"	31



The normal littermates of both obese and adipose showed no evidence of segregation of the Vmax of ATP citrate lyase into two classes. There was therefore no evidence that enzyme's activity differed in heterozygotes and normal homozygotes, and this argues against ATP citrate lyase being the site of the primary lesion, by the second genetic criterion. As there is no significant difference in the Vmax of this enzyme between obese and adipose, the third genetic criterion for the primary lesion is also not fulfilled, which indicates that ATP citrate lyase is not the primary lesion in these animals.

The lack of difference between obese and adipose is confirmed by the results of the breeding programme aimed at producing a double mutant (see chapter 3, part (a)). The proven doubly heterozygous matings in this programme had 66 offspring which fell only into two classes, (31 thin, 35 fat) both in terms of physical appearance and ATP citrate lyase activity (see fig. 10), these classes coinciding for both parameters.

From this experiment it can be seen that (a) no phenotypically recognisable adipose/obese (ad ad/ ob ob) animals have appeared, (b) there are no classes of the Vmax of ATP citrate lyase among the "fat" animals and (c) the probability that the sample of 66 animals taken did not include a double homozygote is small ($P = 0.014$). No further tests of the genetic constitution of these offspring were made.

Therefore, unless the double mutant is lethal, it may be concluded, that the Vmax of ATP citrate lyase from obese = adipose = adipose/obese and that ATP citrate lyase is probably not the primary lesion in obese or adipose animals.

The above results do indicate that the altered activity of ATP citrate lyase is not the only cause of obesity in obese and adipose animals. Recently doubt has been thrown on changes in activity of this enzyme controlling changes in lipogenesis in starvation and re-feeding conditions as well. ATP citrate lyase activity was originally reported as decreased on starvation, (Kornaker and Lowenstein, 1965 a), whereas recent work (Srere, 1965; Srere and Foster, 1967) has shown that, although there is a parallel decrease in ATP citrate lyase activity and lipogenesis after 48 hours starvation, lipogenesis had decreased to zero by 24 hours and at that time the activity of ATP citrate lyase has not changed. This indicates that the decrease in the enzyme in starvation is a result of decreased lipogenesis and not the cause.

At this stage, I wondered whether other enzymes closely involved in lipogenesis, had had their Vmaxs altered in obese and adipose animals. If this were so, not only would it confirm the above conclusion but it also would show that this enzyme may not be the only factor affecting the obesity in these animals.

CHAPTER 4. GENERAL PLAN OF THE INVESTIGATION

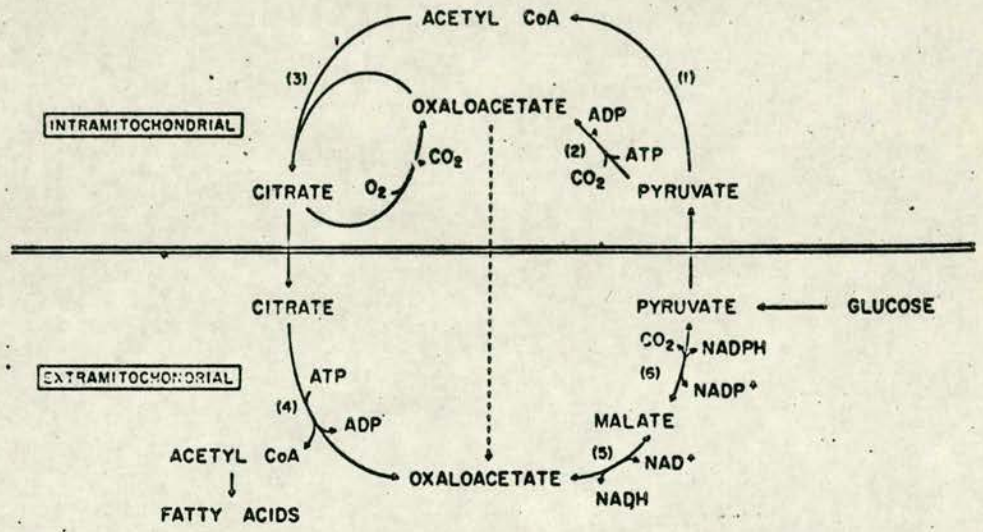
Part (c): THE ACTIVITY OF OTHER ENZYMES IN OBESITY AND METABOLISM

If the Vmaxs of several enzymes catalysing reactions closely connected with lipogenesis have been altered in obese and adipose animals, this not only makes it doubtful that the alteration in ATP citrate lyase is the primary enzymic lesion, in obese mice, but also makes these enzymes candidates for the primary lesion themselves. Then, these other enzymes must also be tested by the same three genetic criteria outlined in chapter 1, first that the primary enzymic lesions are probably different in adipose to obese animals. Secondly, that the primary enzymic lesion should exhibit segregation in apparently normal littermates of obese and adipose animals and thirdly, if the primary lesion is simply an alteration in enzyme activity, then the enzyme will have a lower activity in obese or adipose animals than in their normal littermates.

So the activities of several enzymes were determined for several reasons as outlined below. Malic enzyme (Ec 1.1.1.40, enzyme 21 in fig.5), malic dehydrogenase, (EC 1.1.1.37 enzyme 20) and pyruvate kinase (EC 2.7.1.40, enzyme 3) were assayed because they had been reported to catalyse steps important in the control of lipogenesis. Isocitric dehydrogenase (ICDH) (EC 1.1.1.41, enzyme 15) and fumarase (EC 4.2.1.2, enzyme 16) were assayed as representatives of the TCA cycle, and lactic dehydrogenase (LDH)(EC 1.1.1.27/38, enzyme 31) was assayed as a standard.

The roles of malic enzyme and malic dehydrogenase in lipogenesis can be described with ATP citrate lyase as being involved in an integrated chain of reactions known as the transhydrogenation shunt (fig.11)

Fig.11. The transhydrogenation shunt, (from Kornaker and Ball, 1965).



[redacted].—Postulated scheme for lipogenesis from glucose in adipose tissue. The reactions indicated are catalyzed by (1) pyruvate oxidase; (2) pyruvate carboxylase; (3) citrate condensing enzyme; (4) citrate cleavage enzyme; (5) malate dehydrogenase; and (6) malic enzyme.

(Wise and Ball, 1964; Kornaker and Ball, 1965; Leveille and Hanson, 1966; Ballard and Hanson, 1967; Simpson et al, 1968).

The net result of this transhydrogenation shunt (fig.11) is the conversion of molecule of pyruvate to acetyl CoA, while one molecule of NADH has been converted to NADPH. NADPH is the reduced coenzyme specifically needed for fatty acid synthesis. The cycle should be energetically self sufficient as the two high energy phosphates, that are required, could be met by the reduction of the O_2 by the reduced flavoprotein produced by the conversion of the pyruvate to acetyl CoA. The cycle provides one molecule of NADPH per molecule of acetyl CoA which is half the amount needed to convert the acetyl CoA to fatty acids, the rest coming from the pentose cycle (Kornaker and Ball, 1965). This is in good quantitative agreement with the observation that the pentose cycle can only provide about half the NADPH needed for fatty acid synthesis, (Jungas and Ball, 1961; Flatt and Ball, 1964).

Thus there is a close relationship between lipogenesis and the transhydrogenation shunt. For this reason the specific activity of malic enzyme and malic dehydrogenase were assayed in the<u>obese</u> and <u>adipose</u> mice and their normal littermates.

On the other hand pyruvate kinase activity has been correlated with the nutritional state of the animal in the same way that ATP citrate lyase has been (Krebs and Eggleston, 1965). Moreover, in many cases pyruvate kinase has been reported to be a rate controlling enzyme in glycolysis,

(review, Weber et al, 1967). So the specific activity of this enzyme was assayed in obese and adipose mice as a representative of the "key" glycolytic enzymes, (see chapter 2, part (b) for a discussion of the enzymes and rate control where this enzyme is specifically referred to).

RESULTS

Table 4 shows there is a 2.67 fold increase in the Vmax of malic enzyme, in obese mice over their normal littermates and table 5 shows a 1.77 fold increase in adipose mice. Tables 4 and 5 also show a 1.53 fold increase in the Vmax of pyruvate kinase in obese mice, compared with their normal littermates and a 1.42 fold increase in adipose mice. Thus two other enzymes, both reported to have a rate controlling function, with regard to lipogenesis, have increased Vmaxs in both obese and adipose animals. As has been discussed before, this probably means that these enzymes are not the sites of the primary lesions in these animals.

Tables 4 and 5 also show that there is little difference in the Vmax of MDH, LDH and fumarase in obese, adipose and their normal littermates. There is, however, a slight drop (5 to 15%) in the Vmax of ICDH in both adipose and obese animals.

DISCUSSION

These results suggest, that although there are now three enzymes known to have higher Vmaxs in obese and adipose animals it appears that none of them is the primary lesion and their rise is an affect of some other primary event.

TABLE 4

The Vmax of, malic enzyme, malic dehydrogenase (MDH), pyruvate kinase (PK), lactic dehydrogenase (LDH), isocitric dehydrogenase (ICDH) and fumarase (FUM) expressed as μ moles/hour/mgm protein for obese animals and their normal littermates.

enzyme	Number of pairs of observations	Mean of obese	Mean of normal	Mean difference ±	standard error	Obese as a % of normal	t	P
malic	15	15.6	5.8	9.8 ±	1.57	267	6.2	<0.001
MDH	13	273	255	18 ±	16.1	107	1.1	N.S.
PK	13	16.1	10.5	5.6 ±	1.37	153	4.1	<0.01
LDH	8	106	97	9.0 ±	6.43	109	1.4	N.S.
ICDH	8	16.1	17.0	(-)0.9 ±	0.53	95	1.6	N.S.
FUM	9	20.1	19.7	0.4 ±	0.61	102	0.7	N.S.

TABLE 5

The Vmax of malic enzyme, malic dehydrogenase (MDH), pyruvate kinase (P.K), lactic dehydrogenase (LDH), isocitric dehydrogenase (ICDH) and fumarase (FUM) expressed a μ moles/hr/mgm protein from adipose animals and their normal littermates.

enzyme	Number of pairs of observations	Mean of adipose	Mean of normal	Mean difference ±	standard error	Adipose as a % of normal	t	P
malic	10	21.2	12.0	9.2 ±	2.06	177	4.5	< 0.01
MDH	10	365	382	(-)17 ±	11.3	96	1.5	N.S.
PK	10	12.5	8.8	3.7 ±	0.87	142	4.3	< 0.01
LDH	8	118	103	15 ±	1.83	116	8.1	< 0.001
ICDH	8	18.5	21.7	(-)3.2 ±	0.72	85	4.5	< 0.05
FUM	9	18.1	17.0	1.1 ±	0.83	107	1.3	N.S.

Recently several reports have confirmed and extended my results with obese mice to show the wide alterations of enzyme activity in this syndrome. None of these reports include results of work on adipose animals.

It has been confirmed that liver pyruvate kinase does have about a two fold higher Vmax in obese mice, (Fried, 1967; Seidman et al, 1967), whereas the Vmax's liver lactic dehydrogenase and malic dehydrogenase are only slightly higher, the latter differences being very small or non-existent in other tissue, (Fried and Antopol, 1967). It had earlier been reported that liver phosphorylase had a higher activity in obese mice than in normal ones, whereas muscle and kidney hexokinase (EC 2.7.1.1) (enzyme 1 in fig. 4 and 5) and liver glucose 6 phosphatase (EC 3.1.3.9) (enzyme 7 in fig. 4 and 5) had the same activity in both sort of animals, (Shull, Ashmore and Mayer, 1956). While with a histochemical staining technique, a more intense reaction was seen for acid phosphatase (EC 3.1.3.2), ATPase, (EC 3.6.1.3) and glucose 6 phosphatase (EC 3.1.3.9), in pancreatic tissue from obese mice than in tissue from normal ones, (Hellerstrom et al 1965; Fried and Antopol, 1966).

The activity of glycerolkinase (EC 2.7.1.30)(enzyme 27 in fig.5) is two fold greater in adipose tissue from obese mice IN VITRO, (Lochaya Hamilton and Mayer, 1963; Treble and Mayer, 1963). Mayer and Thomas (1967) consider that this two fold difference IN VITRO would be reflected in a 40 fold difference IN VIVO as the obese mice have 20 times as much adipose tissue as normal mice, and this difference in enzyme activity is the basic alteration in obese animals that can explain all the others.

