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The Reduction of Acetoacetate to β-Hydroxybutyrate in Animal Tissues

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Krebs & Eggleston (1948) reported that minced sheep-heart muscle, when suspended in a saline medium, reduced acetoacetate to β-hydroxybutyrate more rapidly under aerobic than under anaerobic conditions. It is somewhat unexpected that oxygen should promote a reduction and the present investigation was undertaken to throw light on the mechanism by which this effect of oxygen is brought about.

EXPERIMENTAL

Materials

Sodium acetoacetate was prepared as described by Krebs & Eggleston (1945). Stock solutions were stored at −18°. Because of its instability the concentration of the acetoacetate solution used was determined for each experiment.

Sodium DL-β-hydroxybutyrate was obtained from British Drug Houses Ltd. The product was eluted as a single peak when chromatographed on a Celite column (Utter & Swim, 1957) and recoveries of the eluted free acid, based on titration against standard NaOH, were 103% in two successive experiments. Samples of sodium D(−)- and L(+)-β-hydroxybutyrate were kindly given by Dr G. D. Greville (see Lehninger & Greville, 1953).

Adenosine triphosphate (ATP) was obtained from Schwartz Chemical Co., U.S.A. Dicoumarol was the product of Organon Laboratories Ltd.

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Tissue preparations

Homogenates were prepared in a saline medium which consisted of 100 vol. of 0·155M-KCl, 10 vol. of 0·1M-potassium phosphate buffer, pH 7·4, and 1 vol. of 0·1M-$MgCl_2$.

Pig and sheep hearts were obtained from an abattoir. They were placed in ice immediately after removal from the animal and reached the laboratory in 15–20 min. Other tissues were immersed in ice-cooled saline medium immediately after removal from the animal. The tissue was freed from fat and connective tissue, minced in a chilled Fischer mincer (Jouan, Paris) and homogenized in a stainless steel Potter–Elvehjem homogenizer with 6·5 vol. of cold (0°) saline medium.

Incubations were carried out in conical Warburg flasks at 30°. The centre wells of vessels contained 2N-NaOH and filter paper in aerobic experiments, and a stick of yellow phosphorus in anaerobic experiments. The main compartments contained 3·0 ml. of homogenate (approx. 70 mg. dry wt. of tissue). Substrates were placed in the side arm in the form of a 0·1–0·2M-solution of the sodium salt and the total volume was made up to 4·0 ml. with saline medium. The vessels were kept in ice during the preparation of the experiment. Unless stated otherwise the substrates were added after 5 min. incubation at 30° and the vessels were incubated for a further 45 min. At the end of the incubation the vessels were cooled in ice and 2·0 ml. of the contents of each vessel was transferred to a tube containing 0·5 ml. of N-HCl (solution A). These tubes were stored at −18°. To the remaining 2·0 ml. in each vessel 0·5 ml. of 25% (w/v) trichloroacetic acid was added and the

precipitated proteins were removed by centrifuging at 0°. The supernatants (solution B) were stored in a refrigerator and used for acetoacetate determinations on the same day.

Chemical methods

Determination of acetoacetate. Unless otherwise stated the method of Walker (1954) was used. The calibration curve was prepared with a solution of acetoacetate standardized by the aniline citrate method of Edson (1935).

Determination of β-hydroxybutyrate. To 1·0 ml. of solution A (see above) in a 10 ml. conical centrifuge tube were added in turn 2·0 ml. of 0·1 N-NaOH, 2·0 ml. of 0·15 N-$Ba(OH)_2$ and 2·0 ml. of 2·5% (w/v) $ZnSO_4,7H_2O$, and the contents of the tube were mixed and centrifuged. A 5·0 ml. portion of the supernatant was measured into a 10 ml. centrifuge tube, and 0·5 ml. of 20% (w/v) $CuSO_4$, $5H_2O$ and 0·5 ml. of 10% (w/v) $Ca(OH)_2$ suspension were added with mixing. The mixture was kept for 15 min. and then centrifuged. The supernatant was transferred to a 28 mm. × 150 mm. test tube, and a drop of conc. H_2SO_4 and a few alundum chips were added. After weighing the tube and contents the solution was boiled for 10 min. to remove acetoacetate, and the original weight was restored by addition of distilled water. β-Hydroxybutyrate was estimated in 3·0 ml. of this solution by the following modification of the method of Greenberg & Lester (1944) and Lester & Greenberg (1948). Each addition of $K_2Cr_2O_7$ was followed by a 15 min. (instead of a 10 min.) refluxing period. After the oxidation steps, the dichromate was reduced with 0·5 ml. of freshly prepared 30% (w/v) $Na_2SO_3,7H_2O$. The solvent used for the 2:4-dinitrophenylhydrazine was N-HCl as it gave a lower reagent blank and more consistent results than 2N-HCl. The extinction of the phenylhydrazone was measured at 420 mμ.

Isolation of β-hydroxybutyrate. This was carried out according to Utter & Swim (1957) with a Celite column, containing either 5 g. or 50 g. of Celite, the volumes of the fractions being 5 ml. and 50 ml. respectively. β-Hydroxybutyrate was eluted in fractions 22–26. As the elution peak of β-hydroxybutyrate overlaps that of lactate the chromatography had to be repeated several times in order to obtain a sample of β-hydroxybutyrate free from lactate.

Oxygen consumption. The O_2 consumption was measured manometrically at 10 min. intervals, between 10 and 40 min. after adding the contents of the side arm. In calculating the total O_2 consumption during incubation, it was assumed that the rates between 0 and 10 min. were the same as between 10 and 20 min., and that the rates between 40 and 45 min. were the same as between 30 and 40 min. As the O_2 uptake was usually fairly constant, no major errors were introduced by this extrapolation, with the possible exception of some experiments with dinitrophenol concentrations above 0·01 mM where the O_2 uptake fell sharply during the incubation. An error arising from this fall would make values too low.

