SUMMARY

1. The loss of sodium and potassium from isolated rabbit-kidney mitochondria was studied. Addition of ATP and an oxidizable substrate to the incubating medium at 37° caused a significant decrease in the loss of potassium from the mitochondria, but had no effect on the sodium content of the particles.

2. Increasing the pH of incubating medium not containing adenosine triphosphate and substrate by small increments from 6·2 to 7·8 resulted in a significant decrease in the loss of potassium from the mitochondria. These changes in potassium were not observed when adenosine triphosphate and substrate were added to the medium. The sodium content of the particles was not altered by changes in pH.

3. Mitochondria isolated from either the cortex or the medulla of the kidney treated sodium and potassium in a manner similar to those from the whole kidney. However, adenosine triphosphate and substrate did not appear to inhibit loss of potassium from medullary mitochondria as effectively as from cortical mitochondria.

4. Addition of arsenite or 2:4-dinitrophenol to a medium containing adenosine monophosphate and substrate caused a marked increase in the loss of potassium.

5. Possible explanations are presented for the inability to demonstrate decreased loss of sodium in the presence of an oxidizable substrate and adenine nucleotide, and some conflicting results of sodium movements in liver and kidney mitochondria by other investigators are discussed.

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Acetoacetate as Fuel of Respiration in the Perfused Rat Heart

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Kulka, Krebs & Eggleston (1961) found that sheep-heart homogenates readily oxidize added acetoacetate. The ratio (oxygen used/acetoacetate oxidized) reached values of up to 4, indicating that acetoacetate was the sole or main fuel of respiration. It suppressed the oxidation of endogenous substrates.

Experiments on the isolated perfused rat heart are reported in this paper which show that the intact heart of the rat likewise uses acetoacetate as a preferred substrate of oxidation, even in the presence of glucose and insulin.

EXPERIMENTAL

Perfusion method. The perfusion apparatus was similar to that described by Bleehen & Fisher (1954) except that the anti-frothing device was omitted as frothing presented no difficulty. The gas mixture of $O_2 + CO_2$ (95:5, v/v) was saturated with water vapour at 37° before being passed to the gas lifts, in order to avoid loss of water from the perfusion fluid by evaporation. The perfusate volume was usually between 17 and 25 ml. The bath temperature was 37°.

Male rats (Wistar strain) weighing between 220 and 280 g. were used. 'Well-fed' animals had unrestricted

access to food and water. 'Unfed' rats were without food for 24 hr. After the rats were anaesthetized with ether, the heart was rapidly removed and placed in a dish of cold (4°) saline medium (Krebs & Henseleit, 1932). The aorta was stripped of connective tissue and tied on to a cannula through which dripped perfusion medium at room temperature. At this temperature the heart began to beat again, though at a slow rate. A few beats expelled the blood from the coronary vessels, and the cannulated heart was then transferred to the perfusion apparatus. The time between the opening of the thorax and the start of the perfusion was usually less than 2 min.

According to Fisher & Williamson (1961) the O_2 consumption and the coronary flow of the perfused heart preparation falls by 10–20% within the first 15 min. of perfusion and then remains fairly constant for periods of up to 2 hr. In most of the present experiments a period of 15 min. was therefore allowed to elapse before the rate measurements of the metabolic changes began. During this 15 min. period the perfusate equilibrated with the tissue. In most experiments the zero samples (usually 1 ml.) were removed after 15 min. The perfusion was continued for 1 hr., the final samples being collected 75 min. after the start.

Whereas the changes in the perfusate of acetoacetate, β-hydroxybutyrate, glucose and lactate and also the final glycogen content of the tissue could be determined on one and the same heart, the O_2 consumption during perfusion and the glycogen content of the heart after perfusion for 15 min. (zero time) had to be measured on hearts of other animals. Details of the standard error of the means and of the number of experiments are therefore given.

Reagents. Insulin (crystalline insulin containing 23·9 units/mg.) was dissolved in 0·94% NaCl containing 0·01N-HCl to give a concentration of 1 i.u./ml. This stock solution was stored as small samples in the deep-freeze. The final insulin concentration in the perfusion fluid was 2 milliunits/ml. (2 mU/ml.). A molar stock solution of acetoacetate was prepared according to Krebs & Eggleston (1945) and standardized by the method of Edson (1935). It was stored in the deep-freeze.

