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The Influence of Pituitary Growth Hormone on the Catabolism of Fat

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(Received 15 May 1952)

The increase in nitrogen and water and the decrease in fat content of animals treated with growth-promoting anterior pituitary extracts (Lee & Schaffer, 1934; Young, 1945; Li, Simpson & Evans, 1948), and the lowering of the respiratory quotient (Gaebler, 1933*a, b*; Greaves, Freiberg & Jones, 1940; Greenbaum, 1953), clearly indicate that the metabolic pattern of such treated animals is changed, so that a larger proportion of the energy requirement is obtained from the oxidation of fat. Two further lines of evidence point to this same conclusion. The mobilization of fat to the liver within 6 hr. and its subsequent disappearance during the following 18 hr. (Szego & White, 1949; Li, Simpson & Evans, 1949; Greenbaum & McLean, 1953) and the increased level of blood and urinary ketones in rats shortly after growth-hormone treatment (Bennett, Kreiss, Li & Evans, 1948; Bondy & Wilhelmi, 1950) are both indicative of an increased fat catabolism.

There is little information available to indicate whether the influence of growth hormone is on the catabolism of fat in the liver. Bondy & Wilhelmi (1950) have shown that the acute treatment of fasted rats with massive doses of growth hormone failed to increase the rate of acetoacetate production of liver slices from such treated animals, although they did observe an increase in the level of blood ketones. The same authors also found that ketone production by liver slices from hypophysectomized rats, incubated *in vitro* in the absence of added substrate, was greatly reduced, and Tepperman & de Witt (1951), using similar methods, have confirmed this result. Shipley (1944) has claimed that the addition of relatively crude pituitary extracts to

liver slices incubated in serum resulted in an increased acetoacetate production, but Bondy & Wilhelmi (1950) have been unable to confirm this observation using purified growth hormone. There has been no report of the effect of growth hormone on the degradation of fatty acids in the liver.

The influence of growth hormone on the extrahepatic utilization of fat and ketone bodies has also received scant attention. While muscle tissue is known to oxidize ketone bodies rapidly (Harrison & Long, 1940; Stadie, 1945) the evidence of Bennett *et al.* (1948) showing that growth hormone failed to influence their rate of utilization in eviscerated, nephrectomized rats, seems to indicate that this hormone does not stimulate extrahepatic utilization. The direct oxidation of long-chain fatty acids by tissues other than the liver has only recently been conclusively demonstrated (Geyer, Waddell, Pendergast & Yee, 1951; Weinhouse, Millington & Volk, 1950) but, so far, the possible hormonal control of this aspect of fat metabolism has attracted little attention.

A quantitative assay of the overall activity of the liver fatty acid-oxidase system would throw considerable light on the problem of the locus of action of growth hormone in increasing fat oxidation in treated animals and such a study has therefore been initiated. Furthermore, it was of interest to relate the changes in the activity of this enzyme system with the observed mobilization and subsequent loss of neutral fat in the liver. Measurements were also made of the acetoacetate production by the liver since the ketones produced may serve as fuel for the extrahepatic tissues, and because the correlation of liver acetoacetate production with the measured *in vivo* changes in blood and urine ketone bodies is of some interest.

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EXPERIMENTAL

Animals. Female hooded Norway rats, of the Medical Research Council strain, aged approximately 6 months and with initial body weights ranging from 170 to 200 g., were used. The maintenance and diet was as described earlier (Greenbaum & McLean, 1953). Limit feeding was used in preference to the paired-feeding technique, as this has many advantages in experiments in which several groups of rats are treated for differing lengths of time with growth hormone. The uniformity of daily food intake allows the direct comparison between the results obtained on any one group with those from any other, and is essential when measurements of blood and liver constituents are to be made. Further, only one group of control rats is required. The rats were randomly distributed among eight groups, each containing six rats, one group of controls and seven groups of animals treated with purified pituitary growth hormone, 0.5 mg. daily. The periods of treatment were 6, 12 and 24 hr., and 2, 4, 6 and 8 days. Injections were given subcutaneously except on the final day when they were given intraperitoneally. In those experiments where the urinary excretion of ketones was measured, the rats were kept in individual metabolism cages and the urine collected under toluene. The sequence of killings was arranged to be spread as evenly as possible in each group throughout the duration of the experiment. Animals were killed by dislocation of the cervical vertebrae.

Materials. (a) *Growth hormone.* Two samples of growth hormone were used; the first, prepared by the method of Li, Evans & Simpson (1945), was used in one preliminary experiment on ketone excretion, and the second, prepared by the method of Wilhelmi, Fishman & Russell (1948) was used in the majority of the work reported here. This latter preparation is claimed by the authors to be substantially free of adrenocorticotrophin. For injection the hormone was suspended in water and the pH adjusted to 8.0.

(b) *Substrates.* Methyl butyrate was redistilled and the fraction boiling between 102° and 105° was collected. Oleic acid was redistilled under vacuum and stored under nitrogen to prevent autoxidation. Octanoic acid was redistilled and the fraction with a boiling point of 237° collected.

Chemical methods. In the preliminary experiment the urinary ketones were estimated by the method of Denigès & Van Slyke (Peters & Van Slyke, 1932). In the main experiment the method of Lester & Greenberg (1948) was used both for blood and urinary ketones.

Liver-esterase activity. Suspensions of liver were prepared according to the method of Copenhagen, Stafford & McShan (1950) to give a concentration of 1 in 10 and were diluted immediately before use to a final tissue concentration of 10 mg. wet weight/ml. Esterase activity was measured by the method of Connors, Pihl, Dounce & Stotz (1950) using 0.3 ml. of suspension. Preliminary experiments showed that the CO₂ production was proportional to tissue concentration up to 5 mg. wet weight of liver. The results are expressed in the units defined by Connors *et al.* (1950).

Plasma pseudo cholinesterase. This enzyme was measured in plasma, separated from heparinized blood, by the manometric method of Mendel, Mundell & Rudney (1943).