RESULTS

Metabolism of acetoacetate in homogenates of various tissues

Effect of oxygen. Oxygen increased the rate of acetoacetate removal in the four tissues tested, but sheep heart was the only tissue where oxygen also increased the rate of β-hydroxybutyrate formation from acetoacetate (Table 1). In pigeon heart, pig heart and rat-kidney cortex, the amounts of β-hydroxybutyrate formed aerobically represented 15–40% of the acetoacetate removed, whereas in sheep heart it was 50–75% (Tables 1 and 2). The

Table 1. *Acetoacetate removal and β-hydroxybutyrate formation in various tissue homogenates: effects of oxygen, adenosine triphosphate, ethylenediaminetetra-acetic acid and dinitrophenol*

For general conditions see text. The complete system contained 4 μmoles of ATP and 1 μmole of EDTA in 4 ml. and approx. 40 μmoles of acetoacetate. Dinitrophenol was present in the main compartment from the beginning. The acetoacetate was added from the side arm immediately before placing the vessels in the water bath.

				Aerobic changes		Anaerobic changes	
Tissue	Incubation time (min.)	Modification of incubation mixture	Dinitrophenol (mM)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)
Pigeon heart	60	None	0	12·0	1·6	3·3	2·5
		No ATP	0	12·8	2·2	3·8	3·0
Pigeon heart	50	None	0	8·2	1·8	2·9	2·3
		None	0·20	1·4	0·4	1·7	2·3
		No EDTA	0	5·5	1·7	2·5	2·2
Pig heart	30	None	0	4·9	2·1	3·0	3·4
		None	0·20	0·2	0·3	2·1	2·3
		No EDTA	0	5·4	2·1	2·6	3·2
Rat-kidney cortex	45	None	0	10·0	1·8	4·8	2·4
		None	0·20	1·4	0·4	3·8	2·1
		No EDTA	0	6·5	1·4	1·4	—
Sheep heart	45	None	0	11·6	9·4	4·6	6·3
		None	0·20	0·1	1·3	3·4	3·9
		No EDTA, no ATP	0	13·0	9·1	4·6	5·3
		No EDTA, no ATP	0·20	0·9	1·6	4·1	4·3

Table 2. *Time course of the metabolism of acetoacetate in sheep-heart homogenates at different substrate concentrations*

For experimental conditions see text. The data refer to 4 ml. of homogenate. Different homogenates were used for the aerobic and anaerobic incubation.

		Acetoacetate (9·6 μmoles) added		Acetoacetate (19·2 μmoles) added		Acetoacetate (38·4 μmoles) added	
Gas	Incubation time (min.)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
N_2	10	2·5	2·5	3·0	2·6	0·6	3·3
	20	3·2	3·0	3·0	3·3	1·9	3·6
	40	3·7	3·7	3·3	3·7	3·3	4·8
O_2	10	6·0	3·3	6·5	3·3	7·9	3·4
	20	8·0	4·2	9·6	5·7	11·5	5·6
	40	8·5	4·0	13·9	7·4	15·9	8·7

stimulation of β-hydroxybutyrate formation by oxygen, described by Krebs & Eggleston (1948), is thus peculiar to sheep heart. Anaerobically, β-hydroxybutyrate was the principal product formed from acetoacetate in all four tissues.

Effect of 2:4-*dinitrophenol*. A striking effect was obtained. In all the tissues examined, 0·20 mM-dinitrophenol strongly inhibited (80–90%) the aerobic acetoacetate removal and β-hydroxybutyrate formation. Anaerobically, there was a very much smaller inhibition (Table 1).

Effects of ethylenediaminetetra-acetic acid and adenosine triphosphate. Omission of ethylene diaminetetra-acetic acid (EDTA) (0·25 mM) lowered the aerobic acetoacetate removal in rat-kidney cortex and pigeon heart but had no appreciable effect on the formation of β-hydroxybutyrate (Table 1). There were no major effects in pig heart and sheep heart. The omission of ATP, where tested, made no appreciable difference.

Because sheep heart was the only tissue in which oxygen stimulated the formation of β-hydroxybutyrate, it was used for most other experiments. EDTA and ATP were omitted in subsequent experiments (except when otherwise stated) because they had no effect in this tissue.

Acetoacetate metabolism in sheep-heart homogenates

Time course. Anaerobically, the removal of acetoacetate and the equivalent formation of β-hydroxybutyrate usually came to a stop after about 20 min. (Table 2), presumably because of lack of hydrogen donors. Aerobically, both acetoacetate removal and β-hydroxybutyrate formation usually progressed throughout an incubation period of 80 min., though the rates gradually decreased.

Effect of substrate concentration. An increase in substrate concentration from 2·4 to 9·6 mM (Table 2) had no appreciable effect on the rate of the anaerobic metabolism and on the initial aerobic rates, but the rate of the aerobic formation of β-hydroxybutyrate fell when the concentration of acetoacetate became low in the course of the incubation (Table 2).

Metabolism of the optical isomerides of β-hydroxybutyrate. The different effects of dinitrophenol on the aerobic and anaerobic metabolism of the ketone bodies suggested that different isomerides of β-hydroxybutyrate might be involved aerobically and anaerobically. It is known (Lehninger & Greville, 1953; McCann, 1957) that acetoacetate can be directly reduced to D(−)-β-hydroxybutyrate, whereas the reduction to the L(+)-form requires a prior conversion of acetoacetate into its coenzyme A ester. As this conversion depends on the supply of succinyl-coenzyme A through the tricarboxylic acid cycle, it would be expected to be accelerated by respiration. Further, the oxidation–reduction potential of the coenzyme A esters of acetoacetate and β-hydroxybutyrate is estimated to be considerably less negative [E° (pH 7·0), −0·238 v] than that of the free acids [E° (pH 7·0), −0·349 v] (Burton, 1957). This implies that the coenzyme A derivatives are more readily reducible by pyridine nucleotides than are the free acids. Because of these considerations, the behaviour of the D- and L-forms was investigated. Sheep-heart homogenates removed D(−)-β-hydroxybutyrate 3–5 times as fast as L(+)-β-hydroxybutyrate (Table 3). About one-third of the D(−)-isomeride, but very little of the L(+)-isomeride, which disappeared was converted into acetoacetate. These observations are similar to those of McCann (1957) on rat-heart sarcosomes.

The presence of 0·20 mM-dinitrophenol completely inhibited the removal of the L-form, but stimulated the rate of oxidation of the D-form to acetoacetate (Table 3).

