pound is fairly quickly distributed in the body, but that the concentration in the tissues examined falls very slowly. It appears that the rate of elimination of methyl carbamate is such that the time required for the concentration to fall to half the maximum value is 24 hr. for normal rats and almost twice this time for tumour-bearing rats.

DISCUSSION

The data on elimination of ethyl carbamate (Boyland & Rhoden, 1949; Skipper *et al.* 1951) indicated that the time for 50% elimination was of the order of 12 hr. for normal rats or mice and 24 hr. for tumour-bearing animals. The present data indicate that methyl carbamate, like ethyl carbamate or ethanol, is not concentrated by the kidney, so that elimination must be mainly by metabolism. Methyl carbamate appears to be metabolized at about half the rate of ethyl carbamate.

The relationship of metabolism of the methyl and ethyl carbamates to their biological action is difficult to understand. If the radiomimetic action were due to hydrolysis of the carbamate, then the slower metabolism of methyl carbamate should be compensated for by increase of the dose. This, however, is not the case.

If the carcinogenic action of urethane were due to slow liberation of ethanol oxidized to acetaldehyde within the tissues then methanol would be expected to be as effective, as formaldehyde has been found to be mutagenic (Rapoport, 1946).

The discovery that ethyl carbamate is a much more effective inhibitor of transmethylation reactions than is methyl carbamate (McKinney, 1950) suggests that such inhibition may be concerned with the radiomimetic effects produced by urethane.

SUMMARY

1. Methyl carbamate, like urethane, does not appear to be concentrated by the kidney. Methyl carbamate is eliminated from the body by metabolism at about half the rate at which urethane is eliminated.

2. Tumour-bearing rats eliminate methyl carbamate only about half as fast as normal rats.

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Sorbitol Antiketogenesis and the Dehydrogenation and Oxidation of Fatty Acids

By R. L. BLAKLEY*
Department of Biochemistry, Medical School, University of Otago, Dunedin, New Zealand

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Sorbitol is remarkable for its ability to decrease the spontaneous ketogenesis of liver slices from fasted rats, being more efficient in this respect than related hexoses and most other compounds (Edson, 1936*b*; Blakley, 1951). During the investigation of this effect a dehydrogenase was found in rat liver which converts D-sorbitol to D-fructose in the presence of coenzyme I (CoI). The enzyme also converts L-iditol to sorbose, and iditol is as effective as sorbitol in diminishing spontaneous ketogenesis (Blakley, 1951).

A simple explanation of sorbitol antiketogenesis suggested by Edson (1936*b*) is that it results from competition between sorbitol and fatty acids for oxidative enzymes and coenzymes in the liver. When the course of sorbitol metabolism is taken

* Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7.

into account it seems likely that Co I is the object of such competition between sorbitol dehydrogenation and fatty acid oxidation. Although Co I has not been proved essential for the oxidation of fatty acids, octanoate oxidation in washed suspensions of mitochondria is activated by Co I under some conditions (Lehninger, 1945; Lehninger & Kennedy, 1948; Kennedy & Lehninger, 1951). One possibility is that the $\alpha\beta$-dehydrogenation assumed to occur during β-oxidation of fatty acids, and demonstrated in the case of phenyl-substituted acids (Carter, Osman, Levine & Gamm, 1939; Mazza, 1935; Kuhn & Livada, 1933) requires Co I. This paper describes experiments performed with the object of testing this explanation of sorbitol antiketogenesis.

EXPERIMENTAL

Animals. Rats of the Wistar strain, fed as previously described (Blakley, 1951), were used.

Methods. Manometric measurements were made by standard methods. Homogenates were prepared with a stainless-steel instrument similar to the Potter-Elvehjem homogenizer, with a barrel 3 cm. in diameter and 15 cm. long.

Fatty acid oxidizing preparations. Two rat-liver preparations were used for the oxidation of fatty acids. The first was essentially the washed particulate material from a homogenate of rat liver in isotonic KCl (Lehninger, 1945). This is referred to throughout as the 'particulate preparation'. The second consisted mainly of washed mitochondria from rat liver, prepared by an abridged procedure of Lehninger (1949), in which the mitochondria are collected from a sucrose-containing homogenate at 2400*g* after agglutination with isotonic KCl. Considerable numbers of nuclei were present in this preparation, but it was substantially free from erythrocytes.

Sorbitol dehydrogenase. In order that fatty acid oxidation would not be unduly affected by impurities it was desirable to use a dehydrogenase preparation of greater purity than previously described. The enzyme was therefore prepared by an improved procedure as follows. After homogenizing the chilled livers of four rats for 1·5 min. in 6 vol. of ice-cold 0·01 M-potassium phosphate buffer, pH 7·8, in a chilled Waring Blendor, 2 N-HCl was added dropwise while the blending continued, until the pH had fallen to 4·7. The material was then immediately centrifuged at 2° in chilled centrifuge cups. After quickly adjusting the supernatant to pH 7·4, 0·25 vol. ethanol and 0·25 vol. $CHCl_3$ were added and the mixture shaken for 2 min. at room temperature and then immediately centrifuged at 2°. The supernatant, which should be light brown and was usually cloudy, was dialysed without delay against neutralized distilled water at 0° for 24 hr. The dialysed solution (about 200 ml.) was then adjusted to pH 5·0 when a red-brown precipitate usually formed, and was centrifuged off and discarded. The supernatant was treated successively with 30 and 50 ml. of alumina C_γ suspension (16·5 mg./ml.), the solid being centrifuged off after each addition. The sediment from the first centrifugation was used to obtain Co I-cytochrome *c* reductase (see below). To the almost colourless, clear supernatant from the second centrifugation were added 50 ml. of calcium phosphate suspension (24 mg./ml.), and the mixture centrifuged. The sorbitol dehydrogenase was eluted from the gel at room temperature with one 30 ml. and one 20 ml. portion of 0·01 M-Na_2HPO_4 containing $(NH_4)_2SO_4$ to 20% saturation. The dehydrogenase could be further concentrated by treating the dialysed eluate with 1 vol. of acetone at about −20°, rapidly centrifuging off the precipitate and dissolving it in cold 0·01 M-potassium phosphate buffer, pH 7·8. After dialysis against neutral distilled water at 2° the preparation lost little activity when kept at 2° for a week. The preparation was free from Co I-cytochrome *c* reductase and from colouring material and had an activity several times that of preparation *B* described previously (Blakley, 1951).

