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The Redox State of the Nicotinamide-Adenine Dinucleotides in Rat Liver Homogenates

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1. The redox state of the NAD couple of rat liver mitochondria, as measured by the [β-hydroxybutyrate]/[acetoacetate] ratio, rapidly changed in the direction of oxidation during the preparation of homogenates in a saline medium. The value of the [β-hydroxybutyrate]/[acetoacetate] ratio fell from 2·3 to 0·15 in 10min. EDTA diminished the fall and succinate prevented it. 2. The redox state of the rat liver cytoplasm, as measured by the [lactate]/[pyruvate] ratio, changed slightly in the direction of reduction during the preparation of homogenate. This was prevented by succinate. 3. In unsupplemented homogenates the differences in the redox states of mitochondria and cytoplasm decreased. Succinate and EDTA together maintained the differences within the physiological range. A measure of the ability of the mitochondria to maintain different redox states in mitochondria and cytoplasm is the value of the expression [lactate][acetoacetate]/[pyruvate][β-hydroxybutyrate]. If there are no differences in the redox states of the NAD in the two cell compartments the value of the expression is 444 at 37°. The value in the intact rat liver is between 4·7 and 21. 4. α-Oxoglutarate or glutamate were still more effective than succinate in maintaining high [β-hydroxybutyrate]/[acetoacetate] ratios in the homogenates because these substrates supply a reducing agent of NAD^+ and, through succinate, an inhibitor of the oxidation of NADH. 5. When supplemented with α-oxoglutarate and EDTA, homogenates readily adjust the redox state of the β-hydroxybutyrate dehydrogenase system after it has been upset by the addition of either acetoacetate or β-hydroxybutyrate. 6. Amytal and rotenone raised the value of the [β-hydroxybutyrate]/[acetoacetate] ratio. This is taken to indicate that the reduction of acetoacetate in the homogenates was not an energy-linked process. 7. 2,4-Dinitrophenol shifted the [β-hydroxybutyrate]/[acetoacetate] ratio in the presence of succinate in favour of oxidation because it inhibited the oxidation of succinate and accelerated the oxidation of NADH. 8. Rotenone increased the rate of ketone-body formation of liver homogenates, though it decreased the rate of oxygen uptake.

Measurement of the ratios [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] in the intact rat liver can be used to calculate the ratio [free NAD^+]/[free NADH] in the cytoplasm and mitochondria respectively (Williamson, Lund & Krebs, 1967). Experiments reported in the present paper are concerned with the question whether the principles used for the assay of the [free NAD^+]/[free NADH] ratio, which originate from the work of Holzer, Schultz & Lynen (1956) and of Bücher & Klingenberg (1958), can also be applied to tissue homogenates.

The assay of the [free NAD^+]/[free NADH] ratio depends on the existence of equilibria, or near-equilibria, between the substrates and the nicotinamide-adenine dinucleotides in the lactate dehydrogenase and β-hydroxybutyrate dehydrogenase systems. The establishment of equilibrium in turn depends on high activities of the dehydrogenases in the tissue; their activity must be much greater than those of the enzymes that, in the steady state, cause the formation and removal of the substrates. It is further assumed that the intramitochondrial [β-hydroxybutyrate]/[acetoacetate] ratio is equal to that of the incubation medium. There are several facts that justify this assumption. The concentrations of β-hydroxybutyrate and acetoacetate in rat blood are virtually the same as in rat liver (Berry, Williamson & Wilson, 1965). Other physiologically occurring anions like lactate

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are evenly distributed over the whole body water (Hill, Long & Lupton, 1924). In the perfused rat liver the concentrations of lactate and pyruvate (Schimassek, 1963) and of β-hydroxybutyrate and acetoacetate (H. A. Krebs, unpublished work) in tissue and medium are the same.

