The Metabolism of Short-chain Fatty Acids in the Sheep

3*. FORMIC, *n*-VALERIC AND SOME BRANCHED-CHAIN ACIDS

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The production of short-chain volatile fatty acids (VFA's) in the rumen of sheep is well established. Elsden (1945, 1946) used partition chromatography to confirm that acetic, propionic and butyric acids are the principal fatty acids present in rumen contents. Subsequently, other workers, using more efficient chromatographic techniques, have shown that relatively small amounts of formic, *iso*butyric, α-methylbutyric, *iso*valeric and *n*-valeric acids may also occur in the sheep rumen (cf. Annison, 1954).

Acetic, propionic and *n*-butyric acids are readily absorbed from the rumen, and many workers have shown that these acids can be metabolized in the animal body. The metabolism of these acids by various sheep tissues has been studied by Pennington (1952). The present paper concerns the metabolism in the tissues of the other acids found in the rumen. Also, although it could reasonably be assumed that these acids are absorbed from the rumen, this had not been definitely established, and experiments were undertaken to investigate this point.

Apart from their occurrence as products of fermentation, *iso*valeric and α-methylbutyric acids are probably produced during the breakdown of leucine and isoleucine respectively in animal tissues (Bloch, 1944; Coon, 1950). *iso*Butyric acid may arise similarly from valine (Kinnory & Greenberg, 1953).

A preliminary communication of this work was submitted to the Biochemical Society (Annison & Pennington, 1952).

EXPERIMENTAL

Volatile fatty acids. Racemic α-methylbutyric and *iso*valeric acids were synthesized by carbonation of the Grignard complexes of *sec.*-butyl and *iso*butyl chlorides respectively, as described by Gilman & Kirby (1932) for α-methylbutyric acid. Partial resolution of the α-methylbutyric acid was achieved by fractional crystallization of the brucine salt (Schutz & Marckwald, 1896); final preparations showed the following specific rotations: $[\alpha]_D^{22} = +5{\cdot}6 \pm 0{\cdot}01°$; $[\alpha]_D^{22} = -6{\cdot}7 \pm 0{\cdot}01°$. The reported value for the naturally occurring dextrorotatory form is $[\alpha]_D^{20} = +17{\cdot}85°$ (Marckwald, 1899). The other VFA's were 'laboratory reagent' quality (British Drug Houses Ltd.). Each acid had the calculated equivalent wt. on titration with alkali and was homogeneous when examined chromatographically by the method of James & Martin (1952). The sodium salts of the acids were employed.

Incubation of VFA's with various tissues. The general procedures described in previous papers of this series (Pennington, 1952, 1954) were employed. Residual acids were determined by steam-distillation in the Markham still, using 10 ml. samples of the medium + washings, together with 1 ml. of 10 N-H_2SO_4 and 8 g. of $MgSO_4$, $7H_2O$. When formic acid was determined 150 ml. of distillate were collected. The other acids used in the tissue incubation experiments distilled quantitatively in the first 100 ml. of distillate. The steam distillates of the media from duplicate flasks were combined after titration and retained for chromatographic analysis.

Analysis of VFA's. The gas–liquid partition chromatographic method of James & Martin (1952) was used. Improved separations of the valeric acid isomers were obtained by increasing the stearic acid content of the liquid phase (DC 550 silicone, Albright & Wilson Ltd., Oldbury, Birmingham) from 10 to 15% (w/w). The operating temperature was 135° using ethylene glycol monoethyl ether (ethyl cellosolve) in the heating jacket; the flow rate was 15 ml. N_2/min.

Estimation of VFA's in blood. Blood samples (50 ml.) were laked with 150 ml. of water and deproteinized with 25 ml. of 25% (w/v) HPO_3. The filtrates were neutralized with 5 N-KOH, concentrated *in vacuo*, acidified to pH 2–3 with 10 N-H_2SO_4 and steam distilled. The distillates were retained for chromatographic analysis.

Ketone body estimation. The method of Greenberg & Lester (1944), as modified by Pennington (1952), was used.

Estimation of sulphanilamide. Free and total sulphanilamide were determined by the method of Bratton & Marshall (1939).

