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The Effect of Succinate and Amytal on the Reduction of Acetoacetate in Animal Tissues

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It has recently been shown (Kulka, Krebs & Eggleston, 1961) that succinate promotes the formation of β -hydroxybutyrate from acetoacetate in sheep-heart homogenates. An explanation was put forward which is based on the fact that succinate is oxidized in preference to substrates requiring a pyridine nucleotide-linked dehydrogenase. As the electrons from succinate and from reduced pyridine nucleotide travel to oxygen by a common pathway at the cytochrome level the preferential availability of the electrons from succinate blocks the transfer of electrons from reduced pyridine nucleotide to oxygen. At the same time the oxidation of succinate provides a substrate, malate, which reduces pyridine nucleotide. These two circumstances increase the amounts of reduced pyridine nucleotide available for the reduction of acetoacetate. This paper presents further evidence in support of this concept.

METHODS

Materials. Sodium acetoacetate and DL- β -hydroxybutyrate samples were as previously described. Crystalline L-lactic acid was prepared according to Krebs (1960), α -oxoglutaric acid according to Friedman & Kosower (1955); succinate-free crystalline monosodium oxoglutarate was prepared as follows. The crude acid was dissolved in 3 vol. of water and the solution was adjusted to pH 3.7 with solid Na_2CO_3 , pH being checked with a pH meter. The adjustment to this pH is critical as pK_1 was found to be 2.47, pK_2 4.68 (determined electrometrically at 22°). After filtration 1 vol. of ethanol was added slowly and the resultant turbid solution was kept overnight in the refrigerator. The precipitate formed contained mostly impurities. A further volume of ethanol was added to the supernatant and after storage for 24 hr. at 0° crystals of monosodium

α -oxoglutarate were collected. The purity was 96.5%. A third volume of ethanol yielded material of 100% purity. This was used for the experiments. Amytal (5-ethyl-5-isoamylbarbiturate) was added as the sodium salt.

Tissue preparations. Tissue homogenates were prepared and handled as previously described (Kulka *et al.* 1961). Any modifications in incubation time and tissue concentration are stated in the description of the experiments. In most cases the enzymic reactions were stopped by the addition of 1 ml. of N-HCl/4 ml. of homogenate. In other cases HCl was replaced by 15% perchloric acid.

Analytical methods. Acetoacetate was determined according to Edson (1935) or Walker (1954), citrate according to Taylor (1953), succinate according to Rodgers (1961), the sum of malate and fumarate according to Nossal (1952), pyruvate and α -oxoglutarate according to Holzer & Holldorf (1957), malate and lactate according to Hohorst, Kreutz & Bücher (1959), glutamate according to Krebs & Bellamy (1960), β -hydroxybutyrate according to

Table 1. Yield of ' β -hydroxybutyrate' of various compounds treated according to Greenberg & Lester (1944)

The quantities tested correspond to maximum amounts present in the homogenates analysed.

Substance tested (16 μ moles)	' β -Hydroxybutyrate' found (μ mole)
Succinate	0.05
Fumarate	0
L-Malate	0
Citrate	0.25
α -Oxoglutarate	0.16
Pyruvate	0.36
L-Lactate	0.05
Butyrate	0.05
Crotonate	0.14
Glucose	0.09
Amytal (5 μ moles)	0.08

Table 2. *Effect of succinate on the aerobic reduction of acetoacetate in various tissues*

Each vessel contained 4 ml. of medium, including 20 μ moles of acetoacetate. Incubated for 45 min. at 30°; gas space, O₂. The dry weight of the epididymal fat body refers to fat-free tissue.

Tissue	Type of material	Amount of dry tissue (mg./4 ml.)	Concn. of succinate (mm)	Uptake of O ₂ (μ moles)	Acetoacetate removed (μ moles)	β -Hydroxy-butyrate formed (μ moles)	'Acetoacetate oxidized' (μ moles)	O ₂ -equivalents used acetoacetate oxidized
Liver (rat)	Homogenate	120	0	30.0	0.4	0.88	0	—
		120	20	59.1	10.9	10.0	0.9	71
Testis (rat)	Teased-out tissue	160	0	33.2	5.2	0.4	4.8	7.0
		150	20	58.4	7.8	2.64	5.2	11.5
Epididymal fat body (rat)	Whole	28	0	9.9	2.1	0.20	1.9	5.3
		29	20	12.4	2.3	1.23	1.1	11.8
Diaphragm (sheep)	Homogenate	150	0	11.2	12.9	9.88	3.0	5.4
		150	20	30.3	16.4	13.9	2.5	15.0
Diaphragm (cattle)	Homogenate	150	0	9.5	4.9	3.13	1.8	6.2
		150	20	21.1	5.3	4.69	0.6	39
Diaphragm (rat)	Homogenate	120	0	14.3	6.1	1.66	4.4	3.1
		120	20	22.8	6.3	2.44	3.9	6.2
Brain (rat)	Homogenate	90	0	5.4	1.6	0.38	1.2	4.7
		90	20	24.0	2.6	0.63	2.0	12.1
Muscle, neck (sheep)	Homogenate	180	0	6.4	4.2	4.1	~0	—
		180	20	15.8	5.3	5.8	~0	—
Muscle, leg (rat)	Homogenate	150	0	17.0	1.5	0	1.5	11.3
		150	20	32.4	2.5	0.5	2.0	16.4
Muscle, breast (pigeon)	Latapie-mince	150	0	62.0	6.8	0	6.8	9.1
		150	20	102.5	10.5	1.20	9.3	9.1
Kidney cortex (sheep)	Homogenate	180	0	60.0	14.6	1.7	12.9	4.7
		180	20	90.0	18.8	7.7	11.1	8.5
Kidney cortex (sheep)	Homogenate	120	0	21.8	5.8	2.9	2.9	8.1
		120	20	49.9	10.1	3.7	6.4	8.1

Greenberg & Lester (1944) as described by Kulka *et al.* (1961). This method, based on the oxidation of β -hydroxybutyrate to acetone by chromate, is not absolutely specific but interference by added substrates or tissue constituents was negligible. Tests of the reactivity of the added substrates gave the values shown in Table 1. They were very low compared with those of the β -hydroxybutyrate arising in the presence of acetoacetate. The method may therefore be regarded as adequate.