It has been shown in chapter 2, part (b), that this sort of extra-:polation from an IN VITRO to an IN VIVO might be wholly unjustified. Despite this Mayer and Thomas reason that, normally, in the absence of glycerokinase adipose tissue cannot reutilise glycerol released by lipolysis and must depend on glycerophosphate provided by glycolysis to resynthesise triglyceride, (fig.5). Consequently lipogenesis in adipose tissue is normally controlled by the availability of glycerophosphate and indirectly, of glucose. Mayer and Thomas consider that the abnormal presence of glycerokinase activity in the adipose tissue of the obese mice thus provides an explanation of the decreased dependence on carbohydrate metabolism of these animals; thus the concentration of glycerophosphate in the tissues due to lowered rates of conversion may explain why the glucose uptake of adipose tissue is decreased in obese animals, even in the presence of increased insulin. This will cause an accumulation of glucose in the blood which may, in turn, be the reason for the decreased release of free fatty acids during fasting under the influence of epinephrine or FMS. The reaction to this hyperglycaemia could, in turn, cause the hyperplasia of the islet of langerhans, increasing the production of insulin and resulting in the degranulation of the islets/increase in circulating insulin. Finally, Mayer and Thomas suggest that the increased blood sugar and insulin concentration may cause increased synthesis of fat and glycogen in the liver, as is found in obese animals.

The whole of Mayer and Thomas's explanation of the obese syndrome rests on their finding that the activity of glycerolkinase is two fold

higher in adipose tissue from obese mice, IN VITRO, causing a 40 fold rise, IN VIVO. As well as from the theoretical point of view, from the practical one, this proposed 40 fold rise in adipose tissue of glycerol kinase, in obese mice does not seem to be reflected by any large increase in the IN VIVO incorporation of either glycerol-1,3-C_{14} or glycerol-2 H_3 into total carcass fatty acids in obese animals compared with their normal littermates, (Schreeve et al 1967). Also, it has been shown that the metabolic derangements of the liver in obese animals could be more fundamental to the development of the obesity, than the changes seen in extrahepatic tissues, (Fried and Antopol, 1966; Schreeve et al, 1967; Jansen et al, 1967); moreover, there is no difference in the activity of glycerokinase assayed from the livers of normal and obese mice, (Stein, 1968, personal communication). So it appears that any change in glycerokinase activity in obese mice is no more important and probably less significant than the activity changes in the other enzymes.

Lipase activity in adipose tissue from obese mice is about 60% of that from normal mice, (Lochaya Hamilton and Mayer, 1963), whereas the specific 2-monoglyceride lipase has an even lower activity in adipose tissue from obese mice being about 20% of that from normals, (Stein, Anderson and Hollifield, 1967), although there is no difference in the activity of clearing factor lipase, (Robinson, 1967, personal communication).

Acetyl CoA carboxylase (EC 6.4.1.2) (enzyme 22 in fig.5) and fatty acid synthetase (EC 6.2.1.3) (enzyme 23 in fig.5) both have two fold higher Vmaxs when assayed from the livers of obese mice, (Chang et al, 1967). Both these enzymes have been considered to catalyse important

rate controlling steps in fatty acid synthesis (see chapter 2, part (b)), (Wieland et al, 1963; Numa et al, 1961; Lynen et al, 1963; Korchak and Masuro, 1962; Gibson and Hubbard, 1960; Gibson et al, 1960; Gellhorn and Benjamin, 1964).

The specific activity of α-glycerophosphate dehydrogenase, (EC 1.1.1.8) (enzyme 32 in fig.5) was two fold higher in liver supernatant from obese mice, though there was no difference from normal activity when it was assayed from kidney, muscle brain or adipose tissue, whereas glucose 6 phosphate dehydrogenase (EC 1.1.1.49) (enzyme 29 in fig.5) had about 40% higher activity when assayed from both liver and adipose tissue in obese, although succinic dehydrogenase (EC 1.3.99.1)(enzyme 33 in fig.5) was the same in all tissues from both obese and normal animals, (Fried and Antopol, 1966).

Glucokinase (EC 2.7.1.2)(enzyme 1 in fig.5), phosphofructokinase, (EC 2.7.1.11)(enzyme 2 in fig.5) and fructose 1,6 diphosphatase (EC 3.1.3.11) (enzyme 6 in fig.5) all had Vmaxs two to four fold higher in livers from obese animals, while the activity of phosphoglycerate kinase (EC 2.7.2.3) (enzyme 11 in fig.5) was only a little higher, (Seidman et al, 1967).

Recently a third genetically fat mouse has been found. This is also due to the homozygous condition of a recessive gene and has been called diabetes (dbdb). The db gene is non-allelic to ob and ad, although, as well as being physically similar to the other mutants, has several similar alterations in enzyme activity. ATP citrate lyase, Acetyl CoA synthetase and glucokinase, all had two to four fold higher specific activities in

diabetic, although that of glucose 6 phosphate dehydrogenase was the same as in normal animals. The activities of the "key" gluconeogenic enzymes, glucose 6 phosphatase dehydrogenase was the same as in normal animals. The activities of the "key" gluconeogenic enzymes, glucose 6 phosphatase (EC 3.1.3.9) (enzyme 7 in fig.5), fructose 1,6 diphosphatase (EC 3.1.3.11)(enzyme 6 in fog.5) pyruvate carboxylase (EC 6.4.1.1)(enzyme 4, fig.5) and phospho enol pyruvate carboxykinase (EC 2.7.1.40)(enzyme 5 in fig.5) were 20 to 40% higher in diabetic animals (Coleman and Hummel, 1967).

Thus a whole range of enzymes have had their Vmaxs altered in obese animals. Some of these enzymes have also been assayed in adipose and diabetic animals and have been found to have similar higher Vmaxs, in these animals as well.

It can be concluded from these results that the change in Vmax of ATP citrate lyase in obese is not the only cause of the obesity of these animals. The enzymes that do have altered activity in these syndromes mainly have it altered to a higher level. It has already been pointed out that this does not fulfil one of the genetic criteria discussed in chapter 1. Only in this investigation have these enzyme alterations been tested against the other two genetic criteria from chapter 1. However, one of the lipases assayed in obese animals does have a lower activity in this syndrome and this is dealt with in part (e) of this chapter.

It is, however, possible that the range of enzyme alterations seen in obese and adipose mice might be exclusive to this particular kind of obesity syndrome. Fortunately, it is possible to compare the results

on obese and adipose animals with another completely different obese syndrome in mice caused by aurothioglucose injections of normal mice. This work is described in the next part of this chapter.

CHAPTER 4. GENERAL PLAN OF THE INVESTIGATION

Part (d): THE VMAXS OF ENZYMES IN OBESE AND ADIPOSE ANIMALS AND THEIR PHENOCOPIES.

If it can be shown that the same changes that have taken place in the activity of enzymes in obese and adipose animals have also occured in another completely different obese syndrome in mice, then it can be said that the changes in enzyme activity in the genetically fat mice cannot be the only or primary cause of the obesity in these animals. It has been postulated that the hypothalamus is involved in the initiation and termination of feeding, (Anarnd et al, 1955); the ventro-lateral area being involved in initiation, (known as the feeding centre), and the ventro-medial nuclei controlling termination, (known as the satiety centre). Electrolytic and physical destruction of these centres caused aphagia, in the case of the feeding centre, and hyperphagia in the case of the satiety centre (Anarnd et al, 1955; Brobeck, 1946; Mayer et al, 1955). The hyperphagia caused by the destruction of the satiety centre makes rodents extremely obese. Obesity, due to hyperphagia, can also be caused, in rodents, by a 50% lethal intraperitoneal injection of auro-thio-glucose, (Marshall and Mayer, 1954; Mayer and Brecher, 1950; Mayer and Zighera, 1964) which has been shown to cause considerable damage to the ventromedial nuclei of the hypothalamus and therefore is considered to be directly equivalent to their physical destruction, (Mayer et al, 1955; Mayer and Thomas, 1967; Debons et al, 1968). Therefore injections of auro-thio-

glucose provide a simple way of creating obese hyperphagic phenocopies for obese and adipose mice.

Already several physiological parameters have been compared in obese mutants and aura-thio-glucose (ATG) phenocopies, made from their normal littermates. Although ATG and obese animals are physically similar there are many physiological differences between them, the more important ones are summarised in fig.13 from the review by Mayer 1960.

On starvation to normal body weight (25-30 grammes) the ATG animals assume the same body proportions as untreated animals, whereas the obese animals are still obese and laying down fat at the expense of other body tissues. This difference in combination with others in fig.13 have led to Mayer to postulate two completely separate mechanisms for these two obesities. Mayer considers the ATG animals are an example of "regulatory obesity", entirely due to these animals hyperphagia, whereas the obese animals are an example of a "metabolic obesity" due to some metabolic lesion which cause all the available energy to be sucked into lipogenesis at all nutritional states. This is shown by the fact that the ATG animals grow fastest on a high fat diet and the obese animals grow best on a high carbohydrate diet, thus the ATG animals lay down fat most on a diet with highest possible energy intake, whereas the obese animals appear to have some metabolic impairment in the control of lipogenesis from carbohydrates.

Therefore the obesity in ATG animals appears to be a completely different syndrome to that in obese animals, and the injection of

Fig.13. Comparison of obese and ATG mice from the same litter, (from Mayer, 1960)

Object of comparison	obese	ATG
Etiology	Mendelian recessive	1 mg./gm. goldthioglucose
Pathology and mechanism	Pancreatic dysfunction, hyperplasia of islets of Langerhans, increased insulin and glucagon secretion.	Hypothalamic lesions: destruction of cells regulating intake in ventromedial area
Energy balance	Positive during moderate hyperphagia, moderate or small increase in O_2 consumption, activity is drastically decreased	Positive during considerable hyperphagia
Effect of type of diet	Maximum weight gain on high carbohydrate, less on protein, less or decreased on high fat.	Maximum weight gain on high fat diet, less on carbohydrate, decreased on high fat
Effect of weight reduction	Body composition remains obese: i.e. animal loses nitrogen as well as fat, but is still obese when weight is normal or below normal.	Brings body composition back to normal
Resistance to cold	Drastically reduced.	Normal
Blood glucose levels	Generally hyperglycemic; further increased by growth hormone, etc.	Normal
Total levels of blood lipids	Elevated	Elevated
Blood cholesterol levels	Elevated; further elevated by growth hormone, etc.	Normal
Effect of administration of hormones	Abnormal sensitivity to hyperglycemic effects of growth hormone, glucagon, etc. Increased resistance to insulin.	Normal
Mating behavior	Absent	Normal, though less frequent.
Lipogenesis *in vivo*	Increased with hyperphagia and increased during fasting. Fatty acids are broken down and resynthesized abnormally fast.	Increased with hyperphagia, normal during fasting. Normal fatty acid breakdown.
Hepatic lipogenesis *in vitro*	Increased with hyperphagia and increased during fasting.	Increased with hyperphagia, normal during fasting.
Adipose tissue metabolism	Glucose oxidation half of normal, impaired pyruvate metabolism	Glucose oxidation normal, pyruvate metabolism normal
Cholesterogenesis *in vivo*	Increased during fasting	Normal during fasting
Acetate pool and turnover	Increased pool; the rate of turnover was considerably increased	Normal
Liver glycogen turnover	Considerably increased	Normal phosphorylase
Enzymatic activities	Increased liver phosphorylase	Increased in proportion to increase in hyperphagia
Intestinal absorption	Increased in proportion to increase in hyperphagia	High body fat, slightly increased protein; cholesterol content normal; enlarged liver, kidneys, ovaries and uteri
Body composition and the size of specific organs	High body fat, decreased protein, cholesterol content increased with weight, enlarged liver, heart, pancreas, thymus, adrenals; decreased uteri, ovaries, brain.	Increased in proportion to increase in body fat
Retention of steroid hormones	Increased in proportion to increase in body fat	Slightly increased
Ketone levels of fed animals	Slightly increased	Normal elevation of levels of blood ketones
Effect of fasting on levels of blood ketones	Decreased	

aurothioglucose will provide an obese phenocopy with which to compare the enzyme alterations in obese and adipose mice.

