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Genetic control of metabolism: enzyme studies of the *obese* and *adipose* mutants in the mouse

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SUMMARY

The activity of several enzymes has been determined in the livers of homozygous *obese* and *adipose* mice, their normal litter-mates, and phenocopies induced in normal mice by aurothioglucose (ATG) injections.

Obese, *adipose* and ATG mice had higher activities of ATP citrate lyase, malic enzyme (NADP malate dehydrogenase) and pyruvate kinase than normal mice. Heterozygote activities are indistinguishable from wild-type. There was no difference between normal and fat litter-mates in the activity of malate dehydrogenase (NAD-linked), lactate dehydrogenase, isocitrate dehydrogenase and fumarase.

Crosses between mice doubly heterozygous for both the *ad* and *ob* genes produced offspring that were only 'fat' or 'normal' and no offspring could be phenotypically recognized as the double mutant, either physically or in terms of ATP citrate lyase activity.

Gas–liquid chromatography of the fatty acids of the depot fat showed no differences between any of the types of litter-mate.

The alterations found in enzyme activity in *obese* and *adipose* mice are compared to several other enzyme activity differences reported in the literature for *obese* mice. These are discussed in relation to genetical criteria that may be established to assess, from quantitative data, whether an enzyme is the site of the primary lesion in a mutant phenotype. Some general observations are made on genetics and the control of metabolism.

1. INTRODUCTION

Investigations of the control of metabolism are greatly aided if genetic variants affecting different steps in a pathway are available. In particular, if variation in the activity of an enzyme can be correlated with a change in concentration of metabolites and their flux through the pathway, an insight into the importance of specific steps can be obtained (Kacser & Burns, 1968). While micro-organisms have been extensively used to obtain mutants of particular enzyme activities, there have been few attempts to screen for these in mammals. Feinstein and his colleagues (Feinstein *et al.* 1964, 1968) were successful with mice in discovering mutants of catalase by screening the progeny of irradiated males. However, most research on the genetics and metabolism in mice has been carried out with mutants that have been discovered by their gross visible effects. These include symptoms

such as early death, colour, severe neurological and balance defects and skeletal abnormalities. In hardly any of these is the site of the primary lesion known. Moreover little is known of the detailed metabolic consequences of these mutants.

In studying lipid metabolism the *obese* mutant, commonly referred to as the obese–hyperglycaemic syndrome, has been the subject of much research (Mayer, 1960; Jansen, Zanetti & Hutchison, 1967; Mayer & Thomas, 1967; Schreeve *et al.* 1967). The primary alteration in gene product in this mutant is, however, not yet known. It is therefore of importance that Lowenstein and his colleagues (Kornaker & Lowenstein, 1964*a*; Spencer & Lowenstein 1966) have reported that ATP citrate lyase (citrate cleavage enzyme) has a threefold higher specific activity in *obese* mice than in their non-obese litter-mates. Kornaker & Lowenstein (1964) argued that, as this enzyme is the first one in the *de novo* synthesis of fatty acids, it could be 'rate controlling' for the pathway, implying that it may be the gene product of the *obese* locus.

The establishment that this or any other enzyme is the primary change would be valuable in the investigation and understanding of many biochemical and physiological effects of this syndrome. It is, however, not generally accepted that ATP citrate lyase enzyme is the 'rate-controlling step' in lipogenesis. Other enzymes have been investigated for their involvement in the control of *de novo* lipid synthesis from glucose (Wise & Ball, 1964; Lynen *et al.* 1964). Therefore neither the involvement of the *ob* locus with any one enzyme nor the importance of such enzymes in the control of lipid metabolism have been established.

It was therefore thought necessary to investigate the activity of a variety of enzymes in the *obese* mutant and compare them with changes seen in ATP citrate lyase (10). The part of the metabolic map showing the enzymes discussed here is illustrated in Fig. 1. The number after each enzyme refers to its position in this figure. The enzymes chosen were, from the malate transhydrogenation shunt, supernatant malic enzyme (7) and supernatant malate dehydrogenase (6); from glycolysis, pyruvate kinase (5) and lactate dehydrogenase (15); and from the TCA cycle, isocitrate dehydrogenase (8) and fumarase (9). Comparisons are also made with published data on the lipogenic enzymes (acetyl CoA carboxylase (11) and fatty-acid synthetase (12)) and some lipases (13).