Coenzyme I-cytochrome c reductase. The O_2 uptake of earlier preparations of sorbitol dehydrogenase supplied with sorbitol, Co I and methylene blue, was not greatly increased by the addition of Straub's soluble diaphorase (Blakley, 1951). This was explained by the discovery that these dehydrogenase preparations contained an enzyme carrying out the oxidation of reduced Co I by cytochrome *c* and probably by methylene blue also. The enzyme responsible can be eluted from the first portion of alumina C_γ added in the procedure above, using 0·20 M-Na_2HPO_4. The purity of the reductase at this stage depends somewhat on the length of the previous treatment with ethanol-$CHCl_3$. A treatment for 2 min. necessary to eliminate cytochrome and haemoglobin, which are otherwise difficult to separate from sorbitol dehydrogenase, tends to produce larger amounts of brown contaminants in the reductase fraction. Accordingly, a treatment for 1 min. with ethanol-$CHCl_3$ was adopted in preparing reductase. The reductase can be further purified by adsorption on calcium phosphate gel at pH 7·0 and elution with 0·20 M-Na_2HPO_4. The two adsorption steps increased the activity about 12-fold, giving a faintly yellow, clear solution.

Analytical methods. Co I-cytochrome *c* reductase was determined by the method of Hogeboom (1949).

Octanoic acid was determined by the method of Lehninger & Smith (1948) with the following modification. The extractions were performed in rubber-stoppered test tubes and the lower aqueous layer removed after the extraction by means of a small wash-bottle head fitted into the test tube, as described by Weil-Malherbe & Bone (1949). Although the average recovery of octanoate was good, the accuracy obtained was not as high as reported by Lehninger & Smith.

Acetoacetate was determined manometrically (Edson, 1935).

Materials. Sorbitol and cytochrome *c* were the samples previously described (Blakley, 1951).

The octanoic acid used throughout this work was a product of Eastman Kodak Co. Other fatty acids were samples from British Drug Houses Ltd.

Adenosinetriphosphate (ATP) was obtained as the Ba salt from Boots Pure Drug Co. Ltd. The purity was 92–99% as judged by $P_{7\ min.}$, $P_{7\ min.}$/total P and P/N values.

The Co I used in these experiments was a product of Schwarz Laboratories Inc., New York, and assayed 50–60% by the dithionite method.

RESULTS

Effect of sorbitol on ketogenesis in rat-liver homogenates. As a preliminary to searching for a simplified cell-free system in which sorbitol antiketo-

genesis could be tested, it was desirable to examine the effect of sorbitol on ketone production in whole liver homogenates where none of the cell components had been discarded. Failure to produce antiketogensis in such a system could not be due to removal of components essential for the operation of the antiketogenic effect.

greater ketonuria than normal fasted rats. Although the precise mechanism of the effect is unknown, it seemed possible that the livers of rats so treated might provide more favourable material for the investigation of antiketogenesis. Accordingly, female rats, about 200 g. body weight, fasted 2–4 days and injected intraperitoneally with 35 mg.

Table 1. *Effect of co-factors on spontaneous ketogenesis in rat-liver homogenates*

(Female rat, fasted 4 days. Liver homogenized in 2 vol. 0·13 M-KCl containing 0·013 M-sodium phosphate buffer, pH 7·4. Flasks contained 0·13 ml. sodium phosphate buffer, pH 7·4 (0·013 M),* 0·03 ml. KCl (0·06 M), 0·60 ml. homogenate, water to give a final volume of 1 ml. and other components as below. Inseals contained 0·20 ml. 2 N-NaOH. Gas, O_2. Temp., 30°. Time, 75 min.)

Concentrations of components (M)			O_2 uptake	Acetoacetate formed
Mg^{++}	Cytochrome *c* ($\times 10^{-5}$)	ATP	(μmoles)	(μmoles)
0·0000	0·8	0·0012	28·2	1·9
0·0023	0·8	0·0012	20·9	1·7
0·0046	0·8	0·0012	22·1	2·6
0·0046	0·0	0·0012	26·5	2·1
0·0046	0·4	0·0012	20·5	2·2
0·0046	1·3	0·0012	22·0	3·4
0·0046	0·8	0·0000	16·8	1·2
0·0046	0·8	0·0006	22·1	2·0
0·0046	0·8	0·0012	25·2	2·1
0·0046	0·8	0·0032	32·8	2·2

* The figures in parentheses indicate the concentration of the components in the complete reaction medium. This notation is used in Tables 1–11.

Although spontaneous ketogenesis in cell-free homogenates has not been reported previously, homogenates, in isotonic potassium chloride, of livers from fasted rats were found to produce large amounts of acetoacetate endogenously. A necessary precaution for this production of spontaneous ketogenesis in homogenates, however, is that dilution of the tissue suspension, during homogenizing or in mixing it with other solutions in the reaction vessel, must be minimized. The amount of acetoacetate produced varied considerably from one animal to another, but was practically independent of added magnesium ions, cytochrome *c* and ATP (Table 1), as expected in concentrated tissue suspensions of this type.

When sorbitol was added to these concentrated homogenates it produced no significant decrease in the spontaneous ketogenesis (Table 2). If Co I and nicotinamide were also added, sorbitol was vigorously oxidized as indicated by the increase in oxygen uptake, but the production of ketone bodies was still unaffected. The presence of added cytochrome *c*, ATP, magnesium ions, potassium chloride, acetyl phosphate, L-malate, cocarboxylase and yeast extract and combinations of these factors was unable to cause the appearance of any antiketogenic effect. Added fructose and glucose were similarly without effect on ketogenesis.