RESULTS

Fig.14 is the growth curves of three littermates, one an obese, one normal and one normal but injected with aurothioglucose, from the point of the ATG injection. This figure shows that animals injecting with aurothioglucose grow at the same rate as obese animals but never attain the same weight for age. This appears to be due to the "start" the obese animals have over the ATG animals up to the time of injection and this can be seen from fig.14.

Tables 6 and 7 show the weight and the specific activity of ATP citrate lyase, malic enzyme, malic dehydrogenase and pyruvate kinase assayed in obese and adipose animals, and their normal littermates. Tables 6 and 7, like fig.14, also show that ATG mice never quite reach the same weight as obese or adipose mice. These tables also show that the alterations in the Vmax of ATP citrate lyase, malic enzyme, malic dehydrogenase and pyruvate kinase are in the same direction and of the same order in ATG animals as in their obese and adipose littermates.

DISCUSSION

The results in tables 6 and 7 show that the Vmaxs of enzymes, if altered in obese or adipose mice, are also altered in their ATG phenocopies. As ATG mice appear a completely different syndrome to obese and adipose it is unlikely that any of the alterations in Vmax found will be the only

Fig.14. The growth of obese, ATG and normal mice from the same litter.

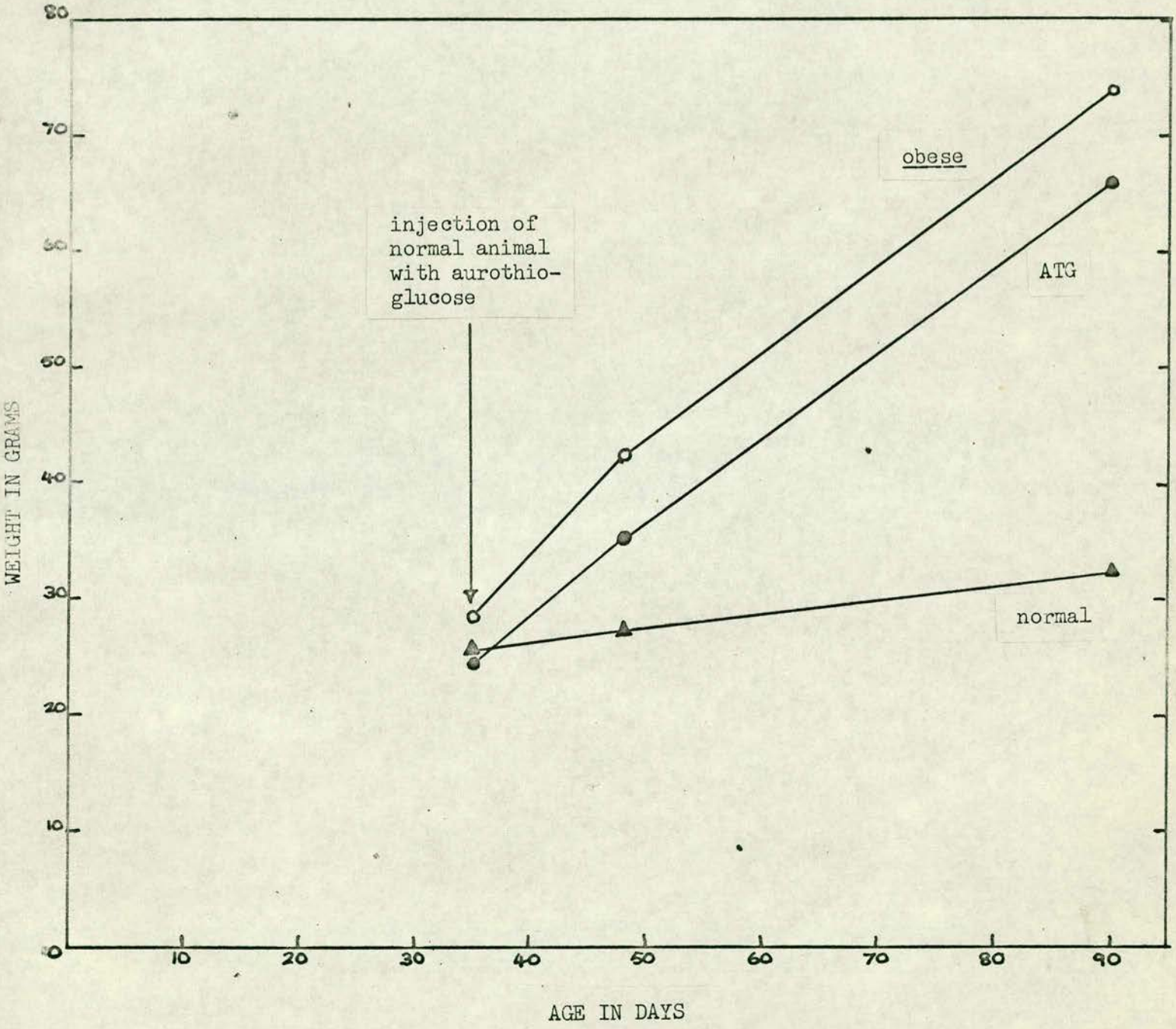


TABLE 6.

The live weight and the Vmaxs of ATP citrate lyase (ATP c.l.), malic enzyme, malic dehydrogenase, (MDH) and pyruvate kinase (P.K.) expressed as μmoles/hr/mgm protein, from obese, ATG and normal littermates.

Comparison	Number of pairs of observations	Mean obese	Mean ATG	Mean normal	percentage difference	mean difference ± standard error	t	P
(a) weight	9							
obese/normal		71.7	-	35.2	(+) 104	36.5 ± 2.75	13.2	<0.001
ATG/normal		-	56.9	35.2	(+) 62	21.7 ± 3.28	6.6	<0.001
obese/ATG		71.7	56.9	-	(+) 26	14.8 ± 4.40	3.4	<0.01
(b) ATP c.l.	9							
obese/normal		0.982	-	0.424	(+) 131	0.558 ± 0.10	5.7	<0.001
ATG/normal		-	0.841	0.424	(+) 98	0.417 ± 0.14	3.1	<0.02
obese/ATG		0.982	0.841	-	(+) 17	0.141 ± 0.07	2.0	N.S.
(c) malic	10							
obese/normal		14.2	-	5.6	(+) 154	8.6 ± 2.21	3.9	<0.01
ATG/normal		-	12.0	5.6	(+) 114	6.4 ± 1.10	6.3	<0.001
obese/ATG		14.2	12.0	-	(+) 18	2.2 ± 2.13	1.1	N.S.
(d) MDH	9							
obese/normal		267	-	222	(+) 20	45 ± 30.5	1.5	N.S.
ATG/normal		-	236	222	(+) 6	14 ± 10.5	1.3	N.S.
obese/ATG		267	236	-	(+) 15	31 ± 31.8	1.0	N.S.
(e) P.K.	9							
obese/normal		18.1	-	12.1	(+) 50	6.0 ± 1.96	3.1	<0.02
ATG/normal		-	18.0	12.1	(+) 49	5.9 ± 2.53	2.3	<0.05
obese/ATG		18.1	18.0	-	(+) 1	0.1 ± 2.48	0.04	N.S.

TABLE 7.

The live weight and the Vmaxs of ATP citrate lyase (ATP c.l) malic enzyme, malic dehydrogenase (MDH) and pyruvate kinase (P.K.) expressed as μmoles/hr/mgm protein, from adipose, ATG and normal littermates.

Comparison	Number of pairs of observations	Mean adipose	Mean ATG	Mean Normal	percentage difference	mean difference ± standard error	t	P
(a) weight	9							
adipose/normal		59.7	-	45.7	(+) 31	14.0 ± 2.18	6.4	<0.001
ATG/normal		-	58.0	45.7	(+) 27	12.3 ± 1.01	12.2	<0.001
adipose/ATG		59.7	58.0	-	(+) 3	1.7 ± 1.92	0.87	N.S.
(b) ATP c.l.	8							
adipose/normal		1.09	-	0.49	(+) 122	0.60 ± 0.070	8.6	<0.001
ATG/normal		-	0.93	0.49	(+) 90	0.44 ± 0.26	16.8	<0.001
adipose/ATG		1.09	0.93	-	(+) 17	0.16 ± 0.092	1.74	N.S.
(c) malic	8							
adipose/normal		14.4	-	8.1	(+) 78	6.3 ± 0.45	14.1	<0.001
ATG/normal		-	19.2	8.1	(+) 137	11.1 ± 0.71	15.6	<0.001
adipose/ATG		14.4	19.2	-	(-) 25	(-)4.8 ± 0.81	(-)6.0	<0.001
(d) MDH	8							
adipose/normal		357	-	352	(-) 1	5.0 ± 20.7	0.24	N.S.
ATG/normal		-	359	352	(+) 2	7.0 ± 4.84	1.40	N.S.
adipose/ATG		357	359	-	(-) 1	(-)2.0 ± 20.3	(-)0.09	N.S.
(e) P.K.	9							
adipose/normal		59.7	-	45.7	(+) 31	14.0 ± 2.18	6.4	<0.001
ATG/normal		-	58.0	45.7	(+) 27	12.3 ± 1.01	12.2	<0.001
adipose/ATG		59.7	58.0	-	(+) 3	1.7 ± 1.92	0.9	N.S.

cause of the obesity in any of these animals. The above conclusions made me decide at this point to search for some agent that might explain the co-ordinated alteration in enzyme activity in obese and adipose mice.

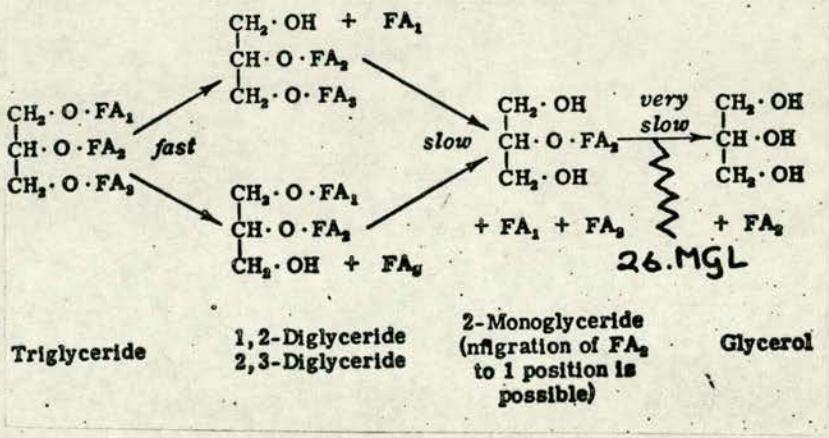
CHAPTER 4. GENERAL PLAN OF THE INVESTIGATION

Part (e): FATTY ACIDS AND THE CONTROL OF LIPOGENESIS

There are many agents that have been reported to have wide effects in controlling lipogenesis. These agents, and their mechanisms of action, were discussed in chapter 2 part (c) to see whether any of them could be involved in the obese or adipose syndromes. Reports of work on obese mice have only indicated, so far, that one of these agents might have an altered metabolism in this syndrome. This agent is oleic acid.

The indication that an alteration in linoleate metabolism might be part of the obese mouse syndrome came from the work of Stein et al (1967). As was mentioned in chapter 4 part (a) (see table 1), this group showed that, on starvation there was a rise in adipose tissue in the proportion of oleic acid (one doublebond 18 carbon atoms in the chain, C 18.1) and no change in the proportion of linoleic acid (C 18.2) in normal mice, whereas, in obese mice, on starvation, there was a rise in the proportion of linoleic acid but no change in the proportion of oleic acid. They also found that oleic and linoleic acid make up 88% of the fatty acid at the 2' position, (i.e. the middle position), of the triglyceride from the fat pad of the obese animals. This led them to assay the activity of 2 monoglyceride lipase (enzyme 26 in fig.5) which specifically attacks the 2' position (see fig.15) of the triglyceride. They found the Vmax of this enzyme to be three times lower when assayed from adipose tissue taken from obese animals than that taken from normal ones.

Fig. 12 Reactions in lipolysis, showing the relative rates of the different steps and the position of 2-monoglyceride lipase, (MGL), (from Wills, 1965).