Several enzymes were found to have altered activities in *obese* mice. It is difficult, however, to establish that an enzyme is the site of the primary lesion of a mutant when data are only available on its altered activity and no information on other kinetic or physical parameters. This problem is discussed in some detail and some criteria are proposed which may help to solve it.

GLC analyses of the lipids were made, since it was possible that the mutant differed in the type as well as the quantity of fatty acid.

The comparison of normal and *obese* animals was extended to two other types of obesity available. The first type is another mutant, *adipose*, discovered in this laboratory (Falconer & Isaacson, 1959). In terms of obesity, *adipose* is indistinguishable from *obese*. They are, however, functionally non-allelic as the double heterozygote is normal. The linkage relationships of the two genes has not been

established and this is discussed below. The third type of obesity is a phenocopy induced in normal mice by aurothioglucose, (ATG), injections (Mayer, 1960).

All three types of animal, *obese*, *adipose* and ATG, were found to have identical enzyme changes when compared with the normal although all three are genetically different. Furthermore it was found that not only ATP citrate lyase but also malic enzyme and pyruvate kinase showed an increase in activity.

These observations, together with the recessive nature of the two mutants, will be discussed in relation to the control of lipogenesis and the question of the site of gene action.

METHODS

(i) *Animals*

The *obese* mice used were from a stock originally obtained from the Jackson Laboratory, Bar Harbor, U.S.A., and have been maintained at this laboratory for some time. The *adipose* mutation appeared in a stock at this laboratory (Falconer & Isaacson, 1959).

(ii) *Quantitative extraction of fat*

Mice were identified by inspection and killed at 6–10 weeks of age and chopped up with solvent in a Waring Blender. The chopped mouse was then washed into a large filter cup and refluxed gently in a Soxhlet for 12 h with diethylether. The dissolved lipid was washed with water, the ether evaporated, and weighed.

(iii) G.L.C. *analysis of lipids*

Lipids were extracted as above and the triglycerides were separated by the method of Goodman, Deykin & Shiratori (1962). The triglyceride was directly saponified with 10% KOH in methanol. Any contaminating non-saponifiables were removed with petroleum ether. The acqueous fraction was acidified with concentrated HCl to pH 1·0, vortexed and extracted three times with petroleum ether. The pooled petroleum ether fractions were washed three times with water and dried over anhydrous sodium sulphate. The extract was concentrated by evaporation and methylated with the boron trifluoride/methanol complex (B.D.H.), which was then destroyed with water. The methylated fatty acids were extracted in petroleum ether and concentrated for gas–liquid chromatography.

(iv) *Enzyme assays*

Mice were identified by inspection and killed at 6–10 weeks of age and a piece of liver immediately homogenized with 3 times (v/w) of freshly prepared 0·25M sucrose at 0–5 °C. Either the crude homogenate was used for the enzyme assay or the homogenate was centrifuged at 60000 g_{av} for 1 h and the supernatant was used.

The enzyme assays used were based on the methods of the following authors ATP citrate lyase (EC 4.1.3.8) (10), Srere (1959); malic enzyme (EC 1.1.1.40) (7), Ochoa (1955*c*); malate dehydrogenase (EC 1.1.1.37) (6), Ochoa (1955*b*);