Fatty acid oxidase. Although there have been intensive investigations of the requirements of the fatty-acid oxidase system (Leloir & Muñoz, 1939, 1944; Muñoz & Leloir, 1943; Lehninger & Kennedy, 1948) and demonstration of its

activity in liver homogenates (Lehninger, 1945*a*), washed mitochondrial suspensions (Lehninger, 1945*b*) and the cyclophorase system (Grafflin & Green, 1948), no satisfactory quantitative measurement of this enzyme has yet been published. The mitochondrial suspensions which have recently been used to study this enzyme system are not entirely suitable for assay purposes. In this investigation suspensions prepared in 0.25 M-sucrose (tissue concentration 1:5) under ice-cold conditions in an all-glass homogenizer (Potter & Elvehjem, 1936) were used. The reaction mixture contained the following components, all of which were measured directly into the main compartment of the Warburg flasks. The values are all given as final molarities, the total volume in each flask being 3.0 ml.; sodium phosphate buffer, pH 7.4, 1.0×10^{-2} M; KCl, 2.5×10^{-2} M; MgCl₂, 6.6×10^{-3} M; succinate, 1.0×10^{-3} M; cytochrome *c*, 2.66×10^{-5} M; adenosinetriphosphate, 2×10^{-3} M; and either oleate, 1.66×10^{-4} M, or octanoate, 1.33×10^{-3} M; 0.25 ml. suspension, equivalent to 50 mg. tissue. All components were adjusted to pH 7.4 with KOH. In the blank determinations all the reagents were present except the substrates, oleate or octanoate. No gassing was required and the reaction was carried out at 37°. The stock 0.005 M-oleate was prepared essentially according to Kennedy & Lehninger (1950) except that the oleic acid was brought completely into solution by the addition of alkali and the pH then adjusted to 8.0 at 60°. This solution was stable for 2 weeks if stored under N₂.

Uniformity of technique and timing is an essential feature for the quantitative assay of this system. Owing to the instability of this enzyme in the absence of its substrate (Grafflin & Green, 1948) only 40–50 sec. were spent in homogenizing and the enzyme was then added directly to the medium in the main compartment of the Warburg flasks. The time elapsing between killing the animal and the beginning of a 10 min. equilibration period was always between 6 and 7 min. Subsequent readings were taken at 5 min. intervals for 20 min., at the end of which the flasks were removed from the bath and the acetoacetate produced during the reaction period was measured.

After removal from the bath, the flask contents were acidified with citric acid and the acetoacetate measured by the method of Edson (1935). An uptake of gas was observed to occur as a result of the action of aniline citrate on the contents of those flasks containing oleate. A blank value was therefore determined using a flask containing oleate and all the other components of the reaction mixture except tissue so that a correction could be made for this factor.

The results of these measurements have been expressed in four ways. (1*a*) As μ moles O₂ uptake/g. wet liver/hr. (total O₂ uptake). The O₂ uptake in the presence of the substrate was corrected only for the endogenous uptake. (1*b*) As μ moles acetoacetate produced/g. wet liver/hr. Here again corrections were made for the production of acetoacetate by liver suspensions in the absence of added substrate. (2) Fatty-acid oxidase may be considered to be the enzyme catalysing the breakdown of fatty acids to two-carbon fragments, and a calculation can be made of that part of the total O₂ uptake used in the formation of these two-carbon fragments. This is termed 'fatty-acid oxidase O₂ uptake' and the results are expressed as μ moles O₂ uptake/g. wet liver/hr. A discussion of the assumptions involved in this calculation is given below. (3) A unit of fatty-acid oxidase is defined as that amount of enzyme which, under the conditions described above, uses 100 μ l. O₂/hr.

In complex systems, such as the fatty-acid oxidase system, an assay procedure only measures the rate-limiting step, but it was felt that even with such limitations this assay would reveal any considerable changes occurring in the oxidation of fat in growth-hormone-treated animals.

The method described here was arrived at as a result of a series of experiments on the optimum levels of substrate concentration and fortification, and the effect of varying the level of the tricarboxylic acid cycle intermediate on acetoacetate production. Octanoate and oleate have both been used as substrate for this enzyme; the former because of its solubility and the wide knowledge of its metabolism by various preparations of liver; the latter because it more nearly represents the substrate normally available to the enzyme. There is, further, a difference in the metabolic behaviour of short- and long-chain fatty acids (Kennedy & Lehninger, 1950).

Fig. 1. O_2 uptake/substrate concentration curves of the oxidation of (a), octanoate and (b) oleate by suspensions of 'homogenized' rat liver, 50 mg. of tissue/flask. Substrate concentrations are $\times 10^{-3}\text{M}$ for octanoate and $\times 10^{-4}\text{M}$ for oleate.

Reaction velocity/substrate concentration curves, determined as described above except only that the substrate concentrations were varied, are illustrated in Fig. 1 (a). The initial reaction velocity of the oxidation of octanoate increased with increasing substrate up to a level of $0.66 \times 10^{-3}\text{M}$ (final concentration) and remained unchanged at the higher concentrations tested. In the present investigation a final concentration of $1.33 \times 10^{-3}\text{M}$ -octanoate was used, a concentration similar to that used by Kennedy & Lehninger (1950) in their study of the oxidation of octanoate by mitochondrial preparations.

The reaction velocity/substrate concentration curves with oleate as substrate are shown in Fig. 1 (b). The experi-

ments demonstrate that the optimum substrate concentration lies between 0.83 and $1.66 \times 10^{-4}\text{M}$. At concentrations above $1.66 \times 10^{-4}\text{M}$ the reaction rate falls off sharply, and at values of $3.3 \times 10^{-4}\text{M}$ and over, not only is the fatty-acid oxidase inhibited but so also is the endogenous respiration. The inhibition increases progressively with time. At substrate concentrations of 0.83 and $1.66 \times 10^{-4}\text{M}$ the O_2 uptake was linear for the first 10 min. but had departed somewhat from linearity by the end of 20 min. Although this departure is quite marked it was not thought to be sufficiently great to vitiate the assay procedure. The condi-

Fig. 2. Relationship between the weight of liver used and the O_2 uptake due to the oxidation of added substrate by the fatty-acid oxidase system of suspensions of 'homogenized' rat liver. The substrates are (a) octanoate at a concentration of $1.33 \times 10^{-3}\text{M}$, and (b) oleate at a concentration of $1.66 \times 10^{-4}\text{M}$. O_2 uptake measured over a period of 20 min.

tions finally employed were a substrate concentration of $1.66 \times 10^{-4}\text{M}$ (compared with $2.5 \times 10^{-4}\text{M}$ used by Kennedy & Lehninger, 1950) and a measurement of O_2 uptake over a period of 20 min. This represents a compromise between two factors; on the one hand an enzyme reaction should be measured so that the initial velocity is determined, while on the other hand a reasonably large O_2 uptake and production of acetoacetate must occur in order to obtain accurate manometric measurements.