These findings support McCann's (1957) conclusion that the L-form requires conversion into

the coenzyme A derivative, whereas the D-form can dehydrogenate as such. As the formation of L-β-hydroxybutyryl-coenzyme A requires ATP, it is expected to be inhibited in homogenates by dinitrophenol.

The differences in the behaviour of the L- and D-isomerides were used to examine the question of whether different isomerides accumulate in homogenates of sheep-heart muscles aerobically and anaerobically. The β-hydroxybutyrate was isolated from a suspension, containing 300 μmoles of acetoacetate and 22·5 ml. of homogenate in a total volume of 30 ml., after aerobic or anaerobic incubation at 30° for 60 min. The incubated suspensions were deproteinized by the addition of 2 vol. of 0·15N-barium hydroxide and 2 vol. of 2·5% (w/v) zinc sulphate heptahydrate. The mixture was centrifuged, and the precipitate was washed with water. Supernatant and washings were then combined and evaporated to dryness. β-Hydroxybutyrate was isolated from the residue on a column containing 5 g. of Celite according to Utter & Swim (1957). The chromatography was repeated without titration, β-hydroxybutyrate being extracted from the chloroform phase with an equivalent amount of sodium hydroxide solution. The aqueous phase was evaporated to dryness, and the residue was dissolved in a small quantity of water. The concentration of β-hydroxybutyrate in this solution was determined according to Greenberg & Lester (1944).

The material isolated from the aerobic and anaerobic incubations behaved identically when added to respiring sheep-heart homogenate: the rate of removal was of the same order as that of D(−)-β-hydroxybutyrate; it was increased by 0·2 mM-dinitrophenol, and the yield of acetoacetate in the presence of dinitrophenol was almost 100%. These results indicate that the D(−)-isomeride was formed both aerobically and anaerobically.

Measurement of the optical rotation of the β-hydroxybutyrate formed aerobically and anerobically. The above conclusion was confirmed by the following experiment. Latapie-minced sheep heart (100 g.) was homogenized with 650 ml. of ice-cold saline medium in a Waring Blendor and the homogenate was diluted to 1000 ml. with saline medium. Equal portions of the homogenate (500 ml.) were placed in two 1 l. flasks, immersed in a bath at 30°, nitrogen being bubbled through one flask and oxygen through the other. After 5 min., acetoacetate (5 mM) was added to each flask and the incubation was continued for 2 hr. At the end of the incubation, 125 ml. of 25% (w/v) trichloroacetic acid was added to each flask and the precipitated proteins were removed by centrifuging. The supernatants were evaporated to dryness, and β-hydroxybutyrate was isolated from the residue by chromatography on a column containing 50 g. of Celite (Utter & Swim, 1957). The β-hydroxybutyrate fraction was rechromatographed three times to remove traces of other organic acids, principally lactate (Krebs & Eggleston, 1945), which might interfere with optical rotation measurements. The procedure for the final chromatographic separation was similar to that described in the preceding section. The optical rotations observed were as follows. β-Hydroxybutyrate formed aerobically: $[\alpha]_D^{19} - 14{\cdot}5° \pm 1{\cdot}0$ in water (c, 8·35); β-hydroxybutyrate formed anaerobically: $[\alpha]_D^{19} - 15{\cdot}1° \pm 1{\cdot}5$ in water (c, 2·65). These values are close to the value ($[\alpha]_D^{19} - 14{\cdot}4°$) reported by Lehninger & Greville (1953) for pure sodium D(−)-β-hydroxybutyrate. Thus all the available evidence demonstrates that the product of both the aerobic and anaerobic reduction of acetoacetate is predominantly if not exclusively D(−)-β-hydroxybutyrate.

Effect of dinitrophenol concentration on acetoacetate metabolism (with acetoacetate as only added substrate). When acetoacetate was the sole substrate, the highest increases in the oxygen uptake (up to threefold) were usually obtained with 0·01 mM-dinitrophenol (Tables 4–9). Higher concentrations gave submaximal increases in most experiments and even inhibitions in some. The rates of oxygen uptake were constant without dinitrophenol or with concentrations up to 0·01 mM. At the higher concentrations, the rates fell during

Table 3. *Metabolism of* D(−)- *and* L(+)-β-*hydroxybutyrate in sheep-heart homogenates: effect of dinitrophenol*

For experimental conditions see text. Incubation time was 45 min.

	β-Hydroxybutyrate		No dinitrophenol added		0·2 mM-Dinitrophenol	
Expt. no.	Isomer used	Amount added (μmoles)	β-Hydroxybutyrate removal (μmoles)	Acetoacetate formation (μmoles)	β-Hydroxybutyrate removal (μmoles)	Acetoacetate formation (μmoles)
1	DL	18·8	6·4	2·0	8·3	8·1
	L(+)	8·9	1·7	0·4	0·2	0·4
2	DL	18·8	6·8	1·6	8·1	9·4
	D(−)	8·9	4·5	1·5	8·0	8·3
	L(+)	8·9	1·0	Nil	Nil	Nil

Table 4. *Effect of dinitrophenol concentration on acetoacetate metabolism in sheep-heart homogenates*

For conditions see text. The data refer to 4 ml. of homogenate to which about 40 μmoles of acetoacetate was added. In Expt. 2, the final dilution of the tissue was 1:8, against 1:10 in the usual procedure. The uncoupling agent was added either from the side arm at the same time as the substrate (procedure A) or to the cup contents before incubation (procedure B) as indicated below.