A major difference between the intact tissue and homogenates with respect to the establishment of an equilibrium is the proportion of substrates to dehydrogenases at a given substrate concentration. To prepare homogenates it is necessary to add relatively large quantities, usually at least ten times the tissue weight, of an aqueous suspension medium that, in effect, dilutes the cytoplasmic phase. The relatively large quantities of metabolites present in the experimental system are liable to delay the re-establishment of the equilibrium when the physiological state has been upset by addition of metabolites. The experiments reported in this paper indicate that liver homogenates under suitable conditions establish equilibria in the β-hydroxybutyrate dehydrogenase and lactate dehydrogenase systems, but it must be emphasized that this does not necessarily apply to other tissues where the activity of the dehydrogenases, especially of the β-hydroxybutyrate dehydrogenase, may be much lower.

EXPERIMENTAL

Homogenates. In the standard procedure 1g. of tissue was homogenized at 0° with 6·5ml. of medium consisting of 95ml. of 0·154M-KCl, 1ml. of 0·1M-$MgCl_2$ and 4ml. of 0·1M-potassium phosphate buffer, pH 7·4. When further solutions were to be added, the volume of the KCl solution was decreased so that the concentrations of the other components remained constant. A 3ml. sample of the homogenate (containing about 400mg. wet wt. and 100g. dry wt. of tissue) was placed in the main compartment of a conical Warburg vessel. Substrates were added in the form of 0·2M neutral solution and the total volume of the reaction mixture was made up to 4ml. with the medium used for making the homogenate. The centre well contained 0·2ml. of 2N-NaOH and the gas space O_2. The temperature of the incubation was 30° or 40° as stated in the Tables. The vessels were rapidly shaken.

Analytical procedures. Acetoacetate and β-hydroxybutyrate were determined enzymically by the method of Williamson, Mellanby & Krebs (1962), and lactate and pyruvate by the method of Hohorst, Kreutz & Bücher (1959). The purified, even crystalline, β-hydroxybutyrate dehydrogenase may contain some malate dehydrogenase and care must be taken that any malate present does not interfere. As long as the concentration of malate is low (below 1mM) there is no danger of interference. Higher quantities of malate, as were present after addition of malate or a ready precursor of malate (fumarate, succinate, α-oxoglutarate), were removed with a suspension of *Lactobacillus arabinosus*, which decarboxylates malate to lactate. The bacterial suspension was prepared as described by Nossal (1951) except that the buffer–cofactor solution was modified by omitting the 3M-sodium acetate buffer. The deproteinized homogenate, neutralized to pH about 6·0 (1ml.), was mixed with 0·2ml. of 0·1M-potassium phosphate buffer, pH 5·8, 0·2ml. of cofactor solution and 0·2ml. of 20% (w/v) bacterial suspension. Water was added to 2ml. The mixture was incubated at 40° for 20min. with shaking. It was then cooled to 0° and the bacteria were centrifuged off. The supernatant was assayed for D-β-hydroxybutyrate in the usual way. Under these conditions 5mM-L-malate was completely removed and added β-hydroxybutyrate was fully recovered. Added acetoacetate did not form β-hydroxybutyrate during the treatment.

RESULTS

Effects of EDTA and succinate. When the basic homogenate was incubated at 30° without further additions the [β-hydroxybutyrate]/[acetoacetate] ratio fell from the value of 2–3 *in vivo* to 0·53 during the preparation of the homogenate and to 0·14 after 10min. incubation (Table 1). On addition of 1mM-EDTA the fall was less: a value of 0·31 was found after 10min. Addition of succinate raised the value of the ratio within 10min. from 0·53 to 2·41, i.e. to the physiological range. EDTA and succinate together raised it more rapidly and the value reached after 10min. was a little higher than with succinate alone. The added succinate was very rapidly oxidized. It was used up within 20min. of the incubation as shown by the determination of succinate with the method of Clark & Porteous (1964) and with the disappearance of the succinate the [β-hydroxybutyrate]/[acetoacetate] ratio fell.