Manometric methods. The effect of the acids on the respiration of rumen epithelium was measured using Warburg manometer vessels gassed with oxygen and with 0·2 ml. of 5% KOH in the centre wells to absorb CO_2. The second method of Dickens & Šimer (1931) was used when measurements were made with a gas phase of 5% CO_2–95% O_2.

RESULTS

Absorption of fatty acids from the rumen

The absorption of the VFA's from the rumen was studied *in vivo* with an ewe anaesthetized with Nembutal. The oesophagus and reticulo-omasal

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orifice were closed by ligatures and the carotid artery and posterior rumen vein exposed for removal of blood samples. The rumen was now emptied and washed out with warm water. A solution (2945 ml.) containing approximately equimolecular amounts of the sodium salts of *iso*-butyric, *n*-butyric, *iso*valeric, α-methylbutyric and *n*-valeric acids (about 0·02M with respect to each acid), potassium dihydrogen phosphate (0·016M), potassium chloride (0·011M) and sodium bicarbonate (0·02M) was warmed to 37°, saturated with carbon dioxide and introduced into the rumen through a permanent fistula. *n*-Butyric acid was included for comparison. Since it is not possible to remove the last traces of VFA from the rumen by washing out with water, a sample of the VFA mixture was removed for pH measurement and VFA analysis immediately after it had been placed in the rumen. Blood samples for ketone body and VFA estimations were taken immediately before the VFA solution was put into the rumen. Samples of blood and rumen fluid were removed for analysis at the times shown in Table 1. The volume of rumen fluid, after 150 min., fell to 2650 ml. (corrected for the fluid removed in sampling) and the pH rose from 6·6 to 7·3.

In a second experiment, under similar conditions, acetate and propionate were included in the solution (3 l.) introduced into the rumen, which again was 0·02M (approx.) with respect to each acid. The changes in the VFA content of the rumen were not followed, but blood samples were analysed as before (Table 1). The final volume of the rumen fluid, the pH of which rose from 6·5 to 7·5, was not recorded.

It will be seen that *iso*butyric, *n*-valeric and α-methylbutyric acids are all absorbed under these conditions. The relative amounts of all the acids appearing in the blood during its passage through the rumen wall may be deduced by subtracting the concentrations in the arterial blood from those in the blood draining the rumen. Values of the same order and comparable to that for *n*-butyric acid are obtained for all four of the acids. There was only a moderate rise in blood ketone bodies. The relatively large amounts of acetic acid appearing in the rumen blood when this acid was originally present in the rumen in the same concentration as the other acids (Expt. 2) is striking.

Table 1. *Changes in the concentrations of volatile fatty acids in the rumen and blood and of ketone bodies in the blood after introduction of a solution of fatty acids into the rumen*

Experiment 1

	Liquid in rumen			Blood from ruminal vein			Carotid blood	
	Initial	90 min.	150 min.	Initial	90 min.	150 min.	Initial	30 min.
Total fatty acid (m-moles/100 ml.)	10·2	8·6	7·8	0·020	0·098	0·152	.	0·035
Mol. percentage of each acid:								
Formic	0	0	0	0	9	10	.	9
Acetic	4	4	10	97	24	33	.	60
Propionic	2	1	0	3	2	1	.	3
n-Butyric	20	19	19	0	16	14	.	8
*iso*Butyric	21	21	19	0	11	10	.	5
n-Valeric	19	19	18	0	13	10	.	5
*iso*Valeric	16	18	17	0	14	11	.	5
α-Methylbutyric	18	18	17	0	12	11	.	5
Ketone bodies (μmoles/100 ml.)	.	.	.	49	100	.	41	107

Experiment 2

	Ruminal vein			Carotid blood		
	Initial	40 min.	180 min.	Initial	50 min.	180 min.
Total fatty acid (m-moles/100 ml.)	0·031	0·10	0·19	0·023	0·041	0·043
Mol. percentage of each acid:						
Formic	9	3	19	14	6	7
Acetic	90	51	44	84	64	86
Propionic	1	13	11	2	3	2
n-Butyric	0	10	5	0	10	2
*iso*Butyric	0	5	6	0	7	1
n-Valeric	0	7	5	0	2	0
*iso*Valeric	0	5	5	0	5	1
α-Methylbutyric	0	6	5	0	3	1
Ketone bodies (μmoles/100 ml.)	30	65	98	38	71	77

