The Effect of Fluoropyruvate on the Respiration of Animal-tissue Preparations

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The study of fluoropyruvic acid was prompted by the expectation that this substance would have potent antimetabolic properties. In particular, it was expected that this fluoroanalogue of pyruvic acid would be able to interfere with some steps of the tricarboxylic acid cycle or with other essential metabolic mechanisms in which pyruvate occupies a key position. This anticipation has been refuted by the outcome of the pharmacological trials, which revealed low toxicity of fluoropyruvate for animals and showed that this compound is not metabolized *in vivo* via fluoroacetic acid (Mager & Blank, 1954; Gal, Peters & Wakelin, 1954). On the other hand, fluoropyruvate proved to be much more effective than fluoroacetate in inhibiting bacterial growth (Mager, Goldblum-Sinai & Blank, 1955). In addition, fluoropyruvate was found to inhibit the oxidation of various substrates by bacterial suspensions (Mager, in preparation). A similar inhibition of respiratory activity was observed also in tissue preparations. The present paper deals with some aspects of the mechanism underlying this inhibition.

MATERIALS AND METHODS

Fluoropyruvic acid was synthesized as described by Mager & Blank (1954). Sodium fluoroacetate was prepared according to the method described by Bergmann & Blank (1953). Oxaloacetic acid was prepared by the method of Heidelberger & Hurlbert (1950). Diphosphopyridine nucleotide (DPN) of 95% purity and triphosphopyridine nucleotide (TPN) of about 40% purity were products of the Sigma Chemical Co. Reduced DPN (DPNH) was obtained by reduction of DPN with crystalline yeast alcohol dehydrogenase as described by Pullman, Colowick & Kaplan (1950). All other chemicals were pure or A.R. products.

Crystalline yeast alcohol dehydrogenase was prepared according to Racker (1950), and purified liver alcohol dehydrogenase was obtained by the method of Bonnichsen & Wassén (1948). In the preparation of pig-heart lactic dehydrogenase, the procedure of Straub (1939) was followed. Malic dehydrogenase and *iso*citric dehydrogenase were crude preparations, obtained by extraction of ox-heart acetone-dried powder as described by Ochoa (1952).

The preparation of the guinea-pig-kidney mitochondria has already been described in detail (Mager & Avi-Dor, 1956), and here only a brief outline of the method is given. The excised kidneys were homogenized with a solution of 0·15 M-KCl–0·05 M phosphate, pH 7·4, in a Potter–Elvehjem tissue grinder. The homogenate was centrifuged at 1500 rev./min. for 10 min., and the sediment containing nuclei and cell debris was discarded. The supernatant was then spun at 12 000 rev./min. for 15 min. in a refrigerated 'International' centrifuge. The sediment was resuspended in the KCl–phosphate solution and again centrifuged at 12 000 rev./min. for 15 min. The final sediment was taken up in the KCl–phosphate solution and added to the reaction mixture in the amounts indicated in the legends.

Manometric experiments were carried out by the conventional Warburg technique. Spectrophotometric examinations were performed in the Beckman model DU spectrophotometer, with silica cells of 1 cm. light path. In experiments with neotetrazolium as final electron acceptor, the amount of mitochondria was one-third of that normally employed in the manometric experiments. The amount of formazan formed was estimated visually in arbitrary units.

The DPN content of the mitochondrial preparation was estimated by the procedure of Holzer, Goldschmidt, Lamprecht & Helmreich (1954), alcohol dehydrogenase being used for the reduction of the extracted DPN.

Urease activity was determined by measuring CO_2 output in the Warburg apparatus, with 50 μmoles of urea, 300 μg. of crystalline urease and 1 ml. of M acetate buffer, pH 4·9; total vol., 3·0 ml.

For quantitative estimation of SH groups, the method of Grunert & Phillips (1951) was adopted. Fluorine was determined by the method of Eger & Yarden (1954).

Citric acid was estimated by the method of Natelson, Pincus & Lugovoy (1948).

RESULTS

Inhibition of the oxidation of pyridine nucleotide-linked substrates

As may be seen from Table 1, fluoropyruvate (0·33 μmole/ml.) produced a strong inhibition of oxygen uptake by guinea-pig-kidney mitochondria with various Krebs-cycle intermediates, except succinate.