Comments on the interpretation of results. Any disappearance of acetoacetate which was not accounted for by the appearance of β -hydroxybutyrate was taken to represent an oxidation of acetoacetate, and if no intermediates of the tricarboxylic acid cycle accumulated the oxidation was assumed to be complete. Reactions other than oxidation and reduction no doubt occurred, such as the decarboxylation to acetone and the synthesis of cholesterol, but the rates of these reactions were probably negligible under the test conditions. This is suggested by the observation that in liver, where the rates of the side reactions are expected to be higher than in other tissues, virtually all acetoacetate was recovered as β -hydroxybutyrate in many experiments.

In order to calculate the contribution which the oxidation of acetoacetate makes to respiration it is necessary to know the amounts of hydrogen acceptors available for the oxidation of acetoacetate. Whilst O_2 is as a rule the only major hydrogen acceptor in animal tissues this is not the case in the presence of acetoacetate, which can act as an additional hydrogen acceptor. In this case the amounts of available hydrogen acceptors are therefore the sum of the O_2 uptake and the β -hydroxybutyrate formation. In subsequent calculations this sum has been expressed as 'O₂-equivalent', 2 moles of β -hydroxybutyrate were equated to 1 mole of O_2 and added to the O_2 uptake.

RESULTS

Effect of succinate on the reduction of acetoacetate in various tissues

The effect of succinate previously described for sheep-heart muscle was also found, in varying degrees, in all other tissues tested (Table 2). Quantitatively the effect of succinate was greatest in liver, where in the example quoted the addition

of succinate increased the rate of β -hydroxybutyrate formation tenfold. In this tissue almost the whole of the acetoacetate removed aerobically in the presence of succinate was accounted for by the formation of β -hydroxybutyrate. In other tissues the amounts of acetoacetate removed were greater than those of β -hydroxybutyrate formed; the excess presumably underwent oxidation.

Acetoacetate as a fuel of respiration

In sheep-heart homogenates the ratio of O_2 -equivalents to acetoacetate oxidized was in many cases near 4 (Kulka *et al.* 1961). If no intermediates accumulate and if the loss of acetoacetate by decarboxylation to acetone is negligible (as was the case under the conditions of the present experiments) a value of 4 indicates that the whole respiration is due to the oxidation of acetoacetate, and if the value is n (where n should be greater than 4) the percentage respiration due to the oxidation of acetoacetate is $100 \times 4/n$.

According to the data in the last column of Table 2 acetoacetate, when added as sole substrate, contributed substantially, i.e. more than 50 %, to the respiration of the diaphragms of the rat, sheep and cattle and of epididymal fat. It also contributed much to the respiration of testis and brain but in these tissues the respiration was rather lower than in the intact tissue owing to damage caused by homogenization or to the absence of glucose or both. No oxidation of acetoacetate occurred in liver. Succinate decreased in most tissues the proportion contributed to respiration by acetoacetate and also decreased the absolute amount of acetoacetate oxidized.

Effect of succinate concentration on the aerobic reduction of acetoacetate

Small quantities of succinate (1 μ mole/4 ml.) had no appreciable effect on the formation of β -hydroxybutyrate. Relatively large quantities of

Table 3. *Effect of succinate concentration on the aerobic reduction of acetoacetate in sheep-heart and rat-liver homogenate*

Incubated for 45 min. at 30°; gas space, O_2 . Data refer to 4 ml. of 10% homogenate. Expt. 1, sheep heart; Expts. 2 and 3, rat liver.

Expt.	Substrates added	Uptake of O_2 (μ moles)	Acetoacetate removed (μ moles)	β -Hydroxybutyrate formed (μ moles)
1	None	19.8	—	0.4
	Acetoacetate (23 μ moles)	34.3	9.5	4.0
	Acetoacetate (23 μ moles); succinate (1 μ mole)	34.5	8.9	4.6
	Acetoacetate (23 μ moles); succinate (10 μ moles)	38.0	11.1	6.1
	Acetoacetate (23 μ moles); succinate (80 μ moles)	65.5	11.8	9.1
2	Acetoacetate (18.7 μ moles)	30.0	1.9	1.5
	Acetoacetate (18.7 μ moles); succinate (10 μ moles)	38.1	8.7	8.9
	Acetoacetate (18.7 μ moles); succinate (80 μ moles)	54.3	13.9	13.7
3	Acetoacetate (20.4 μ moles)	25.1	0	2.2
	Acetoacetate (20.4 μ moles); succinate (1 μ mole)	30.5	0.6	1.7
	Acetoacetate (20.4 μ moles); succinate (2 μ moles)	33.2	1.5	3.2

succinate (80 μ moles/4 ml.) were required for maximum effects (Table 3). There was no simple stoicheiometric relation between the amounts of succinate added and extra β -hydroxybutyrate formed but in all experiments less than 1 molecule of β -hydroxybutyrate was formed per molecule of succinate added. These observations are in accordance with the assumption that the stimulation of β -hydroxybutyrate formation by succinate is due to the blockage of electron transport from reduced diphosphopyridine nucleotide (DPNH₂) to O₂. No stoicheiometry is expected because only a continuous oxidation of succinate can bring about an effective blocking.

Preferential oxidation of succinate
(general considerations)

Data shown in Table 2 already indicate that added succinate is oxidized in preference to acetoacetate. This follows from the fact that succinate increased the rate of oxygen consumption in all tissues, whereas it did not increase or even decrease the amounts of acetoacetate oxidized. The preferential oxidation of succinate also expresses itself in a rise of the value of the ratio O₂-equivalents used/acetoacetate oxidized. The high value of this ratio in the presence of succinate indicates that substances other than acetoacetate served as substitutes of respiration.

In order to examine in more detail the nature of the oxidative reactions in the presence of succinate further experiments were carried out on the following basis. When an intact tissue or a tissue homogenate respire, many oxidizable substrates, endogenous or added, may be available. In the complex system information on the nature of substrate changes can be obtained from simultaneous measurements of the uptake of O₂ and the changes in the concentrations of the starting materials and intermediary metabolites. The chief intermediates which accumulate under most conditions are those of the tricarboxylic cycle, and of the reactions closely

related to it. The following substances were therefore determined: succinate, citrate, α -oxoglutarate, pyruvate, lactate, acetoacetate, β -hydroxybutyrate, acetate and the sum of fumarate and malate. The last-named were not measured individually because they always occur together in animal tissues and have the same oxidation level; the value of the sum of their concentration is therefore sufficient for many purposes. *iso*Citrate and *cis*-aconitate are not easily determined at physiological concentrations but in the presence of acitonate their concentrations may be taken to be 11% of that of citrate (Eggleson & Krebs, 1949), and this amount was added to the observed citrate value so as to include all three tricarboxylic acids. Oxaloacetate was not determined. Unless special precautions are taken it is rapidly decarboxylated to pyruvate, and in the present experiments it was included in the pyruvate determination.