Table 2. *Loss of volatile fatty acid and production of ketone bodies when fatty acids incubated with sheep rumen epithelium*

Tissue incubated for 3 hr. at 39·5°. The flasks contained Ringer–phosphate (Krebs & Henseleit, 1932), or Ringer–phosphate–bicarbonate (Krebs & Henseleit, 1932, modified by Pennington, 1954), together with fatty acid as sodium salt (total vol. = 10 ml.). The flasks containing Ringer–phosphate were gassed with O_2 and those containing Ringer–phosphate–bicarbonate gassed with 80% O_2–20% CO_2. Dry wt. of tissue per flask = approx. 300 mg. The figures are calculated to 100 mg. dry wt. The ketone-body values are derived by subtraction of the corresponding control figures, shown in the table.

		Expt. 1		Expt. 2	
Substrate (100 μmoles)	CO_2	Fatty acid lost (μmoles)	Ketone bodies produced (μmoles)	Fatty acid lost (μmoles)	Ketone bodies produced (μmoles)
*iso*Butyric	–	24·2	−2·3	23·7	−2·2
	+	23·6	−2·4	23·6	−1·7
n-Valeric	–	20·2	5·8	18·3	5·3
	+	27·1	6·0	24·8	5·3
*iso*Valeric	–	1·7	−1·5	0·4	−1·0
	+	5·0	2·9	4·7	5·7
α-Methylbutyric	–	12·2	−1·7	11·9	−1·8
	+	12·5	−2·1	10·8	−1·5
None	–	−1·0*	3·1	−1·9*	3·4
	–	.	.	−1·4*	3·0

* Production of acid.

The apparent presence of formic acid in the sheep's blood was an unexpected feature of these results. In the present paper formic acid is identified by the appearance of a band in the appropriate position on the liquid–gas chromatogram and the identification is, therefore, only tentative. Subsequent work by one of us (Annison, to be published) has established more conclusively the identity of this band and has indicated that under normal conditions 10–30% of the VFA in sheep blood is formate.

Utilization of acids by rumen epithelium

In the first paper of this series (Pennington, 1952) it was shown that *in vitro* the epithelial tissue of the rumen metabolizes acetic, propionic and *n*-butyric acids. Of special interest was the observation that a large proportion of *n*-butyric acid was converted into ketone bodies by this tissue. Table 2 shows the results of similar experiments with the acids under study (except formic acid, which is considered in a later section). Each acid was incubated separately with the tissue sections both in the presence and in the absence of carbon dioxide since it was previously shown that the rate of metabolism of propionate was greatly enhanced by this gas (Pennington, 1952).

Evidently all four acids were metabolized. Whereas the metabolism of *n*-valeric acid produced an increase in ketone bodies over the controls, *iso*-butyric and α-methylbutyric acids were 'antiketogenic'. *iso*Valeric acid increased ketone body formation only when carbon dioxide was present; otherwise there were less ketones formed than in the controls. In another experiment ox rumen epithelium was used, with closely similar results.

The possibility that there might be a breakdown of some of the acids by the tissues into homologues of shorter chain length was next considered. In the experiments recorded in Table 2 only the total VFA was measured after incubation and the presence of new VFA's arising from the acid originally added would remain undetected. Also, in such an event the net loss of VFA shown in Table 2 would not give a true picture of the amount of the added VFA metabolized. In further experiments the residual VFA after incubation was analysed chromatographically. The results are shown in Table 3.

The partial breakdown to lower acids of all the VFA's examined was indicated. The results indicate that *iso*butyric, *n*-valeric and α-methylbutyric acids can give rise to both acetic and propionic acids whilst *iso*valeric acid forms acetic and *iso*butyric acids. The rate of utilization of *iso*valeric acid and possibly of α-methylbutyric acid was increased by the presence of carbon dioxide.

The α-methylbutyric acid used in the experiments described above was optically inactive. In another experiment there was found to be no difference between the rates of disappearance of volatile fatty acid when the partially resolved isomers were incubated, whether at concentrations of 0·005 or 0·04 M.

