The maximal rate constant for association for the anti-D system can be calculated from the gas-collision formula of Moelwyn-Hughes (1947). A value of about 1×10^8 l.mole⁻¹ sec.⁻¹ is obtained if it is assumed that: (1) the size of both combining sites is 700 Å², which is the upper limit given by Campbell & Bulman (1952) for the anti-(arsanilic acid) system; (2) that a collision occurs between combining sites if the lateral displacement between the centres of the combining sites is not more than twice the radius; (3) that every collision between combining sites results in union. This calculation does not take into account any steric factors concerned in the association of the reactants. The observed rate of reaction is thus slower by a factor of approx. 10^{-3} than the maximum theoretical rate.

SUMMARY

1. The reaction between three examples of the human blood-group antibody anti-D and red cells has been investigated by using ¹³¹I-labelled antibody.

2. The range of the equilibrium constants, *K*, extended from 1.4×10^8 to 7.2×10^{10} l.mole⁻¹.

3. The minimum range of the rate constant for association of one example of anti-D antibody was 3.0×10^4 – 6.3×10^4 l.mole⁻¹ sec.⁻¹.

4. The minimum observed ranges of the rate constant for dissociation for each of the three examples of anti-D were 3.4×10^{-4} – 6.1×10^{-5} , 1.5×10^{-4} – 1.0×10^{-5} and 3.6×10^{-5} – 1.1×10^{-6} sec.⁻¹.

5. The following thermodynamic constants were obtained for one example of antibody: standard

enthalpy change, ΔH_0 , 0 ± 0.7 kcal./mole; standard entropy change, ΔS_0 , 41 ± 2 cal./degree/mole.

6. The number of D antigen sites on each red cell of the probable genotype R₁R₁ was estimated to be approx. 24 000.

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The Effect of Calcium Ions on Ketone-Body Production by Rat-Liver Slices

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(Received 12 March 1963)

During a study of the factors responsible for the increased production of ketone bodies caused by starvation it was found that the presence of Ca²⁺ ions in the incubation medium profoundly affected the pattern of ketogenesis in liver slices. Calcium ions influenced the amounts of ketone bodies formed and the ratio of D(–)-β-hydroxybutyrate to acetoacetate, and the effect varied with the

nutritional state of the animal and with the substrate metabolized.

MATERIALS AND METHODS

Chemicals. Sodium pyruvate, prepared according to Robertson (1942), was dissolved in water immediately before use. Sodium *n*-octanoate was prepared by neutralization of redistilled octanoic acid. Acetoacetate, DL-β-hydroxybutyrate, enzymes and coenzymes were as described by Williamson, Mellanby & Krebs (1962).

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Experimental animals. Male albino rats of the Wistar strain weighing 160–220 g. were used. The rats were given either a balanced diet [commercial rat cubes from H. C. Styles (Bewdley) Ltd. containing about 15% of protein, 3% of fat and the residue consisting mainly of carbohydrate, plus mineral and vitamin supplements] or a low-carbohydrate diet (containing 3 parts of casein and 1 part of margarine, plus mineral and vitamin supplements) for at least 5 days before the experiments. To study the effect of starvation, food was withheld from rats previously given the balanced diet for 24 hr. before the experiments.

Manometric experiments. The rats were killed by a blow at the back of the neck and the livers were excised immediately and placed on ice. Tissue slices (0.3–0.4 mm. thick) were cut free-hand and transferred to a Petri dish containing ice-cold phosphate saline (see below) and left in the phosphate saline for about 5 min. to remove the preformed ketone bodies (see below for experimental evidence). The slices were then placed in manometer flasks containing 4 ml. of the appropriate saline (see below). Each experiment was set up in duplicate and the mean values of the results are reported. The manometers were gassed with oxygen and incubated at 37° with shaking (100 oscillations/min.) for 1 hr. Substrates were added to the main compartment of the manometer flasks and, when oxygen uptake was measured, 0.2 ml. of 2*N*-KOH was placed in the centre wells. After incubation the slices were removed from the flasks, blotted and placed in weighed beakers. The dry weights were determined after at least 8 hr. at 110°.