This means that the linoleate is held in a monoglyceride form in the obese animals, instead of being released as free fatty acid. The monoglyceride can then be directly reacylated at the 1' and 3' position back to triglyceride. This would reduce the amount of free linoleate present in these animals. As will be explained later the linoleate level may have an important bearing on the rest of the lipogenic system. From Stein's work it appeared that intermediate values for the Vmax of the lipase were being obtained for some of the littermates of the obese animals while some had the same values as normal mice. This, of course, is highly significant from the genetical point of view, as was discussed in chapter 1, suggesting that the segregation of the three genotypes is observed. The 2-monoglyceride lipase immediately becomes a good candidate for the primary enzymic lesion in the animals. Stein et al do not make this observation and indeed the data are not clear enough to be certain on this point. Another genetic criterion was that, as the gene ob is recessive and the genetic alteration is simply in the Vmax of an enzyme the substitution by the ob gene for the wild type gene should confer a lower activity on an enzyme that is the primary biochemical lesion. Thus the lower activity of 2-monoglyceride lipase in obese animals again makes it a good candidate for the primary lesion. The third genetical criterion that the primary lesion is probably at a different site in obese and adipose animals. Thus the activity of 2 monoglyceride lipase needs further investigation in obese and adipose animals and their normal littermates. However, the assay for this enzyme entails the preparation of 2 monoglyceride as the substrate. This preparation is difficult, especially as the material is metastable and is being worked on by Dr.Stein

in Aberdeen. For these reasons I decided not to investigate the Vmax of this enzyme but rather to see whether the consequences of the low lipase Vmax in obese animals could explain the wide metabolic derangements found in these animals. It is possible that the key result of the lower lipase activity is that it lowers the amount of free linolsate in obese animals by holding it in the monoglyceride form. It was therefore decided to see whether the lower amount of free linoleate in obese animals could be the cause of the increased Vmaxs of the lipogenic enzymes that are found in these animals.

In chapter 2 part (c) I reviewed the evidence indicating the involvment of fatty acids, especially linoleate, in the control of lipogenesis. Briefly summarised it is as follows.

Fatty acids and their acyl-CoA esters have been reported as both inhibiting the activity and repressing the synthesis of lipogenic enzymes. Long chain acyl CoA esters and free fatty acids inhibit the activity of acetyl CoA carboxylase IN VITRO, (enzyme 22 in fig.5) (Robinson et al, 1963; Bortz and Lynen, 1963; Wieland and Weiss, 1963; Tubbs and Garland, 1964) and octanoate IN VITRO inhibits the activity of glucokinase (enzyme 1 in fig.5), phosphofructokinase, (enzyme 2 in fig.5) and pyruvate kinase (enzyme 3 in fig.5) (Weber et al, 1967a). Fatty acids have been suspected for some time to "repress" the synthesis of the lipogenic enzymes, as the addition of fat to a fat free diet sometimes reduces lipogenesis. Recently it has been reported that both acetyl CoA carboxylase (enzyme 22, fig.5) and

fatty acid synthetase (enzyme 23 fig.5) are quickly reduced after the addition of 2% methyl linoleate to a fat free diet of young mice, whereas the addition of 2% methyl palmitate or methyl oleat had no effect. (Allman et al, 1965; Allman and Gibson, 1967; Bartley et al, 1967). Besides having this specific role in lipogenesis, linoleate has been known for many years to be an essential nutrient. Its absence from a diet causes a syndrome that includes caudal necrosis, and if added to a diet it is able to cure a condition known as "fatty livers".

It would, therefore, be significant to establish whether inhibition of the activity or repression of the synthesis of some lipogenic enzymes had been impaired in obese animals, and if so, whether linoleate was implicated in this.

First I tried to see whether enzyme inhibition or deinhibition occurred when extracts of obese and normal animals were mixed.

Secondly, I compared sodium linoleate with sodium octanoate as inhibitors of the lipogenic enzymes. Weber et al (1965d) had previously reported that sodium octanoate was an inhibitor of these enzymes.

Thirdly, I compared enzymes extracted from obese ATO and normal mice in their inhibition properties with sodium linoleate. These inhibition experiments would throw light on the possible effect of linoleate on lipogenesis in so far as it affects the activity of the enzyme in the system. It may therefore contribute to the total syndrome by increasing fat synthesis. The results, however, whatever their nature, could not explain the observed enzyme level (Vmax) changes. If linoleate is

implicated in these changes it must be via the synthesis of a whole battery of enzymes.

I tried, therefore, to see whether the removal or addition of linoleate to different diets had a different effect on the Vmax of enzymes extracted from obese ATG or normal animals.

RESULTS

Table 8 shows the effect of mixing extracts from obese and normal animals on the activity of ATP citrate lyase, (enzyme 18 in fig.5). This Table shows that the mixed extracts give a Vmax which is the sume of that expected from obese and normal extract if these were assayed separately.

Fig.16 shows the inhibition of the Vmax of pyruvate kinase, (enzyme 3 in fig.5), extracted from normal animals, by sodium octanoate and sodium linoleate and compares these results with those for inhibition by sodium octanoate for rat liver pyruvate kinase reported by Weber et al (1965d, 1967a). These results give inhibition constants, (the molarity of the inhibitor at 50% inhibition), of 2.5×10^{-3} for sodium octanoate from the work of Weber's group, 5.1×10^{-3} for sodium octanoate from my work and 0.36×10^{-3} for sodium linoleate.

Fig.17 shows the inhibition by sodium linoleate of the activity of pyruvate kinase, extracted from obese ATG and normal animals. The inhibition constants obtained for the three types of extract were, 3.5×10^{-4} for obese, 3.7×10^{-4} for normal and 3.4×10^{-4} for ATG animals.

All the inhibitions described above, for pyruvate kinase, were non-competitive with the substrate of the reactions, potassium phospho enol pyruvate.

TABLE 8

The Vmax of ATP citrate lyase extracts from obese and normal littermates assayed individually and mixed.

If there is no inhibition present in normal extract then the mixed obese and normal extracts will give the sum of the specific activities of the two individual extracts. If there is an inhibition in normal extract then the Vmax for the mixed extract will deviate, (in fact be lower), than the sum of the two individual extracts.

Vmax in O.D. units/min of individual extracts

		ml of extract	O.D. units/min
obese	(a)	0.02	0.025
	(b)	0.02	0.0245
	(c)	0.02	0.0255
	mean		0.0249
normal	(a)	0.06	0.023
	(b)	0.06	0.023
	(c)	0.06	0.0235
	mean		0.0232

Vmax in O.D. units/min of mixed extracts

ml of mixture			Observed O.D. Units/min	Expected O.D.Units/min from sum of individual assays, i.e. no inhibition
obese		normal		
0.01	+	0.03	0.023	0.024
0.02	+	0.03	0.037	0.037

Fig.16. The inhibition of the Vmax of pyruvate kinase, (enzyme **3** in Fig.5), by sodium octanoate and sodium linoleate, expressed as a percentage of the control value.

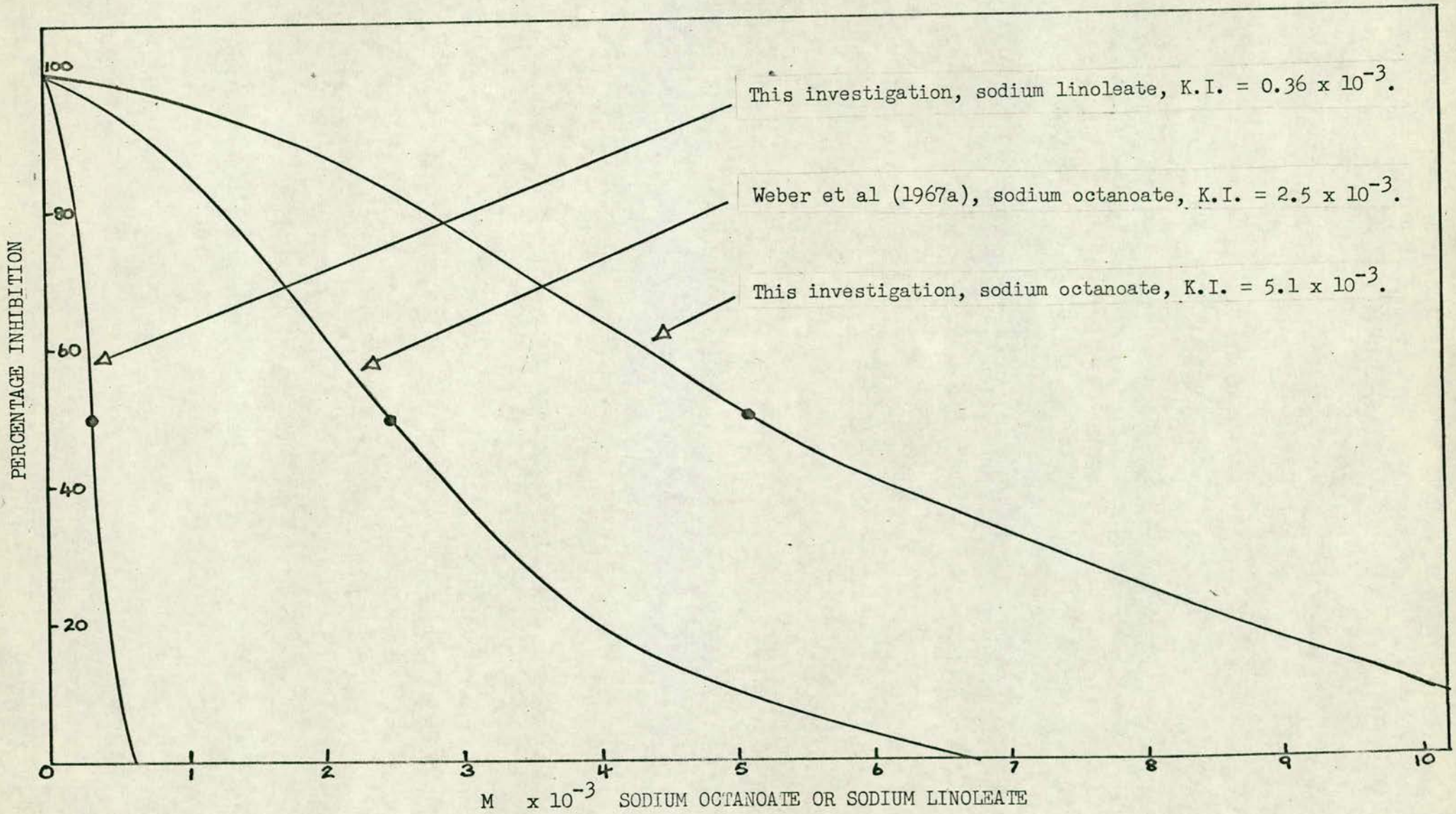


Fig.17. The inhibition of the Vmax of pyruvate kinase, extracted from, obese, ATG and normal animals, by sodium linoleate, expressed as a percentage of the control value.