The appearance of formic acid in most of the media was indicated. The amounts found were never greatly in excess of the quantity appearing in the control flask without substrate. It is therefore unlikely that formic acid was produced by the breakdown of any of the other acids.

Table 3. *Breakdown of volatile fatty acids by rumen epithelium*

Tissue incubated for 3 hr. at 39·5° with fatty acid as sodium salt. The flasks contained Ringer–phosphate (gassed with O_2) or Ringer–phosphate–bicarbonate (gassed with 80% O_2–20% CO_2). Vol. of fluid = 10 ml. Dry wt. of tissue = 290–319 mg. (Expt. 1), 285–296 mg. (Expt. 2). The ketone-body values are derived by subtraction of the corresponding control figures and the figures in parentheses are the percentages of the maximum possible production of ketones from the quantities of fatty acids utilized, assuming that 2 mol. of *n*-valeric give rise to 1 mol. of ketone body and 1 mol. of *iso*valeric gives 1·5 mol. of ketone body (see Discussion).

Substrates	CO_2	Substrate lost (μmoles)	Acids produced (μmoles)					Ketone body produced (μmoles)
			Formic	Acetic	Propionic	*n*-Butyric	*iso*Butyric	
				Experiment 1				
*iso*Butyric	–	117	4·6	7·7	12·2	0·0	0·0	.
(400 μmoles)	+	118	3·0	7·5	7·5	0·0	0·0	.
n-Valeric	–	126	3·2	15·9	47·8	0·0	0·0	.
(400 μmoles)	+	117	2·8	11·2	11·2	0·0	5·6	.
*iso*Valeric	–	14·8	2·0	5·9	0·0	0·0	11·8	.
(400 μmoles)	+	34·0	3·8	11·2	0·0	0·0	3·8	.
α-Methylbutyric	–	62·2	3·4	6·8	3·4	0·0	0·0	.
(400 μmoles)	+	78·0	0·0	10·3	20·6	0·0	0·0	.
None	+		1·4	1·4	0·0	0·0	0·0	.
				Experiment 2				
*iso*Butyric	–	79·7	1·3	6·2	5·0	0·0	0·0	.
(100 μmoles)	+	76·5	1·3	4·3	1·3	0·0	0·0	.
n-Valeric	–	98·0	2·7	9·1	21·8	0·0	0·0	30·1 (62)
(100 μmoles)	+	95·7	1·3	7·9	10·6	0·0	0·0	16·3 (34)
*iso*Valeric	–	0·0	0·0	6·5	0·0	0·0	0·0	−4·2
(100 μmoles)	+	28·0	3·6	10·9	1·8	0·0	0·9	16·3 (38)
α-Methylbutyric	–	44·7	0·0	6·2	5·5	0·0	0·0	.
(100 μmoles)	+	48·2	0·0	10·0	2·7	0·0	0·0	.
None	+	.	1·1	3·4	0·1	0·2	0·0	8·6

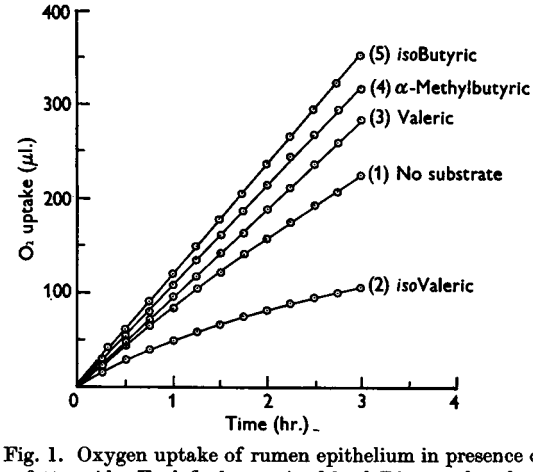

Fig. 1. Oxygen uptake of rumen epithelium in presence of fatty acids. Each flask contained 3 ml. Ringer–phosphate and 30 μmoles of the appropriate substrate. Fatty acids added as Na salts. Gas phase, oxygen. Dry wt. of tissue: 31 mg. (1, 2), 28 mg. (3, 5), 30 mg. (4).