Incubation medium. The liver slices were incubated in either 'phosphate saline A' containing: 100 ml. of 0.154 *M*-NaCl; 4 ml. of 0.154 *M*-KCl; 3 ml. of 0.11 *M*-CaCl₂; 1 ml. of 0.154 *M*-MgSO₄; 12 ml. of 0.1 *M*-phosphate buffer, pH 7.4; or Ca²⁺ ion-free 'phosphate saline B': as phosphate saline A but with CaCl₂ omitted.

Deproteinization of the incubation medium. After removal of the slices, perchloric acid was added to the medium [final concn. 6% (w/v)]. The mixture was kept at 0–4° for 10 min. and was then centrifuged for 5 min. at 3000 *g*. The supernatant fluid was decanted into a 10 ml. graduated centrifuge tube and the volume noted. The solution was neutralized with 20% (w/v) KOH, the volume was again noted and the mixture was kept for 30 min. at 0–4°. It was then centrifuged for 5 min. at 3000 *g* and the supernatant fluid was used for the determination of ketone bodies.

In some experiments the leakage of protein into the incubation medium was determined. The precipitate obtained on the addition of perchloric acid was suspended in 1 ml. of 3% (w/v) NaOH, and 4 ml. of biuret reagent (Layne, 1957) was added. The mixture was kept overnight,

and then any insoluble material was removed by filtration through glass wool and the extinction of the filtrate was read at 540 mμ.

Determination of ketone bodies. Acetoacetate and D(–)-β-hydroxybutyrate were determined enzymically with D(–)-β-hydroxybutyrate dehydrogenase (Williamson *et al.* 1962). A slight modification was necessary when D(–)-β-hydroxybutyrate was determined in samples containing Ca²⁺ ions, since a precipitate formed in the presence of the hydrazine buffer. This gave an increase in the extinction simulating a reduction of NAD. This precipitation was prevented by the addition of EDTA (final concn. 2.5 mM) to the hydrazine buffer. The recovery of ketone bodies from the medium in which the liver slices were incubated was within ±5%. There was no interference from malate, since the amount of malate (determined by the method of Hohorst, Kreutz & Bücher, 1959) formed under these conditions was negligible. The values for ketone-body production given in the present paper represent the total amount formed during the incubation period since any preformed acetoacetate and D(–)-β-hydroxybutyrate rapidly diffused out of the slices before they were placed in the manometer flasks. For example, when 300 mg. of liver slices (fresh wt.) from a starved rat were incubated in 10 ml. of ice-cold phosphate saline A for 5 min., the medium was found to contain 0.2 μmole of acetoacetate and 0.7 μmole of D(–)-β-hydroxybutyrate/100 mg. of tissue (dry wt.). These values are comparable with a value of about 1.2 μmoles/100 mg. dry wt. for the initial ketone-body content of rat liver, derived from values given by Bondy & Wilhelmi (1950).

RESULTS

Endogenous ketone-body production. Though Ca²⁺ ions (2.7 mM) increased the formation of acetoacetate from endogenous sources in liver slices from starved rats (by about 30%), it decreased it in liver slices from fed rats (by about 50%) (Table 1). In liver slices from both starved and fed rats, Ca²⁺ ions increased the amount of D(–)-β-hydroxybutyrate formed. The increase in D(–)-β-hydroxybutyrate production by liver slices from fed rats was not sufficient to balance the decrease in acetoacetate formation. Thus Ca²⁺ ions still caused a 20% fall in the total ketone bodies formed by liver slices from fed rats. When animals were given a diet in which fat was substituted for carbohydrate (low-

Table 1. *Effect of Ca²⁺ ions on the formation of ketone bodies from endogenous substrates by rat-liver slices*

The experimental conditions are given in the text. The results are expressed as μmoles/100 mg. dry wt. of tissue/hr. (±s.d.) and the number of experiments in each series is shown in parentheses.

