small formation of ketone bodies which accompanied the metabolism of acetate was sharply depressed by glucose, in spite of the increased uptake of acetate. The ketone-body production from butyrate was affected to a relatively much smaller extent by glucose.

DISCUSSION

Although the proportions may vary, all three acids used in this study are known to be present in the rumen, on a wide variety of natural diets. It is evident that a reliable quantitative estimate of the importance of the rumen epithelium in metabolizing the fatty acids produced in the rumen cannot be deduced from experiments with the single acids. In the experiments of Table 1, where equal amounts of the acids were present in the mixtures, the most definite effects were the inhibition of propionate uptake by butyrate and of butyrate uptake by acetate. In the normal rumen, with different proportions of the acids, other interrelationships may become more important.

Insufficient information is available to attempt to interpret the results. However, it does not seem that they can be explained by simple competition of the fatty acids for a common activating enzyme. The apparent interconversion of acetate and butyrate in the presence of propionate is of particular interest and merits further investigation. The effect of propionate and glucose upon ketone-body production from acetate accords with their known antiketogenic activity. However, the results

suggest that, even in the presence of these compounds, butyrate will be an important source of ketone bodies in the sheep.

SUMMARY

1. Butyrate lowered the uptake of propionate by sheep-rumen epithelium, and acetate lowered the uptake of butyrate.
2. Ketone-body production from acetate was suppressed by propionate or glucose.
3. Some acetate was produced when propionate and butyrate were metabolized together.

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The Influence of Insulin on Free and Fixed Glycogen in the Diaphragm of the Rat

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Because of a difference in solubility in trichloroacetic acid, tissue glycogen can be divided into two forms, a free and a fixed form (Nerking, 1900; Willstätter & Rohdewald, 1934), recently called free and residual glycogen (Bloom, Lewis, Schumpert & Shen, 1951). The free glycogen is soluble in trichloroacetic acid at room temperature whereas the fixed fraction can be extracted only by heating the tissue for 15 min. at 100° in trichloroacetic acid (Kemp & Kits van Heijningen, 1954), or by destruction of the tissue in boiling potassium hydroxide. Previous investigations (Kits van Heijningen & Kemp, 1955) have made it unlikely that the fixation

of glycogen to protein is an artifact, since extraction with an agent which does not precipitate proteins will yield the same values for both fractions, as does extraction with trichloroacetic acid. An investigation into the influence of muscular contraction on these fractions offered no clue as to their respective physiological functions (Kits van Heijningen & Kemp, 1955).

It is well established that insulin promotes the deposition of glycogen in striated muscle (Best, Dale, Hoet & Marks, 1926; cf. Gemmill, 1940). Should this increase in glycogen content be limited to one of these fractions, it might be argued that

that particular fraction serves primarily as a glycogen store. The incubation of rat diaphragm in a medium containing glucose with or without the addition of insulin, as described originally by Gemmill (1940), seemed particularly suitable to study the effect of insulin on the two glycogen fractions in muscle. Evidence will be presented that this increase in glycogen content is limited to the free fraction.

EXPERIMENTAL

Incubation media. The medium of Krebs & Henseleit (1932), buffered with phosphate to a pH of 7.3 and without CaCl_2 (Umbreit, 1951), was used. If necessary, the pH was adjusted to 7.3 after addition of insulin to the medium. To prevent precipitation of calcium phosphate, the CaCl_2 was added separately to the vessels after the tissue samples.

Insulin. In the first 13 experiments a commercial insulin solution (Novo Terapeutisk Laboratorium, Copenhagen, 40 i.u./ml.) was used; the other experiments were carried out with crystalline insulin (Philips-Roxane, Weesp, Netherlands, 24.3 i.u./mg.).

Manometer flasks. Dickens & Šimer (1930) flasks with a circular trough were used.