Analytical methods. The oxygen consumption was measured polarographically according to R. B. Fisher & J. R. Williamson (unpublished work). Acetoacetate was determined according to Walker (1954) in 0·5 ml. of the 50-fold-diluted perfusate. β-Hydroxybutyrate was estimated in 1 ml. of a fivefold-diluted perfusate according to Greenberg & Lester (1944) and Bessman & Anderson (1957). Acetoacetate was first removed by vigorous boiling of the solution in a test tube (25 mm. × 150 mm.) for 15 min. after the addition of 2 drops of conc. H_2SO_4, and water was added to replace the loss. Glucose when present was removed by copper–lime treatment. The values obtained by the two methods agreed well; the method of Bessman & Anderson was used in most experiments because it is quicker. In a few experiments β-hydroxybutyrate and acetoacetate were determined by an enzymic method based on the use of a soluble D(−)-β-hydroxybutyric dehydrogenase obtained from *Rhodopseudomonas spheroides* (D. H. Williamson & J. H. Mellanby, personal communication). Lactate was determined in 1 ml. of diluted medium by the method of Lehmann (1938) as modified by Wieland (1957), S. R. Elsden (personal communication) and H. A. Krebs & T. Gascoyne (unpublished work). Purified lactic-dehydrogenase preparation (Boeri, Cutole, Luzzati & Tosi, 1955) was used, and ferricyanide replaced by 2:6-dichlorophenol-indophenol, which increased the sensitivity. The perfusate was diluted to contain up to 0·2 μmole of lactate/ml. Glucose was determined with glucose oxidase according to Huggett & Nixon (1957); 1 ml. of a 50-fold dilution of perfusate was used. Citrate was determined according to Taylor (1953), succinate according to Rodgers (1961), malate according to Hohorst, Kreutz & Bücher (1959) and α-oxoglutarate according to Holzer & Holldorf (1957).

For the determination of glycogen in the tissue, the heart was removed from the cannula, cut into two pieces and blotted, and each piece was weighed on a torsion balance. One was used for the determination of the dry weight and the other was dropped into a centrifuge tube, containing 2 ml. of 30% KOH, placed in a boiling-water bath. The glycogen was separated by the method of Good, Kramer & Somogyi (1933) and hydrolysed by adding 3 ml. of 3N-H_2SO_4 and heating in a boiling-water bath for 2·5 hr. The hydrolysate was neutralized with NaOH and made up to a standard volume, and the amount of glucose in the solution was estimated by the glucose-oxidase method.

RESULTS

Disappearance of acetoacetate and formation of β-hydroxybutyrate. As shown in Table 1, acetoacetate at an initial concentration of 5 mM disappeared rapidly from the perfusion medium. The rate remained constant over the first 45 min. by which time the acetoacetate concentration had fallen to about 2·5 mM. The disappearance of acetoacetate was accompanied by the formation of β-hydroxybutyrate, the amounts being about half of the acetoacetate removed. As no appreciable quantities of the intermediates of the tricarboxylic acid

Table 1. *Rate of disappearance of acetoacetate and formation of β-hydroxybutyrate in the perfusion medium*

Heart (121 mg. dry wt.) from well-fed rat was initially perfused with 25 ml. of medium. Samples (1 ml.) were removed for analysis at the times stated.

Time (min.)	0	15	30	45	60	75
Concn. of acetoacetate (mM)	4·80	4·00	3·30	2·55	1·90	1·43
Concn. of β-hydroxybutyrate (mM)	0	0·38	0·71	0·96	1·23	1·28
Acetoacetate removed (μmoles)	—	20·0	36·8	54·0	68·3	78·2
β-Hydroxybutyrate formed (μmoles)	—	9·5	17·4	23·2	29·1	30·2
Acetoacetate 'oxidized' (μmoles)	—	10·5	19·4	30·8	39·2	48·0
Rate of acetoacetate oxidation for consecutive 15 min. periods (μmoles/g. dry wt./hr.)	—	48	296	377	278	292