Janes & Brady (1949) reported that rats injected with nicotinamide during fasting exhibit a much

nicotinamide in saline each day of the fast, were used for the preparation of ketogenic liver slices and homogenates. Although it was confirmed that these animals exhibit high ketonuria, the spontaneous ketogenesis of liver slices and homogenates was not significantly greater than that for normal fasted animals. Moreover, in liver homogenates from such animals, sorbitol was again without effect on spontaneous ketogenesis.

Table 2. *Spontaneous ketogenesis in rat-liver homogenate in presence of sorbitol, glucose and fructose*

(Female rats, fasted 3 days and injected daily during fast with 35 mg. nicotinamide in 1·00 ml. normal saline. Livers homogenized in 2 vol. 0·13 M-KCl containing 0·013 M-sodium phosphate buffer, pH 7·4. Flasks contained 0·13 ml. sodium phosphate buffer, pH 7·4 (0·01 M), 0·06 ml. cytochrome *c* (6×10^{-6} M), 0·08 ml. $MgSO_4$ (0·012 M), 0·06 ml. ATP (0·0024 M), 0·03 ml. nicotinamide (0·01 M), 0·03 ml. Co I (2×10^{-4} M), 0·58 ml. homogenate and water to give a final volume of 1·00 ml. 30°. Gas, O_2. Time, 50 min.)

Additions	Acetoacetate formed (μmoles)
None	4·8
Sorbitol (0·05 M)	4·2
Fructose (0·05 M)	4·7
Glucose (0·05 M)	4·6

Rat-liver homogenates in 0·88 M-sucrose or isotonic potassium chloride rapidly oxidized added octanoate and pyruvate with the formation of

Table 3. *Acetoacetate production from added octanoate and pyruvate in rat-liver homogenate in presence of sorbitol*

(Female rats, fasted 24 hr. Livers homogenized in 2 vol. 0·88 M-sucrose. Flasks contained 0·30 ml. cytochrome *c* ($7·5 \times 10^{-6}$ M), 0·10 ml. ATP (0·001 M), 0·10 ml. $MgSO_4$ (0·0038 M), 0·30 ml. potassium phosphate buffer, pH 7·4 (0·015 M), 1·00 ml. homogenate and water to give a final volume of 4·00 ml. Inseals contained 0·20 ml. 2 N-NaOH. Gas, O_2. Temp., 30°. Time, 35 min.)

Substrate	Nicotinamide (0·01 M) and CoI (2×10^{-4} M)	Sorbitol (0·01 M)	O_2 uptake (μmoles)	Acetoacetate formed (μmoles)
None	Absent	Absent	12·9	4·3
Octanoate (0·001 M)	Absent	Absent	20·4	10·6
Octanoate (0·001 M)	Present	Absent	30·2	11·8
Octanoate (0·001 M)	Present	Present	30·8	9·7
Pyruvate (0·01 M)	Absent	Absent	19·6	8·4
Pyruvate (0·01 M)	Present	Present	24·9	8·7
Octanoate (0·001 M) + pyruvate (0·01 M)	Present	Absent	27·3	13·6
Octanoate (0·001 M) + pyruvate (0·01 M)	Present	Present	29·6	12·6
Octanoate (0·001 M) + α-ketoglutarate (0·001 M)	Present	Absent	28·3	10·2
Octanoate (0·001 M) + α-ketoglutarate (0·001 M)	Present	Present	31·5	8·4

acetoacetate. The production of acetoacetate under these conditions, too, was not significantly affected by the addition of sorbitol, even when CoI and nicotinamide were added to allow rapid sorbitol oxidation (Table 3). Similar results were obtained whether the livers of fasted rats or fed rats were used. Sorbitol is apparently devoid of antiketogenic action in liver homogenates even though it is effective in this respect in liver slices.

Coenzyme I *and the oxidation of octanoate by washed 'particulate preparations'*

In agreement with Lehninger & Kennedy (1948) it was observed that the washed particulate fraction of homogenates of liver in isotonic potassium chloride rapidly oxidizes octanoate (0·001 M) when supplied with magnesium ions, ATP and potassium chloride (0·05 M). Octanoate oxidation was also increased slightly by added cytochrome *c*. It was confirmed that with such preparations suspended either in water or in isotonic potassium chloride, CoI could replace ATP for octanoate oxidation, and in some cases CoI was found to increase octanoate oxidation even when ATP was already present (Table 4). As Lehninger was unable to find any further stimulation of octanoate oxidation by CoI when ATP was already present in the reaction medium, the question was further investigated. It was found that CoI consistently increased octanoate oxidation, both in the presence and absence of ATP, when liver preparations were obtained from fasted animals. When the liver of a recently fed animal was used, however, CoI could replace ATP as a factor for octanoate oxidation, but caused no further stimulation of oxidation if ATP were present.

It is difficult to establish whether the stimulatory effect of CoI on octanoate oxidation is due to the CoI as such or to other compounds present in the CoI preparation. In particular the effect might be due to adenosine-5-phosphate present in the preparation or formed enzymically from CoI during incubation. Such an explanation appears unlikely since the addition of extra ATP did not produce the same stimulation of octanoate oxidation (Table 4).

Table 4. *Effect of added CoI and other co-factors on octanoate oxidation with liver particulate preparations from fasted rats*

(Flasks contained 1·40 ml. of a water suspension of washed 'particulate preparation' from livers of fasted rats, 0·40 ml. octanoate (0·001 M), 0·10 ml. ATP (0·001 M), 0·15 ml. cytochrome *c* (10^{-5} M), 0·10 ml. $MgCl_2$ (0·004 M), 0·20 ml. potassium phosphate buffer, pH 7·4 (0·01 M), 0·10 ml. KCl (0·05 M), 0·50 ml. CoI ($7·8 \times 10^{-4}$ M), 0·13 ml. nicotinamide (0·01 M) and water to give a final volume of 4·00 ml. Inseals contained 0·20 ml. 2 N-NaOH. Gas, air. Temp., 38°. Time; 45 min.)