In order to calculate the nature of the substrate changes from the changes in the amounts of the intermediary metabolites it is necessary to make some arbitrary assumptions. It was assumed that the intermediates found were all derived from succinate when this was added, rather than from endogenous material or the added acetoacetate,

Table 4. *Relations between formation of intermediates and consumption of oxygen and succinate*

The assumption is made that succinate is the sole precursor of the intermediates.

Intermediates formed	Required for the formation of metabolite from succinate	
	O ₂ (μ moles)	Succinate (μ moles)
Fumarate or malate (1 mole)	0.5	1
Pyruvate (1 mole)	1	1
Citrate (1 mole)	2.5	2
α -Oxoglutarate (1 mole)	3	2
Lactate (1 mole)	0.5	1

Table 5. *Competition of succinate with other substrates in sheep-heart homogenate*

Incubated for 60 min. at 30°; gas space, O₂. Data refer to 8 ml. of 10% homogenate.

Vessel no. ...	Metabolic changes (μ moles)			
	1	2	3	4
Substrates added ...	None	Acetoacetate (40 μ moles)	Acetoacetate (40 μ moles) and succinate (160 μ moles)	Succinate (160 μ moles)
O ₂	-24.4	-53.2	-108.8	-109
Acetoacetate	0	-17.0	-17.0	0
β -Hydroxybutyrate	+0.6	+7.8	+18.6	+1.6
Succinate	0	0	-159.0	-143.0
Fumarate + malate	0	0	+112.4	+108.0
α -Oxoglutarate	0	0	+6.4	+3.2
Citrate + <i>isocitrate</i> + <i>cis</i> -aconitate	+0.32	+1.0	+10.8	+4.4

and on this basis the relations between the formation of intermediates and the consumption of O_2 and succinate are as shown in Table 4.

Succinate and acetoacetate in sheep heart and rat livers

When acetoacetate was added to sheep-heart homogenate as the sole substrate a major part of the respiration was due to an oxidation of acetoacetate (Table 5). The amount of acetoacetate oxidized was 9.2 μ moles and as no appreciable

quantities of intermediates were found the acetoacetate was presumably completely oxidized. This accounts for 36.8 μ moles of O_2 or 65 % of the O_2 -equivalents used (53.2 μ moles of O_2 and 3.9 O_2 -equivalents of β -hydroxybutyrate). In contrast there was no acetoacetate oxidized in the presence of succinate: the acetoacetate which had been removed all appeared as β -hydroxybutyrate. From the amounts of fumarate, malate, α -oxoglutarate and citrate formed it follows that the formation of these intermediates accounted for about 90 % of

Table 6. *Analysis of the oxidative reactions in vessels 3 and 4 of Table 5 (sheep heart)*

Metabolite formed	Vessel 3 (acetoacetate and succinate added)		Vessel 4 (succinate added)	
	O_2 required (μ moles)	Succinate required (μ moles)	O_2 required (μ moles)	Succinate required (μ moles)
Fumarate and malate	56.2	112.4	54.0	108.0
Citrate, isocitrate, cis-aconitate	27.0	21.6	11.0	8.8
α -Oxoglutarate	19.2	12.8	9.6	6.4
Sum of all metabolites	102.4	146.8	74.6	123.2
O_2 not accounted for	6.4	—	34.4	—
O_2 -equivalents (including β -hydroxybutyrate) not accounted for	15.8	—	35.2	—
Succinate not accounted for	—	12.2	—	26.2

Table 7. *Competition of succinate with other substrates in rat-liver homogenate*

Incubated for 60 min. at 30°; gas space, O_2 . Data refer to 8 ml. of 10% homogenate.

					Metabolic changes (μ moles)			
Vessel no.	...	...	...	...	1	2	3	4
Substrates added	...	...	...	...	None	Acetoacetate (40 μ moles)	Acetoacetate (40 μ moles) and succinate (160 μ moles)	Succinate (160 μ moles)
O_2					-40.7	-40.4	-79.4	-79.8
Acetoacetate					0	-1.1	-16.3	0
β -Hydroxybutyrate					0	+1.0	+14.8	+0.4
Succinate					0	0	-139.0	-130.2
Fumarate + malate					0	0	+106.4	+117.2
α -Oxoglutarate					+0.42	+0.26	+0.53	+0.81
Citrate + isocitrate + cis-aconitate					+0.37	+1.78	+4.79	+1.70
Pyruvate					-0.22	+0.22	+2.78	+2.43
Lactate					-0.34	-2.76	+0.89	-0.36

Table 8. *Analysis of the oxidative reactions in vessels 3 and 4 of Table 7 (rat liver)*

Metabolite formed	Vessel 3 (acetoacetate and succinate added)		Vessel 4 (succinate added)	
	O_2 required (μ moles)	Succinate required (μ moles)	O_2 required (μ moles)	Succinate required (μ moles)
Fumarate and malate	53.2	106.4	58.6	117.2
Citrate, isocitrate, cis-aconitate	13.6	11.6	4.7	3.7
α -Oxoglutarate	1.5	1.1	2.4	1.6
Pyruvate	2.8	2.8	2.4	2.4
Lactate	1.8	3.6	0	0
Sum of all metabolites	72.9	125.5	68.1	124.9
O_2 not accounted for	6.5	—	11.7	—
O_2 -equivalents (including β -hydroxybutyrate) not accounted for	13.9	—	11.9	—
Succinate not accounted for	—	13.5	—	5.3

the oxygen consumption and 90 % of the succinate removal (Table 6). Thus on addition of succinate plus acetoacetate, or of succinate alone, the oxidation of succinate constituted the main oxidative process, i.e. succinate largely suppressed the oxidation of endogenous material as well as of the added acetoacetate.