Expt. no.	Dinitrophenol concn. (mM)	Procedure	Gas	Oxygen uptake (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
1	Nil	A	O_2	25·0	12·2	5·8
	0·005	A	O_2	40·2	13·0	4·7
	0·01	A	O_2	50·0	14·6	3·4
	0·02	A	O_2	25·1	2·1	0·5
	0·04	A	O_2	21·9	Nil	Nil
	0·08	A	O_2	15·6	Nil	Nil
	0·005	B	O_2	38·6	11·9	4·5
	0·01	B	O_2	48·7	13·5	—
	0·02	B	O_2	22·6	1·5	—
	0·04	B	O_2	16·4	Nil	—
	0·08	B	O_2	14·5	Nil	—
2	Nil	A	O_2	36·3	15·7	7·9
	0·005	A	O_2	51·8	15·7	6·0
	0·01	A	O_2	63·8	16·2	3·4
	0·02	A	O_2	84·0	17·0	3·0
	0·04	A	O_2	60·8	2·8	0·6
	0·08	A	O_2	37·1	1·3	—
	0·16	A		32·3	1·3	—
	Nil	A	N_2	—	6·2	4·9
	0·01	A	N_2	—	6·2	4·9
	0·16	A	N_2	—	4·1	3·8

Table 5. *Comparison of the effect of dinitrophenol and dicoumarol on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate. The 'uncoupling agent' was added from the side arm at the same time as the substrate.

Expt. no.	Uncoupling agent	Concn. (mM)	Gas	Oxygen uptake (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
1	Nil		O_2	20·4	14·8	10·0
	Dinitrophenol	0·01	O_2	54·2	15·9	4·2
	Dicoumarol	0·01	O_2	39·3	13·9	6·6
	Nil		N_2	—	2·7	3·7
	Dinitrophenol	0·01	N_2	—	2·0	3·8
	Dicoumarol	0·01	N_2	—	2·3	3·8
2	Nil		O_2	26·6	15·7	7·2
	Dinitrophenol	0·01	O_2	58·6	16·8	1·7
	Dinitrophenol	0·10	O_2	11·9	0·7	Nil
	Dicoumarol	0·01	O_2	55·0	15·7	2·2
	Dicoumarol	0·10	O_2	10·0	Nil	Nil

the incubation, as had been noted before under other conditions by Tyler (1950) and Simon (1953).

The amounts of acetoacetate removed were not much affected by 0·01 mM-DNP, whereas the ratio of β-hydroxybutyrate formed to acetoacetate removed was decreased from 0·5–0·75 to 0·1–0·35 (Tables 4–9). In contrast, dinitrophenol had no major effects on the anaerobic acetoacetate metabolism.

These effects of dinitrophenol are similar to those on acetate oxidation in rat-kidney homogenates observed by Aisenberg & Potter (1955), who attributed the effect to an inhibition of acetate activation by dinitrophenol. An inhibition by it of the oxidation of acetoacetate and fatty acids in mitochondria has already been reported by Cross, Taggart, Covo & Green (1949) and Witter, Newcomb & Stotz (1953).

Effects of dicoumarol. Dicoumarol, another agent uncoupling oxidative phosphorylation (Martius & Nitz-Litzow, 1953), decreased the aerobic β-hydroxybutyrate formation at a concentration of 0·01 mM to about the same degree as dinitrophenol (Table 5) without appreciably influencing the total

amount of acetoacetate removed. The decreased aerobic β-hydroxybutyrate formation was accompanied by an increased oxygen uptake. 0·1 mM-Dicoumarol, like dinitrophenol, strongly inhibited the aerobic acetoacetate removal.

Effect of adenosine triphosphate. Since uncoupling agents are known to cause a decrease in the ATP concentration, it might be thought that addition of ATP might counteract the effects of dinitrophenol. This did not prove to be the case. 2·5 mM-ATP stimulated aerobic acetoacetate removal and β-hydroxybutyrate formation by 20–35 % without altering the ratio of β-hydroxybutyrate formed to acetoacetate removed (Table 6). This concentration of ATP had no effect on the anaerobic reduction of acetoacetate. In the presence of dinitrophenol (0·05 or 0·2 mM) ATP inhibited the oxygen uptake by about 25 %.

Effect of α-oxoglutarate and dinitrophenol. Dinitrophenol trebled the oxygen consumption in the presence of α-oxoglutarate and caused a still greater increase (up to fivefold) of oxygen uptake when both α-oxoglutarate and acetoacetate were added (Table 7). α-Oxoglutarate stimulated the

Table 6. *Effect of adenosine triphosphate on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate. The substrate (acetoacetate) and dinitrophenol were added from the side arm.

			Aerobic changes				Anaerobic changes	
Expt. no.	ATP concn. (mM)	Dinitrophenol concn. (mM)	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Ratio β-hydroxybutyrate formation/acetoacetate removal	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)
1	0	0	18·1	13·4	8·6	0·64	3·2	3·3
	2·5	0	19·7	16·2	10·4	0·64	3·2	3·4
	0	0·05	15·6	1·3	0·8	—	3·9	3·8
	2·5	0·05	12·0	0·2	Nil	—	3·9	3·3
2	0	0	16·3	14·7	10·9	0·74	—	—
	2·5	0	15·4	19·8	15·3	0·77	—	—
	0	0·20	32·3	2·0	0·8	—	—	—
	2·5	0·20	23·7	1·8	—	—	—	—

Table 7. *Effect of α-oxoglutarate on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. with about 40 μmoles of acetoacetate or 40 μmoles of α-oxoglutarate, or both; dinitrophenol was added from the side arm at the same time as the substrate.

Expt. no.	Substrate	Dinitrophenol concn. (mM)	Gas	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Ratio β-hydroxybutyrate formation/acetoacetate removal
1	Acetoacetate	0	O_2	17·4	16·4	10·7	0·65
		0·01		61·2	16·4	5·4	0·33
		0·05		34·9	1·9	1·0	—
		0·20		32·2	1·9	0·7	—
	α-Oxoglutarate	0		20·5	—	—	—
		0·01		60·8	—	—	—
		0·05		68·0	—	—	—
		0·20		72·3	—	—	—
	Acetoacetate + α-oxoglutarate	0		23·0	21·0	13·5	0·64
		0·01		69·0	24·4	8·6	0·35
		0·05		106·5	21·0	6·9	0·33
		0·20		93·3	21·2	10·0	0·47
2	Acetoacetate	0	O_2	16·3	14·7	10·9	0·74
		0·01		58·8	15·7	5·2	0·33
		0·20		32·0	2·0	0·8	—
	Acetoacetate + α-oxoglutarate	0		19·7	17·7	13·3	0·75
		0·01		70·7	22·7	8·1	0·36
		0·20		99·0	13·9	3·3	0·24
	Acetoacetate	0	N_2	—	5·2	6·6	—
	Acetoacetate + α-oxoglutarate	0		—	14·5	15·3	—
		0·01		—	13·2	12·5	—
		0·20		—	8·2	11·3	—

Table 8. *Effect of fumarate on acetoacetate metabolism in sheep-heart homogenates*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate or 40 μmoles of fumarate, or both. Dinitrophenol was added from the side arm at the same time as the substrate.