	O_2 uptake (μmoles)	Aceto-acetate formed (μmoles)
Exp. 1		
Complete system	11·0	5·5
Octanoate omitted	2·9	0·4
ATP omitted	3·6	2·3
$MgCl_2$ omitted	2·1	1·2
Cytochrome *c* omitted	8·9	4·3
Nicotinamide omitted	10·9	4·8
CoI omitted	4·5	2·9
CoI and ATP omitted	0·2	1·2
CoI and nicotinamide omitted	4·9	2·7
Exp. 2		
Complete system	20·8	11·2
CoI omitted	12·7	7·4
CoI replaced by extra ATP (0·002 M)	12·0	6·7

Lehninger (1945) has stated that CoI could restore the activity of preparations aged 4–6 hr. In the present investigation difficulty was experienced in demonstrating such an effect of CoI because no decrease could be observed in the activity of washed

preparations suspended in water and kept at 0° for 6 hr. If the tissue was suspended in 8 vol. of water the activity immediately disappeared and could not be restored by Co I.

Despite the stimulatory action of Co I on octanoate oxidation in 'particulate preparations', Co I inhibited octanoate oxidation in preparations of mitochondria, both in presence and absence of ATP. The cause of this contrasting behaviour in the different preparations is not apparent. Recombination of the nuclear fraction of homogenates prepared in sucrose solution with the preparation of mitochondria produced a system which was similar to the 'particulate preparations' but in which Co I still inhibited fatty acid oxidation.

antiketogenic effect, both in the presence and absence of Co I and dehydrogenase (Table 5).

In order to test whether the interaction of fatty acid oxidation and sorbitol dehydrogenation occurred through some stage in the reoxidation of reduced Co I, Co I-cytochrome *c* reductase was added to the system with the hope of accelerating reoxidation of reduced Co I. The results obtained in such experiments, however, were essentially the same as those obtained in absence of added Co I-cytochrome *c* reductase.

In further attempts to produce modifications of the above experiments favourable to the demonstration of antiketogenesis, the following changes were made. First 'particulate preparations' were

Table 5. *Octanoate oxidation by washed liver 'particulate preparation' in presence of sorbitol, fructose, Co I and sorbitol dehydrogenase*

(Flasks were filled as in Table 4 except that Co I was omitted unless stated otherwise below. Octanoate (0·0025 M) present unless otherwise stated. Temp., 38°. Gas, air. Time, 45 min.)

Additions	O_2 uptake (μmoles)	Octanoate used (μmoles)	Acetoacetate formed (μmoles)
None	8·8	2·6	4·5
Sorbitol dehydrogenase (0·60 ml.)	10·0	3·8	6·4
Sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	12·6	3·5	7·5
Co I ($3·1 \times 10^{-4}$ M)	12·1	2·8	6·2
Co I ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase	11·7	4·6	5·8
Co I ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (octanoate omitted)	3·0	—	0·1
Co I ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	18·7	3·2	6·7
Co I ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M) (octanoate omitted)	8·9	—	0·2
None	13·3	5·5	8·7
Fructose (0·01 M)	15·3	5·5	9·3
Fructose (0·01 M) + sorbitol dehydrogenase (0·60 ml.)	15·9	6·4	9·8
Fructose (0·01 M) + Co I ($3·1 \times 10^{-4}$ M)	18·1	6·6	8·2
Fructose (0·01 M) + Co I ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.)	15·4	6·4	9·1

Sorbitol and octanoate oxidation in washed 'particulate preparations'

The washed 'particulate preparation' in which Co I was observed to produce some activation of octanoate oxidation promised to be a favourable system for the demonstration of antiketogenesis operating by way of competition for Co I. The effect of the sorbitol system on octanoate oxidation was accordingly tested in such systems, first in washed 'particulate preparations' from fed rats. The results, shown in Table 5, indicate that the sorbitol dehydrogenase preparation itself caused a slight stimulation of oxygen uptake, octanoate disappearance and even acetoacetate formation, but the further addition of Co I and sorbitol causing rapid oxidation of sorbitol, effected no inhibition of fatty acid oxidation. Sorbitol and dehydrogenase, added without Co I, were likewise unable to diminish acetoacetate formation or octanoate disappearance, even though the amount of Co I available would be limiting under these conditions. In a control experiment fructose likewise failed to show any

made from the livers of fasted rats to produce conditions in which Co I has its maximum effect on octanoate oxidation, and secondly stearic acid was oxidized instead of octanoic to approximate more closely to the probable precursor of acetoacetate in slices. In spite of these variations sorbitol dehydrogenation continued to show no sign of diminishing fatty acid oxidation or acetoacetate production (Table 6).

Sorbitol and the 'sparking' phenomenon

Lehninger & Kennedy (1948) have reported that when the particulate preparation from rat liver is washed with isotonic potassium chloride and finally suspended in a large volume of water, octanoate oxidation is not catalysed by the preparation unless an intermediate of the Krebs cycle is present in addition to the other normal co-factors. Later it was found that the oxidation of reduced Co I could replace the oxidation of Krebs-cycle intermediates for this 'sparking' of fatty acid oxidation (Kennedy & Lehninger, 1951). As it seemed possible that this 'sparking' of fatty acid oxidation by the oxidation

of reduced CoI might in some way relate to sorbitol antiketogenesis, attempts were made to produce 'sparking' of octanoate oxidation by sorbitol oxidation.

octanoate oxidation was effected by L-malate (Table 7). In such preparations sorbitol dehydrogenation was quite devoid of any activating effect on octanoate oxidation (Table 7).

Table 6. *Oxidation of stearate and of octanoate in presence of the sorbitol dehydrogenase system*

(Flasks were filled as in Table 4, except that octanoate or stearate were added as indicated below, and CoI was omitted unless otherwise stated. In Exp. 1 the 'particulate preparation' was obtained from a fasted rat, in Exp. 2 from a fed rat. Temp., 38°. Gas, air. Time, 45 min.)