The results of these experiments are of interest in view of the fact that neither Muñoz & Leloir (1943) nor Graffin & Green (1948) were able to obtain the oxidation of higher fatty acids in their system. The former used stearic, palmitic and

oleic acids at a concentration of $3 \times 10^{-3} M$ and found no oxidation, a failure which clearly results from the excessive substrate concentration. Grafflin & Green (1948) noted that while their cyclophorase preparation readily oxidized the higher fatty acids at low concentrations, at higher concentrations the fatty acids were oxidized poorly or not at all.

The effects of varying the concentrations of adenosine-triphosphate (ATP), cytochrome *c* and succinate have also been investigated. The levels of ATP and cytochrome *c* were shown not to be limiting in this system and are about double those used by Kennedy & Lehninger (1950). The level of succinate was also about double that used by these authors and is, in fact, in good accord with the amount found by Lehninger & Kennedy (1948) to be necessary to suppress acetoacetate formation in their *in vitro* system.

The validity of this method as an assay procedure depends on the demonstration of a direct proportionality between the amount of enzyme added and the oxidation of the fatty acid; this is illustrated in Fig. 2. There is a straight-line relationship between O_2 uptake and tissue weight at least up to 80 mg. with octanoate and 60 mg. with oleate. The tissue weight used throughout these experiments, 50 mg., is well within the linear range for both of these substrates.

The rate of acetoacetate production, in liver preparations, from added fatty acid depends on the level of tricarboxylic acid cycle intermediate present (Lehninger & Kennedy, 1948). In order to demonstrate that the observed acetoacetate production in the present experiments was not conditioned by the level of added succinate, O_2 uptake and acetoacetate production from octanoate were measured in systems containing half and double the usual succinate concentration, the tissue weight being constant at 50 mg. In all cases, the extra O_2 uptake due to the oxidation of the substrate and the rate of production of acetoacetate were the same, and therefore independent of the succinate level, thus demonstrating that the changes described below can, in all probability, be ascribed to changes in the enzyme system.

Calculation of O_2 used in the formation of two-carbon fragments and that used in the complete oxidation to CO_2 and water

The O_2 uptake due to the oxidation of octanoate may be considered to consist of two parts; that required for the formation of C_2 fragments, and that used when the C_2 fragments are oxidized to CO_2 and water. The O_2 consumption measured is the sum of the O_2 utilized for both of these steps and is referred to as the 'total O_2 uptake'. The measurement of the total O_2 uptake and the acetoacetate production allows the calculation of that portion of the total O_2 uptake used in the formation of the C_2 fragments, and this is referred to as the 'fatty-acid-oxidase O_2 uptake'.

When octanoate is being oxidized the main reactions occurring may be represented thus:

and the following calculation may be made: The total O_2 uptake equals $A + B + C$. The fatty acid O_2 uptake equals $A + C$. C may be readily calculated from the determined

acetoacetate production. It is apparent that 1 mole of acetoacetate requires 1.5 moles of O_2 for its production and gives rise to 1 mole CO_2 on decarboxylation with aniline citrate. Thus the amount of O_2 used in the formation of acetoacetate, C , is simply 1.5 times the CO_2 produced on decarboxylation. The sum of the O_2 used in reactions A and B is the total O_2 consumption minus C . This quantity can be apportioned between reactions A and B in the ratio of 3:8.

Thus the O_2 used in reaction A is

$$\frac{(\text{total } O_2 \text{ uptake} - C) 3}{11},$$

and that used in reaction B is

$$\frac{(\text{total } O_2 \text{ uptake} - C) 8}{11}.$$

The oxygen used in the formation of the C_2 fragments from octanoate ($A + C$) is then

$$(\mu l. CO_2/\text{flask} \times 1.5) + \frac{[\text{total } \mu l. O_2 \text{ uptake} - (\mu l. CO_2/\text{flask} \times 1.5)] 3}{11},$$

and the O_2 used in the oxidation of the C_2 fragments to CO_2 and water is given by

$$\frac{[\text{total } \mu l. O_2 \text{ uptake} - (\mu l. CO_2/\text{flask} \times 1.5)] 8}{11}.$$

Similar calculation in the case of oleate gives the following equations.

Oxygen used in the formation of the C_2 fragments:

$$(\mu l. CO_2/\text{flask} \times 1.78) + \frac{[\text{total } \mu l. O_2 \text{ uptake} - (\mu l. CO_2/\text{flask} \times 1.78)] 8}{26},$$

and the O_2 used in the oxidation of the C_2 fragments to CO_2 and water is given by

$$\frac{[\text{total } \mu l. O_2 \text{ uptake} - (\mu l. CO_2/\text{flask} \times 1.78)] 18}{26}.$$

The value of these calculations depends on the validity of the assumptions that have been made.

It has been assumed that the C_2 fragments arising from the oxidation of fatty acids are either oxidized via the tricarboxylic acid cycle or react to form acetoacetate, that the products of this oxidation are acetoacetate, CO_2 and water, and that negligible amounts of shorter-chain fatty acids or β -hydroxybutyrate are formed. There are, however, many alternative pathways of metabolism for acetyl groups in animal tissues. They may, for example, participate in the formation of higher fatty acids, cholesterol or the porphyrins (see Bloch, 1947). Nevertheless, it may be expected that these pathways are not large in comparison with oxidation in the present investigation. The synthesis of long-chain fatty acids seems unlikely in view of the experiments of Brady & Gurin (1950) who failed to find such a synthesis with rat-liver suspensions, and although fatty-acid synthesis has been demonstrated in pigeon-liver suspensions (Brady & Gurin, 1951), bicarbonate seems to be an essential

component of the system, a constituent not used here. Lehninger (1946b) has made a quantitative analysis of the products of enzymic fatty-acid oxidation using a washed rat-liver suspension, in the presence of ATP, Mg^{2+} and malonate. Under these conditions octanoate was oxidized completely to acetoacetate, while in the presence of oxaloacetate the yield of acetoacetate was decreased and citrate accumulated. In these experiments there seems to have been no major pathway of metabolism other than acetoacetate formation or oxidation via the tricarboxylic acid cycle, a finding which supports the assumptions made above. Lehninger also reported that when the products of enzymic oxidation of octanoate were analysed for ketone bodies by the aniline citrate method of Edson (1935) and the colorimetric method of Greenberg & Lester (1944), both methods gave results which were in good agreement. This indicated that no β -hydroxybutyrate was formed, and also lends support to the assumption that in the present experiments acetoacetate was the sole ketone body formed.