cycle appeared in the medium or in the tissue (see below), the acetoacetate not accounted for by β-hydroxybutyrate was taken to be completely oxidized, and is therefore referred to as acetoacetate 'oxidized'. The rate of acetoacetate oxidation calculated in this way remained constant over the whole 75 min. period of perfusion (Table 1), although the rate of acetoacetate removal decreased after 45 min., this decrease being paralleled by a decreased β-hydroxybutyrate formation. That the fall of acetoacetate removal was not due to a deterioration of the metabolic capacity of the heart but to a decrease in the acetoacetate concentration was shown by the fact that, in experiments with a larger volume of perfusion fluid, the rate of acetoacetate removal and β-hydroxybutyrate formation remained constant over the whole 75 min. period of perfusion.

Another experiment in which the acetoacetate concentration was nearer the physiological value (initial concentration 0·25 mM) is shown in Table 2. Acetoacetate was still rapidly removed until the concentration fell to about one-tenth of the starting value. Hardly any β-hydroxybutyrate was formed. Whereas the rates of acetoacetate oxidation were not affected by the concentration between 2 and 5 mM, they fell gradually with the concentration between 0·1 and 1 mM. This follows from the values given for the rates of acetoacetate oxidation in Tables 1 and 2.

Oxidation of D(−)-*β-hydroxybutyrate.* An experiment in which D(−)-β-hydroxybutyrate was the substrate added to the perfusate is shown in Table 3. The medium containing D(−)-β-hydroxybutyrate was prepared by perfusing hearts for 75 min. with 25 ml. of medium containing 10 mM-acetoacetate. The perfusate was placed in a boiling-water bath for 60 min. to decompose most of the remaining acetoacetate, and a stream of N_2 was bubbled through the solution to remove the acetone. The $CaCO_3$ formed during this treatment was redissolved after cooling by gassing with $N_2 + CO_2$

Table 2. *Rate of disappearance of acetoacetate and of formation of β-hydroxybutyrate in the perfusion medium at low concentrations of acetoacetate*

Heart (141 mg. dry wt.) from well-fed rat was perfused initially with 50 ml. of medium containing 0·25 mM-acetoacetate. Samples (2 or 4 ml.) were removed for analysis at times stated. The values given in the last horizontal line are $100 \times a/n$ (see Discussion).

Time (min.)	0	10	20	30	40	50	60	70
Concn. of acetoacetate (mM)	0·250	0·175	0·125	0·090	0·065	0·044	0·019	0·012
Concn. of β-hydroxybutyrate (mM)	0	0·004	0·009	0·010	0·013	0·013	0·015	0·013
Acetoacetate removed (μmoles)	—	3·60	5·90	7·37	8·32	9·03	9·78	9·96
β-Hydroxybutyrate formed (μmole)	—	0·19	0·42	0·47	0·58	0·58	0·64	0·59
Acetoacetate 'oxidized' (μmoles)	—	3·41	5·48	6·90	7·74	8·45	9·14	9·37
Rate of acetoacetate oxidation for consecutive 10 min. periods (μmoles/g. dry wt./hr.)	—	145	88	60	35	30	29	10
Contribution of acetoacetate to substrate of respiration (%)	—	36	22	15	9	8	7	3

Table 3. *Rate of disappearance of* D-*β-hydroxybutyrate by the perfused rat heart*

Heart (126 mg. dry wt.) from well-fed rat was initially perfused with 25 ml. of medium containing 2 mM-D-β-hydroxybutyrate. Samples (1 ml.) were removed for analysis at the times stated. The values given in the last horizontal line are $100 \times a/n$ (see Discussion).