Additions	O_2 uptake (μmoles)	Octanoate used (μmoles)	Acetoacetate formed (μmoles)
Exp. 1			
Octanoate (0·001 M)	4·2	2·1	2·1
Octanoate (0·001 M) + CoI ($3·1 \times 10^{-4}$ M)	7·9	3·5	3·6
Octanoate (0·001 M) + sorbitol dehydrogenase (0·90 ml.) + CoI ($3·1 \times 10^{-4}$ M)	9·5	3·7	5·2
Octanoate (0·001 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol (0·01 M) + sorbitol dehydrogenase (0·90 ml.)	22·3	6·8	7·0
Exp. 2			
None	3·1	—	0·6
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.)	4·1	—	0·4
CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	9·0	—	0·6
Stearate (0·00025 M)	7·1	—	2·4
Stearate (0·00025 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.)	7·4	—	2·1
Stearate (0·00025 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + sorbitol (0·01 M)	15·5	—	2·1
Stearate (0·00025 M) + CoI ($3·1 \times 10^{-4}$ M) + sorbitol dehydrogenase (0·60 ml.) + fructose (0·01 M)	7·0	—	1·8

Table 7. *Oxidation of octanoate by suspensions of liver 'particulate preparations' of various dilutions in presence of the sorbitol dehydrogenase system and of malate*

(Flasks filled as in Table 4 except the octanoate, CoI and nicotinamide were absent unless stated otherwise below. Washed particulate preparation of varying dilutions was added as indicated. Temp., 38°. Gas, air. Time, part (1): 60 min.; part (2): 45 min.)

Washed particulate preparation	Additions	Octanoate (0·0025 M)	O_2 uptake (μmoles)	Octanoate used (μmoles)	Acetoacetate formed (μmoles)
Exp. 1					
1·10 ml. diluted 4-fold	None	Absent	1·0	—	0·4
	None	Present	10·1	—	5·8
1·65 ml. diluted 6-fold	None	Absent	0·4	—	0·6
	None	Present	2·0	—	1·3
	L-Malate (0·005 M)	Absent	6·8	—	0·5
	L-Malate (0·005 M)	Present	11·1	—	2·2
2·85 ml. diluted 10-fold	None	Absent	0·6	—	0·0
	None	Present	0·5	—	0·1
	L-Malate (0·005 M)	Absent	2·7	—	0·0
	L-Malate (0·005 M)	Present	3·4	—	0·6
Exp. 2					
2·12 ml. diluted 8-fold	None	Absent	1·4	—	0·9
	None	Present	0·6	0·0	0·4
	Sorbitol (0·01 M) + CoI ($3·1 \times 10^{-4}$ M)	Absent	6·3	—	0·8
	+ sorbitol dehydrogenase + nicotinamide (0·01 M)	Present	6·2	0·0	1·0

In these experiments difficulty was experienced in reproducing the 'sparking' phenomenon. It was found that while preparations in which the wet tissue was diluted with water about fourfold required no addition of a Krebs intermediate for rapid octanoate oxidation, at a tenfold dilution there was negligible octanoate oxidation even when Krebs-cycle intermediates were present. At intermediate dilutions only slight stimulation of

Anaerobic dehydrogenation of fatty acids

The experiments described above showed that in washed 'particulate preparations' sorbitol dehydrogenation was without influence on the overall oxidation of fatty acids. The possibility still remained that if sorbitol antiketogenesis were a result of competition for CoI, and there existed a particular step in fatty acid oxidation for which CoI

was specifically required, then it might be possible to demonstrate competitive inhibition of this isolated reaction by sorbitol in a simplified system. Such a Coi-dependent step occurring in the sequence of reactions involved in fatty acid oxidation is unknown, but the activating effects of Coi on octanoate oxidation are consistent with its existence. A Coi-dependent dehydrogenation of the fatty acid chain would be a reaction of the required type, and a search was therefore made for such a process.

Table 8. *Anaerobic dehydrogenation of octanoate in Thunberg tubes with washed 'particulate preparation' and various co-factors*

(The complete system consisted of 0·20 ml. octanoate (0·001 M), 0·05 ml. $MgCl_2$ (0·004 M), 0·05 ml. ATP (0·001 M), 0·05 ml. KCl (0·05 M), 0·07 ml. nicotinamide (0·01 M), 0·25 ml. Coi ($7·8 \times 10^{-4}$ M), 0·05 ml. methylene blue (0·006%), 0·20 ml. neutral HCN (0·005 M), 0·10 ml. potassium phosphate buffer, pH 7·4 (0·01 M), 0·70 ml. washed 'particulate preparation' and water to give a final volume of 2·00 ml. 38°.)

	Reduction time (min.)
Exp. 1	
Complete system	2·8
Octanoate omitted	8·2
ATP omitted	14·7
Coi omitted	3·0
KCl omitted	3·0
$MgCl_2$ omitted	3·4
Nicotinamide omitted	2·7
Exp. 2. (KCl, $MgCl_2$ and nicotinamide omitted. Preparation obtained from liver of starved rat)	
Complete system	5·5
Octanoate omitted	15·0
Coi omitted	5·5
Exp. 3 (KCl, $MgCl_2$, nicotinamide and Coi omitted. Liver from fed rat. Reaction mixture also contained malonate, 0·01 M)	
Complete system	5·5
Octanoate omitted	∞
ATP replaced by yeast adenylic acid	∞

In Thunberg experiments fatty acids were incubated with washed 'particulate preparations' in the presence of Coi and with methylene blue as hydrogen acceptor. A rapid dehydrogenation of octanoate occurred under these conditions, but it was subsequently found that added Coi was without influence on the rate of the reaction (Table 8), even in liver preparations from fasted rats. Consequently, it was not possible to demonstrate an interaction between this dehydrogenation of fatty acids and sorbitol dehydrogenation. Nevertheless, the fatty acid dehydrogenation was briefly investigated, and the results are reported below.