In rat liver the action of succinate had similar effects (Table 7). As acetoacetate is not appreciably oxidized in this tissue, its only major reaction under the test conditions is the reduction to β -hydroxybutyrate. In the presence of succinate 80–90 % of the oxidation was accounted for by the formation of the intermediates which were determined (Table 8).

There is no definite information on the nature of the reactions of O_2 and succinate not accounted for by the products measured in the two tissues. This fraction is relatively small; it was calculated as the difference of many individual determinations and therefore a considerable experimental error cannot be excluded. Some succinate or endogenous substrate may have undergone complete oxidation.

It is also possible that small quantities of other metabolites that were not determined were formed from succinate.

Effects of substances other than succinate on the reduction of acetoacetate

Among the substances tested in the previous investigation succinate was the only substance which in sheep heart promoted the reduction of acetoacetate aerobically but not anaerobically. As the effect of succinate was much more pronounced in liver than in heart the effect of further substances on the reduction of acetoacetate was examined in liver homogenates. Anaerobically there were no effects on additions of succinate, acetate, propionate, butyrate, crotonate and glucose. Lactate had a slight effect (Table 9); large accelerations of the anaerobic reduction were caused by fumarate, citrate, *isocitrate*, glutamate, α -oxoglutarate and pyruvate. The yield of β -hydroxybutyrate was in most cases somewhat greater than the amount of acetoacetate removed, especially in the presence of pyruvate, and even the

Table 9. *Effects of various substrates on the aerobic and anaerobic removal of acetoacetate in rat liver*

Data refer to 4 ml. of 10 % homogenate. Acetoacetate (20 μ moles) was added to 4 ml. and 80 μ moles of all other substrates. Incubated for 45 min. at 30°; gas space, O_2 .

Expt.	Substrate added other than acetoacetate	Anaerobic changes		Aerobic changes		
		Acetoacetate (μ moles)	β -Hydroxybutyrate (μ moles)	O_2 (μ moles)	Acetoacetate (μ moles)	β -Hydroxybutyrate (μ moles)
1	None	- 5.85	+ 6.8	-27.6	- 1.2	+ 2.2
	α -Oxoglutarate	- 8.55	+10.6	-31.1	-11.7	+14.3
	L-Glutamate	-11.9	+13.3	-38.5	-12.6	+15.9
	Pyruvate	- 7.70	+15.2	-34.5	- 0.8	+ 5.9
	L-Lactate	- 7.20	+ 8.9	-35.2	- 0.5	+ 2.1
2	None	- 4.38	+ 6.25	-22.8	- 1.4	+ 2.9
	Fumarate	-10.0	+10.7	-33.2	- 5.2	+ 3.5
	α -Oxoglutarate	- 9.83	+11.2	-30.1	- 8.3	+ 8.7
	Fumarate + α -oxoglutarate	-10.1	+11.5	-28.4	- 6.0	+ 7.4
3	None	- 4.65	+ 5.9	-24.9	- 0.2	+ 2.0
	Succinate	- 3.45	+ 5.4	-57.4	- 8.9	+12.3
	α -Oxoglutarate	- 9.85	+11.6	-33.4	- 8.7	+11.0
	L-Glutamate	-11.3	+12.4	-37.8	-12.8	+16.3
4	None	- 5.2	+ 7.1	-26.7	- 1.4	+ 3.4
	Fumarate	-11.4	+15.5	-33.6	- 5.4	+ 7.9
	Citrate	-20.0*	+23.6	-28.8	- 8.5	+13.4
	L-Glutamate	-16.4	+19.3	-37.4	-15.0	+20.1
5	None	- 5.95	+ 6.4	-26.9	- 0.5	+ 1.0
	Citrate	-18.3	+20.4	-26.5	- 7.0	+11.6
	Glucose	- 6.1	+ 7.3	-26.7	+ 0.4	+ 1.6
	Pyruvate (no acetoacetate)	+ 0.45	+ 4.1	-34.5	+ 4.9	+ 3.8
6	None	- 5.2	+ 5.38	-24.2	- 0.3	+ 0.8
	Fumarate	- 9.9	+10.7	-34.5	- 4.15	+ 4.9
	L-Malate	- 9.8	+11.7	-31.0	- 8.1	+ 9.1
	Oxaloacetate	- 1.9	+ 2.37	-16.3	- 4.7	+ 5.4
7†	None	- 4.0	+ 5.0	—	—	—
	Citrate	-13.2	+14.0	—	—	—
	<i>iso</i> Citrate	-16.6	+17.9	—	—	—

* All acetoacetate added was removed.

† In Expt. 7, 40 μ moles of citrate were added and the incubation time was 20 min.

Table 10. *Effects of 2:4-dinitrophenol on the removal of acetoacetate and the formation of β -hydroxybutyrate in rat-liver homogenate*

Incubated at 30°; gas space, O₂. Data refer to 4 ml. of 10% homogenate. All suspensions contained 20 μ moles of acetoacetate. 2:4-Dinitrophenol was added with substrate from the side arm at zero time.

Expt.	Further substrates added (μ moles)	Dinitrophenol (μ M)	Period of incubation (min.)	Uptake of O ₂ (μ moles)	Acetoacetate removed (μ moles)	β -Hydroxybutyrate formed (μ moles)
1	None	0	20	11.7	0.4	0.43
	Succinate (80 μ moles)	0		28.5	10.2	10.7
	None	50		5.7	1.3	0.88
	Succinate (80 μ moles)	50		42.3	3.8	3.44
2	None	0	20	16.1	0.5	1.50
	Succinate (80 μ moles) and pyruvate (20 μ moles)	0		31.3	5.9	8.00
	α -Oxoglutarate (80 μ moles)	0		14.8	4.4	5.25
	None	50		6.5	0.6	2.58
	Succinate (80 μ moles) and pyruvate (20 μ moles)	50		44.5	3.9	4.97
	α -Oxoglutarate	50		31.7	0.7	1.25
3	None	0	30	17.6	2.1	1.50
	Succinate (80 μ moles)	0		39.8	10.9	11.4
	None	10		18.3	1.8	1.49
	Succinate (80 μ moles)	10		44.9	9.6	9.12
4	None	0	20	11.7	0.7	0.25
	Succinate	0		27.8	12.4	13.4
	None	25		8.1	0.8	0.88
	Succinate	25		40.2	7.4	6.8
	None	50		7.1	1.1	0.5
	Succinate	50		49.0	2.6	2.3
	None	100		6.8	0.5	0.13
	Succinate	100		41.8	1.6	0.88

Table 11. *Effect of amytal on metabolism of acetoacetate*

Data refer to 4 ml. of 10% homogenate. Incubated for 45 min. at 30°; gas space, O₂. Acetoacetate added: 20 μ moles. Succinate added: 80 μ moles.