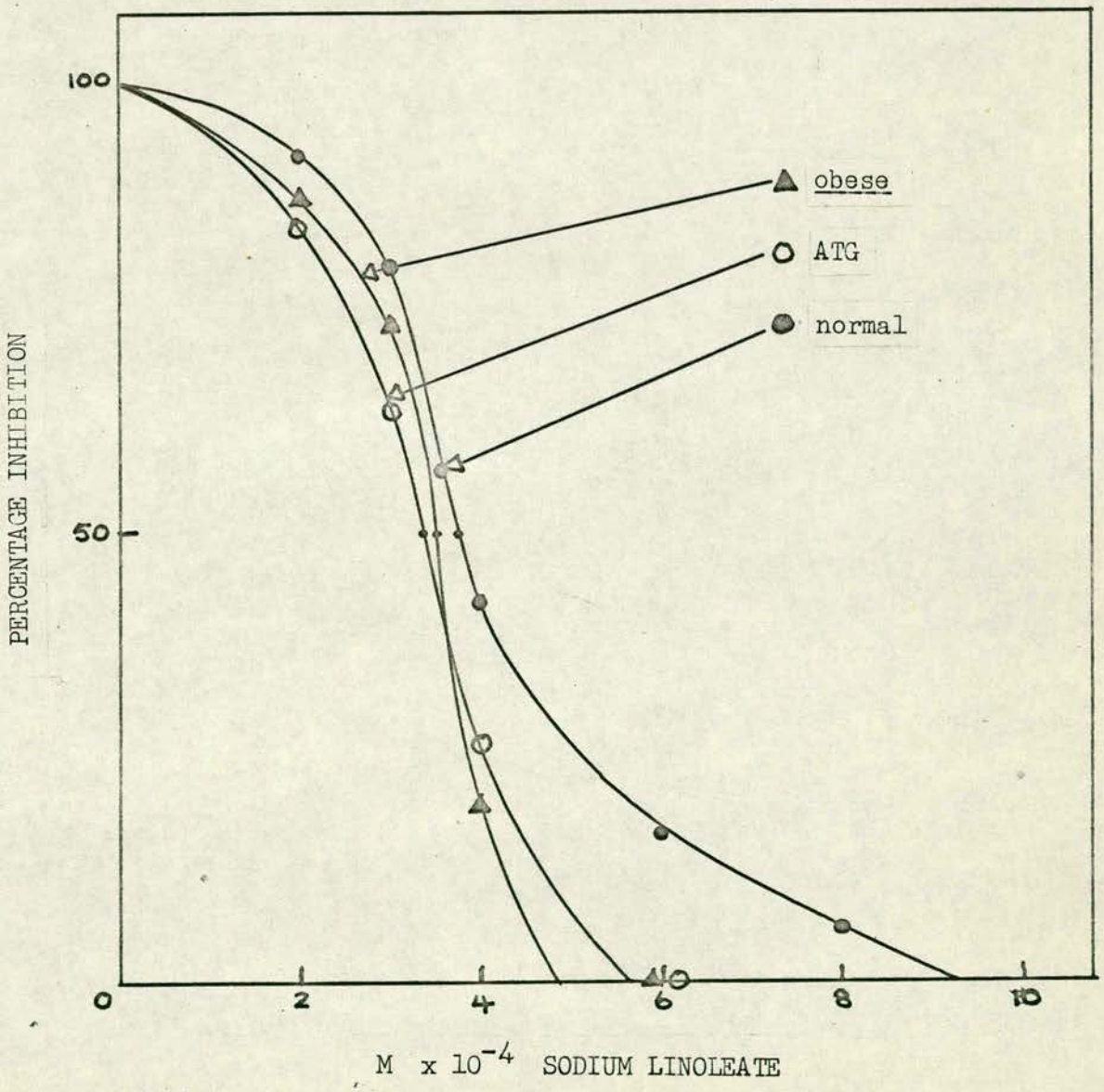


Fig.18 shows the effect, on the Vmax of ATP citrate lyase, of feeding obese and normal animals for four days on a fat free diet and then supplementing the diet with either 2% methyl linoleate or, 2% methyl oleate. Fig.19 shows the effect of the same feeding regeme on the Vmax of malic enzyme. ATP citrate lyase activity (fig.18), increases about five fold, both in obese and normal animals, after four days on a fat free diet. At this point the Vmax is about the same from obese and normal animals. The Vmax of the enzyme from normal animals is quite a lot lower again after 8 days supplementation of the fat free diet with 2% methyl oleate. However, 2% methyl oleate does not reduce the Vmax from the fat free diet level. In obese, 2% methyl linoleate supplementation only slightly lowers the Vmax from the fat free diet level and, again, 2% methyl oleate has no effect.

The same pattern is seen with the Vmax of malic enzyme (fig.19), except that the extract from normal animals never reaches the same Vmax of obese animals, even on the fat-free diet.

Figs. 20 and 21 show the same feeding pattern to fig.18 and 19, with the Vmaxs of same two enzymes, except that this time, after four days on the fat free diet, the animals are only supplemented with 5% methyl linoleate. Figs. 20 and 21 confirm that the Vmax of these two enzymes are depressed more in the extracts from normal animals than from obese animals after 8 days linoleate supplementation.

Fig.22 shows the effect, on the Vmax of ATP citrate lyase, of feeding obese, ATG and normal littermates for seven days on diets of either normal laboratory pellets or, fat free meal or, fat free meal plus

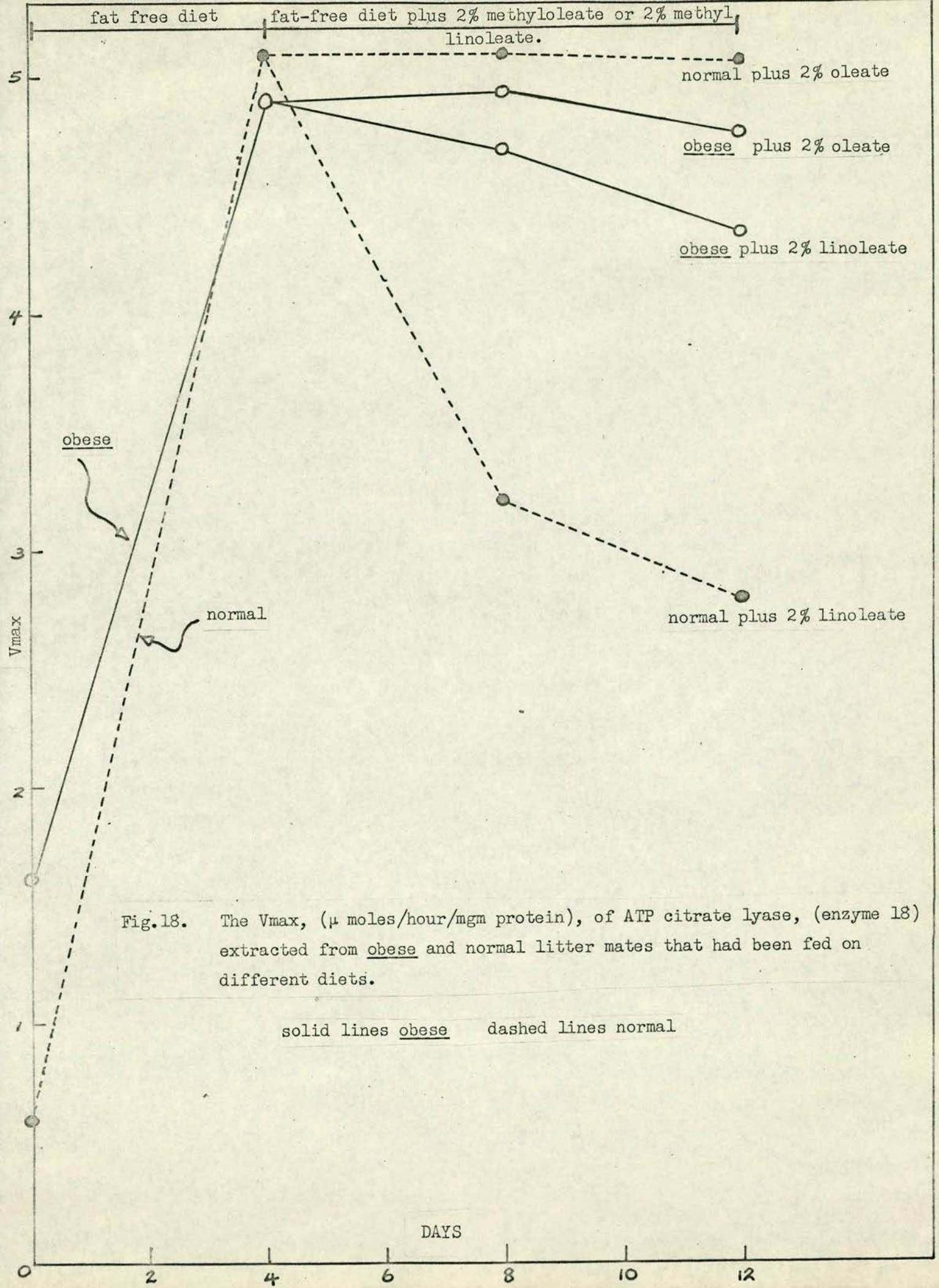
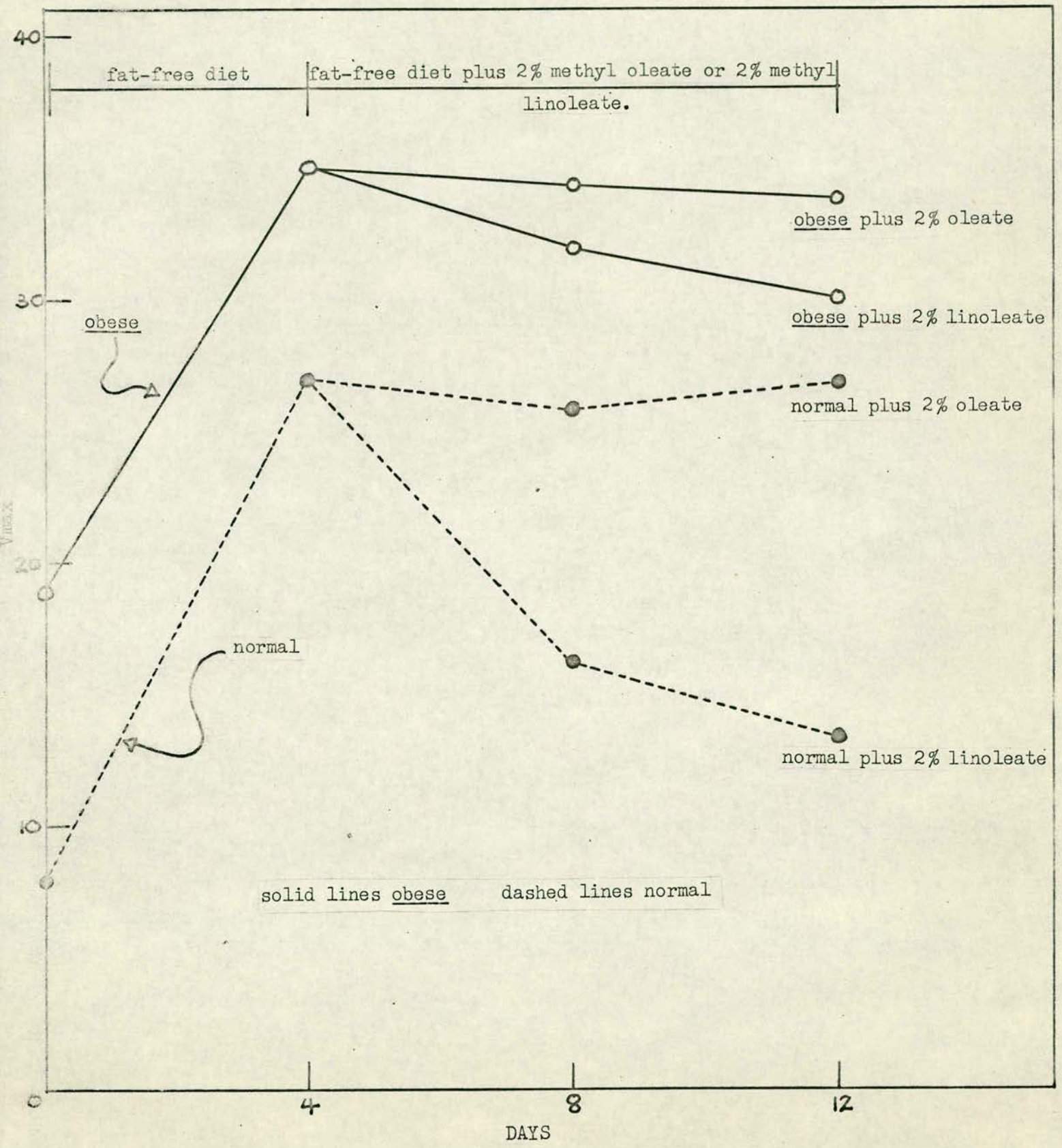


Fig.18. The Vmax, (μ moles/hour/mgm protein), of ATP citrate lyase, (enzyme 18) extracted from obese and normal litter mates that had been fed on different diets.

solid lines obese dashed lines normal

Fig.19 The Vmax, (μ moles/hour/mgm protein), of malicenzyme, (enzyme 21), extracted from obese and normal litter mates that had been fed on different diets.