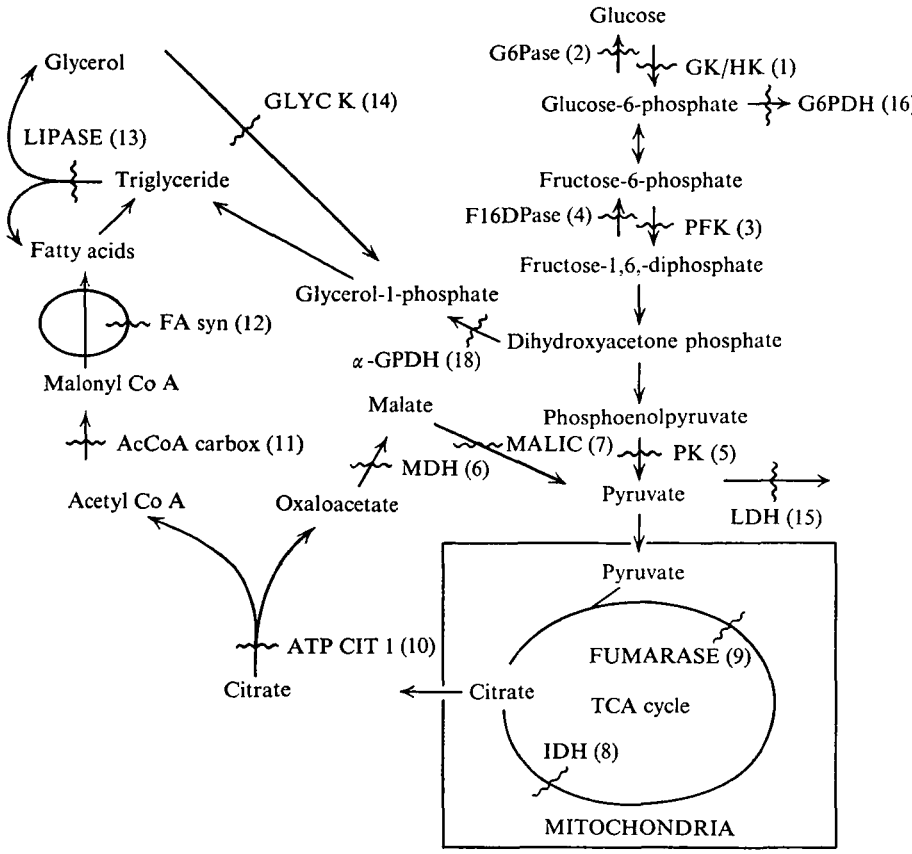

Fig. 1. Area of metabolism and enzymes discussed.

pyruvate kinase (EC 2.7.1.40) (5), Weber, Stamm & Fisher (1965); lactate dehydrogenase (EC 1.1.1.27) (15), Kornberg (1955); isocitrate dehydrogenase (EC 1.1.1.41) (8), Ochoa (1955*a*) and fumarase (EC 4.2.1.2) (9), Racker (1950). Protein was determined by the method of Lowry *et al.* (1951).

(v) *Injection of aurothioglucose*

To destroy the ventromedial nucleii of the hypothalamus and induce phenocopies of *obese* and *adipose*, aurothioglucose was used (Marshall, Barnett & Mayer, 1954). The aurothioglucose, in water, was injected at 1 mg/g body weight, intraperitoneally. This amount is a 50% lethal dose. Dead animals were seen in 2–3 days and the survivors became noticeably fat in 2–4 weeks after injection (see Fig. 2 for an example of the growth of these mice).

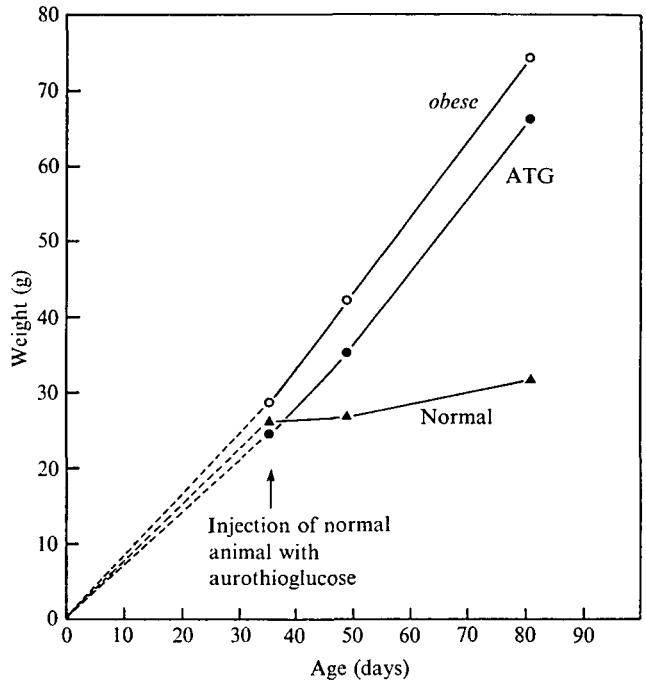

Fig. 2. The growth of *obese*, ATG and normal mice from the same litter.