It is fully appreciated that the assumptions made above may not be entirely valid, nevertheless the approximation they enable us to reach gives a more complete picture of the changes in fat metabolism occurring under the influence of growth hormone. The fact that the ratio of total O_2 uptake: acetoacetate produced varied widely at different periods after growth hormone indicated that the relative rates of the three reactions in the above equations were changing. The calculations not only permit an estimate of the partition of the O_2 uptake among the various reactions, but also allow the calculation of the corrected fatty-acid oxidase activity.

RESULTS

The oxidation of fatty acids and the production of acetoacetate

The results are summarized in Tables 1 and 2 and in Figs. 3 and 4. Two main phases of the changes occurring in fat metabolism may be distinguished.

The earliest change observed is in the inhibition of fatty-acid oxidation and ketone production, when both octanoate and oleate are substrates, which occurs 6 hr. after growth-hormone treatment. When the fatty-acid oxidase oxygen uptake is calculated (Table 2) the inhibition is still apparent thus indicating an actual inhibition of the breakdown of fatty acids to two-carbon fragments. The calculated oxidation of two-carbon fragments to carbon dioxide and water is also decreased (Table 2). At this time interval the endogenous oxygen uptake is raised about 25 % above the control value (Fig. 3).

Twelve hours after the administration of growth hormone the relative values of fatty-acid oxidase activity and acetoacetate production have altered considerably. There is an increase in the total oxygen uptake and the acetoacetate production is restored towards the control level. It is apparent from Table 2 that the increase in the total oxygen uptake is due to a restoration of the fatty-acid oxidase oxygen uptake to the control level, and to a marked stimulation of the complete oxidation of two-carbon fragments to carbon dioxide and water. By this

time the endogenous oxygen uptake has returned to the control level.

The total oxygen uptake/g. liver returns to control level 24 hr. after growth-hormone treatment when the acetoacetate production reaches its peak. The ratio acetoacetate production: total oxygen uptake rises to 0.56 for oleate and 0.54 when octanoate is being oxidized from values of 0.08 and 0.23,

Fig. 3. Oxidation of fatty acids by suspensions of 'homogenized' rat liver from normal and growth-hormone-treated rats. (a) Octanoate as substrate, (b) oleate as substrate. O_2 uptake in the absence of added substrate ($\circ$); O_2 uptake due to oxidation of added fatty acid ($\bullet$) and CO_2 produced by the decomposition of the acetoacetate, formed during the reaction, by aniline citrate ($\ominus$). All values given as μ moles/g. liver wet weight/hr. The vertical lines represent twice the S.E.M. Where no vertical line is shown the S.E.M. is too small to show.

respectively, at 12 hr. (Table 2). This suggests changes in the relative amount of two-carbon fragment being converted to acetoacetate and the amount which is completely oxidized to carbon dioxide and water. It is apparent, therefore, that at this time octanoate and oleate are largely converted to acetoacetate.

When the total oxygen uptake is used as an index of enzyme activity the large increase in acetoacetate output at 24 hr. masks the increased fatty-acid oxidase activity, but if due allowance is made for this and the results are expressed as fatty-acid

Table 1. *The influence of purified growth hormone on the liver fatty-acid oxidase system*

No. of animals	Control rats	6 hr.		12 hr.		1 day		2 days		4 days		6 days		8 days	
		3		3		3		3		3		3		3	
Body weight (g.) Initial	191 ± 4.2	182 ± 3.7		179 ± 5.1		190 ± 3.1		182 ± 7.3		185 ± 9.1		190 ± 7.9		187 ± 6.8	
Body weight (g.) Final	191 ± 4.2	182 ± 3.7		184 ± 3.0		195 ± 3.5		191 ± 5.8		200 ± 10.5		203 ± 6.5		200 ± 6.5	
% change	—	—		3.0 ± 0.7		2.6 ± 0.3		4.0 ± 0.2		8.3 ± 0.7		7.1 ± 1.2		6.7 ± 1.4	
Liver weight (g.)	7.19 ± 0.22	7.68 ± 0.24		7.38 ± 0.30		8.03 ± 0.48		7.21 ± 0.13		7.75 ± 0.14		7.86 ± 0.71		7.45 ± 0.32	
Total O ₂ uptake*															
(1) Octanoate substrate															
(a) Units/g. (wet weight)	21.7 ± 1.1	8.8 ± 2.7		26.8 ± 2.0		18.6 ± 2.2		18.0 ± 1.6		19.2 ± 1.6		23.0 ± 2.6		19.4 ± 0.9	
(b) Total units	156.6 ± 11.4	67.0 ± 23.0		197.9 ± 17.7		150.5 ± 23.1		129.4 ± 9.8		149.2 ± 14.5		182.9 ± 34.1		144.8 ± 11.5	
(2) Oleate substrate															
(a) Units/g. (wet weight)	13.1 ± 2.2	4.4 ± 2.4		21.2 ± 1.6		9.2 ± 2.3		9.8 ± 2.1		8.0 ± 0.9		9.9 ± 0.9		11.4 ± 1.8	
(b) Total units	92.9 ± 13.0	34.0 ± 18.1		156.3 ± 13.0		75.8 ± 21.5		70.2 ± 13.8		62.1 ± 7.4		74.4 ± 4.6		86.1 ± 16.9	
Fatty-acid oxidase O ₂ uptake†															
(1) Octanoate substrate															
(a) Units/g. (wet weight)	12.2 ± 1.3	5.5 ± 2.2		14.1 ± 1.5		16.2 ± 1.5		11.8 ± 3.1		10.9 ± 1.3		14.1 ± 0.8		13.7 ± 0.8	
(b) Total units	87.2 ± 7.9	43.0 ± 17.0		103.2 ± 8.2		131.3 ± 19.3		84.5 ± 20.6		84.5 ± 10.5		111.6 ± 14.5		102.7 ± 10.3	
(2) Oleate substrate															
(a) Units/g. (wet weight)	5.4 ± 0.8	1.4 ± 0.7		8.6 ± 1.8		9.6 ± 1.0		6.0 ± 1.6		4.5 ± 1.1		4.4 ± 0.1		5.5 ± 0.9	
(b) Total units	38.0 ± 5.2	11.1 ± 5.3		62.6 ± 10.8		78.0 ± 12.1		58.1 ± 15.2		34.7 ± 9.1		33.7 ± 9.3		41.4 ± 7.7	