Time (min.)	0	15	30	45	60	75	90	105	120
Concn. of β-hydroxybutyrate (mM)	2·00	1·57	1·28	1·03	0·86	0·72	0·61	0·51	0·39
Concn. of acetoacetate (mM)	0·053	0·140	0·133	0·110	0·103	0·085	0·073	0·060	0·043
β-Hydroxybutyrate removed (μmoles)	—	10·3	17·0	22·5	26·1	28·9	31·0	32·8	34·8
Acetoacetate formed (μmoles)	—	2·09	1·93	1·42	1·27	0·91	0·68	0·45	0·16
β-Hydroxybutyrate 'oxidized' (μmoles)	—	8·23	15·1	21·1	24·8	28·0	30·3	32·3	34·6
Rate of β-hydroxybutyrate 'oxidation' over consecutive 15 min. periods (μmoles/g. dry wt./hr.)	—	261	217	191	118	100	74	64	74
Contribution of β-hydroxybutyrate to substrate of respiration (%)	—	73	61	54	33	28	21	18	21

(95:5, v/v). The concentration of β-hydroxybutyrate in the medium was 2·00 mM and that of acetoacetate 0·053 mM.

On perfusion β-hydroxybutyrate disappeared rapidly at the beginning and more slowly later (Table 3). Some acetoacetate was formed in the first 15 min. but this was subsequently utilized by the heart. The rates of β-hydroxybutyrate oxidation over consecutive 15 min. periods, calculated as the difference between the disappearance of β-hydroxybutyrate and formation of acetoacetate, are shown in the last-but-one horizontal line of Table 3. The rates were comparable with those of acetoacetate oxidation at similar concentrations (Table 2).

Accumulation of intermediates. In order to test whether the oxidation of acetoacetate was complete, the concentrations of the acids of the tricarboxylic acid cycle were measured in the heart tissue and in the perfusate. After 75 min. when another heart had oxidized 48 μmoles of acetoacetate (Table 1) less than 0·1 μmole, i.e. no significant amounts, of α-oxoglutarate, succinate and malate appeared in the perfusate, nor was there an appreciable change in the tissue concentrations of these intermediates (less than 0·1 μmole). On the other hand, 2·2 μmoles of citrate were found in the perfusate at 75 min. About 10% of this could be accounted for by a decrease of the tissue content of citrate. The origin of the citrate in the perfusate was not further investigated. A possible source of the oxaloacetate required for the synthesis of citrate is glutamate, which, according to Hicks & Kerly (1960), disappears from the tissue during perfusion under similar conditions at the rate of 1·2 μmoles in 60 min. Another possible source is CO_2 fixation by pyruvate, catalysed by the malic enzyme.

The findings indicate that by far the greater part of the acetoacetate not accounted for as β-hydroxybutyrate underwent complete oxidation.

Effect of glucose and insulin on acetoacetate metabolism. Glucose alone had no effect on the fate of acetoacetate, whereas insulin (without glucose) decreased the removal of acetoacetate and the formation of β-hydroxybutyrate in well-fed rats (Table 4). The insulin effects were independent of the initial acetoacetate concentration. Insulin decreased the oxidation of acetoacetate by 22–35% under all conditions tested, i.e. with or without glucose, and in unfed or well-fed animals. This effect was statistically significant (P 0·01). In unfed animals the inhibition of acetoacetate removal by insulin was not significant, but a larger proportion of the acetoacetate was reduced to β-hydroxybutyrate so that the amount of acetoacetate oxidized was still significantly decreased.

Table 4 also shows that the mean rate of acetoacetate removal was slightly greater at an initial concentration of 10 mM; but the rate of reduction to β-hydroxybutyrate increased at the same time so that the amounts of acetoacetate oxidized were not significantly affected by the acetoacetate concentration. There was no appreciable difference in the ketone-body metabolism of hearts from well-fed and unfed animals (Table 4).

Effect of acetoacetate on glucose metabolism. According to Bleehen & Fisher (1954), the perfused rat heart removes only small amounts of glucose from the perfused medium unless insulin is added. With insulin the rate of glucose removal becomes very rapid, and some of the glucose removed is recovered as tissue glycogen (Fisher & Lindsay, 1956). As shown in Table 5, insulin also causes a substantial increase in the formation of lactate, and the amount of glucose not accounted for by either glycogen or lactate, and presumably oxidized, was also increased.

In the absence of insulin acetoacetate caused no change in the rate of glucose uptake, but acetoacetate affected the fate of glucose in that less

Table 4. *Removal of acetoacetate and formation of β-hydroxybutyrate by the perfused rat heart: effects of glucose and insulin*

The values shown are means ± S.E.M. and, in parentheses, the numbers of experiments.