The [lactate]/[pyruvate] ratio showed relatively slight variations. There was a small rise in the unsupplemented homogenate and in the presence of EDTA. When succinate alone was added there was no change and with succinate and EDTA together there was a slight fall (Table 1).

The changes in the unsupplemented homogenate, namely a move in the direction of oxidation in the mitochondria and in the direction of reduction in the cytoplasm, meant that the differences in the redox states of the two compartments became very much smaller. A measure of these differences, i.e. the ability of the mitochondrial membranes to maintain different redox states in the two compartments, is the expression [lactate][acetoacetate]/[pyruvate][β-hydroxybutyrate], referred to in Table 1 as *R*. If the lactate dehydrogenase and β-hydroxybutyrate dehydrogenase systems are in equilibrium with a common NAD pool this expression has a value of 444 at 37°, assuming that the equilibrium constant of the lactate dehydrogenase system is $1{\cdot}11 \times 10^{-11}$ and that that of the β-hydroxybutyrate dehydrogenase system is $4{\cdot}93 \times 10^{-9}$ (Williamson *et al.* 1967). From the data of these authors in their Table 1, it can be

Table 1. *Effect of EDTA and succinate on the redox state of the lactate–pyruvate and β-hydroxybutyrate–acetoacetate systems in rat liver homogenates*

Two homogenates were made, one in the standard medium described in the text, the other containing in addition 1mM-EDTA. The liver was from a rat starved for 48hr. L-Lactate (1mM) was added to make the measurements of the concentrations of lactate and pyruvate more accurate. The temperature was 30°. The sum of the concentrations of β-hydroxybutyrate and acetoacetate at zero time was 0·41mM and rose slightly on incubation, reaching the highest value (0·51mM) in the presence of succinate and EDTA. The sum of the concentrations of lactate and pyruvate was initially 0·5mM and remained nearly the same during the incubation. For the definition of *R* see the text.

Time of incubation (min.)	0	5	10	0	5	10	5	10	5	10
Concn. of EDTA (mM)	0	0	0	1	1	1	0	0	1	1
Concn. of succinate (mM)	0	0	0	0	0	0	20	20	20	20
[β-Hydroxybutyrate]/[acetoacetate]	0·53	0·273	0·140	0·79	0·385	0·310	1·66	2·41	2·28	2·66
[Lactate]/[pyruvate]	15·2	20·4	19·0	15·2	18·1	21·2	16·3	15·4	16·3	13·3
R	29	76	135	19	47	69	9·8	6·4	7·2	5·0

Table 2. *Effect of temperature on the maintenance of the redox state of NAD systems in rat liver homogenates*

Two homogenates as described in Table 1 were used. They were tested at 30° and 40°. No substrates were added. The sum of the concentrations of β-hydroxybutyrate plus acetoacetate were initially 0·4mM and rose within 30min. to 0·8mM at 30° and 1mM at 40°. The concentrations of the sum of lactate plus pyruvate was near 0·2mM initially and did not significantly change on incubation. L/P, [lactate]/[pyruvate] ratio; βH/A, [β-hydroxybutyrate]/[acetoacetate] ratio; for definition of *R* see the text.

Time of incubation (min.)	No EDTA						1mM-EDTA					
	30°			40°			30°			40°		
	L/P	βH/A	*R*	L/P	βH/A	*R*	L/P	βH/A	*R*	L/P	βH/A	*R*
0	4·34	1·56	4·07	6·34	1·56	4·07	6·66	1·68	4·00	6·66	1·68	4·00
10	9·83	0·19	52	5·04	0·11	46	9·51	0·41	23	7·23	0·22	33
30	6·98	0·10	69	5·42	0·082	66	6·85	0·47	15	6·69	0·13	51

calculated that in the intact liver *in vivo* the expression had a mean value of 4·8 in the well-fed state, 4·7 in starvation and 21 in the alloxan-diabetic rat liver. These values illustrate the fact that the NAD couples of mitochondria and cytoplasm are not in equilibrium. In the experiment of Table 1 the liver homogenate maintained differences but not to the full extent. When no EDTA was added the value of *R* rose within 10min. from a normal value of about 5 to 135 and this was almost entirely due to a fall of the [β-hydroxybutyrate]/[acetoacetate] ratio. With EDTA the value of *R* rose less (to 69), and with succinate it returned the physiological value from which had fallen during the preparation of the homogenate. This recovery was more rapid when EDTA was also present.