It was thought possible that larger amounts of the breakdown products could be demonstrated by adding a suitable amine and trapping the acids as acyl derivatives, thus preventing their further breakdown. Acetylation of foreign amines by tissue slices is well known, and Schreeve (1952) demonstrated the propionylation of an aromatic amine by liver and kidney slices. Unfortunately, attempts to demonstrate acylation of sulphanilamide by rumen epithelium gave negative results. Sulphanilamide (5 mg.) was incubated with the tissue in the presence of 100 μmoles of either acetic, propionic or *n*-butyric acid. After 3 hr. there was no difference between the free and total sulphanilamide content of the medium. The rate of utilization of the fatty acids was not altered by the presence of the amine. Sulphanilamide in this concentration did not influence the oxygen uptake of the tissue.

Respiration. Fig. 1 shows the curves for oxygen uptake. All of the acids, except *iso*valeric, increased the oxygen uptake of the tissue. Owing to the relatively high endogenous respiration it was not possible to draw any conclusions from a comparison of the oxygen uptakes with the amounts of fatty acid utilized. It is not even possible to deduce minimum values for the number of oxygen atoms taken up per mole of fatty acid metabolized since oxidation of added fatty acids may stimulate the oxidation of endogenous substrates (Grafflin & Green, 1948). *iso*Valeric acid caused a marked and progressive inhibition of respiration. A similar effect had previously been observed with propionic

acid (Pennington, 1954) but only in the absence of carbon dioxide. The effect of *iso*valeric acid was therefore compared in the presence and absence of carbon dioxide, using the Dickens & Šimer (1931) second method for measurements under 5% CO_2–95% O_2 (Table 4). It is clear that like propionic acid, *iso*valeric acid only inhibits the respiration of rumen epithelium in the absence of carbon dioxide.

Utilization of acids by liver slices

Experiments similar to those with rumen epithelium recorded in Table 3 were carried out with liver slices (Table 5). *n*-Butyric acid was included for comparison. It will be seen that each of the acids was readily utilized by liver.

The effect of carbon dioxide was studied only with *iso*valeric acid; its rate of utilization was increased only slightly in the presence of a gas phase of 20% CO_2–80% O_2, when compared to the increased uptake obtained with rumen epithelium under these conditions. The metabolism of α-methylbutyric acid was associated with a small increase in ketone body production by this tissue.

The products formed were both quantitatively and qualitatively different from those obtained with rumen epithelium. Notably, there was, in general, a much smaller production of propionic acid. No *iso*butyric acid resulted from the metabolism of *iso*-valeric acid. The relatively small production of propionic acid from *n*-valeric acid was confirmed by a separate experiment (Expt. 2, Table 5).

Utilization of acids by kidney slices

Table 6 shows that all the acids were metabolized by kidney slices, the rates of utilization per unit weight of tissue being greater than that of the other two tissues, as was found previously with acetic, propionic and *n*-butyric acids. VFA production in the controls without substrate was negligible. The amount of *iso*valeric acid disappearing was about 60% greater in the presence of carbon dioxide. There was no ketone body production from any of the acids.

Utilization of formic acid by various tissues

It was not originally intended to include formic acid in these studies, since it appeared unlikely that it is normally absorbed from the rumen in appreciable quantities. It is known to be rapidly metabolized by the rumen micro-organisms (Claren, 1942). However, in view of the chromatographic evidence for the presence of this acid in both sheep blood (Table 1) and in the media in which tissues had been incubated with VFA's, experiments were carried out to determine whether it could be utilized by the tissues (Table 7). Liver was most

Table 4. *Effect of* iso*valeric acid upon respiration of rumen epithelium in presence and absence of carbon dioxide*

Medium: 3 ml. of Ringer–phosphate or 1·5 ml. of Ringer–bicarbonate (Krebs & Henseleit, 1932). *iso*Valeric acid, 0·01 M (as sodium salt) where added. Temp. 39·5°. Dry wt. of tissue = 25 mg. The values are computed from the readings over a 2 hr. period; Q_{O_2} = μl. O_2 consumed/mg. dry wt./hr.