Methods. Female rats of a cross-bred Wistar strain, weighing 90–115 g., which had not been fasted, were anaesthetized with sodium pentobarbitone (60 mg./kg.) and

bled from their carotid arteries. The diaphragms were removed immediately, care being taken to handle them as little as possible, to prevent contracture of the muscle. For the same reason preliminary soaking in cooled saline was abandoned, although this procedure would increase their glucose uptake (Brown, Park, Daughaday & Cornblath, 1952). The diaphragms were transferred immediately to oxygenated Krebs-Henseleit phosphate solution, pH 7.3, at $37 \pm 0.5^\circ$. The hemidiaphragms of one rat were placed in two separate tubes, and those of the next animal were added to the same tubes in reversed order so as to provide two comparable pairs of a left and a right hemidiaphragm in each tube. Four or six pairs were run at a time. When all the diaphragms had been removed, the pairs were taken from their storage tubes, blotted lightly on filter paper, weighed on a torsion balance, and differences in weight above 10 mg. between two corresponding pairs were corrected by cutting some tissue from the heavier pair. The pairs were transferred to manometer flasks containing 1.95 ml. of medium with 2 mg. of glucose/ml., with or without insulin, 0.05 ml. of 0.1 M- CaCl_2 being added after the tissue samples. Potassium hydroxide (1.8 N, 0.5 ml.) was placed in the surrounding trough. The final concentration of insulin was 0.87 i.u./ml. The vessels were gassed with O_2 for 3 min., equilibrated in the bath at 37.2° for 10 min. before readings were begun, and O_2 uptake was measured at 5 min. intervals for 60 min.

At the end of the incubation period the hemidiaphragms from each flask were blotted on filter paper, placed on top of one another and cut into two or three paired pieces, which

Table 1. *Influence of insulin on free-glycogen content of rat diaphragms incubated in vitro*

Pairs of hemidiaphragms were incubated for 60 min. in Krebs-Henseleit saline buffered with phosphate, containing 2 mg. of glucose/ml. Insulin, final concn. 0.87 i.u./ml., was added to one set, the other serving as a control. Insulin in Expts. 1–13 was Novo commercial insulin soln.; in Expts. 14–24 crystalline insulin (Philips-Roxane) was used. Values for free glycogen have been calculated as described by Kits van Heijningen & Kemp (1955).

Expt. no.	Control		Insulin-treated		Increase in free glycogen (g./100 g. wet wt.)
	Total wt. of pair and wt. of sample (mg.)	Free glycogen (g./100 g. wet wt.)	Total wt. of pair and wt. of sample (mg.)	Free glycogen (g./100 g. wet wt.)	
1	144 (96)	0.35, 0.34	144 (80)	0.41, 0.41	0.06
2	149 (122)	0.15, 0.16	150 (115)	0.40, 0.37	0.23
3	156 (114)	0.20, 0.19	159 (117)	0.37, 0.38	0.18
4	158 (108)	0.38, 0.38	164 (123)	0.42, 0.39	0.02
5	162 (111)	0.25, 0.25	165 (111)	0.42, 0.43	0.17
6	169 (131)	0.12, 0.12	165 (119)	0.41, 0.43	0.20
7	166 (121)	0.23, 0.22	176 (126)	0.40, 0.39	0.17
8	174 (138)	0.13, 0.13	184 (149)	0.31, 0.29	0.17
9	200 (148)	0.23, 0.22	203 (158)	0.39, 0.33	0.13
10	249 (211)	0.02, 0.02	250 (209)	0.12, 0.12	0.10
11	251 (208)	0.04, 0.04	256 (220)	0.22, 0.23	0.18
12	251 (214)	0.09, 0.09	259 (205)	0.08, 0.08	0.00
13	253 (220)	0.00, 0.00	263 (225)	0.27, 0.27	0.27
14	127 (74)	0.09, 0.10	129 (76)	0.52, 0.52	0.41
15	127 (70)	0.01, 0.02	139 (79)	0.31, 0.30	0.29
16	140 (78)	0.22, 0.24	135 (70)	0.44, 0.41	0.20
17	142 (82)	0.04, 0.05	140 (75)	0.44, 0.45	0.40
18	149 (81)	0.08, 0.10	142 (82)	0.52, 0.48	0.41
19	148 (78)	0.00, 0.00	156 (89)	0.18, 0.19	0.18
20	155 (79)	0.33, 0.31	157 (82)	0.50, 0.50	0.18
21	157 (87)	0.19, 0.18	158 (96)	0.47, 0.47	0.28
22	158 (88)	0.56, 0.55	159 (90)	0.66, 0.65	0.10
23	160 (93)	0.26, 0.24	167 (86)	0.50, 0.48	0.24
24	168 (86)	0.02, 0.00	167 (88)	0.20, 0.20	0.19
25	182 (107)	0.47, 0.46	173 (90)	0.55, 0.56	0.09