Expt. no.	Substrate	Dinitrophenol concn. (mM)	Gas	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Ratio β-hydroxybutyrate formation/acetoacetate removal
1	Acetoacetate	0	O_2	23·5	12·2	7·2	0·59
		0·01		52·0	12·9	3·9	0·30
		0·05		23·0	1·1	0·7	—
		0·20		18·6	0·4	0·3	—
	Fumarate	0		25·4	—	—	—
		0·01		38·8	—	—	—
		0·05		35·0	—	—	—
		0·20		25·0	—	—	—
	Acetoacetate + fumarate	0		31·9	16·3	9·6	0·59
		0·01		58·3	18·6	5·6	0·30
		0·05		40·7	2·2	0·7	—
		0·20		33·9	1·1	0·4	—
2	Acetoacetate	0	O_2	23·9	14·5	9·2	0·63
		0·01		57·8	16·6	4·9	0·30
		0·20		15·6	0·9	—	—
	Acetoacetate + fumarate	0		30·9	20·5	12·2	0·60
		0·01		62·2	22·8	7·7	0·34
		0·20		25·6	0	—	—
	Acetoacetate	0	N_2	—	4·3	3·6	—
		0·01		—	4·7	5·3	—
		0·20		—	3·8	5·0	—
	Acetoacetate + fumarate	0		—	21·8	20·2	—
		0·01		—	22·5	22·9	—
		0·20		—	14·5	13·0	—

Table 9. *Effect of pyruvate and lactate on acetoacetate metabolism in sheep-heart homogenates*

Data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate alone or together with 40 μmoles of pyruvate or L(+)-lactate. In Expts. 1 and 2 dinitrophenol was added from the side arm together with the substrate; in Expts. 3 and 4 it was placed in the main compartment.

			Oxygen			Nitrogen	
Expt. no.	Substrate	Dinitrophenol concn. (mM)	Oxygen uptake (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)	Acetoacetate removal (μmoles)	β-Hydroxybutyrate formation (μmoles)
1	Acetoacetate	0	22·1	14·4	7·9	4·2	3·7
		0·01	59·3	13·3	2·6	3·7	3·9
		0·05	16·1	1·1	—	4·6	3·7
	Acetoacetate + pyruvate	0	25·7	12·1	8·9	9·6	8·9
		0·01	62·5	9·8	4·1	7·8	7·5
		0·05	111·5	5·9	2·7	6·4	6·3
2	Nil	0	21·3	—	—	—	—
	Acetoacetate	0	26·0	15·6	11·7	6·7	5·2
	Acetoacetate + pyruvate	0	30·3	12·9	9·7	9·7	10·6
		0·20	83·0	5·9	3·5	4·0	6·4
3	Acetoacetate	0	18·2	15·4	9·2	2·5	3·3
		0·20	11·6	1·6	1·2	2·5	3·5
	Acetoacetate + pyruvate	0	18·8	14·0	10·8	11·2	9·2
		0·20	65·5	4·2	3·6	6·0	4·6
	Acetoacetate + lactate	0	19·8	13·0	8·9	7·4	4·0
		0·20	12·5	−2·2	0·7	4·9	3·5
4	Nil	0	16·5	—	—	—	—
	Acetoacetate	0	20·1	13·3	7·9	3·9	5·2
		0·20	17·7	2·4	1·2	3·3	4·1
	Acetoacetate + lactate	0	23·6	13·1	7·4	6·7	5·4
		0·20	23·2	2·3	1·0	4·4	4·0

able 10. *Effect of succinate on acetoacetate metabolism in sheep-heart homogenate*

The data refer to 4 ml. of homogenate with about 40 μmoles of acetoacetate. Succinate was 80 μmoles, unless otherwise stated.

				Oxygen			Nitrogen	
Expt. no.	Substrate	Temperature	Period of incubation (min.)	Oxygen uptake (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)	Aceto-acetate removal (μmoles)	β-Hydroxy-butyrate formation (μmoles)
1	None	30°	40	16·5	—	—	—	—
	Acetoacetate		40	24·2	9·3	7·0	8·8	4·9
	Acetoacetate + succinate		40	47·2	15·0	12·1	7·3	4·9
2	Acetoacetate	25	20	8·8	3·8	1·7	—	—
	Acetoacetate		40	18·7	5·1	3·8	—	—
	Acetoacetate		60	25·6	4·8	3·8	—	—
	Acetoacetate + succinate		20	21·4	3·3	2·9	—	—
	Acetoacetate + succinate		40	37·8	7·0	5·7	—	—
	Acetoacetate + succinate		60	45·5	8·1	6·6	—	—
3	Acetoacetate	30	45	33·8	9·7	3·4	—	—
	Acetoacetate + succinate		45	65·8	13·0	6·0	—	—
4	None	30	0	0	—	0·6	—	—
	None		45	19·8	—	0·5	—	—
	Acetoacetate		45	34·3	9·5	4·0	—	—
	Acetoacetate + succinate (2 μmoles)		45	34·5	8·8	4·7	—	—
	Acetoacetate + succinate (20 μmoles)		45	38·0	11·1	6·2	—	—
	Acetoacetate + succinate (80 μmoles)		45	60·5	11·8	9·1	—	—

aerobic removal of acetoacetate (see Krebs & Eggleston, 1945) and almost completely reversed the inhibition of the aerobic removal of acetoacetate by dinitrophenol. The aerobic formation of β-hydroxybutyrate was only partially restored by α-oxoglutarate, so that the ratio of β-hydroxybutyrate formed to acetoacetate removed was still decreased in the presence of dinitrophenol and α-oxoglutarate. Anaerobically, α-oxoglutarate substantially increased the removal of acetoacetate (Krebs & Eggleston, 1945), and this reaction was slightly inhibited by dinitrophenol.