The inhibitory effect on the respiration usually attained its maximum without any appreciable time lag. This was in contrast to the course of inhibition observed with fluoroacetate, which even at much higher concentration (8–10 μmoles/ml.) did not affect the rate of mitochondrial respiration

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during a 60–90 min. observation period. Accumulation of citrate, a characteristic feature of fluoroacetate poisoning (Liébecq & Peters, 1949), did not occur in the case of fluoropyruvate inhibition. These results provide further evidence in favour of the conclusion that fluoropyruvate itself acts, and is not converted into fluoroacetate (cf. Mager & Blank, 1954).

Since all the oxidative systems which have been found susceptible to the action of fluoropyruvate are known to be linked to pyridine nucleotide coenzymes, it appeared likely that fluoropyruvate might interfere with the catalytic function of these coenzymes. This assumption was supported by the finding that the inhibition pattern remained unaltered when oxygen was replaced by a suitable dye (methylene blue, 1 μmole/ml.; 2:6-dichlorophenol-indophenol, 1 μmole/ml.; or neotetrazolium chloride 20 μg./ml.) as an electron acceptor, thus replacing the cytochrome system.

Table 1. *Effect of fluoropyruvate on the oxidation of different substrates by guinea-pig-kidney mitochondria*

Each flask contained 0·5 ml. of suspension of the kidney particles (15–20 mg. dry wt.), 50 μmoles of phosphate (pH 7·4), 6 μmoles of ATP, 6 μmoles of $MgCl_2$, 50 μmoles (or 100 μmoles of racemates) of the different substrates (as sodium salts). DPNH was generated *in situ* by adding to the above reaction mixture the following system: 0·33 μmole of DPN, 0·1 ml. of 95% ethanol, 0·3 ml. of yeast alcohol dehydrogenase (approx. 200 000 units), and 90 μmoles of semicarbazide. Total vol., 3 ml. Centre well contained 0·2 ml. of 20% KOH. Incubation at 37°. Gas phase: air. Equilibration time: 10 min. (The mitochondrial suspension was added last to the chilled flasks.) Readings were taken at 5–10 min. intervals for 90 min.

Substrate	Fluoropyruvate (μmoles)	O_2 uptake (μmoles/hr.)	Inhibition (%)
Succinate	0·0	21·6	—
	0·6	21·8	—
	1·3	21·2	2
	2·5	20·8	4
DL-Malate	0·0	23·0	—
	0·6	0·9	96
	1·3	1·0	96
	2·5	0·8	97
Fumarate	0·0	21·5	—
	0·6	16·4	24
	1·3	12·1	44
	2·5	3·7	83
Citrate	0·0	21·5	—
	1·3	14·3	33
	2·5	7·3	66
DL-*iso*Citrate	0·0	20·0	—
	0·6	20·1	0
	1·3	16·0	20
	2·5	9·0	55
L-Glutamate	0·0	23·0	—
	0·6	22·0	4
	1·3	9·8	57
	2·5	7·4	68
DPNH	0·0	9·8	—
	2·5	0·9	92

Effect of dehydrogenases

Soluble dehydrogenases were incubated with fluoropyruvate for 15–20 min. at 37°, and their activities compared afterwards with controls by following the rate of reduction of DPN (yeast and liver alcohol dehydrogenase, malic dehydrogenase), or TPN (*iso*citric dehydrogenase). Alternatively, the rate of oxidation of the reduced pyridine nucleotide was measured in the presence of a suitable substrate. No significant inhibition was observed with either of these systems. Similarly, no interference by fluoropyruvate was noted when mitochondrial malic dehydrogenase was assayed by determining the rate of DPNH oxidation in the presence of oxaloacetate in the following system: 0·3 μmole of DPNH, 50 μmoles of phosphate (pH 7·4), mitochondria (1–2 mg. dry wt.) and 10 μmoles of oxaloacetate added last at the start of the spectrophotometric measurements. At the low mitochondrial concentration used in this assay the DPNH oxidase activity was negligible and did not interfere with the dehydrogenase determination.

While testing lactic dehydrogenase under similar conditions, it was observed that fluoropyruvate underwent rapid reduction by this enzyme. This was demonstrated by determining both the oxidation of DPNH and the concomitant disappearance of the carbonyl group, as estimated by the Friedemann & Haugen (1943) test for α-keto acids. The product of this reduction (presumably fluorolactic acid) did not inhibit mitochondrial respiration.