3. RESULTS

(i) *Live weight and percentage fat*

Table 1 shows that the *adipose* mutant has just as much extractable fat in its carcass as the *obese* mutant. *Adipose* was found to be slightly lighter than *obese* but this may be due to a difference in genetic background.

(ii) *Fatty-acid composition*

The relative amounts of the different fatty acids in fat tissue of *obese* mice had been determined (Stein, Anderson & Hollifield, 1967; Haessler & Crawford, 1965) but was not found to be significantly different from normal mice. In the present investigation no difference between *obese*, *adipose* and normal mice has been detected (Table 2). Therefore there is no alteration in the metabolism of *obese* and *adipose* mice which affects the type of fatty acid produced. The effects of the genetic alterations are therefore not concerned with the steps synthesizing specific fatty acids but must be concerned with the synthesis or breakdown of total lipids.

(iii) *Enzyme activities*

Tables 3–9 show the results of determination of enzyme activity in *obese*, *adipose*, ATG and normal mice. Each table presents the results of within-litter comparisons for one enzyme. The ATG injections were made about 5–6 weeks of age after animals had been classified as normal (Fig. 2). Since no attempt was made to

Table 1. *The live weight, and the weight of fat expressed as a percentage of live weight, of* obese adipose *and their normal litter-mates*

	No. of pairs of observations	Mean of *obese*	Mean of normal	Mean difference ± S.E.	*obese* as a % of normal
Live weight	19	60·7	32·4	28·3*** ± 2·6	187
% of fat	10	33·4	12·0	21·4*** ± 2·1	—
		Mean of *adipose*	Mean of normal		*adipose* as a % of normal
Live weight	13	49·8	34·6	15·2*** ± 2·1	144
% of fat	10	36·8	20·2	16·6*** ± 2·3	—

*** $P < 0{\cdot}001$.

Table 2. *Composition of fat*

(Amounts of six fatty acids, expressed as a percentage of total fatty acids, in the triglycerides of *obese adipose* and their normal litter-mates.) Source of data (reference below)

	(1)		(2)		(3)		(4)		(4)	
Fatty acid	*obese* (%)	Normal (%)	*obese* (%)	Normal (%)	*obese* (%)	Normal (%)	*obese* (%)	Normal (%)	*adipose* (%)	Normal (%)
Myristic (14:0)	2	2	1	2	1	1	1	1	1	1
Palmitic (16:0)	27	24	20	26	20	20	20	24	21	20
Palmitoleic (16:1)	8	5	11	6	8	4	6	4	6	4
Stearic (18:0)	3	2	2	5	1	5	3	3	3	4
Oleic (18:1)	43	39	39	37	39	45	40	39	38	40
Linoleic (18:2)	17	29	26	22	30	22	30	29	30	31

(1) Haessler & Crawford (1965): *ad lib.*
(2) Stein *et al.* (1967): fed mice.
(3) Stein *et al.* (1967): starved mice.
(4) Means of present work: *ad lib.*

equalize the genetic background of the *ad* and *ob* carrying strain, slight differences between the normal values for these strains were observed, but these do not affect the conclusions which can be reached. For enzymes where a difference in activity was observed between the mutant and the normal, only two non-overlapping classes were found. These classes coincided with scoring done on the basis of 'fat' or 'normal' phenotypes. This is relevant to the discussion of dominance relationships in a later section.

Table 3 shows that ATP citrate lyase (10), malic enzyme (7) and pyruvate kinase (5) activity fall into two classes. The activities of the enzymes were at a similar level in *obese*, *adipose* and ATG when compared with normal litter-mates. However, the activities of malate dehydrogenase (6), lactate dehydrogenase (15), isocitrate dehydrogenase (8) and fumarase (9) showed no difference between litter-mates (Table 3).