Nutritional state of animal	In Ca ²⁺ ion-free phosphate saline B			In Ca ²⁺ ion-containing phosphate saline A		
	Acetoacetate formed	D(–)-β-Hydroxybutyrate formed	Total	Acetoacetate formed	D(–)-β-Hydroxybutyrate formed	Total
Balanced diet (5)	2.50±0.80	0.42±0.11	2.92	1.61±0.51	0.72±0.17	2.33
Low-carbohydrate diet for 5 days (4)	2.12±0.19	0.18±0.17	2.30	2.58±0.10	1.20±0.18	3.78
Starved for 24 hr. (4)	3.98±0.77	0.20±0.10	4.18	6.05±0.38	0.90±0.22	6.95

carbohydrate diet), results intermediate between those with the normal and the starved animals were obtained. Thus the inclusion of Ca^{2+} ions in the incubation medium increased the formation of both acetoacetate and $\text{D}(-)\text{-}\beta\text{-hydroxybutyrate}$, although the effect was not so marked as with livers from starved rats (Table 1). The time-course of ketone-body production was also dependent on the presence of Ca^{2+} ions and on the nutritional state of the animals. The rate of acetoacetate production by liver slices from fed rats was constant during the first hour of incubation in the Ca^{2+} ion-free phosphate saline B. In contrast, when liver slices from starved rats were incubated in the same phosphate saline B, the rate of acetoacetate formation fell after

the first 15 min. of incubation (Table 2). If Ca^{2+} ions were included in the medium (phosphate saline A) the production of acetoacetate was almost linear for at least 1 hr.

Ketone-body production with added octanoate and pyruvate. The addition of pyruvate or octanoate is known to increase the yield of ketone bodies formed by liver slices. The total amount of ketone bodies formed from octanoate was increased by Ca^{2+} ions, and this effect was much more marked in starved liver (Table 3). When pyruvate was the substrate, Ca^{2+} ions depressed the formation of ketone bodies by liver slices from both fed and starved rats (Table 3). As with ketone-body formation from endogenous substrates, Ca^{2+} ions increased the ratio

Table 2. *Time-course of the production of ketone bodies by liver slices from a starved rat*

The experimental details are given in the text. The results are expressed as $\mu\text{moles}/100\text{ mg. dry wt. of tissue/hr.}$

Time of incubation (min.)	In Ca^{2+} ion-free phosphate saline B		In Ca^{2+} ion-containing phosphate saline A	
	Acetoacetate formed	$\text{D}(-)\text{-}\beta\text{-hydroxybutyrate}$ formed	Acetoacetate formed	$\text{D}(-)\text{-}\beta\text{-hydroxybutyrate}$ formed
20	2.30	0.32	2.60	0.31
40	2.85	0.30	4.22	0.62
60	3.52	0.33	7.02	0.75

Table 3. *Effect of Ca^{2+} ions on the formation of ketone bodies by rat-liver slices in the presence of pyruvate or octanoate*

The experimental conditions are given in the text. The results are expressed as $\mu\text{moles}/100\text{ mg. dry wt. of tissue/hr.}$, and are from typical experiments. The changes (%) in ketone-body formation refer to the increase or decrease found when Ca^{2+} ions (2.7 mM) were included in the incubation medium.

Substrate added	Nutritional state of animal	Acetoacetate			$\text{D}(-)\text{-}\beta\text{-hydroxybutyrate}$			Total		
		- Ca^{2+} ions	+ Ca^{2+} ions	Change (%)	- Ca^{2+} ions	+ Ca^{2+} ions	Change (%)	- Ca^{2+} ions	+ Ca^{2+} ions	Change (%)
Pyruvate	Balanced diet	2.15	1.45	-33	1.65	1.95	+18	3.80	3.40	-11
Octanoate		6.50	3.35	-48	5.85	11.45	+96	12.35	14.75	+20
Pyruvate	Starved for 24 hr.	5.00	4.10	-18	1.30	1.35	+4	6.30	5.45	-13
Octanoate		8.95	12.90	+44	2.90	8.50	+195	11.85	21.40	+81

Table 4. *Effect on ketone-body formation of incubation in a Ca^{2+} ion-free medium followed by subsequent incubation in a medium containing Ca^{2+} ions*