Effect on the mitochondrial DPNH-oxidase system

The effect of fluoropyruvate on mitochondrial DPNH oxidase was studied both manometrically and spectrophotometrically. In the manometric experiments, where DPNH was generated by an ethanol–alcohol dehydrogenase system, fluoropyruvate produced a strong inhibition of oxygen uptake (see Table 1). The degree of this inhibition was considerably diminished by ethylenediaminetetraacetic acid (EDTA), which, according to Slater & Cleland (1952), increases the stability of heart-muscle sarcosomes. The protective effect of this agent was especially marked if it was included in the suspending medium (KCl–phosphate solution) used for the preparation of the kidney particles (see Methods). Addition of nicotinamide to the EDTA-treated particles caused a further decrease in the extent of fluoropyruvate inhibition (Fig. 1).

When, however, the effect of fluoropyruvate on the DPNH oxidase was tested by following the rate of decrease of extinction of the added DPNH, at 340 mμ. (with 0·33 μmole of DPNH, 0·5 ml. of

0·1 M phosphate buffer, pH 7·4, water to 3·0 ml.) no inhibition could be demonstrated.

Under the conditions of the manometric experiments, part of the DPNH added was found to be in the oxidized state (cf. Lehninger, 1951), and this part would be liable to destruction by the DPN-splitting enzyme (DPNase) present in the mitochondrial preparation. On the other hand, the DPNH used in the spectrophotometric tests is not susceptible to DPNase action (McIlwain & Rodnight, 1949). The discrepancy between the results obtained by the two methods, as well as the protective effect of nicotinamide, a potent inhibitor of DPNase (Mann & Quastel, 1941), suggested therefore that fluoropyruvate might act by enhancing the destruction of the intramitochondrial DPN, as was previously found in the case of chloretone inhibition (Mager & Avi-Dor, 1956). In fact, repeated determinations showed that a brief exposure (10–15 min. at 37°) of the mitochondrial preparation to fluoropyruvate under the usual test conditions resulted in a substantial lowering of its DPN content. Thus in one experiment the value decreased from 0·68 to 0·10 μmole of DPN/g. dry wt.

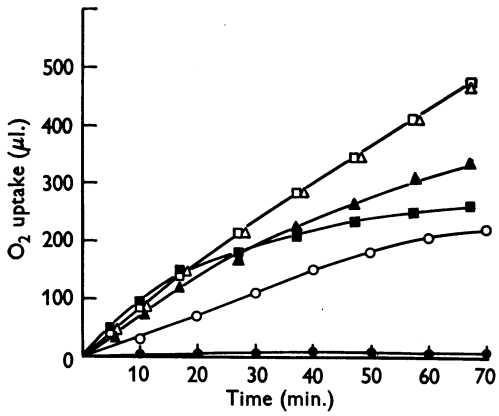

Fig. 1. Influence of ethylenediaminetetraacetic acid and nicotinamide on the fluoropyruvate-induced inhibition of DPNH oxidation in guinea-pig-kidney mitochondria. The system for generation of DPNH and the conditions of the experiment are described in Table 1. Open symbols: controls without fluoropyruvate; filled symbols: 3·75 μ-moles of fluoropyruvate/3 ml. of reaction mixture. ○ or ●, mitochondria (22 mg. dry wt./3 ml.) prepared by standard procedure; □ or ■, mitochondria (20 mg. dry wt./3 ml.) prepared with ethylenediaminetetraacetic acid (5×10^{-4} M); △ or ▲, mitochondria (20 mg. dry wt./3 ml.) prepared with ethylenediaminetetraacetic acid (5×10^{-4} M) and 30 μmoles of nicotinamide included in the reaction mixture.

Effect on the DPNH-oxidase system of a non-mitochondrial heart-muscle preparation

In order to obtain further information on the mode of action of fluoropyruvate on the DPNH-oxidase system, its effect on a particulate DPNH oxidase, prepared from pig heart muscle according to Edelhoch, Hayaishi & Teply (1952), was studied.

This preparation oxidizes DPNH and succinate rapidly, but shows no activity towards other tricarboxylic acid cycle intermediaries. The DPNH oxidase of the heart-muscle preparation was found to be strongly depressed by fluoropyruvate, whereas the oxidation of succinate was not significantly impaired (Table 2). The degree of inhibition of the DPNH oxidase decreased with rising amounts of the heart-muscle preparation used (Table 3).