Tissue	Substrates added	Sodium amytal (final concn.) (mM)	Uptake of O ₂ (μ moles)	Acetoacetate (μ moles)	β -Hydroxybutyrate (μ moles)
Rat liver	Acetoacetate	0	-27.3	-2.2	+1.3
	Acetoacetate; succinate	0	-56.5	-11.8	+11.6
	Acetoacetate	1	-11.7	-7.1	+6.1
	Acetoacetate; succinate	1	-53.8	-14.6	+15.7
	Acetoacetate	2	-10.3	-6.3	+5.8
	Acetoacetate; succinate	2	-54.8	-16.6	+17.2
Sheep heart	Acetoacetate	0	-27.2	-10.9	+4.3
	Acetoacetate; succinate	0	-56.0	-15.1	+11.4
	Acetoacetate	1	-21.0	-13.4	+8.3
	Acetoacetate; succinate	1	-43.0	-16.8	+17.3
Sheep heart	Acetoacetate	0	-21.0	-10.6	+2.5
		1.25	-8.3	-8.3	+5.4
		2.5	-4.33	-10.5	+10.0
		5.0	-3.12	-9.4	+8.25
	Acetoacetate; succinate	0	-46.0	-12.7	+9.25
		1.25	-41.5	-10.9	+10.0
		2.5	-35.2	-13.7	+13.5
		5.0	-36.4	-12.5	+13.3

homogenate to which no acetoacetate had been added formed small amounts of β -hydroxybutyrate. Whilst the initial β -hydroxybutyrate value could have been due to the lack of specificity of the analytical method this is unlikely to apply to the additional β -hydroxybutyrate formed on incubation. Presumably acetyl-coenzyme A formed anaerobically, especially from pyruvate (see Expts. 1 and 5), condensed to yield acetoacetate which was reduced by the DPNH₂ formed in the conversion of pyruvate or other substrates into acetyl-coenzyme A. The most rapid anaerobic formation of β -hydroxybutyrate occurred on addition of citrate and of glutamate. The effects of α -oxoglutarate and fumarate were not additive.

Succinate was again the only substance which strongly stimulated the formation of β -hydroxybutyrate under aerobic conditions but had no effect anaerobically. In some experiments α -oxoglutarate and glutamate generated more β -hydroxybutyrate aerobically than anaerobically but the differences were relatively small and can be accounted for by the formation of succinate from these two substrates.

Effects of 2:4-dinitrophenol. As in the previous experiments on sheep heart (Kulka *et al.* 1961), addition of dinitrophenol to liver homogenates affected the reduction of acetoacetate whenever it affected respiration (Table 10). An increase in respiration was accompanied by a decrease in the yield of β -hydroxybutyrate (Expts. 1 and 2). At lower concentrations of dinitrophenol (10 μ M) which did not stimulate respiration appreciably (Expt. 3) there was no major effect on the reduction of acetoacetate. When acetoacetate was the only added substrate 50 μ M-dinitrophenol inhibited the O₂ uptake (Expts. 1 and 2).

Action of amytal on the reduction of acetoacetate. Very large effects on the reduction of acetoacetate were caused by amytal (Table 11). When acetoacetate was the only added substrate the rate of reduction was increased more than fourfold in rat liver whereas the respiration was decreased. With acetoacetate and succinate there was also a marked increase in the rate of reduction of acetoacetate. Sheep heart showed similar effects (Table 11).

Amytal and other barbiturates are known to be respiratory inhibitors, affecting the oxidation of most substrates except that of succinate. It blocks the transfer of electrons from DPNH₂ to flavo-protein (Jowett & Quastel, 1937; Michaelis & Quastel, 1941; Eiler & McEwen, 1949; Ernster, Jalling, Löw & Lindberg, 1955). If this were the only effect of amytal and if the block were complete [as in certain mitochondrial preparations (Ernster *et al.* 1955)] then it would be expected that in the presence of sufficient amytal the reactions of acetoacetate would be the same as under

anaerobic conditions (because O₂, though present, would not be utilizable). In fact the block by amytal is not complete. According to Ernster *et al.* (1955) and Lehninger (1953-54), two pathways of oxidation of DPNH₂ exist in animal tissues and only one of these is inhibited by amytal. The relative proportion of the amytal-insensitive pathway in the homogenate used in the present work is shown in Table 12. Irrespective of the substrates used (which did not include succinate) the maximal inhibition was reached when the amytal concentration was between 1 and 6 mM. The residual respiration was 20-30 % of the respiration without amytal and the removal of fumarate, α -oxoglutarate, pyruvate and glutamate was reduced to about the same extent. With citrate the residual substrate removal was still 55 % of that found in the absence of amytal. These experiments show that under the test conditions the amytal-insensitive pathway carried electrons to O₂ at about one-quarter of the rate of electrons transported in the absence of amytal.

In order to examine in detail the metabolic changes occurring in the presence of amytal the intermediate metabolites were measured in an experiment which was set up parallel with that recorded in Table 7 (see Table 13). The main effect of amytal occurred in the absence of suc-

Table 12. *Effect of amytal on the oxygen uptake and substrate removal in rat-liver homogenate*

Incubated at 30° for 60 min.; gas space, O₂. Substrate added: 40 μ moles. Data refer to 4 ml. of 10 % homogenate.