* The results are expressed as total O₂ uptake corrected only for the endogenous O₂ uptake.† The results are expressed as 'fatty-acid oxidase O₂ uptake', and refer only to that part of the total O₂ uptake used in the formation of the C₃ fragments.Table 2. *Relative rates of degradation of fatty acids to C₃ fragments, to CO₂ and water and to acetoacetate*

Group	Octanoate oxidation (μmoles/g. liver (wet weight)/hr.)				Oleate oxidation (μmoles/g. liver (wet weight)/hr.)			
	Total O ₂ uptake	μmoles O ₂ used for fatty acid → C ₃ *	μmoles O ₂ used for C ₃ → CO ₂ + H ₂ O	Acetoacetate Total oxygen	Total O ₂ uptake	μmoles O ₂ used for fatty acid → C ₃ *	μmoles O ₂ used for C ₃ → CO ₂ + H ₂ O	Acetoacetate Total oxygen
Control	97.1 ± 4.9	60.0 ± 7.5	37.0 ± 15.0	0.32	58.4 ± 9.6	23.9 ± 4.1	34.6 ± 4.3	0.08
6 hr.	39.3 ± 12.0	24.6 ± 10.3	15.3 ± 10.2	0.33	19.7 ± 10.5	6.4 ± 2.9	13.2 ± 2.5	0.02
12 hr.	119.7 ± 8.9	62.7 ± 7.2	57.8 ± 13.8	0.23	94.7 ± 7.3	38.6 ± 10.3	56.3 ± 3.0	0.08
1 day	83.1 ± 9.7	72.3 ± 9.6	12.9 ± 7.7	0.54	41.1 ± 10.3	42.9 ± 7.1	4.8 ± 0.4	0.56
2 day	80.4 ± 7.1	52.8 ± 17.4	27.6 ± 15.1	0.35	43.7 ± 9.4	25.5 ± 7.9	18.2 ± 4.0	0.22
4 day	85.8 ± 7.1	48.7 ± 6.8	36.7 ± 17.1	0.27	35.7 ± 3.9	19.0 ± 6.7	15.3 ± 1.5	0.18
6 day	102.7 ± 11.8	63.2 ± 6.2	37.9 ± 12.1	0.31	44.2 ± 4.0	30.0 ± 5.1	14.2 ± 1.3	0.20
8 day	86.7 ± 3.9	61.1 ± 4.0	57.0 ± 13.1	0.40	50.9 ± 8.2	24.7 ± 3.9	26.2 ± 1.8	0.14

* Fatty-acid O₂ uptake.

oxidase oxygen uptake then it can be seen clearly that the enzyme reaches its maximum activity 24 hr. after growth-hormone treatment (Table 2).

After administration of growth hormone for 2 days, and at all time intervals up to 8 days, the fatty-acid oxidase activity expressed either as total oxygen, as fatty-acid oxidase oxygen uptake, or even as total liver units, falls within the range of the control values (Fig. 3, Table 2). The acetoacetate production/g. liver is also unchanged throughout this period when either oleate or octanoate is substrate. It is interesting to note that when oleate is being oxidized the ratio acetoacetate production: total oxygen uptake is consistently higher than the control values at all times after 24 hr. treatment, a point of difference from octanoate. This would

Fig. 4. Production of acetoacetate by the entire liver of normal rats and rats treated with growth hormone. The ordinate represents μ moles acetoacetate produced by the entire liver/hr. from oleate (○) and octanoate (●). The vertical lines are equal to the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

seem to indicate that a larger proportion of the two-carbon fragments are being converted to acetoacetate at these times and may be correlated with the tendency for the total acetoacetate production by the entire liver to be increased when oleate is being oxidized (Fig. 4). Calculation of the mean daily output of acetoacetate by the entire liver between 4 and 6 days of growth-hormone treatment (294 mg./24 hr.) compared with the output of control animals (152 mg./24 hr.) reveals the scale of this increased production.

The *in vitro* changes in total acetoacetate production by the entire liver are shown in Fig. 4, the two most striking points being the inhibition of production at 6 hr. followed by a stimulation at 24 hr. Treatment of the intact animal with purified growth hormone also results in ketonaemia and ketonuria (Fig. 5). As soon as 3 hr. after growth-hormone administration the level of blood ketones had increased more than threefold, rising from $1.15 \text{ mg.} \pm 0.07$ to $3.56 \text{ mg.} \pm 0.26/100 \text{ ml.}$ plasma. The level of blood ketones remained about three

times that of the control throughout the entire period. The level of urinary ketones was also increased in growth-hormone-treated rats. It is of interest to note a quantitative difference in response in two experiments, both of which employed the same strain of rat on the same diet. In August 1950, injections of 0.5 mg./day of growth hormone prepared by the method of Li *et al.* (1945) induced a ketonuria eight times as great as that found in the control rats. In December 1950, animals treated

Fig. 5. The level of ketone bodies in (a) blood and (b) urine of normal rats and rats treated with growth hormone. In the lower graph the excretion of ketone bodies over 24 hr. periods in August (○) and December (●) is shown. The control levels for these two periods are shown by horizontal interrupted lines. In the upper graph showing the level of ketone bodies in the blood the vertical lines represent twice the S.E.M.; where no vertical line is given the S.E.M. is too small to show.

with an identical dose of growth hormone prepared by the method of Wilhelmi *et al.* (1948) exhibited a much smaller response, the highest values being approximately three times the control value.