Table 3. *The specific activity (μmoles/mg protein/h) of enzymes in* obese *and* adipose *mice, their normal litter-mates and phenocopies induced in normal litter-mates by aurothioglucose (ATG) injections*

Enzyme	*n*		Specific activity	% of normal	Mean difference ± S.E.	
ATP citrate lyase (10)	9	*obese*	0·98	231	*obese-normal*	0·56*** ± 0·10
		Normal	0·42	100	ATG-normal	0·42** ± 0·14
		ATG	0·84	198	*obese*-ATG	0·14 ± 0·07
	8	*adipose*	1·09	222	*adipose*-normal	0·60*** ± 0·07
		Normal	0·49	100	ATG-normal	0·44 ± 0·15
		ATG	0·93	190	*adipose*-ATG	0·16 ± 0·09
Malic enzyme (7)	10	*obese*	14·2	254	*obese*-normal	8·6** ± 2·2
		Normal	5·6	100	ATG-normal	6·4*** ± 1·1
		ATG	12·0	214	*obese*-ATG	2·2 ± 2·1
	8	*adipose*	14·4	178	*adipose*-normal	6·3*** ± 0·45
		Normal	8·1	100	ATG-normal	11·1*** ± 0·71
		ATG	19·2	237	*obese*-ATG	(−)4·8*** ± 0·81
Pyruvate kinase (5)	9	*obese*	18·1	150	*obese*-normal	6·0** ± 1·96
		Normal	12·1	100	ATG-normal	5·9* ± 2·53
		ATG	18·0	149	*obese*-ATG	0·1 ± 2·48
	10	*adipose*	12·5	142	*adipose*-normal	3·7** ± 0·87
		Normal	8·8	100	ATG-normal	3·1** ± 0·91
		ATG	11·9	135	*obese*-ATG	0·6 ± 1·1
Malate dehydrogenase (6)	9	*obese*	267	120	*obese*-normal	45 ± 30·5
		Normal	222	100	ATG-normal	14 ± 10·5
		ATG	236	105	*obese*-ATG	31 ± 31·8
	8	*adipose*	357	101	*adipose*-normal	5·0 ± 20·7
		Normal	352	100	ATG-normal	7·0 ± 4·8
		ATG	359	102	*adipose*-ATG	(−)2·0 ± 20·3
Lactate dehydrogenase (15)	8	*obese*	106	109	*obese*-normal	9·0 ± 6·43
		Normal	97	100	—	—
	8	*adipose*	118	116	*adipose*-normal	15*** ± 1·83
		Normal	103	100	—	—
Iso-citrate dehydrogenase (8)	8	*obese*	16·1	95	*obese*-normal	(−)0·9 ± 0·53
		Normal	17·0	100	—	—
	8	*adipose*	18·5	85	*adipose*-normal	(−)3·2 ± 0·72
		Normal	21·7	100	—	—
Fumarase (9)	9	*obese*	20·1	102	*obese*-normal	0·4 ± 0·51
		Normal	19·7	100	—	—
	8	*adipose*	18·1	107	*adipose*-normal	1·1 ± 0·83
		Normal	17·0	100	—	—

* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

The activities in ATG-treated mice were indistinguishable from either *obese* or *adipose* for those enzymes which showed higher activities. Similarly, for an enzyme unaffected by the genotype (MDH) no alteration in activity was observed for the ATG treated mice. There is no reason to believe that the other enzymes shown in Table 3 for which no ATG valves are available would have behaved any differently.

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It therefore appears that the ATG phenocopies mimic the mutants at the level of enzyme activity as well as by their physical appearance.

(iv) *Dominance relationships*

Where the activity of an enzyme had been altered only two activity classes were found. This clearly means that dominance is shown at the enzyme level. Therefore a ratio of 3:1 is expected in offspring from a heterozygous mating. This was tested in a larger scale experiment with ATP citrate lyase (10) activity. The observed ratio of normal:high was 72:23 in litters from the *ob*/+ cross and was 43:13 in litters from the *ad*/+ cross. Neither of these ratios is significantly different from 3:1. The absence of an intermediate enzyme activity class – that is, recessivity at the enzyme level – is a significant observation relevant to the question of primary gene action. This lack of a gene dosage effect will be dealt with in the discussion.

(v) *The relationship between* ob *and* ad

The genes *ob* and *ad* are non-allelic, Falconer & Isaacson (1959) having found no mutant phenotypes in 72 progeny from test crosses between proved *ad* heterozygotes and proved *ob* heterozygotes. The two genes may therefore code for different gene products. However, *ad* and *ob* may be in the same structural gene (and hence very closely linked), but may complement each other.