Liver slices from a starved rat were incubated in the presence of octanoate (2.5 mM) at 37° in O_2 with or without Ca^{2+} ions (2.5 mM) as indicated. After 60 min. sufficient Ca^{2+} ions were added to two of the flasks containing the Ca^{2+} ion-free phosphate saline B to give a final concentration of 2.5 mM. All the flasks were incubated for a total of 120 min. The results are expressed as $\mu\text{moles}/100\text{ mg. dry wt./hr.}$

Saline	O_2 uptake		Acetoacetate 0-120 min.	$\text{D}(-)\text{-}\beta\text{-hydroxybutyrate}$ 0-120 min.	Total 0-120 min.
	0-60 min.	60-120 min.			
Ca^{2+} ion-free phosphate saline B	53.3	23.1	14.6	5.7	20.3
Ca^{2+} ion-free phosphate saline B	54.0	21.0	14.5	6.2	20.7
+ Ca^{2+} ions added after 60 min.					
Ca^{2+} ion-containing phosphate saline A	44.6	33.0	17.8	17.2	35.0

of D(-)- β -hydroxybutyrate to acetoacetate formed in the presence of added octanoate or pyruvate (see Table 5).

An experiment was carried out to see whether the lowered ketone-body production from added fatty acids in the absence of Ca^{2+} ions could be reversed by the subsequent addition of Ca^{2+} ions. Liver slices from a starved rat were incubated with octanoate in the Ca^{2+} ion-free phosphate saline B for 60 min., Ca^{2+} ions were then added and the incubation was continued for a further 60 min. The amount of ketone bodies formed was compared with the amount produced by liver slices from the same rat after incubation for 120 min. in the Ca^{2+} ion-free phosphate saline B and in the Ca^{2+} ion-containing phosphate saline A (Table 4). There was no difference between the amount formed in the Ca^{2+} ion-free phosphate saline B and in the Ca^{2+} ion-free phosphate saline B to which Ca^{2+} ions were subsequently added.

Leakage of protein into the incubation medium. The leakage of protein from liver slices described by Kaufmann & Wertheimer (1957) was decreased by Ca^{2+} ions. Thus, though without Ca^{2+} ions the ratio of protein found in the incubation medium to the dry weight of tissue was 0.24 ± 0.06 , with Ca^{2+} ions it was 0.16 ± 0.04 (20 experiments).

DISCUSSION

Effect of Ca^{2+} ions on ketone-body production. The presence of physiological concentrations of Ca^{2+} ions in the medium: (1) decreases the endogenous ketone-body formation by liver slices from animals given a balanced diet; (2) increases the endogenous ketone-body formation by liver slices from animals that have been starved or given a low-carbohydrate diet; (3) increases the ketone-body formation from added octanoate by liver slices from starved rats, and to lesser extent by liver slices from well-fed rats; (4) decreases ketone-body formation from added pyruvate by liver slices of both well-fed and starved rats. The effects of Ca^{2+} ions thus depend on the state of nutrition and on the nature of the added substrate. A stimulating effect of Ca^{2+} ions on the ketone-body production appears to occur whenever fatty acids are the sub-

strate of respiration, either in starved animals or when octanoate was added, which suggests that Ca^{2+} ions may play a role in the degradation of fatty acids.

Calcium ions are known to have many other effects on tissue preparations *in vitro*. Their presence in the incubation medium decreased the loss of protein nitrogen from kidney-cortex slices (Cutting & McCance, 1947; Robinson, 1949), and this has been shown in the current work to apply also to liver slices. Calcium ions are also known to cause swelling of isolated mitochondria. The respiration of tissue preparations *in vitro* is lowered by the absence of Ca^{2+} ions in some cases (Lasnitski, 1934) and increased in other cases (Gore & McIlwain, 1952). Whether all these effects have a common basis (such as an action on membranes) is an open question.