	Additions to perfusion medium (initial concn.)			Metabolic changes (μmoles/g. dry wt./hr.)		
Nutritional state	Acetoacetate (mM)	Glucose (mM)	Insulin (mU/ml.)	Acetoacetate	β-Hydroxybutyrate	Acetoacetate 'oxidized'
Well-fed	5	—	—	503±14 (8)	211±17 (8)	292±11 (8)
	5	—	2	377±18 (6)	172±21 (6)	205±15 (6)
Well-fed	10	—	—	566±12 (4)	224±11 (4)	322±6 (4)
	10	—	2	481±31 (6)	229±27 (6)	252±10 (6)
Unfed for 24 hr.	5	—	—	499±24 (10)	187±16 (10)	312±21 (10)
	5	—	2	461±23 (4)	257±23 (4)	204±22 (4)
Well-fed	5	5	—	515±5 (4)	205±11 (4)	310±12 (4)
	5	5	2	361±15 (7)	147±6 (7)	214±11 (7)

Table 5. *Effects of acetoacetate and insulin on glucose removal, lactate formation and glycogen synthesis in the perfused rat heart*

Glucose, lactate and the 75 min. value for glycogen were measured in one heart preparation simultaneously. Consumption of O_2 and the glycogen value for 15 min. were obtained from other preparations. Well-fed animals were used. The values shown are means ±S.E.M. and, in parentheses, the numbers of experiments. Glycogen is expressed as glucose equivalents.

Additions to medium (initial concn.)			Metabolic changes (μmoles/g. dry wt./hr.)							
					Glycogen			Glucose not accounted for		
Glucose (mM)	Acetoacetate (mM)	Insulin (mU/ml.)	Glucose	Lactate	Found at 15 min.	Found at 75 min.	Diff.	by glycogen or lactate	O_2 uptake	Acetoacetate 'oxidized'
5	—	—	72±8 (5)	0 (5)	77±6 (4)	72±5 (5)	0	72	1680±27 (23)	—
5	—	2	266±7 (6)	114±14 (6)	95±6 (5)	140±9 (6)	+45	164	1734±30 (18)	—
5	5	—	71±13 (9)	83±22 (4)	78±10 (4)	96±5 (10)	+18	12	1591 (3)	310±12 (4)
5	5	2	129±16 (13)	95±21 (3)	108±11 (4)	146±14 (10)	+38	43	1637 (2)	214±11 (7)

Table 6. *Summary of data on oxygen consumption and substrate changes (acetoacetate, β-hydroxybutyrate, glucose, glycogen) in the perfused rat heart (contribution of acetoacetate and glucose to fuel of respiration)*

The data are compiled from Tables 4 and 5 and from R. B. Fisher & J. R. Williamson (unpublished work).

	Nutritional state of animal										
	Well fed	Well fed	Well fed	Well fed	Unfed	Unfed	Well fed	Well fed	Well fed	Well fed	Well fed
Additions to perfusion medium											
Acetoacetate (mM)	5	5	10	10	5	5	5	5	—	—	—
Glucose (mM)	—	—	—	—	—	—	5	5	5	5	—
Insulin (mU/ml.)	—	2	—	2	—	2	—	2	—	2	—
Acetoacetate unaccounted for as β-hydroxybutyrate (μmoles/g. dry wt./hr.)	292	205	322	252	312	204	310	214	—	—	—
Glucose unaccounted for as glycogen or lactate (μmoles/g. dry wt./hr.)	—	—	—	—	—	—	12	43	72	164	—
O_2 uptake (μmoles/g. dry wt./hr.)	1455						1607		1704		1700
Glycogen change (μmoles/g. dry wt./hr.)	+5	−16	−18	−54	−47	−29	+18	+38	−5	+45	−78
β-Hydroxybutyrate formed (μmoles/g. dry wt./hr.)	211	172	244	229	187	257	205	147	—	—	—
O_2 equivalents (μmoles/g. dry wt./hr.)	1561	1541	1577	1570	1549	1583	1710	1681	1704	1704	1700
$\frac{O_2 \text{ equivalents}}{\text{Acetoacetate oxidized}}$ (*n*)	5·33	7·54	4·90	6·22	4·96	7·75	5·52	7·86	—	—	—
$\frac{O_2 \text{ equivalents}}{\text{Glucose oxidized}}$ (*n*)	—	—	—	—	—	—	70	26	23	10	—
Contribution of acetoacetate as fuel (%)	75	53	82	64	81	52	73	51	—	—	—
Contribution of glucose or glycogen as fuel (%)	0	6	7	20	29	12	4	15	24	58	27