Effect of temperature on the maintenance of the mitochondrial redox state. When no EDTA was added to the homogenate, the [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] ratios were about the same after incubation for 30min. at 30° and 40°. The effect of EDTA was more marked at 30° than at 40°, the *R* values for 30min. being 15 at 30° and 51 at 40° (Table 2).

Replacement of phosphate by N-tris(hydroxymethyl)methyl-2-aminoethanesulphonate buffer. Since phosphate can increase the permeability of mitochondria (Kaufman & Kaplan, 1960) it was replaced by *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonate buffer (Good *et al.* 1966), and the maintenance of the mitochondrial redox state was measured. No significant difference between the two buffers was found.

Effect of α-oxoglutarate and glutamate. When added aerobically, 8mM-α-oxoglutarate or 8mM-glutamate was more effective than succinate in maintaining a high [β-hydroxybutyrate]/[acetoacetate] ratio (Table 3). This is accounted for by the fact that the two former substrates provide, through α-oxoglutarate, a powerful reducing agent of NAD^+, and at the same time, through succinate, an inhibitor of the oxidation of NADH (Kulka, Krebs & Eggleston, 1961; Krebs, Eggleston & d'Alessandro, 1961). It is true that malate formed

Table 3. *Effects of α-oxoglutarate, glutamate and succinate on the redox state of the β-hydroxybutyrate dehydrogenase and lactate dehydrogenase systems in rat liver homogenates*

A 4ml. sample of homogenate containing 0·5mM-EDTA was incubated for various periods at 40°. The concentration of the added substrate was 8mM. The rat was starved for 48hr. The values refer to 4ml. of homogenate.

Substrate added	None				α-Oxoglutarate			L-Glutamate			Succinate		
Time of incubation (min.)	0	10	20	40	10	20	40	10	20	40	10	20	40
O_2 uptake (μmoles)			24·3	38·1		29·7	55·2		28·2	51·7		26·6	51·2
Sum of β-hydroxybutyrate and acetoacetate (μmoles)	1·33	2·17	2·72	3·25	2·65	3·04	3·29	3·31	4·39	5·14	2·45	2·47	2·51
[β-Hydroxybutyrate]/[acetoacetate]	1·11	0·13	0·07	0·05	2·32	3·04	2·61	3·02	3·83	2·47	1·04	0·67	0·48
Sum of lactate and pyruvate (μmoles)	1·10	1·12	1·16	1·25	1·44	1·61	1·73	1·27	1·12	1·18	1·56	2·05	2·32
[Lactate]/[pyruvate]	5·40	6·79	6·79	6·24	3·15	2·35	2·85	10·0	8·68	8·00	2·35	1·49	1·20

from succinate is also a hydrogen donor for NAD^+, but owing to the unfavourable position of the redox potential malate is a weak generator of NADH and any reduction of NAD^+ occurring on addition of malate is largely due to the secondary products of malate degradation (pyruvate and isocitrate), which can act as hydrogen donors. This conclusion is borne out by the stereospecificity of the hydrogen transfer by malate dehydrogenase (Griffiths & Roberton, 1966). The [β-hydroxybutyrate]/[acetoacetate] ratio fell during the later stages of the incubation, especially where succinate was added, because of the disappearance of the added substrate. The rapid oxygen uptake removed most of the succinate within 15min.