	No substrate		*iso*Valerate	
Gas phase	Q_{O_2}	R.Q.	Q_{O_2}	R.Q.
O_2	4·26	.	1·56	.
95% O_2–5% CO_2	4·12	0·60	3·90	0·64

Table 5. *Breakdown of volatile fatty acids by sheep liver slices*

Tissue incubated for 3 hr. at 39·5° with fatty acid as sodium salt. The flasks contained Ringer–phosphate (gassed with O_2) or Ringer–phosphate–bicarbonate (gassed with 80% O_2–20% CO_2). Vol. of fluid = 10 ml. Dry wt. of tissue = 364–387 mg. The ketone body values are derived by subtraction of the corresponding control figures, and the figures in parentheses are the maximum possible production of ketones from the quantities of fatty acids utilized assuming that 2 mol. of *n*-valeric or α-methylbutyric acid or 1 mol. of *n*-butyric acid give rise to 1 mol. of ketone body and 1 mol. of *iso*valeric acid gives 1·5 mol. of ketone body.

Substrate (100 μmoles)	CO_2	Substrate lost (μmoles)	Acids produced (μmoles)			Ketone bodies produced (μmoles)
			Formic	Acetic	Propionic	
		Experiment 1				
*iso*Butyric	–	65·0	1·2	4·5	1·6	−5·0
n-Valeric	–	73·0	4·0	20·0	1·0	6·0 (16)
*iso*Valeric	–	59·0	0·0	7·5	0·0	35·7 (40)
	+	72·5	0·7	4·1	0·0	50·0 (41)
α-Methylbutyric	–	43·5	1·2	3·7	0·0	3·7 (18)
n-Butyric	–	62·7	0·0	6·4	0·4	34·3 (55)
None	–	.	1·7	2·1	0·0	9·9
		Experiment 2				
n-Valeric	–	38·5	3·9	14·0	0·8	.
None	–	.	1·9	4·7	0·5	.

Table 6. *Breakdown of volatile fatty acids by sheep kidney slices*

Tissue incubated for 3 hr. at 39·5° with fatty acid as sodium salt. The flasks contained Ringer–phosphate (gassed with O_2) or Ringer–phosphate–bicarbonate (gassed with 80% O_2–20% CO_2). Vol. of fluid = 10 ml. Dry wt. of tissue = 122–134 mg.

Substrate (100 μmoles)	CO_2	Substrate lost (μmoles)	Acids produced (μmoles)		
			Formic	Acetic	Propionic
*iso*Butyric	–	47·2	2·4	4·8	4·8
n-Valeric	–	43·2	0·6	3·0	1·2
*iso*Valeric	–	17·8	1·3	1·3	0·0
	+	28·5	0·7	0·7	0·7
α-Methylbutyric	–	27·8	2·6	10·5	0·0

Table 7. *Utilization of formic acid by sheep and rat tissues*

Tissue incubated for 3 hr. at 39·5° in Ringer–phosphate containing sodium formate (100 μmoles). Vol. of fluid = 10 ml.; dry wt. of tissue = approx. 300 mg. The figures are calculated to 100 mg. (dry wt.) of tissue.

	Tissue	Formic acid utilized (μmoles)
Sheep	Rumen epithelium	1·5
	Liver	9·3
	Kidney	6·8
Rat	Liver	7·2
	Kidney	4·0

active in this respect. Kidney, which utilizes the other short-chain fatty acids more rapidly than liver, was less active, whilst rumen epithelium showed a negligible rate of utilization. There were no marked differences between the sheep and rat tissues in the rates of utilization. Ketone-body production by liver slices was not influenced by formate.

DISCUSSION

The metabolism of the acids studied in the experiments described above were the subject of many earlier investigations, in which interest was largely confined to ketone-body production. Jowett & Quastel (1935) found that *n*-valeric acid increased the Q_{O_2} (μl. O_2 consumed/mg. dry wt. tissue/hr.) and acetoacetate production of slices of guinea pig liver. Edson (1935) showed that both *n*- and *iso*-valeric acids were ketogenic when incubated with liver slices from well fed rats, whereas Cohen (1937) found that only the latter acid increased ketone body production. Geyer & Cunningham (1950) demonstrated the production of labelled carbon dioxide and acetoacetate by liver and kidney slices from *n*-valeric acid labelled with ^{14}C in the carboxyl group. Liver produced much more labelled acetoacetate relative to carbon dioxide than did kidney.