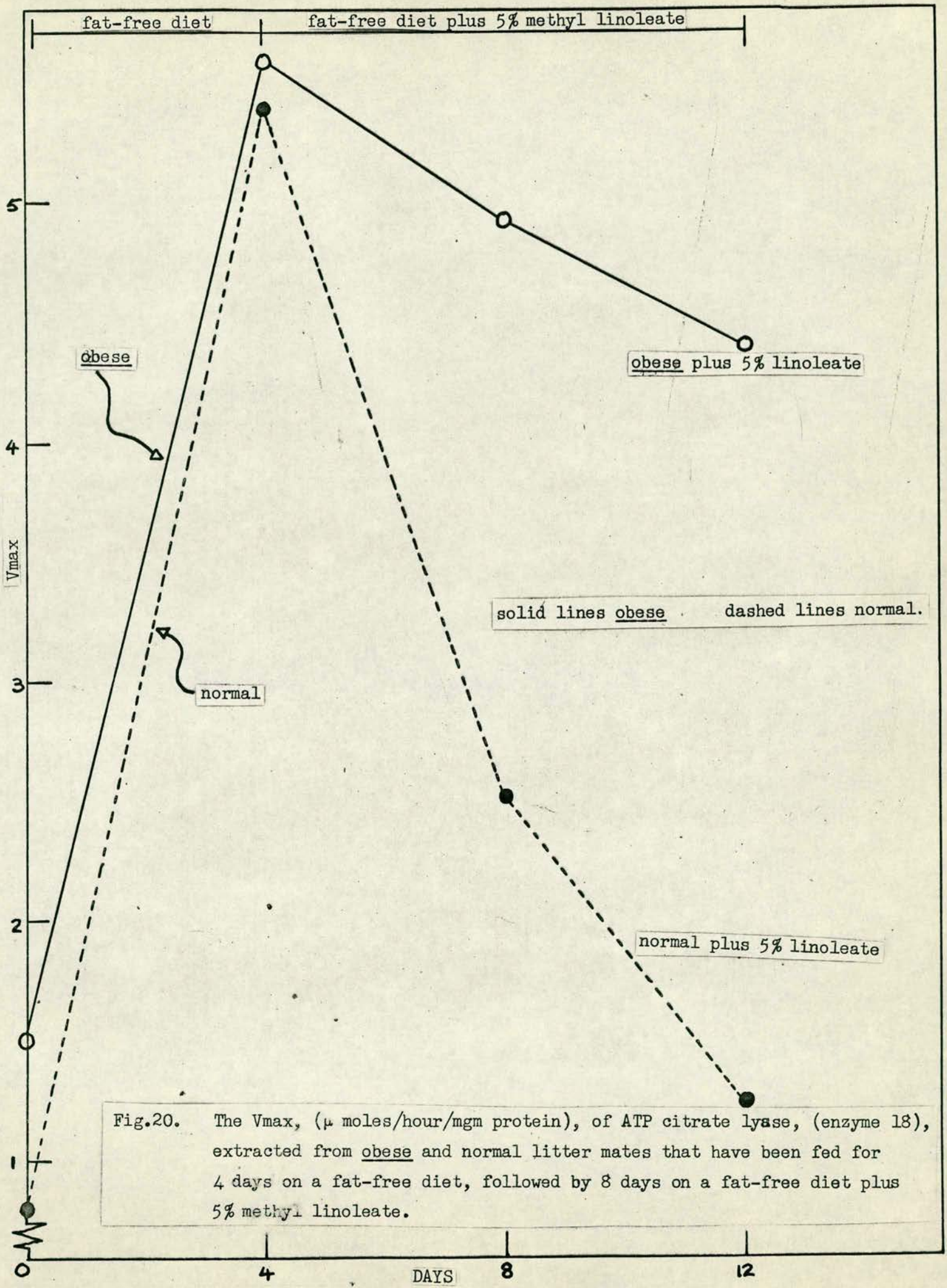


Fig.20. The Vmax, (μ moles/hour/mgm protein), of ATP citrate lyase, (enzyme 18), extracted from obese and normal litter mates that have been fed for 4 days on a fat-free diet, followed by 8 days on a fat-free diet plus 5% methyl linoleate.

Fig.21. The Vmax, (μ moles/hour/ mgm protein), of ATP citrate lyase, (enzyme 18), extracted from obese and normal litter mates that have been fed for 4 days on a fat-free diet, followed by 8 days on a fat-free diet plus 5% methyl linoleate.

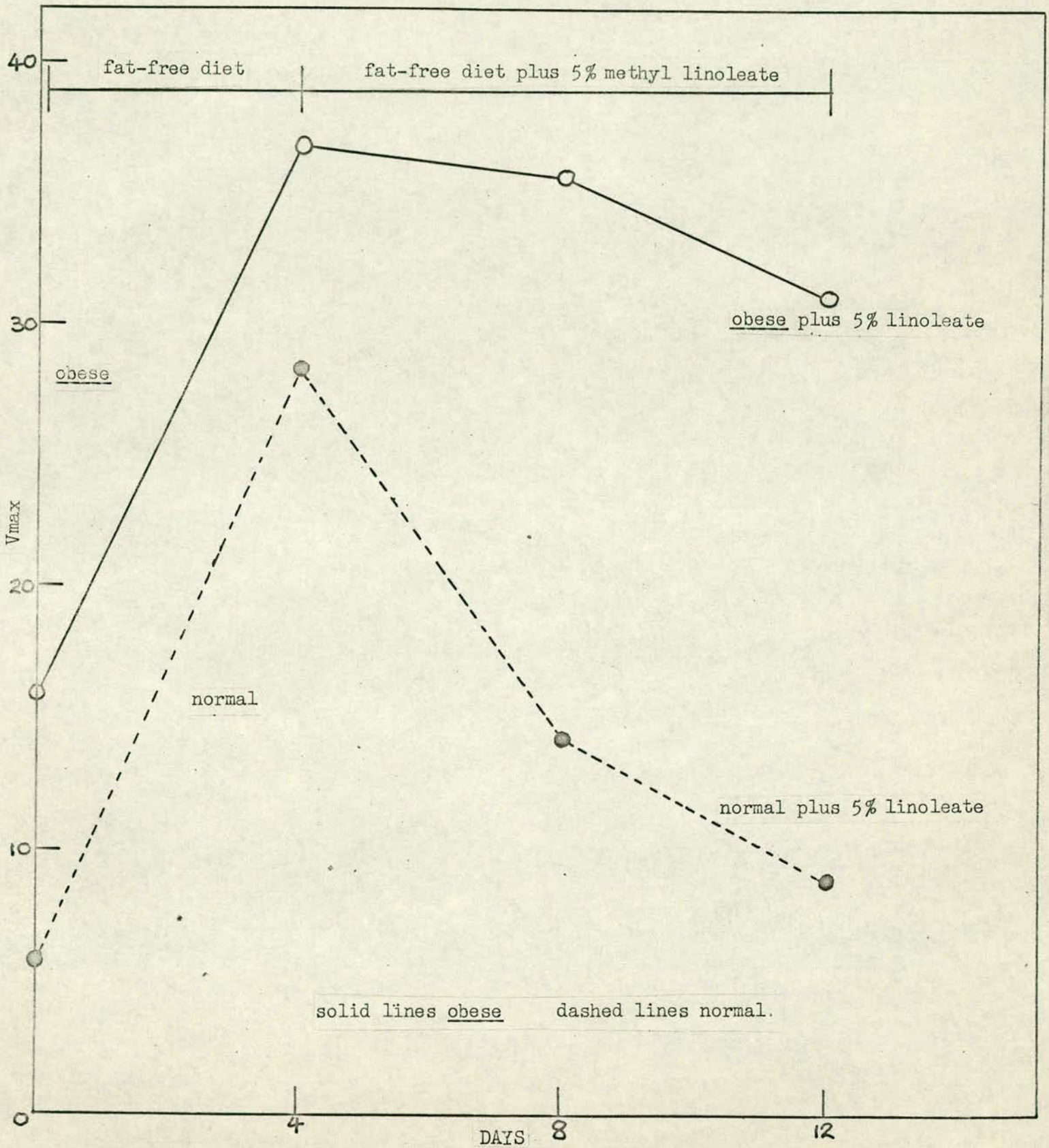
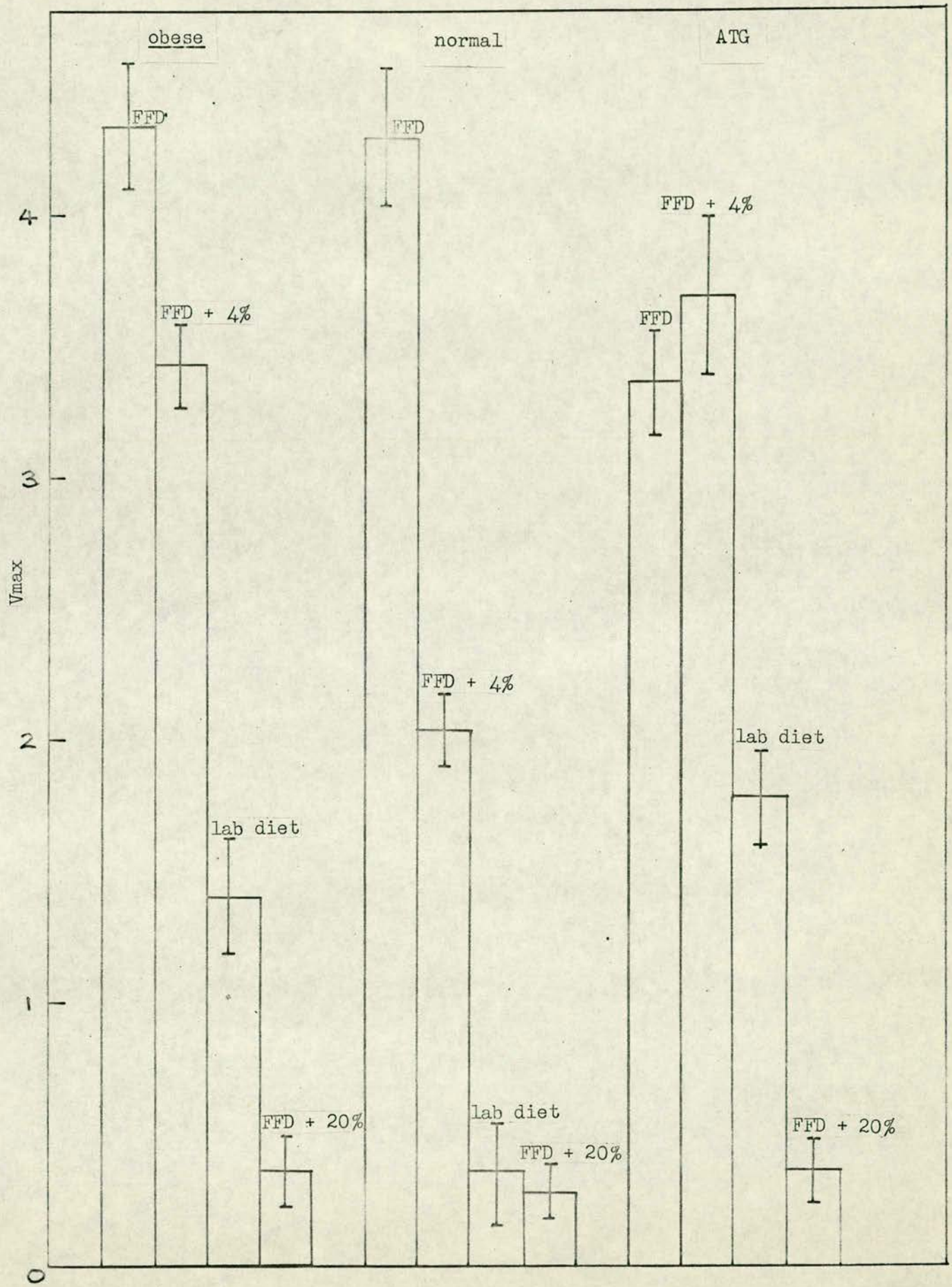


Fig.22. The Vmax, (μ moles/hour/mgm protein), of ATP citrate lyase, (enzyme 18), from obese, ATG and normal animals maintained for 8 days on, (a) fat-free diet, (FFD), (b) fat-free diet plus 4% methyl linoleate, (FFD + 4%), (c) fat-free diet plus 20% methyl linoleate, (FFD + 20%), or (d) laboratory diet, (lab diet). All values show the means and standard errors of four animals.


obese
normal
ATG
FFD
FFD + 4%
lab diet
FFD + 20%
Vmax
0
1
2
3
4

4% linoleate or, fat free meal plus 20% linoleate. Fig.23 shows the effect of the same four feeding regemes on the Vmax of malic enzyme, and fig. 24 on the Vmax of malic dehydrogenese.