The [lactate]/[pyruvate] ratio (Table 3) was very low after addition of α-oxoglutarate (between 2·35 and 3·15) and of succinate (between 1·2 and 2·35). These low values were not caused by a fall in the concentration of lactate but by an increase of that of pyruvate. The latter rose threefold with α-oxoglutarate and about sixfold with succinate. Pyruvate accumulated because it is formed from α-oxoglutarate via malate and oxaloacetate and its removal is competitively inhibited by α-oxoglutarate (Haslam & Krebs, 1963) and succinate (Krebs, 1967, Table 7). There was no increase of the pyruvate concentration after addition of glutamate because the oxaloacetate formed from glutamate reacted with excess of glutamate to form aspartate (Krebs & Bellamy, 1960; Borst & Slater, 1960).

A remarkable feature is the stimulation of ketone-body formation by the addition of glutamate. Though the control and the homogenate supplemented with α-oxoglutarate formed 1·9μmoles of ketone body in 40min., glutamate caused this value to rise to 3·8. Succinate inhibited ketone-body formation.

[*β-Hydroxybutyrate*]/[*acetoacetate*] *ratio under anaerobic conditions.* Anaerobically, as shown previously (Krebs & Eggleston, 1945; Kulka *et al.* 1961; Krebs *et al.* 1961), acetoacetate is readily reduced in tissue homogenates by substrates of mitochondrial NAD-linked dehydrogenases, e.g. isocitrate, glutamate, pyruvate and malate. In previous work in this Laboratory, an excess of acetoacetate was added so that no equilibrium in the β-hydroxybutyrate dehydrogenase system was attained on incubation. In the experiments recorded in Table 4 it was the amount of acetoacetate present that was rate-limiting and the reduction ceased when equilibrium in the β-hydroxybutyrate dehydrogenase system was reached. Under these conditions the differences in the amounts of β-hydroxybutyrate formed on addition of reducing substrates were relatively small. In all cases the formation of β-hydroxybutyrate exceeded the disappearance of aceto-

Table 4. *Anaerobic reduction of acetoacetate in rat liver homogenates*

The homogenates were prepared as described in the text. At zero time 1mM-acetoacetate and 10mM other substrate were added. The temperature was 30° and the period of incubation 30min. The gas space contained N_2 and the centre well yellow phosphorus. The values refer to 4ml. of homogenate. Initially 4ml. contained 4·44μmoles of acetoacetate and 0·34μmole of β-hydroxybutyrate. These values were taken into account in the calculation of the changes.

Other substrate added	None	Citrate	L-Malate	α-Oxoglutarate	L-Glutamate	Pyruvate
Acetoacetate removed (μmoles)	2·11	3·55	3·49	3·75	3·40	3·14
β-Hydroxybutyrate formed (μmoles)	2·93	4·37	3·96	4·23	4·08	5·67
[β-Hydroxybutyrate]/[acetoacetate]	1·40	5·27	4·53	6·62	4·26	4·63

Table 5. *Adjustment of redox state of β-hydroxybutyrate system after addition of acetoacetate or β-hydroxybutyrate*

The standard homogenate was supplemented during its preparation with 5mM-α-oxoglutarate, and at zero time either 1mM-acetoacetate or 2mM-DL-β-hydroxybutyrate was added. 'Sum' refers to the sum of the concentrations of acetoacetate and D-β-hydroxybutyrate, and 'Ratio' to the quotient [D-β-hydroxybutyrate]/[acetoacetate]. The rat was starved for 48hr. The temperature was 30°.

Time of incubation (min.)	No ketone bodies added		DL-β-Hydroxybutyrate (2mM) added		Acetoacetate (1mM) added	
	Sum (mM)	Ratio	Sum (mM)	Ratio	Sum (mM)	Ratio
0	0·41	1·70	1·58	8·05	1·42	0·23
5	0·59	2·66	1·75	4·22	1·51	1·91
10	0·70	2·15	1·85	3·25	1·67	1·98
20	0·78	0·89	1·96	1·73	1·73	1·00

acetate, indicating that ketone bodies were formed on incubation. With pyruvate, where the discrepancy between acetoacetate removal and β-hydroxybutyrate formation was much greater (2·5μmoles) than in all the other cases (between 0·47 and 0·82μmole), the pathway of formation of ketone bodies from pyruvate (via acetyl-CoA and acetoacetyl-CoA) is known. In the other cases the anaerobic source of ketone bodies (or of acetyl-CoA) is obscure. An anaerobic formation of β-hydroxybutyrate from pyruvate has already been described by Krebs & Johnson (1937). Under the test conditions lactate and succinate, as expected, did not promote the reduction of acetoacetate (not recorded in Table 4).