The changes in plasma pseudo cholinesterase and liver esterase

These enzymes were measured to find if they were associated with the changes in mobilization and oxidation of fat occurring in growth-hormone-treated animals.

Plasma pseudo cholinesterase was measured 3 hr. after growth-hormone treatment, that is at the time when a rapid mobilization of fat to the liver occurs. No changes in the activity of the enzyme were observed, the values for the control and growth-hormone-treated animals being 34.7 ± 2.7

and 33.1 ± 1.1 units respectively. The difference is not significant ($P = 0.512$). The level of the enzyme also remained unchanged in rats treated for 10 days with growth hormone.

The effect of growth hormone on liver esterase was also investigated, and the results of this experiment are shown in Fig. 6 expressed as units of esterase/g. liver and as total units of liver esterase. There is a gradual decline of this enzyme throughout the experimental period. By the fourth day the

Fig. 6. Changes in the level of esterase activity in the livers of growth-hormone-treated rats. The horizontal broken line represents the control level. The vertical lines represent twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

values are significantly different ($P = 0.023$), and after 8 days of treatment the total liver esterase has fallen to 60% of the control value. In another experiment liver esterase was measured 3 hr. after growth-hormone treatment. No difference was observed between the two groups, total liver esterase units being 1439 ± 153 and 1440 ± 134 for the hormone-treated and control groups respectively. Thus, while the esterase remained unchanged throughout the 24 hr. period when a rapid mobilization and oxidation of fat in the liver occurs, there was, nevertheless, a gradual decline in activity throughout the period of treatment with growth hormone.

DISCUSSION

Two points of difference may be observed in the behaviour of the fatty-acid oxidase system when oxidizing either octanoate or oleate. First, more

oxygen is taken up in the presence of octanoate than when oleate is substrate, a difference which may be due to the higher molar concentration of the former substrate. Secondly, the acetoacetate production is considerably higher from octanoate than from oleate. That the reduced rate of acetoacetate production when oleate is being oxidized is not due to a general decrease, resulting from a lower rate of oxidation of a long-chain fatty acid, is evidenced by the fact that the ratio acetoacetate production: total oxygen uptake in control animals is 0.08 with oleate and 0.32 when octanoate is substrate for the enzyme. This observation is in accord with that of Kennedy & Lehninger (1950) who found, using rat-liver mitochondria, that the length of the carbon chain modified the nature of the products of fatty-acid oxidation. The ratios of acetoacetate production: total oxygen uptake, found above are in good agreement with those reported by Kennedy & Lehninger (1950), 0.03 and 0.51, respectively, when oleate and octanoate were being oxidized.

Two distinct phases in the activity of the liver fatty-acid oxidase system after growth-hormone treatment may be recognized. There is a marked inhibition after 6 hr. followed by a period of increased activity and within 48 hr. the values have returned to the control level. The nature of the initial inhibition of the oxygen uptake and acetoacetate production is not clear, but it would seem to result from an actual inhibition of fatty-acid oxidase activity as evidenced by the calculated reduction in the rate of production of two-carbon fragments (Table 2).

It is of considerable interest that there is a simultaneous increase in the endogenous respiration. This probably cannot be ascribed to the oxidation of endogenous neutral fat, since the endogenous oxygen uptake had returned to normal levels 12 hr. after growth-hormone treatment, at which time the neutral fat and lipid of the liver was still higher than control values. It is possible that an increased secretion of insulin could account for these changes in fat oxidation and endogenous oxygen uptake. Evidence for an increased insulin secretion after growth-hormone treatment comes from several laboratories. Richardson & Young (1937) observed that pituitary extracts could elicit proliferation of the islet tissues in the rat, while an increase in the insulin content of the rat pancreas was observed by Marks & Young (1940*a, b*). More recently, Milman & Russell (1950) found that a moderate hypoglycaemia, lasting at least 8 hr., followed administration of purified growth hormone. They suggested that this hypoglycaemic effect was due to an increased secretion of insulin, since alloxan-diabetic rats, similarly treated, responded with an elevation of the blood glucose level. Thus the increased endogenous oxygen uptake can, probably,

be ascribed to an increased carbohydrate oxidation. If such a mechanism were brought into play, it might well explain in part the inhibition of fat oxidation by stimulating the anabolism, as opposed to the catabolism, of fats (Brady & Gurin, 1950; Brady, Lukens & Gurin, 1951).

A consideration of the results in Fig. 3 and Table 2 relating to the second phase, 12–24 hr., shows considerable changes in the pattern of fat oxidation. At the earlier time interval the total oxygen uptake is increased, but the oxidation of fatty acid to two-carbon fragments (fatty-acid oxidase activity) and the production of acetoacetate is normal. There is, however, a great increase in the amount of two-carbon fragments totally oxidized to carbon dioxide and water, and it is the oxygen necessary for this ultimate step which enhances the total oxygen uptake. At 24 hr. the oxygen uptake has returned to normal but there has been a striking modification of the pattern of fat oxidation. Although there is a considerable increase in the fatty-acid oxidase activity, a negligible amount of fat is being oxidized to carbon dioxide and water, and instead there is a considerable conversion of the two-carbon fragments to acetoacetate.

Since the endogenous oxygen uptake in the liver 24 hr. after growth-hormone injection is the same in experimental as in control animals, it is likely that equal amounts of oxaloacetate are available for condensation with the two-carbon fragments. At the same time almost equal amounts of two-carbon units are produced from long-chain fatty acids by both control and growth-hormone-treated rats; nevertheless, a much larger proportion is completely oxidized in the controls. The conclusion emerges, therefore, that growth hormone in some way directly controls the fate of the two-carbon fragments, possibly by inhibiting their oxidation through the tricarboxylic acid cycle.

When growth hormone is administered for 2 or more days, the values for the total oxygen uptake return to control levels while the acetoacetate production decreases but does not reach control level, and thereafter remains constant. At the longer time intervals there is a marked tendency, most clearly seen in the case of oleate, for the conversion of two-carbon fragments to carbon dioxide and water to be reduced, while the oxidative breakdown of fatty acids to this stage remains relatively constant.