glucose underwent oxidation; instead almost all the glucose removed was converted into glycogen or lactate. In the presence of insulin, acetoacetate halved the glucose uptake, this decrease being almost entirely due to a suppression of glucose oxidation. The amounts of lactate and glycogen formed from glucose were not affected by acetoacetate.

Oxygen consumption. Data on the O_2 consumption are included in Table 5. Those for the medium without acetoacetate are taken from R. B. Fisher & J. R. Williamson (unpublished work). Values obtained in the presence of acetoacetate and glucose were not significantly different from those obtained in the presence of glucose alone. When acetoacetate was the sole added substrate slightly lower values were obtained [$1455 \pm 44(18)$ μmoles/g. dry wt./hr.]. Insulin or deprivation of food had no effect.

DISCUSSION

Acetoacetate and glucose as fuels of respiration in rat heart. The mean values of substrate disappearance based on Tables 4 and 5 and additional data, together with the oxygen consumption in the presence of the substrates, are summarized in Table 6. When acetoacetate was added it served as a hydrogen acceptor in addition to oxygen, and the total oxidative capacity was therefore the sum of oxygen consumption and β-hydroxybutyrate formation. This sum is expressed in Table 6 as 'O_2 equivalents'. The contribution made by β-hydroxybutyrate formation to the sum was about 5–8 %. If the observed ratio (O_2 equivalents/substrate oxidized) is n and the O_2 equivalent required for complete oxidation of this substrate is a, the value $100 \times a/n$ expresses, as a per cent, the contribution that the substrate makes to the total respiration, provided that the oxidation is complete and does not lead to accumulation of intermediates. For acetoacetate a is 4, and for glucose a is 6.

The last-but-one vertical column of Table 6 shows that in the absence of insulin acetoacetate contributed 73–82 % to the respiration. This was independent of the nutritional state of the rat or the presence of glucose. Insulin decreased the contribution made by acetoacetate to 51–64 % and this decrease was independent of the presence of glucose. In other words, glucose did not suppress the oxidation of acetoacetate, even in the presence of insulin, but acetoacetate suppressed the oxidation of glucose; in the presence of acetoacetate most of the glucose removed was recovered either as glycogen or as lactate.

When glucose was the sole added substrate it contributed no more than 24 % to the respiration. Insulin raised the contribution to 57 %.

Under the test conditions three kinds of substances provided the fuel of respiration: (1) added acetoacetate, (2) added glucose, (3) endogenous substrates. As no ammonia, urea, glutamate or aspartate was formed (unpublished data; see also Hicks & Kerly, 1960), amino acids may be excluded as endogenous substrates. These substrates were presumably carbohydrate and fat. In the hearts from well-fed animals glycogen contributed at most 20 % to the fuel of respiration when acetoacetate was present. Without insulin the contribution was even smaller (Table 6). Thus acetoacetate, when available, was the preferred fuel, though it did not completely suppress the use of endogenous substrates. Insulin shifted the proportion of acetoacetate to endogenous fuel in favour of the latter, raising it from 18–27 % to 36–49 %. The endogenous substrates were next to acetoacetate in the order of preference as fuel. The fact that the oxygen consumption of the heart was not raised by the addition of substrates indicates that endogenous material can maintain the normal rate of respiration for considerable periods. Glucose without insulin did not compete effectively with endogenous substrates, but when insulin was also added glucose contributed about half the fuel of respiration.