Early German workers (Baer & Blum, 1906; Embden, Salomon & Schmidt, 1906; Wirth, 1910), by feeding and perfusion studies, concluded that both *iso*valeric and α-methylbutyric acids were ketogenic. Demethylation was thought to be the first step in their metabolism. However, Lang & Adickes (1940), who measured ketone body production by liver slices from a wide range of branched-chain acids, found that *iso*valeric but neither α-methylbutyric nor *iso*butyric acids produced ketone bodies. Similar results were obtained by Wick (1941) when these acids were injected into rabbits. More recently, interest has centred upon the detailed mechanism of breakdown of these acids and of the amino acids from which they are derived. Bloch (1944) administered deuterium-labelled acids to rats and found that *iso*valeric but not *iso*butyric acid could provide acetyl groups for acetylation of phenylaminobutyric acid. The breakdown of *iso*valeric acid by rat-liver slices was studied further by Zabin & Bloch (1950) and, in greater detail, by Coon (1950). By labelling the molecule in various positions Coon showed that carbon atoms 1 and 2 are split off, the 2-carbon fragments combining in pairs to give acetoacetate. This can now probably be interpreted to occur through acetyl coenzyme A formation. The remaining 3-carbon portion of the molecule apparently combines with a molecule of carbon dioxide to form acetoacetate. The stimulating effect of carbon dioxide upon *iso*valerate utilization, shown above, provides additional evidence for a pathway involving carbon dioxide fixation. The fact that this effect was less marked in liver and kidney than in rumen epithelium may be a result of a higher endogenous carbon dioxide production masking the effect. The formation of acetate from *iso*valeric acid provides more direct evidence for the occurrence of a 2-carbon product. No explanation is offered for the apparent production of *iso*butyric acid from *iso*valeric acid.

The marked inhibition of the respiration of rumen epithelium by *iso*valeric acid in an oxygen atmosphere (Fig. 1) parallels the effect of propionic acid (Pennington, 1954) and explanation along the lines of that suggested for the latter may be tentatively advanced. A complex may be formed between a moiety of the *iso*valeric acid molecule and coenzyme A or another coenzyme, which requires carbon dioxide for further metabolism. When the

concentration of carbon dioxide is low the coenzyme thus bound would be unable to function in the oxidation of endogenous substrates. It is of interest that propionic and *iso*valeric acids, both of which are metabolized more rapidly in the presence of carbon dioxide, have similar effects upon the respiration of the tissue.

Since the experiments described here were carried out, Coon and co-workers (Coon & Abrahamsen, 1952; Coon, Abrahamsen & Greene, 1952) have presented evidence, obtained from isotope studies, for a pathway of breakdown of α-methylbutyric acid by liver slices. It is postulated that β-oxidation and cleavage of the longer carbon chain occur to produce 'acetate' (which gives rise to acetoacetate) and propionate. The formation of propionate was demonstrated by isotope dilution, in which an excess of unlabelled propionate was added during the metabolism of labelled α-methylbutyric acid; about 3% of the added ^{14}C was recovered as propionate. Using rumen epithelium we were able to demonstrate the net formation of propionic acid (Table 3), but we are unable to explain why much more propionic acid is formed in the presence of carbon dioxide in Expt. 1 (Table 3).

The suppression of ketone-body formation in rumen epithelium by α-methylbutyric acid is probably connected with the formation of propionic acid, which has been shown to suppress ketogenesis from acetic acid by this tissue (Pennington & Pfander, unpublished results).

*iso*Butyric acid was studied by Atchley (1948). It was shown to be partly broken down to propionic acid by kidney 'cyclophorase' system and a mechanism involving β-oxidation to methyl malonic semialdehyde followed by decarboxylation to propionaldehyde was suggested. Recent support for breakdown via propionic acid was provided with isotope studies by Peterson, Fones & White (1952) and Kinnory & Greenberg (1953). Such a mechanism does not explain the simultaneous formation of acetic acid, strongly suggested by the data in Tables 3, 5 and 6, and possibly alternative mechanisms of breakdown exist. Valdigué & Séguélas (1946) claim to have demonstrated the formation of acetone from *iso*butyric acid, by a mechanism other than the decarboxylation of acetoacetate.