Table 2. *Fixed-glycogen content and O₂ uptake of the same pairs of hemidiaphragms as in Table 1*

Expt. no.	Control			Insulin-treated		
	Fixed glycogen (g./100 g. wet wt.)	O ₂ uptake (μ l./mg. wet wt./hr.)	O ₂ uptake (μ l./mg. dry wt./hr.)	Fixed glycogen (g./100 g. wet wt.)	O ₂ uptake (μ l./mg. wet wt./hr.)	O ₂ uptake (μ l./mg. dry wt./hr.)
1	0.19, 0.18	0.84	—	0.20, 0.20	0.85	—
2	0.13, 0.12	1.79	—	0.10, 0.11	1.65	—
3	0.16, 0.16	1.50	—	0.16, 0.17	1.43	—
4	0.18, 0.18	1.49	—	0.17, 0.17	1.55	—
5	0.14, 0.14	0.84	—	0.20, 0.17	0.80	—
6	0.17, 0.18	1.50	—	0.17, 0.17	1.47	—
7	0.16, 0.16	1.21	—	0.15, 0.16	1.31	—
8	0.09, 0.09	1.50	—	0.11, 0.10	1.37	—
9	0.21, 0.16	1.25	—	0.13, 0.14	1.17	—
10	0.07, 0.07	0.95	—	0.09, 0.09	0.95	—
11	0.10, 0.09	0.90	—	0.08, 0.08	0.85	—
12	0.07, 0.07	0.93	—	0.09, 0.09	0.95	—
13	0.09, 0.09	0.85	—	0.09, 0.09	0.86	—
14	0.21, 0.23	1.40	5.90	0.22, 0.22	1.38	5.95
15	0.20, 0.21	1.33	5.95	0.16, 0.16	1.25	5.25
16	0.14, 0.14	1.67	7.58	0.17, 0.19	1.77	7.18
17	0.17, 0.18	1.32	5.66	0.23, 0.22	1.44	6.55
18	0.17, 0.22	1.26	5.34	0.17, 0.18	1.26	5.30
19	0.16, 0.16	1.36	5.99	0.18, 0.18	1.35	5.80
20	0.30, 0.30	1.41	5.97	0.26, 0.25	1.34	5.32
21	0.14, 0.14	1.95	8.25	0.15, 0.13	1.75	7.30
22	0.26, 0.23	1.50	6.47	0.23, 0.23	1.47	6.62
23	0.23, 0.24	1.74	7.75	0.21, 0.21	1.65	7.10
24	0.21, 0.20	1.16	4.94	0.19, 0.20	1.26	5.58
25	0.21, 0.20	1.48	6.33	0.28, 0.28	1.54	6.65

were weighed on a torsion balance. One pair was transferred to 5 ml. of 80% (w/v) methanol to determine the free glucose and glucose 1-phosphate, another pair to 5 ml. of 5% (w/v) trichloroacetic acid for the determination of free and fixed glycogen, and, if present, the third pair was used to determine the dry weight. Free and fixed glycogen and the free glucose and glucose 1-phosphate concentrations were determined in duplicate in the trichloroacetic acid and methanol extracts by the method described in a previous paper (Kemp & Kits van Heijningen, 1954; cf. Mendel, Kemp & Myers, 1954).