The [β-hydroxybutyrate]/[acetoacetate] ratio rose anaerobically to values not found under aerobic conditions in homogenates, slices or the intact rat liver. The highest value, 6·6, was found on addition of α-oxoglutarate, and this is no doubt connected with the very negative redox potential of the α-oxoglutarate dehydrogenase system. The higher values found anaerobically indicate that molecular oxygen is one of the factors that determine the value of the ratio under physiological conditions.

Adjustment of the redox state of the β-hydroxybutyrate dehydrogenase system after addition of acetoacetate or β-hydroxybutyrate. To test the capacity of the mitochondria in liver homogenates to regulate the redox state of the β-hydroxybutyrate dehydrogenase system and to maintain it near the physiological value, the [β-hydroxybutyrate]/[acetoacetate] ratio was measured at various intervals after the addition of either acetoacetate or β-hydroxybutyrate (Table 5). α-Oxoglutarate (1mM) was also added to stabilize the initial metabolic activities of the homogenate. The values for the sum of acetoacetate and β-hydroxybutyrate indicate that the concentration of the ketone bodies in the homogenate slowly increased on incubation, as expected for starved liver, and that increases were not influenced by the presence of added acetoacetate or β-hydroxybutyrate. The addition of β-hydroxybutyrate caused an immediate 4·7-fold rise in the [β-hydroxybutyrate]/[acetoacetate] ratio, whereas the addition of acetoacetate caused a 7·4-fold fall. Thus the additions caused a 35-fold difference in the ratios. Within 5min. this difference had decreased to 2·2 and after 10min. to 1·6. Subsequently the ratios fell in all three homogenates because the added α-oxoglutarate had been used up. These observations, i.e. the fact that the values of the ratios after 10min. were of

Table 6. *Effect of Amytal and 2,4-dinitrophenol on the redox state of the β-hydroxybutyrate dehydrogenase and lactate dehydrogenase systems in rat liver homogenates*

The standard homogenate prepared from the liver of a rat starved for 48hr. was incubated for 10min. at 30°. The values of the O_2 uptake were obtained from a parallel experiment incubated for 30min. For definition of R see the text.

Additions	[β-Hydroxybutyrate]/[Acetoacetate]	[Lactate]/[Pyruvate]	R	O_2 uptake (μmoles/g. dry wt./hr.)
None (not incubated)	2·23	9·8	4·4	
None	0·18	16·0	90	539
Amytal (1mM)	2·41	12·6	5·2	318
Succinate (10mM)	3·28	5·4	1·64	830
Succinate (20mM), Amytal (1mM)	4·74	7·9	1·67	783
Dinitrophenol (0·1mM)	0·17	12·6	74	137
Succinate (20mM), dinitrophenol (0·1mM)	0·62	6·7	10·7	493
Succinate (20mM), Amytal (1mM), dinitrophenol (0·1mM)	1·66	7·0	4·3	416

the same order in the three homogenates, indicate a substantial capacity of the mitochondria to adjust the redox state of the mitochondrial NAD system when it has been artificially upset. As already emphasized in the introduction, the homogenate contained 90% of medium and 10% of tissue. Thus the medium/tissue ratio was at least four times the blood plasma/liver ratio in the intact animal and the upset caused by the sudden addition of 1mM-ketone body was greater than anything likely to occur *in vivo*.