The values for the ratio (O_2 equivalents/acetoacetate oxidized) were of the same order as those obtained on sheep-heart homogenates (Kulka *et al.* 1961), as were the ratios (acetoacetate oxidized/β-hydroxybutyrate formed). That heart muscle, like other tissues (Snapper & Grünbaum, 1927, 1928; Mirsky & Broh-Kahn, 1937; Jowett & Quastel, 1935; Barnes, Drury, Greely & Wick, 1940; Wick & Drury, 1941), can oxidize ketone bodies has long been established qualitatively, especially by the work of Bing, Siegel, Ungar & Gilbert (1954) and Bing (1954–55). What has not been realized before is the preferential oxidation of the ketone bodies.

It may not be unexpected that sheep heart readily utilizes ketone bodies since in ruminants a large proportion of the foodstuff reaches the organs in the form of short-chain fatty acids or ketone bodies. But it is surprising that a non-ruminant heart also uses acetoacetate preferentially. Pigeon-heart homogenates behave similarly to rat heart (H. A. Krebs & L. V. Eggleston, unpublished work).

The observations on the effects of insulin differ from those reported by Beatty, Marco, Peterson, Bocek & West (1960) for striated muscle fibres of pancreas-diabetic rats. In this material insulin appears to accelerate acetoacetate removal. However, the experimental conditions and the type of tissue were very different and a comparison with the present results is not warranted.

Ketone bodies as fuels in vivo. In well-fed rats the concentration of ketone bodies in the blood is

below 0·5 mM. A concentration of 1 mM is regarded as mildly ketotic and in severe ketosis the value can rise to 20 mM (see Harrison & Long, 1940; Beatty *et al.* 1960). The data in Tables 2 and 3 therefore indicate that ketone bodies serve as fuel at concentrations that occur *in vivo*.

Formation of lactate. On addition of glucose plus insulin some lactate appeared in the medium. Acetoacetate also increased lactate formation and in the presence of acetoacetate insulin had no additional effect (Table 5). But this aerobic glycolysis was low (about 100 μmoles of lactate/g. dry wt./hr.) compared with the oxygen uptake (about 1600 μmoles of oxygen/g. dry wt./hr.). It was also low compared with the maximum rate of anaerobic glycolysis under the same conditions [2800 μmoles of lactate/g. dry wt./hr. (unpublished data; see also Morgan, Randle & Regen, 1959)]. The increased lactic acid production on addition of insulin supports the view that the rate of glucose entry into the cells may play some part in the control of the rate of glycolysis, i.e. the Pasteur effect (Randle & Smith, 1958; Morgan *et al.* 1959).

Ottaway & Sarkar (1958) also recorded an increased lactate formation in rat heart, perfused with acetoacetate, and in diaphragm, and Beatty *et al.* (1960) made similar observations on rat-muscle fibres. It is unlikely that lactate arises directly from acetoacetate. The effect is probably an indirect one. If acetoacetate suppresses the oxidation of glucose it leaves more intracellular glucose available for lactate formation. The stimulating effects of acetoacetate and of insulin on lactate formation have thus probably a common basis: both acetoacetate and insulin increase the amounts of intracellular glucose albeit by different mechanisms.

Effect of insulin. The fact that insulin in the absence of glucose decreased the oxidation of acetoacetate suggests that it has a direct stimulating effect on the combustion of endogenous material, besides its well established action on the entry of sugars. The values for the glycogen changes indicate that only part of the endogenous material made available for combustion by insulin is carbohydrate, and that insulin in the absence of glucose may increase the oxidation of substrates other than carbohydrates.

Inhibition of glucose uptake by acetoacetate. The slow uptake of glucose by the heart that occurred when insulin was not added was not affected by acetoacetate, but the rapid uptake of glucose in the presence of insulin was almost halved by 5 mM-acetoacetate. This observation is of relevance to the insulin assay based on the stimulation by insulin of the uptake of glucose by rat diaphragm (Groen, Kamminga, Willebrands & Blickman, 1952; Marsh & Haugaard, 1952; Randle, 1954, 1956; Vallance-Owen & Hurlock, 1954). Like rat heart, rat diaphragm oxidizes acetoacetate in preference to glucose (Krebs, Eggleston & d'Alessandro, 1961). If acetoacetate depresses the uptake of glucose in rat diaphragm as it does in rat heart, it would be expected to interfere with the insulin assay. Discrepancies noted by Steinke, Taylor & Renold (1961) in the assay of serum of untreated diabetics would then be accounted for by the presence of acetoacetate in this material.