When methylene blue was used as hydrogen acceptor, the inhibitory effect of fluoropyruvate on the heart-muscle preparation, contrary to the findings with mitochondrial preparations, was completely abolished (Table 2). It appears therefore that fluoropyruvate inhibits the DPN–cytochrome *c* reductase activity but does not affect the diaphorase function of the DPNH-oxidase system.

The DPNase activity of the heart-muscle preparation was much weaker than that of the guinea-pig mitochondria, and, under the conditions of the

Table 2. *Effect of fluoropyruvate on the DPNH oxidase and succinoxidase activity of pig heart-muscle preparation*

Each flask contained 0·2 ml. of the heart-muscle preparation (4 mg. dry wt.) and 0·8 ml. of 0·5 M phosphate buffer, pH 7·4; total vol., 3·0 ml. For the measurement of succinoxidase activity 0·5 ml. of 0·5 M succinate was added. DPNH was generated as described in Table 1. To the flasks indicated by MB, 15 μmoles of methylene blue and 10 μmoles of KCN were added. The substrates were tipped in from the side arm following pre-incubation of the otherwise complete reaction mixture for 10 min. at 37°. Other conditions of experiment were as in Table 1.

	O_2 uptake (μmoles/hr.)					
			Fluoropyruvate			
	Control		2·5 μmoles		3·75 μmoles	
Substrate	MB absent	MB present	MB absent	MB present	MB absent	MB present
DPNH	14·0	8·5	7·0	7·8	0·4	8·6
Succinate	12·7	—	12·0	—	10·1	—

experiment, added DPN could be recovered almost quantitatively even after 30 min. incubation, both in the presence and in the absence of the inhibitor. Therefore, the inhibitory action of fluoropyruvate on the DPNH oxidase of the heart-muscle preparation could not be ascribed to the destruction of DPN.

Prevention of the inhibitory effect of fluoropyruvate by thiol compounds

Cysteine or mercaptosuccinate, added before or simultaneously with fluoropyruvate, prevented its inhibitory effect on the oxidative activity of both mitochondrial and heart-muscle preparations (Table 4).

However, the inhibition, if once established, could not be reversed by subsequent addition of thiol compounds. These findings pointed to the possibility that fluoropyruvate might be reacting with SH groups. Since urease is known to be very sensitive to SH reagents (Hellerman, Chinard & Deitz, 1943), the effect of fluoropyruvate on this enzyme was examined. It was found that fluoropyruvate (3·75 μmoles/3 ml.) caused an 85 % inhibition of the urease activity. Here, too, cysteine or mercaptosuccinate prevented the inhibition when added before or simultaneously with fluoropyruvate.

The assumption that fluoropyruvate acts as an SH reagent was further supported by the finding that addition of the fluoro compound to some mercapto derivatives (e.g. mercaptosuccinic acid, ethanethiol) resulted in liberation of one equivalent of HF (as determined titrimetrically and by fluorine estimation), with the simultaneous disappearance of one equivalent of SH group. The reaction products showed a characteristic absorption in the ultraviolet region with a maximum at 270–275 mμ. (type I spectrum). If the thiol compound contained an unsubstituted amino group in the α- or β-position relative to the SH group (e.g. cysteine, mercaptoethylamine, homocysteine), its reaction product with fluoropyruvate exhibited an absorption peak at 300 mμ. (type II spectrum), with a molar extinction nearly ten times that obtained with the type I spectrum compounds (Fig. 2).

Table 3. *Effect of fluoropyruvate on the oxidation of DPNH by varying amounts of pig heart-muscle preparation*

Each flask contained pig heart-muscle preparation as indicated and 0·8 ml. of 0·5 M phosphate buffer, pH 7·4. DPN was added from the side arm after 10 min. incubation of the enzyme preparation with fluoropyruvate. The system for the generation of DPNH and other conditions of the experiment were as in Table 1.