Substrate added	Amytal (mM)	Uptake of O ₂ (μ moles)	Substrate removed (μ moles)
None	0	-32.4	—
	0.25	-28.4	—
	1.25	-10.4	—
	6.25	-9.3	—
Fumarate	0	-61.7	-9.5
	0.25	-59.9	-8.9
	1.25	-23.2	-3.2
	6.25	-16.2	-2.8
α -Oxoglutarate	0	-54.8	-25.3
	0.25	-51.9	-30.4
	1.25	-14.5	-5.0
	6.25	-12.1	-5.0
Pyruvate	0	-62.6	-34.0
	0.25	-49.2	-27.0
	1.25	-14.0	-19.8
	6.25	-12.8	-7.6
Glutamate	0	-53.7	-14.7
	0.25	-51.2	-18.7
	1.25	-17.3	-5.3
	6.25	-15.2	-3.2
Citrate	0	-53.5	-22.4
	0.25	-46.5	-26.4
	1.25	-13.7	-12.8
	6.25	-13.4	-12.3

Table 13. *Effect of amytal on oxidations in rat liver*

The experiment was carried out on the same liver and under the same conditions as those of Table 7. Amytal, 2.5 mm.

Substrates added	...	Metabolic changes (μmoles)			
		None	Acetoacetate (40 μmoles)	Acetoacetate (40 μmoles) and succinate (160 μmoles)	Succinate (160 μmoles)
O ₂		- 10.5	- 10.6	- 74.8	- 78.5
Acetoacetate		0	- 8.6	- 17.1	0
β-Hydroxybutyrate		0	+ 9.8	+ 16.0	+ 0.5
Succinate		0	0	- 137.2	- 133.8
Fumarate + malate		0	0	+ 100.9	+ 97.8
α-Oxoglutarate		+ 0.15	+ 0.33	+ 0.49	+ 0.73
Citrate		0	0	+ 4.54	+ 0.42
Pyruvate		0	0	+ 3.02	+ 1.04

Table 14. *Balance sheet of the reactions taking place in rat-liver homogenate anaerobically on addition of citrate and acetoacetate*

Data refer to 4 ml. of homogenate containing 10% of tissue. Free CO₂ formed was measured manometrically and bound CO₂ was determined at the end of the incubation period by adding 1 ml. of N-HCl from a side bulb. The initial amounts of bound CO₂ (5.75, 5.04 and 5.08 μmoles respectively in cups 1-3) were determined in separate cups and their values have been deducted in the data given below. Temp., 30°. Gas phase, N₂. Yellow phosphorus in centre well. Incubation time, 60 min.

Vessel no.	...	...	...	Changes		
				1	2	3
Acetoacetate added (μmoles)	...			40.9	0	40.9
Citrate added (μmoles)	...	...		0	36.2	36.2
Acetoacetate				- 8.90	0	- 31.20
β-Hydroxybutyrate				+ 9.45	+ 0.63	+ 33.70
Free CO ₂				+ 3.30	+ 2.23	+ 17.6
Bound CO ₂				+ 0.50	- 0.19	+ 14.1
Total CO ₂				+ 3.80	+ 2.04	+ 31.7
Citrate				0	- 3.4	- 15.4
Succinate				+ 0.39	- 0.21	+ 11.4
α-Oxoglutarate				0	0	+ 3.52
L-Glutamate				- 1.11	- 0.84	+ 1.54
Fumarate, malate, pyruvate				0	0	0

inate and consisted of an inhibition of about 75 % of the O₂ uptake and an eight- to nine-fold increase in the removal of acetoacetate and the formation of β-hydroxybutyrate. The non-accumulation of other intermediates suggests that the substrate changes of respiration (mainly those of the tricarboxylic acid cycle) proceeded in the normal order though at a reduced rate, with acetoacetate instead of O₂ serving as an additional terminal electron acceptor. When succinate was present, the amounts of intermediates formed were not appreciably changed by amytal (except for β-hydroxybutyrate).

Anaerobic interaction between citrate and acetoacetate

In an earlier paper (Krebs & Eggleston, 1945) it was stated that the tricarboxylic acids did not accelerate reduction of acetoacetate in animal tissues. As shown in Table 9, Expts. 5 and 7, this

statement is not valid, citrate being in fact a very rapid hydrogen donor. Thus all dehydrogenases of the tricarboxylic acid cycle involve pyridine nucleotides, i.e. all oxidative steps except that leading from succinate to fumarate can provide hydrogen atoms for the reduction of acetoacetate. It is noteworthy that the rapid reaction of citrate occurs in liver homogenates although most of the isocitric dehydrogenase of liver is triphosphopyridine nucleotide-linked. The rapid rate indicates that either the transhydrogenase activity of the tissue is high or that the diphosphopyridine nucleotide (DPN)-linked isocitric dehydrogenase is also very active (see Ernster & Glasky, 1960; Ernster, 1960).

A full analysis of the products of the interaction between citrate and acetoacetate (Table 14) shows that the main reaction can be described by the scheme:

The data are in general agreement with this, the only appreciable deviation being a somewhat low value for succinate and the formation of some α -oxoglutarate and glutamate. The presence of these products indicates that the following reactions also occurred:

(Krebs, Eggleston & Hems, 1948; Bartley, Sobrinho-Simões, Notton & Montesi, 1959). In addition some acetoacetate must have been reduced at the expense of endogenous reducing substrates, the presence of which is indicated by the data obtained with vessel 1.

Maximum rates of anaerobic reduction of acetoacetate

In relation to the role of the reduction of acetoacetate in electron transport (see Discussion) information is required on the maximum rates at which acetoacetate can be reduced anaerobically. From the experiments already reported in Table 9 it follows that the reduction can be coupled with the oxidation of citrate, of fumarate, of pyruvate and of glutamate. Maximum rates may therefore be expected when several substrates are available. This proved to be the case (Table 15). Combinations, e.g. of citrate and fumarate, gave higher β -hydroxybutyrate values than the separate substrates but the effects were not fully additive. The β -hydroxybutyrate yield again exceeded the acetoacetate removal, especially in the presence of pyruvate.

Effect of creatine phosphate

If the reversal of oxidative phosphorylation played a role in the reduction of DPN, and through DPN of acetoacetate, addition of adenosine triphosphate or creatine phosphate would be expected to increase the rate of β -hydroxybutyrate formation. To test this, Expt. 1 (Table 10) was repeated with the addition of creatine phosphate (0.01 M final concentration). This had no effect in the absence of dinitrophenol. In its presence it abolished completely the inhibition of respiration. The reduction of β -hydroxybutyrate was unaffected. These results argue against a role of energy-rich phosphates in the reduction of acetoacetate (see Discussion). The fact that creatine phosphate counteracted the inhibition of respiration by dinitrophenol is in accordance with the view that this inhibition is connected with a fall in concentration of adenosine triphosphate in the presence of dinitrophenol.