SUMMARY

1. The removal of acetoacetate and glucose, the formation of β-hydroxybutyrate and lactate, the consumption of oxygen and the changes in the concentrations of glycogen were measured in the perfused heart preparation described by Bleehen & Fisher (1954). Acetoacetate, β-hydroxybutyrate, glucose and insulin, alone and in combinations, were added to the perfusate.

2. Acetoacetate was rapidly removed from the perfusate. At concentrations above 4 mM about half was recovered as β-hydroxybutyrate. At low concentrations (below 0·25 M) acetoacetate also disappeared rapidly but little was reduced to β-hydroxybutyrate. No intermediates of oxidation accumulated and the acetoacetate not accounted for as β-hydroxybutyrate may therefore be taken to be completely oxidized.

3. Added D(−)-β-hydroxybutyrate was as readily oxidized as acetoacetate.

4. Insulin with or without glucose somewhat lowered the rate of oxidation of acetoacetate. It is calculated from the rates of oxygen consumption and acetoacetate oxidation that in the absence of insulin acetoacetate (5 mM) contributed 73–82 % to the fuel of respiration. Insulin decreased this value to 51–64 %, in the presence or the absence of glucose.

5. Acetoacetate decreased the oxidation of glucose in the presence or in the absence of insulin. It halved the uptake of glucose by the heart in the presence of insulin.

6. Glucose when added as the sole substrate contributed 24 % to respiration and insulin raised this to 57 %. When acetoacetate and glucose were added, glucose contributed 4 % in the absence, and 15 % in the presence, of insulin.

7. The data indicate that acetoacetate is oxidized in preference to glucose and endogenous substrates.

8. Insulin increased the contribution made to respiration by endogenous substrates. This is taken to indicate that insulin has effects other than accelerating the entry of sugars into the tissue.

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Determination of Maltase and Isomaltase Activities with a Glucose-Oxidase Reagent

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Glucose oxidase is a valuable tool for the assay of glycosidase activities, especially when the substrate itself is a reducing sugar, and so the common reductometric methods cannot be used for estimation of the hydrolysis products. However, the commercially available glucose-oxidase preparations also attack maltose and isomaltose in addition to glucose, probably as an effect of contaminant glycosidases. These contaminating enzymes prohibit the use of glucose oxidase for the assay of maltase and isomaltase activities, and necessitate the use of more complicated methods (Dahlqvist, 1960*c*).

This paper describes a modification of the glucose-oxidase reagent so that it may also be used for the determination of maltase and isomaltase activities.

MATERIALS AND METHODS

Glucose-oxidase preparations. The following preparations were used: glucose oxidase 'crude' and glucose oxidase 'purified' (Sigma Chemical Co., St Louis, U.S.A.), glucose oxidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), glucose oxidase–Dawes (obtained from Pfanstiehl Laboratories Inc., Waukegan, Ill., U.S.A.), glucose oxidase pure (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), notatin Leo (Leo Pharmaceutical Prod., Copenhagen, Denmark) and glucose oxidase (C. F. Boehringer und Soehne, Mannheim, Germany).

Sugars. The commercial preparations used were of analytical-grade purity, glucose (dextrose) from Baker Chemical Co. (Phillipsburg, U.S.A.) and maltose monohydrate from Pfanstiehl Laboratories. Isomaltose was prepared in the laboratory by the method previously described (Dahlqvist, 1960*b*). α-Glucose refers to Baker Chemical Co. ordinary glucose (see above) dissolved in water at 20° less than 2 min. before use. Initial $[\alpha]_D^{20}$ +112° (5% in water). β-Glucose was obtained from Nutritional Biochemicals Corp. The sugar was dissolved in water at 20° less than 2 min. before use. Initial $[\alpha]_D^{20}$ +19° (5% in water). 'Equilibrium glucose' was obtained by keeping an appropriate solution of glucose at 20° for 18–20 hr. After that time $[\alpha]_D^{20}$ was +52·5° (10% in water). The solution