Heart-muscle preparation (mg. dry wt.)	Fluoro-pyruvate (μmoles)	O_2 uptake (μmoles/hr.)	Inhibition (%)
1·35	0	5·4	0
	0·125	3·3	45
	0·25	1·9	65
2·25	0	11·0	0
	0·125	6·5	41
	0·25	5·3	52
4·5	0	14·6	0
	0·125	15·0	0
	0·25	10·7	26
9·0	0	22·8	0
	0·125	21·0	7
	0·25	21·2	7

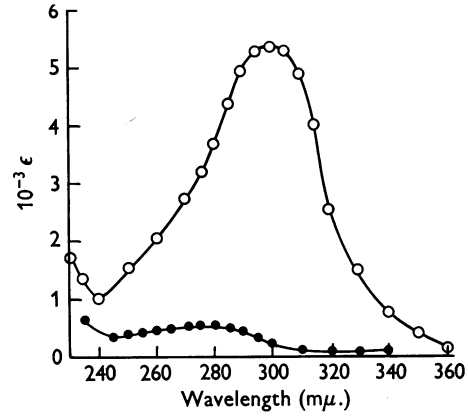

Fig. 2. Absorption spectra of the reaction products between fluoropyruvate and thiol compounds. Reaction mixture: 100 μmoles of aminotrishydroxymethylmethane buffer, pH 8; 10 μmoles of fluoropyruvate, 0·2 μmole of the thiol compound. Total vol., 3 ml. Control cuvette: thiol compound omitted. ○, Cysteine; ●, mercaptosuccinate. Molarity was calculated for the thiol compound (0·2).

Table 4. *Prevention of fluoropyruvate inhibition by thiol compounds*

The system for generation of DPNH was as in Table 1. (*a*) Guinea-pig mitochondria (25 mg.); (*b*) heart-muscle preparation (8·5 mg.). Cysteine (5 μmoles) was added either (1) before the addition of the particles, or (2) from the side arm after 15 min. incubation of the reaction mixture. Mercaptosuccinate (5 μmoles) was added before the particles. Other conditions were as in Table 1.

	O_2 uptake (μmoles/hr.)							
	Control				Fluoropyruvate (3·75 μmoles)			
	No addition	Cysteine (1)	Cysteine (2)	Mercapto-succinate	No addition	Cysteine (1)	Cysteine (2)	Mercapto-succinate
(*a*)	16·0	7·8	15·0	13·5	1·1	7·1	3·1	12·4
(*b*)	22·0	21·0	—	21·5	6·4	15·2	—	21·0

Table 5. *Effect of alkylating* SH *reagents on the respiration of guinea-pig-kidney mitochondria*

Conditions of experiments as in Table 1.

Substrate	Inhibitor (μmoles/3 ml.)	O_2 uptake (μmoles/hr.) Fluoropyruvate	Iodoacetate	Iodoacetamide
Succinate	0·00	21·7	21·7	21·7
	0·62	19·7	20·5	—
	1·25	19·8	19·8	21·6
	2·50	21·8	18·7	21·6
DL-Malate	0·00	9·9	9·9	9·9
	0·62	8·4	4·9	—
	1·25	7·9	2·8	0·8
	2·50	1·8	2·5	0·4

Comparison of the inhibitory effect of fluoropyruvate with known alkylating SH *reagents*

The stoicheiometric nature of the reaction between fluoropyruvate and thiol compounds seemed to be most readily accounted for by the assumption that fluoropyruvate acts as an alkylating agent. It appeared interesting, therefore, to examine the effects of some known alkylating thiol reagents on the respiration of the guinea-pig mitochondria. As can be seen from Table 5, iodoacetic acid and iodoacetamide inhibited the oxidation of malate, but did not affect significantly the succinoxidase activity.

DISCUSSION

The action of fluoropyruvate on the respiration of isolated tissue particles resembles in many essential points the inhibition pattern of chloretone and other narcotic substances, as described by Quastel and his co-workers (Quastel & Wheatley, 1932; Michaelis & Quastel, 1941) in homogenates, and recently studied by Mager & Avi-Dor (1956) in fractionated tissue preparations. Both chloretone and fluoropyruvate affect selectively the oxidative systems linked to the pyridine nucleotide coenzymes. In both instances the inhibition is correlated with an increased destruction of the intramitochondrial DPN.