Ratio of D(-)- β -hydroxybutyrate to acetoacetate. The variations of this ratio were very much greater in the present experiments than they are in the blood of the intact organism (Williamson *et al.* 1962). The ratio depends on the nutritional state of the animal, the substrate added and the presence of Ca^{2+} ions. The measurements and calculations by Bücher and his associates (see Hohorst *et al.* 1959; Hohorst, Kreutz & Reim, 1961) of the concentrations *in vivo* of the reduced and oxidized forms of the substrates of certain dehydrogenases (e.g. malate dehydrogenase and lactate dehydrogenase) indicate that the reactants of these dehydrogenase systems and the free NAD and NADH_2 are effectively in equilibrium in the cytoplasmic compartment of the liver cell. On the other hand, the concentrations *in vivo* of D(-)- β -hydroxybutyrate and acetoacetate (M. N. Berry, personal communication) and the equilibrium constant of the β -hydroxybutyrate-dehydrogenase system (Krebs, Mellanby & Williamson, 1962) suggest that the substrates of this system are not in equilibrium with the cytoplasmic NAD and NADH_2 . However, the D(-)- β -hydroxybutyrate:acetoacetate ratio may be a reflection of the prevailing NAD:NADH₂ ratio at the site where the inter-conversion of the two ketone bodies occurs, i.e. within the mitochondria (see also Wieland, Matschinsky, Löffler & Müller, 1961). Hence the

Table 5. Ratios of β -hydroxybutyrate to acetoacetate formed in rat-liver slices

The data given in this Table are derived from Tables 1 and 3.

Nutritional state of animal	No substrate		With pyruvate (2.5 mM)		With octanoate (2.5 mM)	
	- Ca^{2+} ions	+ Ca^{2+} ions	- Ca^{2+} ions	+ Ca^{2+} ions	- Ca^{2+} ions	+ Ca^{2+} ions
Balanced diet	0.17	0.44	0.77	1.35	0.90	3.00
Low-carbohydrate diet for 5 days	0.08	0.46	—	—	—	—
Starved for 24 hr.	0.05	0.15	0.26	0.33	0.32	0.66

D(-)- β -hydroxybutyrate:acetoacetate ratios reported in the present paper (Table 5) indicate that in incubated liver slices from well-fed rats the NAD:NADH₂ ratio within the mitochondria is lower than in slices from starved rats. Some support for this conclusion is provided by the observation of Frunder & Richter (1955) and Kayne, Taylor & Alpert (1963) that during incubation the NAD:NADH₂ ratio increases more rapidly in liver slices from starved rats or rats given a low-carbohydrate diet than in liver slices from rats given a balanced diet.

Calcium ions increase the D(-)- β -hydroxybutyrate:acetoacetate ratio (and presumably therefore decrease the NAD:NADH₂ ratio) (Table 5), but there seems to be no correlation between this effect and the effects of Ca²⁺ ions on total ketone-body production.

SUMMARY

1. Calcium ions decrease the total amount of ketone bodies formed by liver slices from fed rats, but increase the total formed by liver slices from starved rats and rats given a low-carbohydrate diet.

2. Calcium ions enhance ketone-body production by liver slices from fed and starved rats in the presence of octanoate, but the effect is more marked with starved animals. In contrast, Ca²⁺ ions depress the formation of ketone bodies from both fed and starved animals when pyruvate is the substrate.

3. The ratio of D(-)- β -hydroxybutyrate to acetoacetate formed is increased by Ca²⁺ ions (about threefold); the value for the ratio is higher

in liver slices from fed animals than in liver slices from starved animals.

4. Leakage of protein from rat-liver slices during the incubation is lower in the presence of Ca²⁺ ions.

We thank Professor Sir Hans A. Krebs, F.R.S., for useful discussions and advice in the preparation of this paper. J.M. was supported by a Medical Research Council scholarship. This work was aided by grants from the Rockefeller Foundation and from the National Institutes of Health, United States Public Health Service.

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Studies on the Metabolism of the Protozoa

10. THE MOLECULAR STRUCTURE OF THE RESERVE POLYSACCHARIDES FROM *OCHROMONAS MALHAMENSIS* AND *PERANEMA TRICHOPHORUM**

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(Received 27 February 1963)

Although several protozoa synthesize iodophilic reserve polysaccharides of the glycogen, amylopectin or starch type (see, for example, Manners &

Ryley, 1955; Archibald, Hirst, Manners & Ryley, 1960), others contain iodophobic reserve substances. We now describe the structural analysis of such materials from the flagellated protozoa *Ochromonas malhamensis* and *Peranema tricho-*

* Part 9: Ryley (1962).