Effect of fumarate and dinitrophenol. The action of fumarate differed in several respects from that of α-oxoglutarate (Tables 7 and 8). The stimulation of oxygen consumption by dinitrophenol was smaller with fumarate than with α-oxoglutarate. The higher concentrations of dinitrophenol (0·05 and 0·2 mM), which did not abolish the stimulation of oxygen consumption produced by 0·01 mM in the presence of α-oxoglutarate, did so in the presence of fumarate, and of acetoacetate and fumarate. In contrast with α-oxoglutarate, fumarate failed to restore aerobically the inhibitory effects of 0·2mM-dinitrophenol on the acetoacetate removal. Anaerobically, fumarate greatly accelerated the removal of acetoacetate and the formation of β-hydroxybutyrate, as was already shown by Krebs & Eggleston (1945). This anaerobic reaction was inhibited (30 %) by 0·2 mM, but was not affected by 0·01 mM-dinitrophenol.

Effects of pyruvate, lactate and dinitrophenol. Dinitrophenol caused very large increases in the oxygen uptake when both pyruvate and acetoacetate were present, but not in the presence of L(+)-lactate and acetoacetate (Table 9). Unlike α-oxoglutarate, pyruvate did not fully abolish the inhibition by dinitrophenol of the aerobic removal of acetoacetate, though it decreased the inhibition slightly at 0·2 mM-dinitrophenol. Lactate had no effects on the aerobic acetoacetate removal or β-hydroxybutyrate formation.

Anaerobically, pyruvate and lactate increased both acetoacetate removal and β-hydroxybutyrate formation. Lactate increased the former but not the latter. 0·20 mM-Dinitrophenol largely abolished the accelerating effects of pyruvate and lactate.

Effect of succinate. α-Oxoglutarate, fumarate and pyruvate raised the rate of reduction of acetoacetate aerobically and anaerobically and, in the presence of these substrates, the anaerobic rate was always greater than the aerobic one. In the experiments reported so far, the only material in which the reduction was more rapid aerobically than anaerobically was the suspension unsupplemented by substrates. In further experiments many other substrates were tested, and succinate was the only one which augmented the

Table 11. *Relationships between amount of oxygen consumed and acetoacetate oxidized in sheep heart*

The data are calculated from other tables, as indicated. 'Acetoacetate oxidized' is taken to be the difference between acetoacetate removal and β-hydroxybutyrate formation. The ratio given in the last vertical column is calculated by considering two molecules of β-hydroxybutyrate equivalent to one molecule of O_2. DNP, dinitrophenol.

Table no.	Expt. no.	Substances added	Oxygen uptake (μmoles)	Aceto-acetate oxidized (μmoles)	Oxygen used / Acetoacetate oxidized	β-Hydroxy-butyrate formation (μmoles)	O_2 equivalents used / Acetoacetate oxidized
4	1	Acetoacetate	25·0	6·4	3·9	5·8	4·4
		Acetoacetate + DNP (0·01 mM)	50·0	11·2	4·5	3·4	4·6
4	2	Acetoacetate	36·3	7·8	4·7	7·9	5·1
5	1	Acetoacetate	20·4	4·8	4·3	10·0	5·3
5	2	Acetoacetate	26·6	8·5	3·1	7·2	3·6
7	1	Acetoacetate	17·4	5·7	3·1	10·7	4·0
		Acetoacetate + α-oxoglutarate	23·0	7·5	3·1	13·5	4·0
		Acetoacetate + α-oxoglutarate + DNP (0·01 mM)	69·0	15·8	4·4	8·6	4·6
7	2	Acetoacetate	16·3	3·8	4·4	10·9	5·5
		Acetoacetate + α-oxoglutarate	19·7	4·4	4·4	13·3	6·0
		Acetoacetate + α-oxoglutarate + DNP (0·01 mM)	70·7	14·6	4·8	8·1	5·1
8	1	Acetoacetate	23·5	5·0	4·7	7·2	5·4
		Acetoacetate + fumarate	31·9	6·7	4·8	9·6	5·4
8	2	Acetoacetate	23·9	5·3	4·5	9·2	5·4
		Acetoacetate + fumarate	30·9	8·3	3·7	12·2	4·5
9	1	Acetoacetate	22·1	6·5	3·4	7·9	4·0
		Acetoacetate + pyruvate	25·7	3·2	8·0	8·9	9·6
		Acetoacetate + pyruvate + DNP (0·01 mM)	62·5	5·7	11·0	4·1	11·2
9	2	Acetoacetate	26·0	3·9	6·7	11·7	8·2
		Acetoacetate + pyruvate	30·3	3·2	9·5	9·7	11·0
		Acetoacetate + pyruvate + DNP (0·02 mM)	83·0	2·4	34·5	3·5	35·2
9	4	Acetoacetate	20·1	5·4	3·7	7·9	4·4
		Acetoacetate + lactate	23·6	5·7	4·1	7·4	4·8
10	4	Acetoacetate	34·3	5·5	6·2	4·0	6·6
		Acetoacetate + succinate (80 μmoles)	60·5	2·7	22·4	9·1	24·0

difference between the aerobic and anaerobic reduction rates. Anaerobically, succinate had no effect but, aerobically, increases of up to 100% occurred (Table 10).

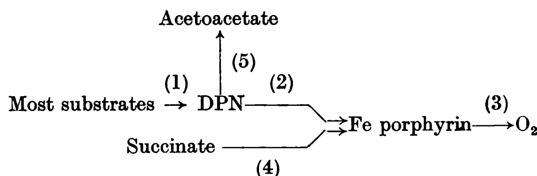
DISCUSSION

The experiments show that the fate of acetoacetate in sheep-heart preparations is readily influenced by environmental conditions, the main factors determining the fate being the presence or absence of oxygen, the concentration of other metabolites such as α-oxoglutarate, fumarate, pyruvate or succinate, and the addition of agents uncoupling oxidative phosphorylation. Many of the observations can be accounted for on the basis of the established enzymic reactions of acetoacetate and the known effects of the uncoupling agents.