Co-factors for fatty acid dehydrogenation. Table 8 shows that Coi, nicotinamide or magnesium sulphate were unnecessary for the reaction, and it was also independent of the electrolyte (potassium chloride) concentration. The only co-factor necessary was ATP and this requirement was specific; ATP could not be replaced by adenosine-3-phosphate. (Adenosine diphosphate and adenosine-5-phosphate were not available.)

Substrate and reaction rates. When a series of straight-chain fatty acids was tested with the washed 'particulate preparation' and ATP by the Thunberg technique, all the saturated acids from

Table 9. *Substrate specificity of the fatty acid dehydrogenase system of rat liver*

(Tubes contained fatty acid, 0·05 ml. ATP (0·001 M), 0·50 ml. potassium phosphate buffer, pH 7·4 (0·025 M), 0·05 ml. methylene blue (0·0125%), 0·10 ml. neutral HCN (0·005 M), 0·10 ml. malonate (0·01 M), 0·60 ml. washed 'particulate preparation' and water to give a volume of 2·00 ml. In Exp. 2 the reaction volume was 4·00 ml., but concentrations of compounds other than fatty acid were kept the same as in (1). Temp. 38°.)

Fatty acid (0·001 M)	Reduction time (min.)
Exp. 1	
None	∞
Acetate	∞
Propionate	∞
Butyrate	Partial reduction in 60 min.
n-Pentanoate	19·0
n-Hexanoate	5·2
n-Heptanoate	7·0
n-Octanoate	5·5
Nonanoate	8·5
Decanoate	9·2
Laurate	Partial reduction in 60 min.
Myristate	Partial reduction in 60 min.
Palmitate	10·0
Stearate	11·0
Undecylenate	Partial reduction in 60 min.
Oleate	14·0
Exp. 2	
Fatty acid (0·00025 M)	
None	40·0
Octanoate	5·0
Laurate	6·2
Myristate	9·8
Palmitate	6·5
Stearate	5·8
Undecylenate	6·5
Oleate	8·7

C_4 to C_{18} were dehydrogenated (Table 9). The reaction proceeded most rapidly with hexanoate and the rate fell off quickly with shortening of the carbon chain. Acids with carbon chains shorter than that of butyrate were not attacked. Heptanoate, octanoate and nonanoate were attacked at about the same rate as hexanoate and decanoate at about half this rate. Acids of greater chain length than C_{10} reacted more slowly still, but stearate and palmitate reacted more rapidly than laurate and myristate. The slower reaction of the higher acids,

especially C_{12} and C_{14}, was apparently due to the surface effects of these compounds, for when the concentration of fatty acid was lowered from 0·001 to 0·00025 M their reaction rates were considerably increased. At this concentration laurate, palmitate and stearate reacted almost as rapidly as octanoate, while myristate reacted at approximately half this rate.

The only unsaturated acids tested were undecylenate and oleate, which reacted at rates similar to those of saturated acids of similar chain length. The participation of oleate in the reaction is significant and is discussed below.

methylene blue in absence of substrate without inhibiting dehydrogenation of fatty acids, the dehydrogenation was normally carried out in the presence of 0·01 M-malonate and 0·005 M-HCN.

Intracellular distribution of the enzyme system. When a homogenate of rat liver in 0·88 M-sucrose was fractionated by centrifuging according to the abridged method of Lehninger (1949), the fatty acid dehydrogenase activity was found only in the mitochondrial fraction (Table 11). The soluble fraction was difficult to test for activity because of the rapid reduction of the methylene blue by endogenous substrates.

Table 10. *Effect of malonate and cyanide on aerobic dehydrogenation of fatty acids*

(Tubes were filled as for Table 8, Exp. 1. Temp., 38°.)

	Reduction time (min.)
Complete system	2·8
Octanoate omitted	4·3
Complete system + 0·20 ml. neutral HCN (0·005 M)	4·0
Octanoate omitted; 0·20 ml. neutral HCN (0·005 M)	11·0
Complete system + HCN (0·005 M) + malonate (0·01 M)	3·8
Octanoate omitted; HCN (0·005 M) + malonate (0·01 M)	34·0

Stability of the dehydrogenase. The enzyme system responsible for the dehydrogenation was rather rapidly inactivated at the incubation temperature of 38°, so that after 60 min. no further reduction of the methylene blue occurred. This explains why slow dehydrogenations did not reach completion at all (Table 9).

Table 11. *Intracellular distribution of the enzyme system causing anaerobic dehydrogenation of fatty acids*

(Tubes were filled as for Table 9, Exp. 1 except that the homogenate fraction added was as indicated below. Temp., 38°.)

Homogenate fraction	Octanoate (0·001 M)	Reduction time (min.)
Mitochondria	Absent	∞
Mitochondria	Present	7·0
Nuclear fraction	Absent	∞
Nuclear fraction	Present	∞
'Soluble fraction'*	Absent	3·2
'Soluble fraction'*	Present	3·2
Mitochondria + nuclear fraction	Absent	∞
Mitochondria + nuclear fraction	Present	6·7

* Submicroscopic particles and soluble material.

Effect of inhibitors. The anaerobic dehydrogenation of fatty acids was not inhibited by 0·005 M-potassium cyanide, nor by 0·01 M-malonate (Table 10). Arsenite (0·003 M), dinitrophenol (0·00025 M), fluoride (0·01 M) and arsenate (0·005 M, tested in absence of phosphate) increased the reduction time two- to three-fold. Since malonate and cyanide increased the time for reduction of

DISCUSSION

The main object of the present investigation has been to determine whether the antiketogenic effects of sorbitol and iditol are explicable in terms of a competitive inhibition of fatty acid oxidation. Of the possible factors for which sorbitol dehydrogenation and fatty acid oxidation might compete, Co I and ATP seem the most likely. Co I is essential to the enzymic oxidation of sorbitol and L-iditol, and ATP is probably involved in the further metabolism of the fructose and sorbose produced respectively by the dehydrogenation of these compounds. It has been shown, on the other hand, that the presence of Co I or ATP is essential for fatty acid oxidation, and with liver preparations from fasted animals maximal oxidation was found to occur only when both Co I and ATP were supplied. A situation in which both Co I and ATP would limit fatty acid oxidation would arise if they were involved in a rate-limiting synthesis of Co II, and the latter were essential for some step in fatty acid oxidation.