DISCUSSION

Aerobic reduction of acetoacetate. All observations on the rate of reduction of acetoacetate described in this paper can be accounted for by the hypothesis put forward previously (Kulka *et al.* 1961). This is based on Scheme I, which illustrates diagrammatically the relevant stages of electron transport between substrates and oxygen, including the joint pathway between cytochrome and oxygen.

If both acetoacetate and β -hydroxybutyric dehydrogenase are present a reduction of acetoacetate occurs when the transfer of electrons to DPN (reaction 1) is more rapid than the transfer of electrons from DPN to oxygen (reactions 2 and 3). Agents which have been found to affect the aerobic yield of β -hydroxybutyrate in a major way are succinate, amytal and dinitrophenol. Succinate accelerates the reduction of acetoacetate because reaction (4) is more rapid than reaction (2), and the electrons from succinate therefore compete successfully with those from DPNH_2 . They thus inhibit competitively the transfer of electrons from DPNH_2 to oxygen. At the same time succinate, because of its rapid oxidation, provides hydrogen donors for DPN such as malate, pyruvate, isocitrate and α -oxoglutarate. As shown in anaerobic experiments (Table 9), these oxidation products of succinate readily reduce acetoacetate.

Amytal stimulates β -hydroxybutyrate formation because it inhibits reaction (2) without blocking it completely. It still permits the oxidative formation of reducing agents through the tricarboxylic acid cycle. Dinitrophenol, on the other hand, decreases the yield of β -hydroxybutyrate because it accelerates reactions (2) and (3) and therefore decreases the amount of DPNH_2 available for reaction (5).

As the reduction of β -hydroxybutyrate is preceded by the reduction of DPN the observations by Chance and others (Chance & Williams, 1955; Chance & Hollunger, 1960; Klingenberg, Slenczka & Ritt, 1959; Birt & Bartley, 1960) on the rise of the concentration of DPNH_2 on addition of succinate are related to the findings reported in this paper. In fact the formation of β -hydroxybutyrate is a magnified reflexion of the formation of DPNH_2 .

The explanation of the succinate effect offered here differs from that put forward by Chance & Hollunger (1960), who assume that the increased

rate of reduction of DPN in the presence of succinate involves energy-rich intermediates. They suggest that adenosine triphosphate or possibly enzyme-bound, energy-rich phosphate formed as a result of the oxidation of succinate causes a reversal of oxidative phosphorylation. The coupling of phosphorylation with the oxidation of DPNH_2 may be formulated:

(where FAD and FADH_2 represent flavoprotein and reduced flavoprotein, ADP is adenosine diphosphate and ATP adenosine triphosphate). If this reaction is reversible any increase in the concentration of adenosine triphosphate would drive the reaction from right to left. There is evidence [the occurrence of the adenosine triphosphate-phosphate-exchange reaction (Boyer, Luchsinger & Falcone, 1956; Cooper & Lehninger, 1957; Löw, Siekevitz, Ernster & Lindberg, 1958)] that reaction (6) is in fact reversible. The mechanism proposed by Chance & Hollunger is therefore a feasible one. However, it cannot be responsible for the reduction of DPN in the present experiments because reaction (6) is completely inhibited by higher concentrations of amytal (Löw *et al.* 1958). The fact that amytal, on the contrary, greatly stimulates acetoacetate reduction is incompatible with the hypothesis of Chance & Hollunger, and supports the concept favoured in the present paper. The failure of creatine phosphate to promote the reduction of acetoacetate also argues against the involvement of energy-rich phosphate.

Anaerobic reduction of acetoacetate. Anaerobically only reactions (1) and (5) are expected to occur. The data on the anaerobic rate of formation of β -hydroxybutyrate show that (1) and (5) are relatively slow when no substrate has been added to the liver homogenate. Fumarate (malate), citrate (isocitrate), α -oxoglutarate, glutamate and pyruvate greatly accelerate the reduction of acetoacetate whereas lactate has a slight effect. The low rates of reduction in the unsupplemented homogenate and the fact that most of the mitochondrial pyridine nucleotide is normally present in the oxidized form must be ascribed to the low concentrations of substrates capable of reducing DPN, i.e. of intermediates of the tricarboxylic acid cycle and of related reactions. The only intermediate which may occur in relatively high concentrations is lactate and this is not an effective reducing agent even at high concentrations. This is presumably due to the location of lactic dehydrogenase in relation to DPN within the cell's compartments which does not permit the effective coupling with the mitochondrial DPN or the carriers

between the DPN of mitochondria and other regions.

Effect of oxygen. Oxygen increased the rate of reduction of acetoacetate in the presence of succinate but decreased it in the presence of fumarate and citrate. This decrease can also be explained on the basis of Scheme I. The rate of reduction of acetoacetate depends on the amounts of available DPNH_2 , and these in turn depend on the rate of generation of DPNH_2 (reaction 1) and the rate of reaction (2). Given an excess of reducing substrate such as fumarate or citrate, reaction (1) may be expected to be the same aerobically and anaerobically but reaction (2) is zero anaerobically and greater than zero aerobically. Hence aerobically less DPNH_2 will be available for reaction (5). The inhibition by oxygen of the reduction of acetoacetate can thus be ascribed to a competition of the electron-transport chain with acetoacetate for DPNH_2 . This situation applies to all pyridine nucleotide-linked substrates. In the presence of glutamate and α -oxoglutarate, which yield succinate, the position is less clear-cut because of the inhibition of reaction (2) by the succinate formed. It is therefore not unexpected that oxygen does not appreciably decrease the reduction of acetoacetate in the presence of glutamate or α -oxoglutarate.

Partial inhibition of electron transport by amytal. According to Jalling, Lindberg & Ernster (1955), 1.8 mM-amytal inhibits the mitochondrial oxidation of pyruvate, citrate and malate completely. In the homogenates used in the present experiments (and in minced material not homogenized; unpublished experiments) the inhibition of respiration in the presence of these substrates was no more than 75%, indicating that about 25% of tissue respiration passed through the 'extramitochondrial' pathway. The fact that the inhibition of substrate utilization by amytal was lowest with citrate (45%) is in agreement with the well-established observation that a large proportion of isocitric dehydrogenase resides extramitochondrially. The partial inhibition of tissue respiration suggests that at least two enzymes catalysing the same step but differing in the sensitivity to amytal exist in the tissue preparations. The enzymes may either differ in their location and accessibility or in their physicochemical properties. Multiple enzymes ('isoenzymes'; Markert & Möller, 1959) have in fact been discovered in liver and other tissues (Delbrück, Schimassek, Bartsch & Bücher, 1959; Wieland, Pfeleiderer, Haupt & Worner, 1959; Thorne, 1960; Wieme, 1959).