In all cases the activity of the enzymes in *obese* and *adipose* differed only slightly from each other. These slight differences were probably due to *ob* and *ad* having arisen in different strains. No attempt was made to cross these genes into the same background. The slight enzyme differences, however, became undetectable in the breeding programme aimed at producing double mutants when genetic backgrounds tended to be equalized.

Three matings were constructed where both the male and female had been progeny tested and were known to be heterozygotes for both the *ad* and *ob* genes. In genetical nomenclature the matings were

$$\frac{ob}{+},\frac{ad}{+}\,♀♀ \times \frac{ob}{+},\frac{ad}{+}\,♂♂.$$

There were 66 offspring from these matings which fell into two-non-overlapping classes, 31 being 'normal' and 35 'fat'; these classes coincided for both physical appearance and ATP citrate-lyase activity (Fig. 3).

If the genes are closely linked they would be present in the above matings in repulsion. Therefore a ratio of 1:1, normal:fat, would be expected among the 66 progeny. The observed ratio of 31:35, tested against this expectation, gives a $\chi^2 = 1{\cdot}24$ with $P = 0{\cdot}5$, and therefore the data are consistent with close linkage.

If *ad* and *ob* are not linked we have different expectations. The products of the two genes may interact – that is, the gene dosage at one locus may influence the expression of the other. For although it is known that the double heterozygote is 'normal' because the parents were of this genotype

$$\left(\frac{ad}{+},\frac{ob}{+}\right),$$

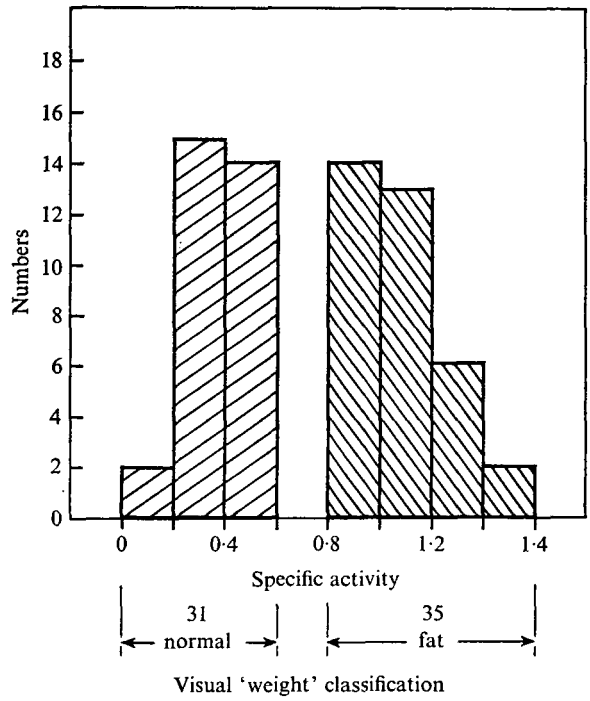

Fig. 3. Frequency distribution of the specific acivity (μmoles/mg protein/h), of ATP citrate lyase (10), of animals from litters, both of whose parents were known to be heterozygous for both the *ad* and *ob* genes $\left(\frac{ob}{+}, \frac{ad}{+}\right)$.

it is not known if the homozygous expression of one gene is affected by the heterozygosity of the other gene; that is, in the genotypes

$$\frac{ad}{ad}, \quad \frac{ob}{+} \quad \text{and} \quad \frac{ad}{+}, \quad \frac{ob}{ob}.$$

Similarly, the phenotype of

$$\frac{ad}{ad}, \quad \frac{ob}{ob},$$

is not known. Any of these phenotypes may be prenatal lethal, normal, fat or 'superfat'. No 'superfat' animals appeared in these 66 progeny and the most reasonable assumption to make is that the three unknown genotypes are all fat. In that case, the assumption of no linkage would predict a digenic ratio of 9 normal:7 fat. The observed ratio of 31:35 tested against this expectation gives a $\chi^2 = 2{\cdot}31$ with $P > 0{\cdot}1$. It is therefore not possible to distinguish between linkage and no-linkage on the basis of the data.