The breakdown of *n*-valeric acid into C_2 and C_3 units is presumably a result of β-oxidation. Support for this mechanism is provided by Siegel & Lorber (1951). Ketone-body formation would arise from condensation of the C_2 units in pairs.

The extensive participation of formate in intermediary metabolism has been shown by recent investigations indicating, for example, that isotope-labelled formate is incorporated into purines and pyrimidines (Lavik, 1953), acetoacetate (Plaut & Lardy, 1950), serine (Sakami, 1948; Plaut, Betheil & Lardy, 1950) and the methyl groups of choline (du Vigneaud, Verly & Wilson, 1950). Formate has been shown to be an intermediate in histidine metabolism (Soucy & Bonthillier, 1951), and the production of formate from methyl compounds incubated with liver slices was reported by Sievekitz & Greenberg (1950).

In conclusion, it seems safe to presume that any of the above-mentioned acids produced in the sheep's rumen will be utilized in the tissues and will not represent a loss of the energy of the foodstuffs to the animal.

SUMMARY

1. *iso*Butyric, *n*-valeric, *iso*valeric and α-methylbutyric acids, which are known to occur in the sheep's rumen, can be absorbed into the blood from the rumen.

2. All these acids are metabolized to some extent when incubated with rumen epithelial tissue. Small amounts of acetate and propionate were produced from *iso*butyric, *n*-valeric and α-methylbutyric acids under these conditions, whilst *iso*valeric acid gave rise to acetate and possibly *iso*butyrate. *n*-Valeric and *iso*valeric acids increased ketone-body formation.

3. The rate of metabolism of *iso*valeric acid was increased by the presence of carbon dioxide. In the absence of carbon dioxide this acid inhibited the respiration of rumen epithelium.

4. Liver and kidney slices also metabolized these acids, with breakdown to acids of shorter chain length.

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Some Esterases of the Rat

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There is much confusion in the literature on the identity of esterases and lipases. Much of this has arisen from the different conditions under which they have been examined and in particular to the variety of substrates which have been used. In most cases the preparations probably contained more than one esterase whose activities against different substrates have not been separated. For instance, although it was pointed out over 20 years ago (Cherry & Crandall, 1932) that tributyrin is not a specific substrate for pancreatic lipase, this fact has not always appeared to be appreciated. It is shown in this paper that tributyrin is an excellent substrate for both lipase and esterase.

The esterases which are discussed in this paper are not cholinesterases and do not hydrolyse choline esters. It is first necessary to try to separate the activities of the esterases (including lipases) so that the effects of alterations in the chemical nature and physical state of the substrates may be examined under strictly comparable conditions. Previous work (Aldridge, 1953*a*, *b*) has shown that the esterases of serum may be separated into two distinct types (A and B) by their sensitivity to organophosphorus inhibitors. This method has since been used for the esterases of erythrocytes (Mounter & Whittaker, 1953) and for tissue esterases (Mendel, Myers, Uyldert, Ruys & Bruyn, 1953). Using this method an initial examination of the esterases of the intestinal mucosa and pancreas of the rat soon showed that they differed markedly from those present in rat serum. A detailed examination has been made of the A-type esterases of rat serum and pancreas and the B-type esterases of intestinal mucosa. After the activities of esterases and pancreatic lipase had been clearly separated, an examination of the effect of the physical state of the substrate on the activity of both enzymes was made. This has provided a clue to the essential difference between these two enzymes. The characterization and a knowledge of the distribution of esterases should provide clues to the physiological function of this group of enzymes. The techniques described in this paper should help in approaching this problem.

MATERIALS AND METHODS

In the methods used for determining the activity of the esterases examined in this paper no claim is made that optimum conditions have been produced. No attempt has been made to determine the activity of the enzymes at their optimum pH because where several substrates are being used the pH optima may vary considerably from substrate to substrate. This has been well shown for alkaline phosphatase by Delory & King (1943) and for pancreatic lipase by Schønheyder & Volqvartz (1945*a*).