The sequence of events leading to the coenzyme inactivation is not yet well understood. In the case of fluoropyruvate it appears likely that this compound acts primarily by blocking some SH groups which are essential for the maintenance of the structural integrity of the mitochondrial unit. The hypothesis is favoured by the findings that fluoropyruvate shows chemical properties of a typical alkylating SH reagent and that known reagents of this type (e.g. iodoacetate) affect mitochondrial respiration in a closely analogous manner. The partial protection afforded by EDTA against fluoropyruvate inhibition seems to be attributable to the suppression of the ATPase activity resulting from chelation of a metal activator (calcium) (see Slater & Cleland, 1952). This interpretation is consistent with the view that ATP is required for the preservation of the intramitochondrial coenzymes (Raaflaub, 1953*a*, *b*; Brenner-Holzach & Raaflaub, 1954; Mager & Avi-Dor, 1956), and points to the possibility that the primary event in the fluoropyruvate inhibition may be an interference with the regenerative cycle of ATP. The assumption that this interference is due to the SH-binding capacity of fluoropyruvate may be pertinent to the postulated participation of sulphydryl bonds in the phosphorylative mechanism associated with DPNH oxidation (Lehninger, 1953–4).

The similarity in action pattern between fluoropyruvate and chloretone is also striking in respect of their effect on cytochrome *c* reductase (cf. Mager & Avi-Dor, 1956). Both compounds inhibit the cytochrome reductase of the non-mitochondrial heart-muscle preparation, but do not significantly affect the corresponding system in the mitochondria. Further work is needed to decide whether the discordant behaviour of the two functionally analogous systems reflects an intrinsic difference in their enzymic make-up, or depends rather on some physicochemical factors which differ in the two tissue preparations.

SUMMARY

1. In a mitochondrial preparation from guinea-pig kidney, fluoropyruvate inhibits the activity of the pyridine nucleotide-linked oxidative systems.

2. Accumulation of citrate, a characteristic feature of fluoroacetate poisoning, does not take place.

3. The inhibition is correlated with the destruction of endogenous pyridine nucleotide.

4. Ethylenediaminetetraacetic acid diminishes the degree of inhibition induced by fluoropyruvate. Nicotinamide enhances the protective action of the chelating agent.

5. In the presence of lactic dehydrogenase, fluoropyruvate is rapidly reduced by reduced diphosphopyridine nucleotide (DPNH), yielding a compound devoid of inhibitory properties.

6. In a non-mitochondrial heart-muscle preparation, fluoropyruvate blocks the DPNH-oxidase

system at the stage of cytochrome *c* reductase; the diaphorase activity is not affected.

7. Thiol compounds react chemically with fluoropyruvate, thereby preventing its inhibitory activity. The reaction products show characteristic spectra in the ultraviolet region.

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The Metabolism of Short-chain Fatty Acids in the Sheep

4. THE PATHWAY OF PROPIONATE METABOLISM IN RUMEN EPITHELIAL TISSUE*

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Propionic acid is metabolized by the epithelial tissue of the rumen, and it is likely that a substantial part of the propionic acid produced by microbial activity in the rumen is metabolized by this tissue. In a previous paper in this series (Pennington, 1954) it was shown that the rate of metabolism of propionic acid by sections of sheep-rumen epithelium increased with the concentration of carbon dioxide/bicarbonate in the system. It was accordingly suggested that propionic acid (or propionyl coenzyme A) may be carboxylated by addition of carbon dioxide. The possibility that the observed effect was due to a recognized fixation reaction, namely addition of carbon dioxide to pyruvate formed from propionate, appeared less likely from certain other evidence which was presented. Conversion of propionate into succinate in liver and kidney would provide an explanation for the results of the Cleveland investigators (Lorber, Lifson, Sakami & Wood, 1950; Shreeve, 1952), who found that the α- and β-carbon atoms of propionate were completely randomized during the formation of glycogen or acetyl groups. On the other hand, Wisconsin workers (Huennekens, Mahler & Nordmann, 1951; Mahler & Huennekens, 1953) postulate a pathway of α-oxidation via lactate in liver.

The present paper is concerned with further investigations with carbon-14, into the pathway of metabolism of propionate in sheep-rumen epithelial tissue. The results obtained support the theory that succinate is formed from propionate and carbon dioxide. In addition, some studies were made on the 'antiketogenic' action of propionate with this tissue and a mechanism is suggested to account for the suppression of ketone-body formation by propionate.

* Part 3: Annison & Pennington (1954).