The experimental results indicate, however, that under conditions where competition for Co I or ATP or both might occur, sorbitol dehydrogenation was without influence on fatty acid oxidation. Neither the complete system for sorbitol dehydrogenation, nor various combinations of its components, showed any consistent tendency to diminish the oxidation of octanoate or stearate in washed 'particulate preparations' from rat-liver homogenates.

An attempt has also been made to isolate some step of fatty acid oxidation which requires Co I, in

the expectation that such a reaction would be competitively inhibited by sorbitol dehydrogenation. This investigation revealed an anaerobic dehydrogenation of straight-chain fatty acids with 4 to 18 carbon atoms, but the essential co-factor proved to be ATP and not CoI, so that the reaction is not relevant to the antiketogenesis problem.

This dehydrogenation of fatty acids is of interest, however, since it seems to be different from the 'desaturation' reaction described by Lang (1939) and by Annau, Eperjessy & Felszeghy (1942, 1943). Hypoxanthine and adenosine-5-phosphate are the coenzymes for the desaturation reaction (Lang & Mayer, 1939*a*, *b*), in which an olefinic linkage is introduced into the centre of the carbon chain of higher fatty acids, and the purified enzyme is reported to require CoI (Burton, 1948). The dehydrogenation described here was activated by addition of ATP, but adenosine-5-phosphate would be formed under the experimental conditions. Properties of the dehydrogenation system which distinguish it from the desaturation system, however, are its apparent independence of CoI, its ability to attack fatty acids of short and intermediate chain length, its insensitivity to cyanide (cf. Lang & Mayer, 1939*a*) and its association with subcellular particles. Furthermore, oleic acid is readily dehydrogenated, whereas it inhibits the desaturation enzyme (Burton, 1948) and accumulates during stearate desaturation (Lang & Adickes, 1939).

It is possible that the anaerobic dehydrogenation of fatty acids described in these experiments represents the initial step or steps of the aerobic process of β-oxidation. Consistent with this suggestion are the requirement for ATP, the location of the enzyme system in subcellular particles (probably mitochondria), and the wide range of fatty acids attacked. If this is the case $\alpha\beta$-unsaturated acids may be produced in the reaction, although the unexpected participation of ATP may imply more profound changes in the structure of the fatty acid.

The role of CoI in fatty acid oxidation remains obscure, for although added CoI stimulated octanoate oxidation in particulate liver preparations, the dehydrogenation of fatty acids appeared to be independent of added CoI. It is clear that this does not eliminate the possibility that CoI is a cofactor in fatty acid dehydrogenation, for bound CoI is present in mitochondria (Cross, Taggart, Covo & Green, 1949); nevertheless, the dehydrogenation is apparently not the site of the action of added CoI. Added CoI may act by accelerating the synthesis of some other essential factor, or by supplying energy through its enzymic reduction and re-oxidation.

To return to the problem of antiketogenesis, another possibility investigated was that sorbitol dehydrogenation is able to initiate or accelerate fatty acid oxidation under conditions in which the 'sparking' effect of malate and other Krebs-cycle intermediates has been reported to occur. In these experiments the 'sparking' effect of malate could be observed only with 'particulate preparations' suspended in water to give tissue concentrations within rather narrow limits. Under conditions in which some 'sparking' by malate did occur, sorbitol dehydrogenation was ineffective as a 'sparker'. A possible explanation of the non-requirement of 'sparker' under most conditions by these preparations is that they contained traces of oxidizable substrates. The oxygen uptake of these preparations in absence of added substrate was very low, however, amounting to about 10% of that in the presence of fatty acid. Figures given by Lehninger & Kennedy (1948) for rat-liver preparations, and by Grafflin & Green (1948) for rabbit-kidney preparations which required 'sparking', show about the same oxygen uptake in absence of added substrate compared with that in presence of fatty acid. Furthermore, it was found that a fourth washing of the preparation with isotonic potassium chloride produced no change in the preparation as regards requirement of 'sparker'.

It has always been assumed that the ketone bodies produced in the diabetic or fasted animal are largely formed by hepatic oxidation of the fatty acids mobilized to the liver as carbohydrate reserves are depleted. Consequently, the antiketogenic effect has been sought by looking for a suppression of hepatic oxidation of fatty acids. But it now seems probable that antiketogenic agents inhibit the formation of acetoacetate arising, not from the oxidation of fatty acids, but from some other source, for the following reasons. Antiketogenic agents do not diminish the production of acetoacetate from fatty acids added to liver slices (Edson, 1936*b*; Weinhouse, Millington & Friedman, 1949). Furthermore, it has been found that in cell-free systems sorbitol dehydrogenation has no effect on fatty acid oxidation. Finally, although sorbitol and fatty acid oxidation proceed unaffected after the tissue has been homogenized, antiketogenesis no longer occurs. It is possible that absence of the antiketogenic effect in homogenates is due to destruction of essential factors, or that disorganization of the cell stops equilibration between bound mitochondrial CoI and CoI outside the mitochondria. Alternatively, disruption of the cell may be considered to dislocate a complex series of metabolic events with which ketogenesis is fundamentally concerned. The synthesis of fatty acids may be suggested as such a coordinated process which is disrupted by destruction of the cell, and which might be intimately connected with antiketogenesis.