Effects of Amytal and 2,4-dinitrophenol. Amytal (1mM), like succinate, promoted the reduction of acetoacetate to β-hydroxybutyrate (Krebs *et al.* 1961; Krebs & Eggleston, 1962; Azzone, Ernster & Weinbach, 1963), and maintained a [β-hydroxybutyrate]/[acetoacetate] ratio near the physiological value of 2–3 (Table 6). When succinate and Amytal were added together the effects were partly additive, so that an exceptionally high [β-hydroxybutyrate]/[acetoacetate] ratio, 4·74, was found. Under the test conditions Amytal inhibited the oxygen consumption by about 40% when no substrate was added to the homogenate, but in the presence of succinate the inhibition of the oxygen uptake by Amytal was slight. 2,4-Dinitrophenol (0·1mM) inhibited the oxygen uptake, but had no effects on the [β-hydroxybutyrate]/[acetoacetate] ratio when no substrate was added and when the value was already low. It decreased the value of the ratio in the presence of succinate from 3·28 to 0·62 and this decrease was partly abolished by Amytal, which raised it to 1·66. The effects of Amytal and 2,4-dinitrophenol on the [lactate]/[pyruvate] ratio were relatively small. The ratio was always low on addition of succinate. As already mentioned this was due to a formation of pyruvate from succinate and inhibition of the oxidation of pyruvate by the excess of succinate. 2,4-Dinitrophenol inhibited the oxygen consumption with and without succinate. The marked fall of the [β-hydroxybutyrate]/[acetoacetate] ratio in the presence of succinate that occurred on addition of 2,4-dinitrophenol can be accounted for by the acceleration of the oxidation of NADH caused by 2,4-dinitrophenol, and by the inhibitory effect of 2,4-dinitrophenol on the oxidation of succinate (see Krebs, 1962).

Table 7. *Effect of rotenone on the formation and redox state of ketone bodies in rat liver homogenates*

The liver homogenate used was prepared from a rat that had been starved for 48hr. A 4ml. sample of the homogenate contained 105mg. dry wt. of tissue and initially 0·52μmole of acetoacetate and 0·49μmole of β-hydroxybutyrate. The values refer to 4ml. of homogenate incubated for 40min. at 30°.

Concn. of rotenone (mM)	0	0·001
O_2 uptake (μmoles/4ml.)	29·0	10·6
Acetoacetate formed (μmoles/4ml.)	1·2	0·8
β-Hydroxybutyrate formed (μmoles/4ml.)	0·9	2·3
Total ketone bodies formed (μmoles/4ml.)	2·1	3·1
Final [β-hydroxybutyrate]/[acetoacetate] ratio	0·83	2·2

Effect of rotenone. At suitable concentrations (1μM under the test conditions) the effects of rotenone were similar to those of Amytal. Rotenone inhibited the oxygen consumption and raised the value of the [β-hydroxybutyrate]/[acetoacetate] ratio (Table 7). This is to be expected because, like Amytal, rotenone inhibits the oxidation of NADH by the carriers of the respiratory chain (Ernster, Dallner & Azzone, 1963; Burgos & Redfearn, 1965; Horgan & Singer, 1967*a*,*b*). Rotenone also increased the rate of ketone-body formation in spite of a decreased oxygen consumption. Thus in the

presence of rotenone a major part of the remaining cell respiration was due to an oxidation of fatty acids to ketone bodies. In the experiment recorded in Table 7, the ratio oxygen used/total ketone bodies formed in the presence of rotenone was $10{\cdot}6/3{\cdot}1 = 3{\cdot}4$.

DISCUSSION

Maintenance and regulation of the redox state of the NAD couple in liver homogenates. The main result of the experiments is that under suitable conditions rat liver homogenates maintain in the mitochondria and in the cytoplasm (i.e. the medium bathing the mitochondria) [free NAD^+]/[free NADH] ratios that are similar to those in the liver *in vivo* where the value for the mitochondrial ratio is 50–100-fold lower than that for the cytoplasm. The maintenance of the different redox states for the two compartments in homogenates depends on the presence in the medium of EDTA and certain oxidizable substrates such as succinate, α-oxoglutarate or glutamate. Without these supplements the redox states of the two compartments tend to approach equilibrium. The supplements are not necessarily identical with those operating *in vivo* but functionally replace them.