RESULTS

In Table 1 the effect of insulin on the free-glycogen content of the diaphragms is shown. The pairs have been arranged in order of increasing weight. In the last 12 experiments the dry weight of a sample of each pair of hemidiaphragms was determined, and the O₂ consumption calculated/mg. dry wt. This was done to exclude any influence that insulin might have had on the wet weight of the tissues. In 22 out of these 25 experiments there was a rise in glycogen as compared with the controls, which was entirely accounted for by the increase in free glycogen. As shown in Table 2, there was also an increase in fixed-glycogen content in Expt. 25; in the control pair a very high free-glycogen content was found. In Expts. 4 and 12 there was no appreciable increase in glycogen content, only an increase in free glucose and glucose 1-phosphate (Table 3). The

weight of the sample in Expt. 12 was 251 mg., one of the heaviest of the series.

The values for free glucose plus glucose 1-phosphate, and the difference between the insulin-treated pairs and their controls, are given in Table 3. In a number of rats of the same weight and sex as used in these experiments, free glucose plus glucose 1-phosphate was determined in the diaphragm immediately after killing the animals; these values are given in Table 4.

DISCUSSION

The effect of insulin on the glycogen content of the diaphragm of the rat appears to be limited to the free-glycogen fraction. In the only experiment which gave a rise in fixed glycogen as well, the free-glycogen concentration found in the control pair was one of the highest, not only of this series but of all values found in diaphragms tested so far. In this case the free fraction might have reached its maximum.

The oxygen uptake of the diaphragms was in agreement with the values reported by Stadie & Zapp (1947), but higher than those reported by Gemmill (1940), except for Expts. 10–13. In these experiments, however, the wet weight of the tissue per flask was higher than that used by Gemmill. In one of these four experiments the insulin-treated pair showed no increase in glycogen. It seems likely

Table 3. *Free glucose plus glucose 1-phosphate concentrations in the same pairs of hemidiaphragms as in Table 1*

Expt. no.	Control		Insulin-treated		Difference between controls and insulin-treated pairs
	Wt. of muscle sample (mg.)	Free glucose + glucose 1-phosphate (g./100 g. wet wt.)	Wt. of muscle sample (mg.)	Free glucose + glucose 1-phosphate (g./100 g. wet wt.)	
1	20	0.18, 0.20	33	0.22, 0.22	+0.03
2	27	0.12, 0.13	35	0.06, 0.11	-0.04
3	42	0.11	42	0.16	+0.05
4	50	0.03	41	0.07	+0.04
5	29	0.08, 0.14	45	0.20, 0.17	+0.07
6	38	0.16	46	0.09	-0.07
7	45	0.13	50	0.10	-0.03
8	36	0.11, 0.11	35	0.11	0.00
9	53	0.14	45	0.12, 0.11	-0.02
10	38	0.11	41	0.18	+0.07
11	43	0.17	36	0.18	+0.01
12	37	0.09	54	0.19	+0.10
13	33	0.20	38	0.15	-0.05
14	30	0.22, 0.19	31	0.15, 0.12	-0.07
15	27	0.27	27	0.18	-0.09
16	32	0.07, 0.07	28	0.08, 0.07	+0.01
17	24	0.15, 0.15	26	0.15, 0.15	0.00
18	38	0.15, 0.18	38	0.11, 0.16	-0.03
19	52	0.19, 0.20	38	0.17, 0.17	-0.03
20	42	0.27, 0.27	40	0.22, 0.22	-0.05
21	29	0.07, 0.11	32	0.04, 0.06	-0.04
22	37	0.12, 0.13	38	0.16, 0.17	+0.04
23	38	0.19, 0.17	50	0.20, 0.22	+0.03
24	37	0.20, 0.23	36	0.17, 0.20	-0.03
25	43	0.15, 0.15	41	0.19, 0.18	+0.04

Table 4. *Free glucose plus glucose 1-phosphate content of normal rat diaphragms*

Female rats, 80-115 g. body wt., were killed by bleeding under pentobarbitone anaesthesia. Mean values of three determinations are given $\pm$ s.e.