In the liver of the fasted animal synthesis of long-chain fatty acids practically ceases (Masoro, Chaikoff, Chernick & Felts, 1950), a similar failure of

fatty acid synthesis being also observed in the livers of diabetic animals (Brady & Gurin, 1950; Chernick, Chaikoff, Masoro & Isaeff, 1950). The question arises, what is the fate, in the diabetic or fasted animal, of the metabolic intermediates which normally act as precursors of long-chain fatty acids? One possibility which must be considered is that they are stabilized as acetoacetate. In this event, that is, if ketone bodies are produced both by oxidation of fatty acids and from fatty acid precursors, antiketogenic agents may act by restoring fatty acid synthesis and consequently diminishing formation of acetoacetate from the precursors of fatty acids. Such a hypothesis is consistent with the facts discussed above, and offers an explanation of the fact that antiketogenic agents do not suppress more than 70% of acetoacetate formation and usually much less, and that their effect is rather variable (Blakley, 1951).

There remains the question of the manner in which an antiketogenic agent such as sorbitol could assist the restoration of fatty acid synthesis. Sorbitol antiketogenesis is attributable, at least in part, to the dehydrogenation of sorbitol, because sorbitol is more effective than fructose in diminishing acetoacetate production (Blakley, 1951). It is possible that the effect of sorbitol dehydrogenation is to furnish a direct and readily available source of energy through the reoxidation of reduced CoI, a process yielding two energy-rich phosphate bonds per molecule (Lehninger, 1949). Glycerol and L-iditol, which are strongly antiketogenic *in vitro*, and lactate, which is moderately so, may also act by providing a ready supply of energy in this way. Ethanol and malate, although dehydrogenated by liver enzymes requiring CoI, are not antiketogenic in rat-liver slices (Edson, 1936*a*, *b*), but this is explained by their failure to be oxidized appreciably under these conditions as shown by the figures for oxygen uptake. On such a hypothesis oxidation of reduced CoI is considerably more efficient than glucose or fructose oxidation in supplying energy for fatty acid synthesis in liver slices since sorbitol and iditol are more antiketogenic *in vitro* than glucose, fructose or sorbose. This may be explained in part by assuming that the oxidation of reduced CoI is in fact a good source of energy for synthetic reactions especially when it is rapidly formed by a vigorous dehydrogenation such as the oxygen uptake figures show sorbitol to undergo in liver slices. It may also be due in part to unfavourable conditions for glucose oxidation in the livers of diabetic or fasted animals (Stetten & Stetten, 1951). An alternative explanation of the effect of sorbitol dehydrogenation is that reduced CoI is used to reduce acetyl radicals ('active acetate') to fatty acid.

To test these hypotheses techniques are required which were outside the scope of the present investigation. If they are correct, the *in vitro* antiketogenic effect of sorbitol involves a fundamental change in the intermediary metabolism of fat which may operate in the intact animal in spite of the rapid conversion of sorbitol to glucose *in vivo* (Stetten & Stetten, 1951).

SUMMARY

1. Spontaneous ketogenesis has been demonstrated in liver homogenates of fasted rats, and has been found independent of added co-factors. Such spontaneous ketogenesis and the formation of acetoacetate from added substrates in homogenates is not affected by added sorbitol even when the latter is being rapidly oxidized.

2. For the activation of octanoate oxidation in washed mitochondria suspensions, coenzyme I (CoI) can replace adenosinetriphosphate (ATP); when livers from fasted rats are used for the preparation of mitochondria the activating effects of ATP and CoI are additive.

3. The oxidation of octanoate with acetoacetate formation in washed mitochondria suspensions is unaffected by the simultaneous dehydrogenation of sorbitol by purified dehydrogenase preparations. The results are unchanged by the presence or absence of CoI and of CoI-cytochrome *c* reductase.

4. In the presence of methylene blue and ATP, saturated fatty acids (C_4 to C_{18}), undecylenate and oleate are dehydrogenated anaerobically in suspensions of the washed particles from rat-liver homogenates. No other co-factors are required; adenosine-3-phosphate cannot replace ATP.

5. The anaerobic fatty acid dehydrogenase system is probably located in the mitochondria. It is not inhibited by 0·005M-hydrocyanic acid or 0·01M-malonate.

6. The significance of these results for theories of the mechanism of sorbitol antiketogenesis is discussed.

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Sedimentation and Diffusion of Human Albumins

2. NEPHROTIC HUMAN ALBUMINS AT A LOW CONCENTRATION

By P. A. CHARLWOOD
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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Marked deviations from normal in the quantitative distribution of the serum proteins of nephrotic cases have been demonstrated by several methods, notably electrophoresis (Longsworth, Shedlovsky & MacInnes, 1939; Longsworth & MacInnes, 1940; Luetscher, 1940; Thorn, Armstrong, Davenport, Woodruff & Tyler, 1945). The presence of abnormally large amounts of lipid (Longsworth & MacInnes, 1940; Thorn *et al.* 1945), indicated the possibility of qualitative changes also. A number of different approaches have been made to decide whether there are any such differences between normal and nephrotic serum proteins, or between these and nephrotic urinary proteins.

Hewitt (1927) was unable to differentiate between serum and urinary albumin by determinations of optical rotatory power and dispersion. Similarly, Widdowson (1933) found no differences in the optical rotation, specific refractive increments, or racemization curves. None of these properties would, however, be likely to depend very critically on molecular weight, unless the alteration in molecular weight were associated with the incorporation of material other than protein.

The finding of Alving & Mirsky (1936), that the 'albumin' fraction, separated by half-saturation of nephrotic sera with ammonium sulphate, had a low cystine content is almost certainly due to the presence, in the supernatant 'albumin' solution, of a large proportion of α-globulin rich in lipid. As Gutman (1948) pointed out, the incompleteness of such separations must be taken into account when assessing results obtained with the fractions. (See also Bradley & Tyson, 1948.)

As regards electrophoretic properties, Longsworth & MacInnes (1940) noted that the mobilities of the urinary albumins were lower than those of normal or the corresponding serum albumins. It was discovered by Luetscher (1939) that the two components which separated from both nephrotic serum and nephrotic urinary albumin during electrophoresis at pH 4 were in approximately the same relative proportions, but their ratio differed from that found with normal human albumin.