Since the concentration of free NADH is very much lower than that of free NAD^+, changes in the [free NAD^+]/[free NADH] ratio are mainly caused by variations in the concentration of NADH. This concentration depends on the balance of the reactions that remove and generate NADH. The removal of NADH is mainly brought about by the electron-transport chain, and, on a smaller scale, by the transfer of the hydrogen atoms to a few special substrates such as acetoacetate and oxaloacetate. Generation of NADH is mainly brought about by the intramitochondrial dehydrogenases of the tricarboxylic acid cycle and associated reactions and, on a smaller scale, possibly by transhydrogenation. What regulates the balance between removal and generation is not adequately understood. It is, however, obvious why the inhibition of the electron transfer by the respiratory chain through anaerobiosis or addition of Amytal shifts the redox state towards reduction, and why acceleration of the electron transfer by the respiratory chain by uncouplers can cause changes in the direction of oxidation.

Role of energy-linked reduction in the formation of β-hydroxybutyrate. Azzone *et al.* (1963) have considered the question whether an energy-linked reduction may play a role in the formation of β-hydroxybutyrate from acetoacetate under physiological conditions. Though it is true that this can occur under special circumstances, i.e. when an excess of succinate is available (Klingenberg & Häfen, 1963), the effects of Amytal and rotenone in the present experiments argue against the occurrence of an energy-linked reduction in liver homogenates under the test conditions. In experiments on the perfused rat liver, Scholz, Schwarz & Bücher (1966) also found an increased formation of β-hydroxybutyrate on addition of barbiturates. Since Amytal and rotenone inhibit energy-linked reductions of NADH, it is widely believed (Azzone *et al.* 1963) that inhibitions of reductions by these agents indicate that energy-linking is involved, whereas the non-inhibition indicates that the reduction is due to a dismutation. If energy-linked reduction does not provide NADH then endogenous hydrogen donors must be responsible for the reduction of acetoacetate that occurs in the unsupplemented controls. The concentrations of such endogenous substrates of sufficiently negative potential as are present in measurable quantities (isocitrate, α-oxoglutarate, pyruvate) are too low to account for the observed extent of reduction. It is therefore likely that the following dismutation reaction takes place:

$$\text{Acetoacetate} + \beta\text{-hydroxyacyl-CoA} \rightleftharpoons \beta\text{-hydroxybutyrate} + \beta\text{-oxoacyl-CoA}$$

The values obtained by Krebs, Mellanby & Williamson (1962) and Burton (1957) indicate that the redox potential of the β-hydroxyacyl-CoA–β-oxoacyl-CoA system is 28mv more positive than that of the β-hydroxybutyrate–acetoacetate system. Thus the equilibrium of the above reaction is against the reduction of acetoacetate under standard conditions, but the conditions in the liver cell may be very different. Here the concentration of β-oxoacyl-CoA is exceedingly low because it enters the rapid thiolase reaction, the equilibrium constant of which is about 10^{-5} (Goldman, 1954) and thus favours the removal of β-oxoacyl-CoA.

Anaerobic reduction of acetoacetate on addition of malate. It is a remarkable fact, already noted in previous experiments (Krebs & Eggleston, 1945), that malate can accelerate anaerobic reduction of acetoacetate. As the redox potential of the malate dehydrogenase is about 100mv more positive than that of the β-hydroxybutyrate dehydrogenase system, a significant reduction by malate can occur only if the steady-state concentration of oxaloacetate is kept below 0·1% of that of malate. This postulate can be met by the rapid conversion of any oxaloacetate formed into pyruvate, acetyl-CoA, citrate, α-oxoglutarate and succinate. The ready occurrence of these reactions in animal tissues has already been demonstrated (Krebs & Eggleston, 1945).

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