It is of interest to calculate the amount of fatty acid that the enzyme is potentially capable of oxidizing, and to correlate this figure with the measured changes in neutral fat in the liver at different times (Greenbaum & McLean, 1953). Using the figures from Table 2 of the fatty-acid oxidase oxygen uptake, such a calculation has been made and is given in Table 3.

It is clear that there is an accumulation of lipid in the liver when the liver has a relatively low ability to oxidize fats, while the subsequent disappearance of fat can be accounted for largely by oxidation in the liver quite apart from transport from the liver in the form of phospholipins.

Table 3. *Comparison of the changes in fat content and the potential fat-oxidizing capacity of the livers of rats treated with 0.5 mg. of purified growth hormone*

Time after injection of growth hormone (hr.)	Fat-oxidizing capacity of the fatty-acid oxidase system (mg. oleic acid/g. wet liver weight)	Change in neutral fat content (mg./g. wet liver weight)
0–6	3.2	14.1 accumulated
6–12	4.8	3.6 disappeared
12–24	17.4	14.6 disappeared

The failure of the liver to show an increased oxidation of fat after growth-hormone treatment lasting 2 or more days is difficult to reconcile with the low respiratory quotient observed, and clearly means that the liver cannot be the site of the extra fat oxidation, and therefore directs attention to other sites of fat catabolism. Evidence in support of this may be adduced from some values of the fat content of the carcasses of a composite group of similarly treated rats drawn from the data of Greenbaum (1953). It may be calculated from these values, that, over an equivalent period, the loss of fat from the body was 390 mg./day/100 g. body weight, corresponding to a loss of 370 mg. of free fatty acid. The data presented above show that during this same period the average daily oxidation of fat by the liver could not have amounted to more than 80–100 mg. on the assumption that the *in vitro* measurements approximate to the rate of oxidation in the body. Thus, nearly 300 mg./day of fat is being oxidized in tissues other than the liver and it is suggested, therefore, that much of the increased oxidation of fat occurs in the extrahepatic tissues.

The direct oxidation of long-chain fatty acids by heart muscle was shown by Lehninger (1946*a*), while Grafflin & Green (1948) demonstrated that kidney enzyme preparations rapidly oxidize straight-chain monocarboxylic fatty acids up to tridecanoic acid. Geyer, Matthews & Stare (1949), using labelled trilaurin and octanoate, and Weinhouse *et al.* (1950), using labelled palmitate, have substantiated and extended the proof of the oxidation of fatty acids by extrahepatic tissues such as kidney, spleen, lung and heart. There is, however, some disagreement over the oxidation of fatty acids by skeletal muscle; the former investigators reported that fatty acids were oxidized by this tissue,

while Weinhouse *et al.* did not find any oxidation of palmitate by rat skeletal muscle. On the other hand, Geyer *et al.* (1951), from experiments using hepatectomized or nephrectomized rats injected with isotopically labelled fats or fatty acids, have emphasized the probable importance of lipids as an energy source in the extrahepatic tissues.

Considering the results of the influence of growth hormone on the blood and urine ketones, it may clearly be seen that treatment of normal rats with 0.5 mg. of purified growth hormone produces a marked ketonaemia and ketonuria. The rapid rise in blood ketone to a value three times the control value 3 hr. after growth-hormone treatment is particularly striking. These results are in good agreement with those of Bennett *et al.* (1948) and Bondy & Wilhelmi (1950) who found that 2 or 3 hr. after treating normal fasting rats with growth hormone, there was a marked increase in blood and urine ketones. The level of ketone bodies in the blood and urine in the present experiment was very much lower than the values reported by the latter authors, largely because their effect was a potentiation of an already existing fasting ketosis by exogenous growth hormone.

It is difficult to correlate the *in vivo* changes in blood ketones with the changes in acetoacetate production by liver suspensions *in vitro*, for it is found that the blood ketones rise to their highest level just at the time when the *in vitro* production by the liver falls to its lowest, that is 6 hr. after growth-hormone treatment (see Figs. 4 and 5). Furthermore, no peak in blood ketones is observed at the time when the *in vitro* production by the liver rises to its highest level at 24 hr. after growth-hormone treatment. Parallel observations were made by Bondy & Wilhelmi (1950), who found an increased blood ketone level associated with no change in the rate of acetoacetate production in liver slices from fasted rats treated with growth hormone.

It is realized that measurements of acetoacetate production *in vitro* may bear little relation to that obtaining *in vivo*. In the present investigation the suspensions were fully supplemented with the known cofactors including ATP and succinate, and consequently the supply of oxaloacetate for condensation with the two-carbon fragments arising from fatty acids is unlikely to be a limiting factor in this system. In the intact animal the supply of oxaloacetate might have been a limiting factor at this time interval and resulted in an increased acetoacetate production.

If it is assumed that the ketone body formation in suspensions of 'homogenized' liver from oleate is an indication of the changes occurring in the liver of the intact animal, then the likeliest explanation of the coincidence of a maximum blood ketone level with a minimal hepatic production, is that utilization by

the extrahepatic tissues is inhibited at this time. However, no evidence, either from the present experiments, or from the literature, can be cited in support of this view. Indeed, Bennett *et al.* (1948) have produced evidence that extrahepatic utilization is unchanged in growth-hormone-treated animals, and that the increased ketone level in blood and urine is due to overproduction by the liver.

The level of a metabolite in the blood represents the resultant effect not only of the rate of production, but of the rate of utilization and excretion. A calculation of the approximate rates of these reactions in control and growth-hormone-treated rats may be made from the results presented here. Comparison is best made between the control rats and the animals treated with growth hormone for 5 or 6 days, at which time the rapid initial changes in fatty-acid oxidase activity and acetoacetate production are over, and the general tendency for the level of ketone-body production to be elevated above the control level is clearly seen (Fig. 4). The results of this calculation are given in Table 4.