Van der Kroon & Buis (1970) have shown that the genes *ob* and *dw* (*dwarf*) are linked and about 33 map units apart. Falconer & Isaacson (1959) obtained one double *adipose/dwarf* mutant from an incidental cross. This is not inconsistent with *ad* being loosely linked to *dw* but does not give further information on the linkage of *ob* and *ad*.

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Table 4. *Percentage enzyme activity in* obese *and lean mice reported in the literature and this investigation*

Author	Enzyme	Position in fig. 1	Tissue	*Obese* as a % of lean
Elevated				
Lochaya *et al.* 1963	Glycerol kinase	14	Adipose	371
Seidman *et al.* 1967	Glucokinase	1	Liver	357
Chang *et al.* 1967	Acetyl CoA carboxylase	11	Liver	335
Chang *et al.* 1967	Fatty-acid synthetase	12	Liver	267
This investigation	Malic enzyme	6	Liver	254
This investigation	ATP citrate lyase	10	Liver	231
Seidman *et al.* 1967	Fructose-16-diphosphatase	4	Liver	213
Freid & Antopol, 1966	α-Glycerol P D.H.	18	Liver	210
Slightly elevated				
Seidman *et al.* 1967	Glucose-6-phosphatase	2	Liver	163
Seidman *et al.* 1967	Phosphofructosekinase	3	Liver	160
Freid & Antopol, 1966	Glucose-6-P D.H.	16	Adipose	151
This investigation	Pyruvate kinase	5	Liver	150
Fried & Antopol, 1966	Glucose-6-P D.H.	16	Liver	139
Unchanged				
This investigation	Malate D.H.	6	Liver	120
Seidman *et al.* 1967	Phosphoglycerate kinase	19	Liver	111
This investigation	Lactate D.H.	15	Liver	106
Freid & Antopol, 1966	Succinate D.H.	17	Liver	103
This investigation	Fumarase	9	Liver	102
This investigation	Isocitrate D.H.	8	Liver	95
Depressed				
Lochaya *et al.* 1963	Hormonal-sensitive lipase	13	Adipose	73
Stein *et al.* 1967	Monoglyceride lipase	13	Adipose	20

4. DISCUSSION

There is no alteration in the activity of four enzymes in *obese adipose* and, where determined, ATG mice, whereas there is a higher level of three others (Table 3). Other workers have determined the activity of several other enzymes in *obese* but not in *adipose* and these are shown in Table 4. So altogether eight enzymes have much higher activities in *obese*, four enzymes have slightly elevated activities and six enzymes have unaltered activities. Where the activity has been altered in *obese* only two classes of activity are reported for *obese* and their normal litter-mates. There is therefore no gene dosage affect at the level of the activity of these enzymes.

Two further enzymes have been investigated in *obese*, namely hormone-sensitive lipase (Lochaya, Hamilton & Mayer, 1963) and 2-monoglyceride lipase (Stein *et al.* 1967) (13). Contrary to the behaviour of all other investigated enzymes, both these show lowered activity in the *obese* mutant.

Glucose oxidation is another phenotype with lowered values in *obese*. It has further been reported that the fat pads from the *obese* heterozygote has intermediate rates of glucose oxidation, although no information was given on any of the enzymes involved (Yen, Lowry & Steinmitz, 1968).

As described in the Methods section, physical phenocopies of the *obese* and

adipose can be induced by aurothioglucose injections. These ATG mice are known to be, in part, physiologically different from *obese* (Mayer, 1960). In terms of enzyme pattern, however, they are very similar to the mutants and therefore a different biochemical lesion induces the same complex enzyme pattern. The observed enzyme pattern therefore appears to be a secondary phenomenon.

A further interesting aspect of these observations is that there appears to be no simple pattern in the distribution of 'affected' and 'non-affected' enzymes. Thus both classes occur in gluconeogenesis as well as in glycolysis. Both enzymes in lipogenesis are elevated. The primary effect in both mutants and the lesion in the phenocopy therefore has widespread consequences.