Rat no.	Wt. of samples (mg.)	Free glucose + glucose 1-phosphate (g./100 g. wet wt.)
1	28-41	0.13 $\pm$ 0.01
2	28-56	0.13 $\pm$ 0.03
3	28-43	0.13 $\pm$ 0.04
4	31-37	0.13 $\pm$ 0.03
5	36-60	0.11 $\pm$ 0.03
6	57-60	0.10 $\pm$ 0.03
7	51-70	0.22 $\pm$ 0.04
8	47-76	0.19 $\pm$ 0.03
9	49-72	0.22 $\pm$ 0.02
10	39-42	0.19 $\pm$ 0.03

that the comparatively low oxygen consumption in these experiments and the lack of increase in glycogen content in one of them is related to the greater weight and presumably greater thickness of these pairs, whereby the inner cells of the diaphragm might have taken no part in the metabolism (cf. Gemmill, 1940). This might also explain why the greatest increase in glycogen is found in the diaphragms with the lowest weight (see also Liébecq, 1954). For diaphragms of greater weight

less insulin/mg. of tissue was available for fixation (Haugaard, Vaughan, Haugaard & Stadie, 1954) and this might have exerted an influence in the same direction.

Calculation of the oxygen consumption/mg. dry wt. showed no discrepancies compared with the consumption/mg. wet wt. which might have pointed to an influence of insulin on the wet weight of the tissue.

Since the animals were on ordinary laboratory diet, which contains about 60% of carbohydrate, and had access to food for 16 hr. a day, it was considered unlikely that insulin would cause a significant increase in oxygen consumption (Kerly & Ottaway, 1954). Therefore the agreement between oxygen consumption of insulin-treated pairs and their controls was used as a criterion for the similarity of circumstances. Pairs showing significant differences were rejected, though such differences did not materially alter the insulin effect.

The fixed glycogen remains remarkably constant in all experiments except in Expt. 25. It does not seem very likely therefore that the increase in free glycogen could have been derived from an initial increase in fixed glycogen, which would be abolished subsequently by transfer of an equal amount of glycogen from the fixed to the free fraction. A similar increase in free glycogen under the influence

of insulin is reported by Meyer, Russel, Platner, Purdy & Westfall (1955) in breis of rat's cardiac muscle. As pointed out by these authors, the normal intracellular relationship between the two fractions is disrupted in this case; this makes a quantitative transfer of the synthesized glycogen from the fixed to the free fraction even more unlikely. Therefore, direct storage of the extra glycogen produced under the influence of insulin in the free fraction seems at present the more simple explanation of the results obtained. In the beginning of a tetanic contraction Bloom & Knowlton (1953) demonstrated a decrease in free glycogen only. These authors (cf. Russell & Bloom, 1955) together with Meyer *et al.* (1955) take the view previously formulated by Przyłęcki & Majmin (1935), that it is the free fraction which takes part actively in the metabolism of glycogen, whereas the fixed fraction, which in their experiments remains more or less constant under various circumstances, would act as a depot. On the other hand, in experiments similar to those of Bloom & Knowlton (1953), but with less-frequent stimuli, either a decrease in both glycogen fractions or no effect at all has been obtained (Kits van Heijningen & Kemp, 1955). Neither in the experiments of Bloom nor in our own on contracting rat muscle could a transfer of glycogen from the free to the fixed form be excluded. Such a transition does not seem unlikely *a priori* if one assumes that one of these fractions acts as a depot. It has been demonstrated by Szent Györgyi (1951) that glycogen and myosin form an insoluble complex *in vitro*, which dissociates upon addition of adenosine triphosphate. A similar effect was obtained by the author with rat myosin and glycogen prepared from rat liver by extraction with trichloroacetic acid at room temperature. This does not, of course, prove that the fixed glycogen in intact muscle is also bound to myosin and released by adenosine triphosphate. Szent Györgyi (1951) assumes that this myosin-glycogen complex and its dissociation by adenosine triphosphate might play a part in the regulation of carbohydrate metabolism in muscle. Some light might be thrown on the sequence of events during normal muscular activity if it were possible to block the supposed transition from the free to the fixed form. From the present experiments it appears that conditions which promote glycogen deposition in muscle provoke an increase in free glycogen only.

SUMMARY

1. The effect of insulin on free and fixed glycogen of rat diaphragm has been investigated *in vitro*.

2. The increase in glycogen content brought about by insulin is limited to the free fraction.

3. The significance of this finding and the possible roles of both glycogen fractions in muscular contraction are discussed.

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