Stimulation of β-hydroxybutyrate formation by oxidizable substrates. The acceleration by α-oxoglutarate, fumarate and pyruvate of the anaerobic formation of β-hydroxybutyrate may be ascribed to coupled oxidation–reduction, in which the oxidative process maintains a supply of reduced diphosphopyridine nucleotide (DPN) (see Green, Needham & Dewan, 1937; Krebs & Eggleston, 1945). Such reactions can also take place aerobically, but a simple coupled oxidation–reduction cannot explain the ready aerobic reduction of acetoacetate in the presence of succinate, as the oxidation–reduction potential of the succinate–fumarate couple is more than 300 mv more positive than that of the acetoacetate–β-hydroxybutyrate couple. This accounts for the fact that succinate does not interact with acetoacetate under anaerobic conditions.

Any explanation of the stimulating effect of succinate on the reduction of acetoacetate must be based on the fact that this reduction requires reduced DPN. Thus it follows that succinate in-

creases the supply of reduced DPN. It is already known from the observations of Chance & Williams (1955*a, b*), Chance & Hollunger (1960), Klingenberg, Slenczka & Ritt (1959) and Birt & Bartley (1960) that the level of reduced DPN in mitochondrial suspensions rises when succinate is added. Chance & Hollunger assume that a reduction of DPN by succinate is achieved by what amounts to a reversal of oxidative phosphorylation. They suggest 'that high-energy intermediates formed in the cytochrome portions of the chain by succinate oxidation can be used to reduce DPN' (see also Davies & Krebs, 1952; Krebs & Kornberg, 1957). An alternative explanation is the assumption that in the presence of succinate the transfer of hydrogen from reduced DPN to molecular oxygen is blocked at the iron porphyrin stage, owing to the rapid dehydrogenation of succinate. It is known that hydrogen transport from succinate and from reduced DPN to oxygen shares a common pathway from the iron porphyrin level onwards. Thus the electrons from succinate must compete with those from reduced DPN for the same carrier, and the great increase in the oxygen consumption occurring on addition of succinate (Table 11), as well as the rapid conversion of succinate into fumarate (Krebs, 1960), indicate that the electrons from succinate win in this competition. More reduced DPN therefore becomes available for the conversion of acetoacetate into β-hydroxybutyrate, the supply of reduced DPN being maintained by the dehydrogenation of the products of oxidation of succinate such as malate, pyruvate, *iso*citrate and α-oxoglutarate. The situation is illustrated by Scheme 1.



Scheme 1. Pathways of electron transport.

To sum up it is suggested that a reduction of acetoacetate occurs when the transfer of electrons to DPN [reaction (1)] is more rapid than the transfer of electrons from DPN towards oxygen [reactions (2) and (3)]. Succinate accelerates the reduction of acetoacetate because reaction (4) is more rapid than (2). This causes a competitive inhibition of the transfer of electrons from DPN to oxygen. At the same time, on account of its rapid oxidation, succinate supplies hydrogen donors for DPN.

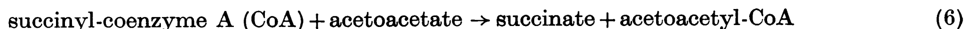
The accelerating effect of oxygen on the reduction of acetoacetate in sheep-heart homogenates unsupplemented by other substrates is presumably due to the same set of circumstances.

Effects of dinitrophenol. Particularly large increases in the rate of oxygen consumption were caused by dinitrophenol when α-oxoglutarate and acetoacetate, or pyruvate and acetoacetate were present, and with these substrates the higher concentrations of dinitrophenol (0·2 mM) did not appreciably decrease the stimulation caused by lower concentrations, as they did when acetoacetate alone or acetoacetate plus fumarate were added substrates. In other words the effects of dinitrophenol on the oxygen uptake depend on the nature of the substrates present (Tyler, 1950; Krebs, 1959).

A stimulation of respiration by dinitrophenol is due to increased concentrations of adenosine diphosphate (ADP) arising from the uncoupling of oxidative phosphorylation (Lardy & Wellman, 1952; Aldridge, 1957), and the inhibition of respiration by the higher concentration of dinitrophenol is probably due to loss of ADP, owing to the combined action of adenylate kinase and adenosine triphosphatase. The former causes the formation of adenosine monophosphate (AMP), and, once formed, this is not readily rephosphorylated owing to the low concentration of ATP. In the presence of α-oxoglutarate some ADP can be prevented from entering the adenylate-kinase reaction by conversion into ATP through the dinitrophenol-insensitive α-oxoglutarate-dehydrogenase system (see Hunter & Hixon, 1949). Hence α-oxoglutarate, unlike other substrates of respiration, is expected to prevent, or at least delay, the loss of ADP, a rate-limiting component of respiration.

When dinitrophenol increased the oxygen uptake it also decreased the aerobic β-hydroxybutyrate formation, whereas the aerobic removal of acetoacetate was not affected, and even rose in some cases. The ratio of β-hydroxybutyrate formation to acetoacetate removal therefore fell on addition of dinitrophenol (e.g. Tables 4, 5 and 7–9). This is expected on the basis of Scheme 1. The rate of reaction (5) depends on whether (1) is faster than (2) and (3). Dinitrophenol raises the rate of (2) and (3), and is therefore liable to reduce the rate of (5).

Higher concentration of dinitrophenol (0·2 mM) strongly inhibited the oxidation of acetoacetate when this was the only added substrate or when added together with fumarate or lactate, and this inhibition was much greater than the inhibition of respiration. The oxidation of acetoacetate is known to be mainly initiated by the coenzyme A-transferase reaction (see Stern & Ochoa, 1951; Green, Goldman, Mii & Beinert, 1953; Stern, Coon, del Campillo & Schneider, 1956):


succinyl-coenzyme A (CoA) + acetoacetate → succinate + acetoacetyl-CoA (6)

As two molecules of succinyl-coenzyme A are produced in the course of the complete oxidation of one molecule of acetoacetate, there is no obvious reason why dinitrophenol should inhibit acetoacetate oxidation (if its only primary effect is an interference with the synthesis of energy-rich phosphate bonds). However, an explanation for the dinitrophenol effect can be offered on the basis of the fact that succinyl-coenzyme A takes part in a second series of reactions involving ATP, the overall effect of which is:

$$\text{succinyl-CoA} + \text{ADP} + \text{phosphate} \rightarrow \text{succinate} + \text{ATP} + \text{CoA}. \quad (7)$$

Since dinitrophenol causes a fall in the concentration of ATP and a rise in that of ADP, reaction (7) may be expected to be accelerated by it. In consequence succinyl-coenzyme A would react according to (7) rather than to (6). This could account for the observed inhibition of acetoacetate removal. The fact that α-oxoglutarate counteracts the inhibitory effect of dinitrophenol is explained by the ready formation of succinyl-coenzyme A from α-oxoglutarate.