Before this investigation was started it had been reported (Kornaker & Lowenstein, 1964*a*; Spencer & Lowenstein, 1966) that *obese* mice had a higher ATP citrate lyase activity. This could be interpreted to mean that the enzyme might be the site of the primary lesion, although the evidence from *obese* (and partly from *adipose* and ATG) that 12 enzymes show elevated activities makes it unlikely (although not impossible) that the primary alteration is in one of them.

Although the primary lesion could be at a regulatory step rather than in an enzyme, it is useful to discuss, in the absence of structural data, other criteria that can be applied to a situation like the present one, in which there have been changes in the activity of several enzymes. There are three criteria that may be used to establish from alterations in activity whether an enzyme is the site of a mutant gene's action. Although each of them is not solely decisive, they can at least together be used to devise fairly firm conclusions. The criteria are:

(i) *The allelic criterion*

In the case of two functionally allelic genes the problem is only to find the enzyme with altered structure or kinetic parameters. When two genes are functionally non-allelic (as in the case of *ob* and *ad*), if the two mutations are in two structural genes, it should be possible to pinpoint a separate altered enzyme for each mutant. However, the two mutants may affect different polypeptides in the same enzyme and therefore only one enzyme will be affected. Alternatively, they may be complementing alleles at one locus, and the primary effect may be similar for both genes. The observations that the same enzyme pattern appears in both mutants makes this criterion inapplicable in the present case.

(ii) *The dosage criterion*

In cases where the gene–enzyme relationship has been established, dominance at some phenotypic level has always been shown to be associated with intermediate values at the enzyme level. The finding of intermediate values in heterozygotes is therefore a criterion that the locus involved controls the enzyme measured. This has been shown, for example, in the many human inborn errors of metabolism whose primary enzymic lesion is known (Harris, 1970, pp. 174–175). The absence of a third and intermediate enzyme activity class in all heterozygotes

examined in the present investigation makes it unlikely that the *ad* or *ob* locus is directly concerned with the structural genes for any of these enzymes.

The obverse statement, that the presence of an intermediate class of enzyme activity in heterozygotes constitutes unambiguous evidence of the primary gene-enzyme relationship, is not true. It is possible that one gene product can change the activity of several enzymes in the heterozygote to an intermediate level.

(iii) *The recessivity criterion*

From much work on auxotrophs in micro-organisms, the study of inborn errors in man where the enzymic lesion is known, and from general consideration about the kinetic properties of multi-enzyme systems, we expect that a recessive structural allele will correspond to lower activity at the enzyme level. In *obese* and *adipose*, all enzymes altered had higher activity in the recessive homozygote. This argues against any of the 12 enzymes being primarily affected by the loci in question.

It may be concluded that none of the enzymes so far examined fulfil all the above three criteria in either the *obese* or the *adipose* mutants. However, of all the metabolic data published on *obese* only two alterations fulfil any of the criteria. The hormone-sensitive lipase (Lochaya *et al.* 1963) and 2-monoglyceride lipase (Stein *et al.* 1967) have lower activity in *obese* than normal and therefore fulfil criterion no. (iii); the intermediate rate of glucose oxidation by *obese* heterozygotes fulfils criterion no. (ii). It is not known whether these two sets of observations fulfil other criteria and they require closer investigation. Should the site of primary gene action be found, the problem of the elevation of a large number of other enzymes as secondary consequences would remain to be elucidated.

Lowenstein and his co-workers have also established that ATP citrate lyase activity correlates with lipogenesis under different nutritional and hormonal conditions as well as in the *obese* mouse (Kornaker & Lowenstein, 1964*b*, 1965*a*, *b*). They suggest that this might be the 'rate controlling' enzyme with respect to lipogenesis. 'Rate control' is often investigated in mammals by altering nutritional and hormonal conditions and recording correlated responses of enzymes, a response being thought of as consistent with rate control. However, in the *obese*, *adipose* and ATG mouse many enzymes are altered simultaneously. This would seem to indicate the hardly acceptable conclusion that all these enzymes are 'rate controlling'. With so many parameters altered in complex syndromes like *obese* and *adipose* it appears impossible to evaluate the 'rate controlling' properties of any enzyme. To simplify this problem Kacser & Burns (1968 and 1972) have developed a 'sensitivity coefficient' of flux to enzyme activity, which can be established for each enzyme using mutants in micro-organisms where the lesion is known and affects the activity of one enzyme.

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