From figs. 22 and 23 it can be seen that Vmaxs of ATP citrate lyase and malic enzyme respond to the fat free diet and the fat free diet plus 4% linoleate in the same way as they did in the previous experiments. On the 20% supplemented diet, however, the Vmaxs of both enzymes descend to a very low level in all animals. However, the Vmaxs of these enzymes extracted from ATG animals appear to have been altered in a different manner to those of obese and normal in response to the four diets. The Vmaxs are in fact higher for animals on the 4% supplemented diet than from animals on the fat free diet.

From fig.24 it can be seen that no consistent pattern is obtained for the Vmax of malic dehydrogenase for any type of animal on the four diets.

DISCUSSION

From table 8, it can be seen that mixing the extracts of ATP citrate lyase from obese and normal animals gives a Vmax which is the sum of that expected from the obese or normal extract, if assayed separately. If there was an inhibitor in the normal extract then one would expect the mixed extracts to give a Vmax nearer the normal value. Therefore there does not appear to be an inhibitor in the normal extract.

It was, however, thought that an investigation of the role of linoleate as an inhibitor might throw some light on the problem.

Fig.23. The Vmax, (μ moles/hour/mgm protein), of malic enzyme, (enzyme 22) from obese, ATG and normal animals maintained for 8 days on, (a) fat-free diet, (FFD), (b) fat-free diet plus 4% methyl linoleate, (FFD + 4%), (c) fat-free diet plus 20% methyl linoleate, (FFD + 20%) or (d) laboratory diet, (lab diet). All values show the means and standard errors of four animals.

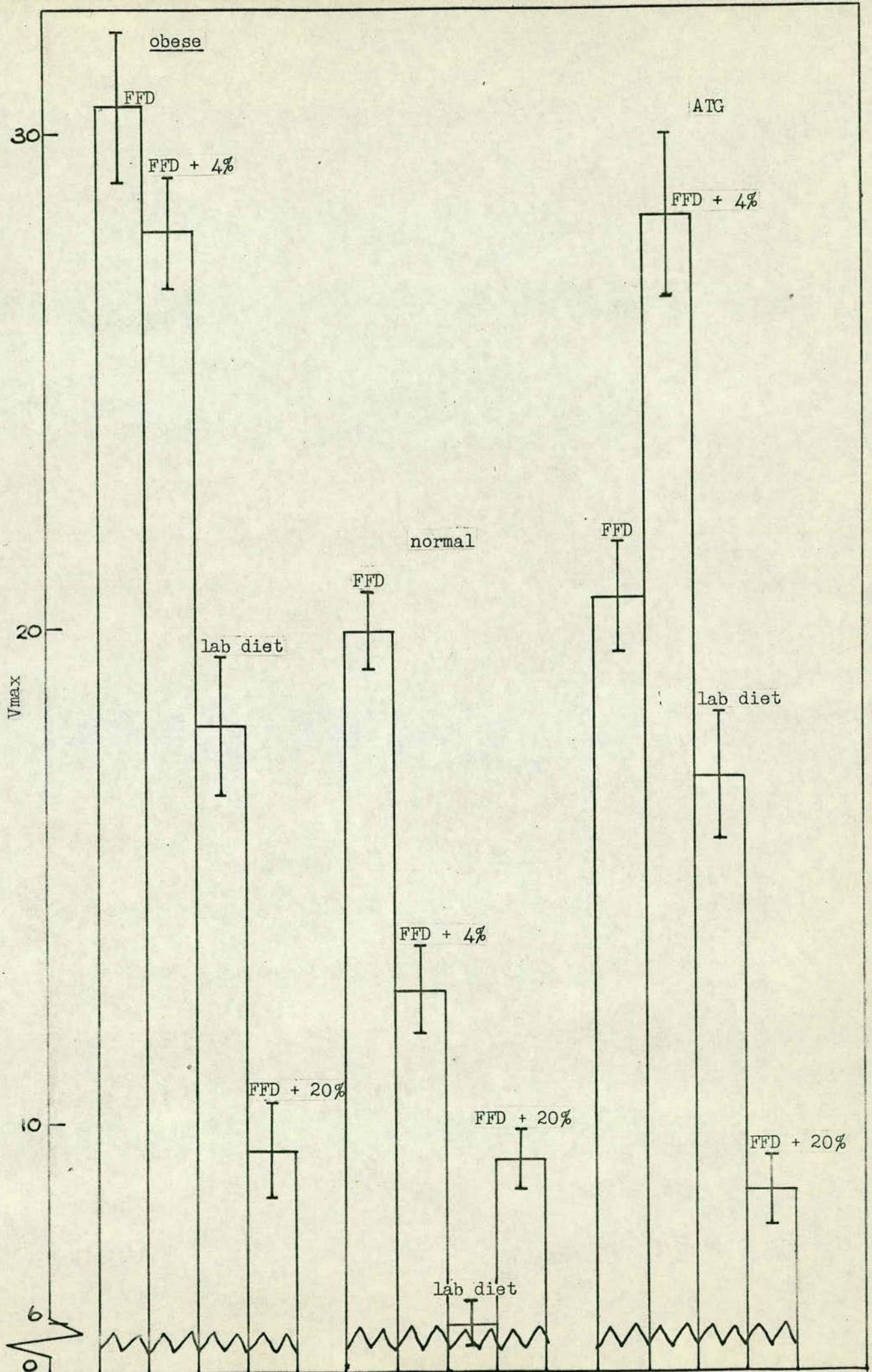
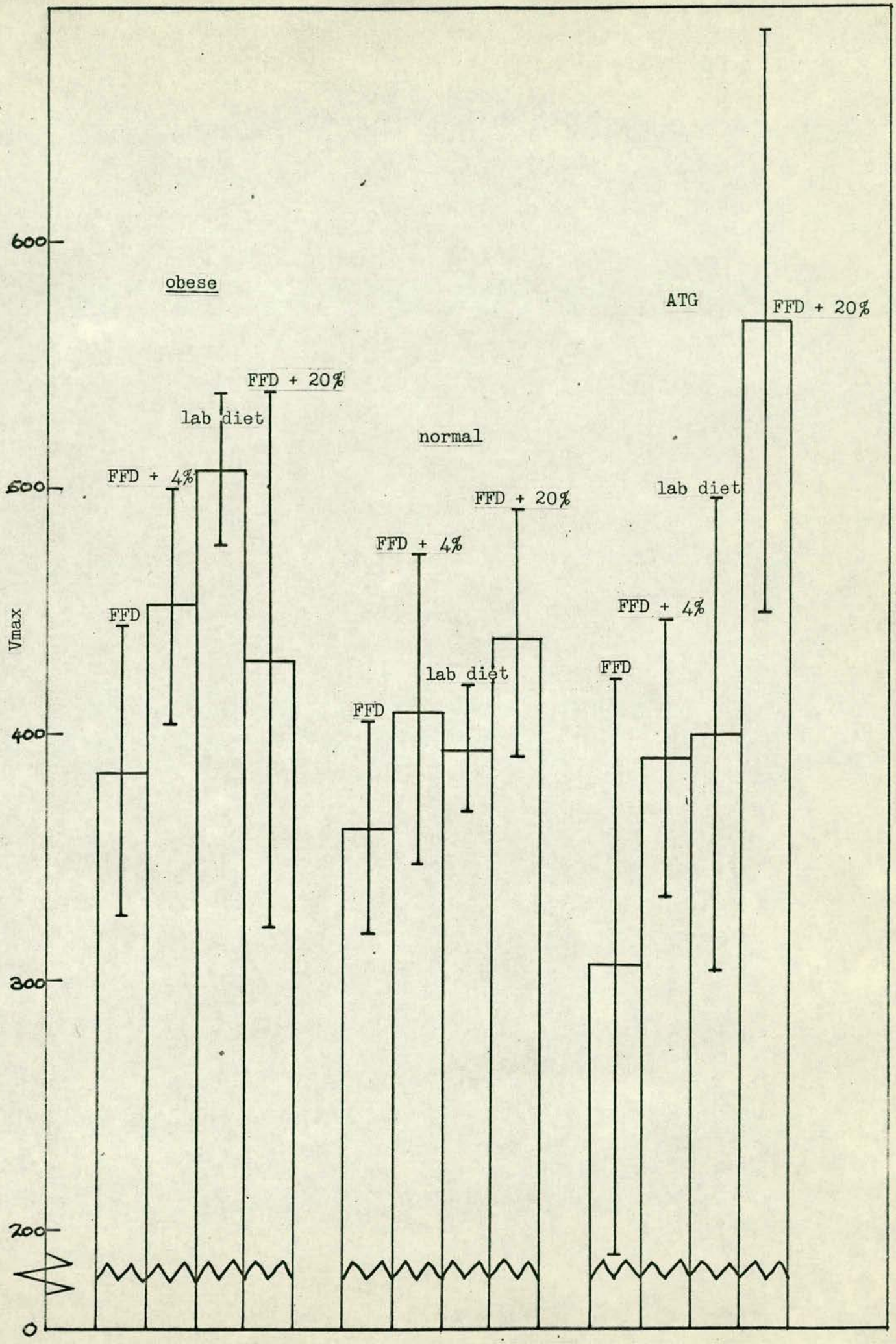

obese
FFD
FFD + 4%
lab diet
FFD + 20%
normal
FFD
FFD + 4%
lab diet
FFD + 20%
ATG
FFD
FFD + 4%
lab diet
FFD + 20%
Vmax
30
20
10
6
0

Fig.24. The Vmax, (μ moles/hour/mgm protein), of malic dehydrogenase, (enzyme 20), from obese, ATG and normal animals maintained for 8 days on, (a) fat-free diet, (FFD), (b) fat-free diet plus 4% methyl linoleate, (FFD + 4%), (c) fat-free diet plus 20% methyl linoleate, (FFD + 20%) or (d) laboratory diet (lab diet). All values show the means and standard errors of four animals.


600
500
400
300
200
0
Vmax
obese
normal
ATG
FFD
FFD + 4%
lab diet
FFD + 20%
FFD
FFD + 4%
lab diet
FFD + 20%
FFD
FFD + 4%
lab diet
FFD + 20%

Unfortunately, malic dehydrogenase, the link in the assays of ATP citrate lyase, is inhibited by lower concentrations of sodium linoleate than is ATP citrate lyase. It is, therefore, not possible to show the action of sodium linoleate on the latter enzyme. Instead it was decided to look at the inhibition characteristics of pyruvate kinase, (enzyme 3 in fig.5), which also has an elevated Vmax in obese animals. Fig.16 shows the inhibition of pyruvate kinase, extracted from normal animals, by sodium octanoate and sodium linoleate, and compared with the results with sodium octanoate reported by Weber et al (1967). These results indicate that sodium linoleate is 8-15 times a more powerful inhibitor of pyruvate kinase than is sodium octanoate. All these inhibitions were non-competitive with the substrate of the reaction, K phospho enol pyruvate.

Fig.17 shows that the inhibition constants for pyruvate kinase from obese ATG or normal animals with sodium linoleate, are of the same order by normal inhibition tests. Therefore, it is not possible to show that linoleate differentially affects the activity of the enzyme, IN VITRO, taken from obese ATG and normal animals. This confirms that the different level of activity in obese animals is due to a quantitative change in enzyme amount occasioned by some other signal.

It is, however, possible that linoleate, or some derivative, acts as this signal. Thus the effect of linoleate on the activity of some lipogenic enzymes IN VIVO, must be investigated ~~at~~ to see whether there is any difference between obese ATG and normal animals.

Preliminary experiments showed that large supplements of linoleate will keep the enzymes, ATP citrate lyase (enzyme 18), malic enzyme (20) and

and malic dehydrogenase, (21). Now it has been shown by Gibson's group (Allman et al 1965a,b) that if normal young mice were placed on a fat free diet the Vmax of some lipogenic enzymes increased four fold, whereas when this diet was supplemented with a small amount of methyl linoleate (2%) the Vmaxs of the enzyme returned to the control level in four days, although methyl oleate and methyl palmitate did not have this effect. Now I have postulated that free linoleate is less available in obese animals due to the lower activity of 2 monoglyceride lipase. So, if obese animals are fed on a fat-free (therefore also linoleate-free) diet, and then this diet is supplemented with a small amount of methyl linoleate, it is to be expected that less of this linoleate will be available in obese animals to play a metabolic role, such as lowering the quantity of some of the lipogenic enzymes. Hence the lipogenic enzymes would stay at a high level in the obese animals, but not in normal ones. To see whether there was this effect with enzymes extracted from obese mice, I repeated the experiment of Gibson's group with obese and normal mice.