Table 4. Comparison of the rate of acetoacetate production in liver suspensions and of the level of blood and urinary ketones in normal rats and in rats treated with 0.5 mg. growth hormone daily for 5 days

	5 days treatment with growth hormone	
	Control	100
Acetoacetate produced (mg./ 24 hr./entire liver/100 g. body weight)	53	100
Blood ketone level (mg. acetone/100 ml. blood)	1	3.5
Urine ketone level (mg. acetone/24 hr./100 g. body weight)	0.5	1.0

Weinhouse & Millington (1951) have made very reliable estimates of the absolute rates of acetoacetate formation and utilization in individual tissues by isotopic methods. Using 'fed' liver slices they estimated that the liver would liberate up to 24 mg. acetoacetate/100 g. body weight into the blood in 24 hr. The close correspondence between the measured acetoacetate production of normal animals in the present experiment using suspensions of 'homogenized' tissue, those of Weinhouse & Millington (1951) using liver slices, and those of Crandall, Ivy & Ehni (1940) who employed the angiotomy technique in whole animals, indicates that the *in vitro* measurements do constitute a reasonable approximation to the production of ketone bodies in the whole animal. If the *in vitro* measurement of acetoacetate does indeed reflect changes in the *in vivo* production by the liver, it is clear that the treatment of normal rats with purified

growth-hormone results both in an increased production of ketone bodies and in an increased extrahepatic utilization. That the extrahepatic tissues can readily oxidize the extra ketones produced by the liver during periods of treatment greater than 2 days is apparent from the figures quoted by Stadie (1945), showing that the mean basal ketone utilization of peripheral tissues is 300 mg./24 hr./100 g. body weight.

There is a marked difference in the rate of excretion of ketone bodies after growth-hormone treatment in summer and winter (Fig. 5). Since identical conditions were used in the two experiments, the two main factors which might be responsible for the difference are probably the preparation of growth hormone used or a seasonal variation in response to growth hormone. While it is possible that the two growth-hormone preparations were contaminated to a different extent with adrenocorticotrophic hormone and this could affect the degree of ketosis (Tepperman & Tepperman, 1951), there is, perhaps, more evidence in favour of a variation in seasonal response. Cori & Cori (1927) have observed such a variation in their study of fasting ketosis in rats, and from a consideration of the glucose tolerance curves and rate of glucose utilization in summer and winter, concluded that the differences could be attributed to changes in the functional activity of the pancreas. It is possible that such a seasonal change in the activity of the pancreas could account for the difference in ketone excretion after growth-hormone treatment found in the present investigation in August and December.

SUMMARY

1. A method of assay for the overall activity of the fatty-acid oxidase system in suspensions of 'homogenized' liver has been described, and measurements have been made of the activity of this enzyme and of acetoacetate production in control and growth-hormone-treated adult female rats at intervals up to 8 days of treatment.

2. Liver fatty-acid oxidase activity and acetoacetate production were inhibited at 6 hr. after

treatment of the animal with growth hormone, and greatly stimulated between 12 and 24 hr. With periods of treatment longer than 48 hr. the fatty-acid oxidase activity had returned to normal.

3. The acetoacetate production from oleate by suspensions of 'homogenized' liver has a marked tendency to be increased 2, 4, 6 and 8 days after treatment with pituitary growth hormone.

4. Calculation of the partition of the oxygen uptake between the reactions, the oxidation of fatty acids to two-carbon fragments, and the oxidation of the two-carbon fragments to carbon dioxide and water, and the measurement of the rate of acetoacetate production permits a study of the changes occurring on treatment with growth hormone. It is suggested that the fate of the two-carbon fragments arising from fat oxidation in the liver is in some way directly controlled by growth-hormone administration.

5. Calculations have been made correlating the activity of the fatty-acid oxidase system with the accumulation and subsequent disappearance of fat from the liver. Further calculations from the potential ability of the liver to oxidize fats have shown that this tissue is not capable of catabolizing the amount of fat disappearing from the body, and it is concluded that growth hormone stimulates the direct extrahepatic utilization of fat.

6. It has been demonstrated that growth-hormone treatment results in a definite ketonaemia in normal adult female rats.

7. Liver esterase and plasma pseudo cholinesterase have been measured. While plasma pseudo cholinesterase remained unchanged liver esterase showed a gradual decline to a value only 60 % of the control level over a period of 8 days of treatment.

It is a pleasure to acknowledge our gratitude to Prof. F. G. Young for his advice and encouragement; to Dr J. Judah for his invaluable advice on fatty-acid oxidase measurements, and to Miss E. I. Large for her skilful assistance. We must also acknowledge our gratitude for a grant from the Medical Research Council to one of us (P.M.) during the tenure of which this work was done, and to the Royal Society for an expenses grant (A.L.G.).

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Excretion of Formate after Methanol Ingestion in Man

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(Received 22 November 1952)

Experiments which demonstrated the ability of ethanol to depress the metabolism of methanol in man were described briefly by Zatman (1946) and have recently been reported in detail (Leaf & Zatman, 1952). In these experiments urinary methanol concentration was used as an index of methanol concentration in the body water. It was shown that the decline in methanol concentration, subsequent to the attainment of a peak concentration after dosage, could be very greatly retarded by administration of ethanol. Agner & Belfrage (1947) determined the level of methanol in the blood of rabbits which had been dosed with the alcohol, and showed that the decline in methanol concentration was slower when ethanol was also given. Bartlett (1950), by studies of the radioactivity of expired carbon dioxide after administration of ^{14}C -labelled methanol, proved that the oxidation of methanol to carbon dioxide in the rat was depressed by the administration of ethanol. Bastrup (1947*a*), using dogs and rabbits, has confirmed an earlier observation of Asser (1914) that the excretion of formate

which follows methanol administration is considerably reduced when ethanol has also been given to the animal. In these experiments on the dog, rabbit and rat, the dose of methanol (1–2 g./kg. body weight) was above the range which is of practical interest in relation to man. So far as we are aware no studies of formate excretion after the experimental administration of methanol to man have been reported; in this paper the result of a short series of such studies are presented.

SUBJECTS

The subjects were two adult males. Both (A.H.G. and L.P.K.) had been used 4 years before in studies of methanol elimination, and the data then obtained are to be found in the paper of Leaf & Zatman (1952). At least a week was allowed to elapse between successive experiments on the same subject.

METHODS

The dose of methanol was 4 ml., i.e. about 0.05 g./kg. body weight, in all the experiments. It was diluted to about 100 ml. with water, and taken orally immediately after the