Physiological significance of the reduction of acetoacetate. The question arises of the physiological significance of the ready reduction of acetoacetate. Devlin & Bedell (1960) found that DPNH_2 added to mitochondria is not oxidized but

Table 15. *Maximum anaerobic rates of removal of acetoacetate and formation of β -hydroxybutyrate in rat-liver homogenate*Rat-liver homogenate, 10 %. Temp., 30°. Gas phase, N₂. Incubation time, 20 min. Data refer to 4 ml.

Substrates added (all 40 μ moles)	Acetoacetate removed (μ moles)	β -Hydroxybutyrate formed (μ moles)
Acetoacetate	3.0	5.8
Acetoacetate; fumarate	7.8	8.4
Acetoacetate; pyruvate	6.1	11.9
Acetoacetate; citrate	11.2	13.8
Acetoacetate; citrate; fumarate	16.3	17.9
Acetoacetate; citrate; pyruvate	12.2	17.3
Acetoacetate; citrate; pyruvate; fumarate	13.4	18.6
Acetoacetate; pyruvate; fumarate	14.3	17.9
Pyruvate	0	1.7

reacts with oxygen when catalytic quantities of acetoacetate or β -hydroxybutyrate are present. β -Hydroxybutyrate is taken to be a hydrogen carrier between the 'extramitochondrial' and 'intramitochondrial' regions. If acetoacetate played a major role as a hydrogen carrier the rate of its reduction, and of the reoxidation of β -hydroxybutyrate, should be of the same order of magnitude as the overall rates of respiration.

The maximum figures for the reduction of acetoacetate in Table 15 (16 μ moles) represent a rate of 120 μ moles/g. wet wt./hr. at 30°. The corresponding rate of consumption of oxygen under similar conditions was about 115 μ moles (calculated from data in Table 9). As 1 oxygen molecule is equivalent to 2 acetoacetate molecules the observed maximum rate of reduction would be sufficient for about half the electron transport to pass through the acetoacetate- β -hydroxybutyrate system. Since only the pyridine nucleotide-linked dehydrogenases, not succinic dehydrogenase, would be coupled to the acetoacetate system, five of six of the electrons reaching oxygen could maximally involve acetoacetate when carbohydrate serves as the substrate. These considerations lead to the conclusion that a substantial part but not the whole of the electron transport in liver homogenate could pass through acetoacetate. In liver the rates would be adequate for dealing with any DPNH₂ generated in the cytoplasm, e.g. by isocitric or triose phosphate dehydrogenases. It should be mentioned that analogous roles as hydrogen carriers have been considered for the glycerophosphate-dihydroxyacetone systems (Estabrook & Sacktor, 1958; Lee, Takemori & Lardy, 1959; Bücher & Klingenberg, 1958; Zebe, Delbrück & Bücher, 1959) and for the malate-oxaloacetate system (Delbrück *et al.* 1959; Szent-Gyorgyi, 1935).

SUMMARY

1. In many animal tissues (see Table 2) succinate accelerated the aerobic rate of reduction of

acetoacetate as previously demonstrated for sheep heart. This effect was especially pronounced in liver.

2. Acetoacetate served as a major substrate of respiration in the diaphragm of various species and also in epididymal fat, testis and brain of the rat.

3. The determination of intermediate metabolites formed in respiring sheep heart and rat liver on addition of succinate and acetoacetate indicates that succinate was oxidized in preference to all other substrates.

4. Under conditions where 2:4-dinitrophenol stimulated respiration the rate of reduction of acetoacetate was diminished.

5. Amytal greatly increased the aerobic rate of reduction of acetoacetate in rat liver and sheep heart.

6. In liver homogenates the inhibition of respiration by amytal did not exceed 75 %. The remaining 25 % represent the capacity of the amytal-insensitive or 'extramitochondrial' pathway of electron transport.

7. Anaerobically fumarate, glutamate, α -oxoglutarate, pyruvate and citrate accelerated the rate of reduction of acetoacetate in rat-liver homogenates. With these substrates the aerobic rates of reduction were lower than the anaerobic rates. Succinate was the only substrate in rat liver that increased the rate aerobically but not anaerobically.

8. Citrate reacted anaerobically with acetoacetate according to the scheme:

9. Maximum rates for the reduction of acetoacetate were found on addition of mixtures of substrates such as citrate plus fumarate. The rates measured permit the assumption (Devlin & Bedell, 1960) that in the respiration of rat liver a substantial part of the electrons reaching oxygen passes through the acetoacetate- β -hydroxybutyrate system.

10. Creatine phosphate had no effect on the rates of acetoacetate reduction but abolished the inhibition of respiration by 2:4-dinitrophenol.

11. The observations on the formation of β -hydroxybutyrate from acetoacetate summarized in paragraphs 1, 4, 5, 7 and 10 are in agreement with Scheme I, according to which the reduction takes place whenever reaction (1) is more rapid than reaction (2).

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Oligosaccharides in Dextran-Producing Cultures of *Streptococcus bovis*

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Species of *Leuconostoc mesenteroides* and *Streptococcus bovis* produce dextran and fructose when grown in media containing sucrose (Hehre, 1951; Tsuchiya *et al.* 1952; Bailey, Barker, Bourne & Stacey, 1957; Dain, Neal & Seely, 1956; Bailey & Oxford, 1958a). In addition, reducing disaccharides containing glucose and fructose units are formed. One of these, leucrose (5-O-D-glucopyranosyl-D-fructopyranose), has been isolated from cultures of

L. mesenteroides (NRRL B-512F) and characterized by Stodola, Sharpe & Koepsell (1956), who also mentioned the formation of isomaltulose (Sharpe, Stodola & Koepsell, 1954). Bailey & Bourne (1959) have isolated leucrose from dextran-producing cultures of *S. bovis* and noted that a second disaccharide was produced. The present paper reports a closer examination of the oligosaccharides produced in cultures of *S. bovis*.