Figs. 18 and 19 show that with ATP citrate lyase and malic enzyme respectively, a supplement of 2% methyl linoleate does bring the Vmaxs of these enzymes down from the fat-free diet level, far more in normal than in obese animals. The 2% methyl oleate had little affect in either type of animal. It is also interesting to note from figs 18 and19 that, on the fat-free diet, the Vmax of ATP citrate lyase, from normal animals, is similar to that from obese animals, wherease the Vmax of malic enzyme extracted from animals on the fat-free diet, is still much lower in normal than in obese animals. No explanation can be offered for this.

The Vmax of the enzymes extracted from the normal animals, are less depressed by the supplement of 2% methyl linoleate, in figs 18 and 19 than they were in the experiment of Gibson's group, where they were brought right down to the control level in four days. So I decided to repeat my experiment, this time using a larger 5% supplement of methyl linoleate.

Figs. 20 and 21 show the more dramatic difference between the obese and normal animals on the 5% supplemented diet than in the previous experiments. The Vmaxs of the enzymes extracted from normal animals are nearly depressed to normal level in 8 days. Therefore, the results from these experiments of supplementing a fat-free diet with different levels of methyl linoleate, does indicate that the Vmaxs of the enzymes extracted from obese animals are more resistant to the depressing action of dietary linoleate than are those of the enzymes extracted from normal animals. This may be due to their being less free linoleate available in the obese animals caused by the lower activity of 2 monoglyceride lipase.

Next I decided to compare enzymes extracted from ATD animals with those extracted from obese and normal animals on linoleate supplemented fat free diets. It can be seen from fig.24 that malic dehydrogenase activity reacts to the different diets in a way which does not show any consistent pattern in any of the three types of animal. It may be noted that this enzyme is one which normally has a high Vmax and is the same in obese adipose and their normal littermates, and is one of those that is alone in catalysing a particular metabolic change.

Whereas, from figs. 22 and 23 it can be seen that obese and normal animals respond to the fat-free diet plus 4% linoleate in a similar way as

they did in previous experiments. On the 20% diet, however, the Vmaxs of both enzymes descend to a very low level in all animals. Not only is this probably due to the large amounts of linoleate in these diets but intake is also depressed due to the large amount of linoleate. However, the Vmax of the enzyme extracted from ATG animals appear to have been altered in a different manner, as the Vmax of both ATP citrate lyase and malic enzyme are slightly higher on the fat-free diet supplemented with 4% linoleate than they are on the straight fat-free diet, (although the Vmaxs are very low again on the 20% diet). This result may be due to the nature of the syndrome in the ATG animals. Because the ATG animals are grossly hyperphagic on normal diets they have elevated Vmaxs for these two enzymes. The signal causing this rise in Vmax is probably quite different in ATG from obese animals, and it has been postulated that, on the 4% diet there is not as much free linoleate available in obese animals to bring the enzymes down, as there is in normal ones. On the other hand, the hyperphagic ATG mice will probably still be ingesting large amounts of carbohydrates on the 4% diet, whereas obese mice has a near normal intake, and thus enzymes extracted from ATG are still high on the 4% diet. However, it is probably that the 20% diet is just too much for even the ATG mice and their intake and enzyme activity are reduced.

This is, of course, a preliminary observation on the difference in behaviour of the Vmax of different enzyme extracts taken from ATG obese and normal animals on linoleate supplemented diets. All the same, from what is known of the physiology and biochemistry of these two obese

syndromes the above interpretation of the results appears to be the most reasonable one at this stage.

Of course, all the work that I have done on the function of linoleic acid in obese animals is based on the expectation that the lower activity of 2-monoglyceride lipase is responsible for a lower amount of free linoleate in these animals, which in turn causes the animals to have a higher Vmax. One way that this hypothesis can be tested in the future is to assay the amount of free linoleate that is present in obese animals. However, whatever the level of free linoleate in obese animals this investigation has uncovered a phenomenon involving linoleate in obese animals that has to be explained. Also, I explained earlier that this lower activity in obese mice was significant because of the recessive nature of the ob gene, this being one genetic criteria for the primary lesion. The other two were that the enzyme activity should segregate among littermates, (and there is some suspicion that this does occur with this enzyme), and that the primary lesion would probably be different in obese animals from adipose animals.

Because of the difficulty in preparing the substrate for the assay for this enzyme I was not able to follow up these leads. However, Dr. Stein in Aberdeen has been routinely determining the Vmax of this enzyme for some time and originally found the difference in Vmax between obese and normal animals (Stein et al, 1967). I suggested to Dr. Stein that she might concentrate on assaying the Vmax of the enzyme in littermates of the obese mice and also compare obese and adipose, as this approach might

elucidate whether this enzyme was indeed the site of the primary biochemical lesion in obese mice. I sent Dr. Stein several litters from the strains containing obese and adipose animals and she has kindly sent me her preliminary results so that I may quote them here.

Dr. Stein has confirmed her original finding that 2 monoglyceride lipase (enzyme 26 in fig.5) has 1/5th of the Vmax when assayed from adipose tissue from obese mice compared with that from normal ones. There is also a large spread of Vmaxs in the normal animals which could be interpreted as falling into two groups, although far more data is needed. What is significant, however, is that Dr. Stein cannot find any difference between the Vmax of enzymes extracted from adipose mice and their normal littermates. However, the Km of this enzyme could have been changed in adipose mice and this could result in the same Vmax in normal and adipose mice but a lower effective activity in adipose at lower substrate levels. Therefore, this preliminary work indicates that an intensive study of this enzyme's behaviour in obese, adipose, ATG animals and their normal littermates might show that this enzyme is the site of the primary biochemical lesion in obese animals. If this is found to be so, it would give more validity to the interpretation I have put on the results of my work with these animals on linoleate supplemented diets. It appears from Dr. Stein's provisional results that 2-monoglyceride lipase has the same Vmax in adipose as in normal animals and therefore is probably not the primary lesion in these animals. This raises the problem of the nature of the lesion in adipose. Also, adipose animals ought to be able to be used as controls for obese in linoleate feeding experiments and dietary work on this mutant has yet to be done.

CHAPTER 5. GENERAL DISCUSSION AND CONCLUSION

Few biochemical studies in mammals have used genetical techniques. Even when a syndrome, like *obese* and *adipose*, that is known to be caused by a single simple genetic alteration, is under study, the genetical criteria that are readily available have rarely been used to evaluate the biochemical information that is collected.

In the *obese* mouse the elevated V_{max} of ATP citrate lyase was implied as being the primary lesion, mainly because this enzyme was "rate-controlling" with respect to lipogenesis. From the genetical point of view it is not necessary just to show that there has been an alteration in a "rate-controlling" enzyme to prove that enzyme to be the site of the primary lesion. Proof for the site of the primary lesion needs the application of more exact criteria based on genetic knowledge. Three criteria were discussed in the Introduction: first, the primary lesion is probably in a different enzyme in *adipose* and *obese*; second, the primary lesion should exhibit segregation in normal littermates of *obese* animals, and, third, if the primary lesion is simply an alteration in enzyme activity, then the enzyme should have a lower activity in *obese* animals because the *ob* gene is recessive. Application of these criteria to the enzymic differences between *obese* or *adipose* animals and their normal littermates indicates that none of the 7 enzymes investigated is the primary lesion in litters of these syndromes.

Furthermore, the examination in chapter 2 part (b) of the theoretical assumptions underlying the assignation of one enzyme in a metabolic pathway as "rate-controlling" to the pathway, were found in most reported work to

be erroneous. This is true for the information claiming to indicate ATP citrate lyase as a "rate-controlling" enzyme.

From this investigation it is possible to make two general conclusions, the first regarding the search for the primary enzymic lesion in obese and adipose animals and the second regarding the application of genetical criteria and techniques to the study of the control of mammalian metabolic processes, such as lipogenesis.

First, studies attempting to elucidate the primary lesion in obese or adipose animals should now be concentrated on examining the Vmax of 2-monoglyceride lipase in these animals and their normal littermates. The genetical criteria already discussed can be used to ascertain whether this enzyme is indeed the site of the primary lesion in litters of these animals. It is also important to know whether there is actually less free linoleate circulating in obese animals than in their normal littermates. If there is less free linoleate in obese, than the interpretation I have placed, in this thesis, on the action of dietary linoleate in these animals is made more meaningful.

Secondly, there are different genetical criteria and techniques available, other than studying obvious syndromes caused by single gene mutation, to investigate the control of mammalian metabolic processes like lipogenesis. One way would be to see if any genes that alter the activity of individual lipogenic enzymes could be found from studying inbred strains and to try and see if altering the activity of an enzyme does have an influence on the flux of lipogenesis, as described in chapter 2 part (b).

It might be possible to find a whole range of genetic differences for each of these enzymes and by breeding work, study the influence of different genetical combinations on the lipogenic flux and obesity. This sort of study might be used in conjunction with selective breeding work for lipogenic parameters. So far there has been no attempt to use genetics in this sort of way to study biochemical control mechanisms. It is, of course, possible that no genetical alterations in a single enzyme's activity might be found and in fact fat inbred strains might just have the activity of their lipogenic enzymes co-ordinately elevated under the influence of some other agent, (for example hormones). This type of result would also be very significant in the search for the nature of biochemical control and would definitely indicate that other agents are more important in control than the level of the individual enzymes. But, no matter to what kind of biochemical process or moiety the search for control mechanisms in metabolism may lead, genetical techniques will be extremely valuable.

CHAPTER 6. SUMMARY

This investigation has attempted to find the primary biochemical lesion in *obese* and *adipose* mice by applying genetical criteria and elucidating the consequences of these lesions.

The physiology and biochemistry of the *obese* mouse syndrome has been reviewed, as has the theoretical and practical considerations involved in the concept of rate controlling enzymes, as well as the agents and mechanisms of the control of lipogenesis.

It has been found that the fatty acid profile of the triglyceride of the fat pad was very similar in *obese*, *adipose*, and normal animals. This indicates that there has been an increase in the synthesis of all the fatty acids in the genetically fat mice rather than any particular group of them.

The activity of several enzymes has been assayed in these animals. ATP citrate lyase, malic enzyme and pyruvate kinase have higher Vmaxs in *obese*, *adipose*, and their phenocopies, caused by auro-thio-glucose injections, than in their normal littermates, whereas there is little difference in the Vmax of isocitric dehydrogenase, lactic dehydrogenase, fumarase and malic dehydrogenase. By applying genetical criteria to these results, it is concluded that none of these enzymes can be the only or primary lesion in any of these animals.

However, another enzyme, 2 monoglyceride lipase, has been reported to have a lower activity in *obese* mice and possibly to exhibit other

properties that would make it the site of the primary enzymic lesion in these animals on genetical criteria. It was not possible to assay the activity of this enzyme in this investigation, but it was possible to examine one of the consequences of this lower activity, the low amount of free linoleic acid that may be available as a metabolic agent, in these animals.

The significance of free linoleic acid as an inhibitor of the activity and as a repressor of the synthesis of the lipogenic enzymes was discussed. It was shown that linoleate was less able to reduce the activity of the lipogenic enzymes in obese than in normal animals when it was fed as a supplement to a fat free diet. It was considered that this effect might be due to the greater amount of linoleate being held in a glyceride form in obese animals, due to the lower activity of 2 monoglyceride lipase. Thus the low amount of free linoleate available in obese does not reduce the lipogenic enzymes as much as the large amounts available in normal animals.

Linoleate is also a powerful inhibitor of the activity of pyruvate kinase. However, there was no difference in its ability to inhibit the activity of this enzyme taken from obese ATC or normal animals.

It was concluded, that, as the preliminary work on 2 monoglyceride lipase in obese, adipose and normal animals has shown that this enzyme might possibly be the primary lesion, in obese mice, then this enzyme's behaviour should be studied in great detail in all these animals to test the validity of these primary observations.

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Fig 5. Chart of metabolic pathways, showing enzymes, substrates and intra-cellular barriers.