Relationship between oxygen uptake and acetoacetate oxidation. The amounts of acetoacetate which disappeared but were not converted into β-hydroxybutyrate must have undergone complete oxidation, since no appreciable quantities of intermediates accumulated. Table 11 indicates that in many experiments in which the amounts of acetoacetate oxidized (i.e. the difference between acetoacetate removed and β-hydroxybutyrate formed) and the oxygen consumed were measured simultaneously, the ratio of oxygen used to acetoacetate oxidized was about 4. Since the complete oxidation of one molecule of acetoacetate requires 4 molecules of oxygen, it follows that acetoacetate served as a chief substrate for respiration. Since β-hydroxybutyrate acted in addition to oxygen as a terminal hydrogen acceptor in these experiments the ratio given in the last vertical column of Table 11 is a more accurate value of the relationship between the oxidative reactions and acetoacetate degradation. 'O_2 equivalents' in this column represent the sum of oxygen used and β-hydroxybutyrate formed, two molecules of the latter being taken as equivalent to one of oxygen.

Since addition of acetoacetate did not cause a large increase in oxygen consumption it must have suppressed the utilization of endogenous substrates.

Substrate competition. Acetoacetate was not only the preferred substrate of respiration when added as sole substrate, but also when the respiration was accelerated by 0·005 or 0·01 mM-dinitrophenol (e.g. Tables 4, 5 and 7–9). But at higher concentrations of dinitrophenol (0·08 to 0·2 mM) acetoacetate no longer serves as the main substrate (e.g. Tables 4, 7, 9). This can be explained by the fact that acetoacetate depends on ATP for its competition with other substrates. As already mentioned, dinitrophenol interferes with conversion of acetoacetate into acetoacetyl-coenzyme A. On the other hand the initiation of the oxidation of many other substrates such as glucose or acetate is dependent on the ATP concentration.

When the rate of acetoacetate oxidation is accelerated by α-oxoglutarate (Table 7) or by fumarate (Table 8) the ratio of oxygen equivalents used to acetoacetate oxidation remained of the same order (Table 11), which indicates that acetoacetate was still the preferred substrate when α-oxoglutarate or fumarate is present. However, with pyruvate there was a considerable decrease in oxidation of acetoacetate, the ratio of oxygen equivalents used to acetoacetate oxidized reaching values of 11. This means that only 36 % of the oxygen uptake was accounted for by oxidation of acetoacetate. Succinate suppressed the oxidation of acetoacetate almost completely.

SUMMARY

1. Acetoacetate, when added aerobically to sheep-heart-muscle homogenates, is partly oxidized to carbon dioxide and water, and partly reduced to D(−)-β-hydroxybutyrate. The ratio of oxygen equivalents used to acetoacetate oxidized is near 4. This suggests that acetoacetate is oxidized in preference to endogenous substrates.

2. In sheep heart, but not in the hearts of rats or pigeons and various other tissues tested, more β-hydroxybutyrate is formed aerobically than anaerobically when acetoacetate is the sole added substrate.

3. α-Oxoglutarate, fumarate and pyruvate accelerate both the anaerobic or aerobic conversion of acetoacetate into β-hydroxybutyrate. This can be explained by coupled oxidation–reduction mediated by diphosphopyridine nucleotide (DPN).

4. Succinate increases the rate of the aerobic reduction of acetoacetate but not of the anaerobic reduction. This is discussed and it is suggested that succinate, on account of its ready dehydrogenation, blocks the transfer of electrons from reduced DPN to the cytochrome system and thereby increases the supply of reduced DPN available for the reduction of acetoacetate.

5. The action of dinitrophenol on acetoacetate metabolism varied with the presence of other substrates. 0·01 mM-Dinitrophenol caused large increases in the oxygen uptake and acetoacetate oxidation, especially when α-oxoglutarate and pyruvate were also added. At 0·2 mM, it inhibited

the oxygen uptake and acetoacetate oxidation when added alone or with fumarate, but not in the presence of α-oxoglutarate. The reasons for these differences are discussed. Anaerobically, relatively slight inhibitions of the reduction of acetoacetate were observed.

6. The oxidation of L(+)-β-hydroxybutyrate is inhibited by dinitrophenol, whereas that of the D(−)-form is not. This is related to the fact that only the L(+)-form requires conversion into the coenzyme A derivative. The differences in the behaviour of the L- and D-forms towards dinitrophenol were used to examine the configuration of the β-hydroxybutyrate formed in sheep-heart muscle aerobically and anaerobically. In both cases the D-form only was found.

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Studies on Glucosaminidase

2. SUBSTRATES FOR *N*-ACETYL-β-GLUCOSAMINIDASE*

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Methods available for the estimation of the activity of *N*-acetyl-β-glucosaminidase have been limited by a lack of suitable substrates. Those with an alkyl *N*-acetyl-β-glucosaminide as substrate (Neuberger & Pitt Rivers, 1939; Kuhn & Tiedemann, 1954) depend on the relatively non-specific estimation of reducing sugar liberated. Phenyl (Helferich & Iloff, 1933) and *p*-nitrophenyl (Westphal & Schmidt, 1952) *N*-acetyl-β-glucosaminide were used as substrates by Watanabe (1936), Neuberger & Pitt Rivers (1939) and Pugh, Leaback & Walker (1957*b*), and by Conchie, Findlay & Levvy (1959) respectively, in methods depending on the colorimetric or spectrophoto-

* Part 1: Pugh, Leaback & Walker (1957*b*).