- Coxon, R. V. (1953). *Biochem. J.* **55**, 545.
 Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 Green, D. E., Loomis, W. F. & Auerbach, V. H. (1948). *J. biol. Chem.* **172**, 389.
 Judah, J. D. (1951). *Biochem. J.* **49**, 271.
 Knodt, C. B. & Peterson, W. E. (1946). *J. Dairy Sci.* **29**, 115.
 Korkes, S., del Campillo, A., Gunsalus, I. C. & Ochoa, S. (1951). *J. biol. Chem.* **193**, 721.
 Krebs, H. A. (1943). *Advanc. Enzymol.* **3**, 191.
 Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
 Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.
 Liébecq, C. & Peters, R. A. (1949). *Biochim. biophys. Acta*, **3**, 215.
 Melrose, D. R. & Turner, C. (1953). *Biochem. J.* **53**, 296.
 Meyerhof, O. (1927). *Biochem. Z.* **183**, 176.
 Moore, R. O. & Nelson, W. L. (1952). *Arch. Biochem. Biophys.* **36**, 178.
 Natelson, S., Pincus, J. B. & Lugovoy, J. K. (1948). *J. biol. Chem.* **175**, 745.
 Neuberg, C., Strauss, E. & Lipkin, L. E. (1944). *Arch. Biochem.* **4**, 101.
 Ostern, P. (1933). *Hoppe-Seyl. Z.* **218**, 160.
 Peeters, G., Coussens, R. & Sierens, G. (1953). *Arch. int. Pharmacodyn.* **95**, 153.
 Potter, V. R. (1946). *J. biol. Chem.* **165**, 311.
 Potter, V. R. & Elvejehm, C. A. (1936). *J. biol. Chem.* **114**, 495.
 Pucher, G. W., Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.
 Shaw, J. C. (1942). *J. biol. Chem.* **142**, 53.
 Slater, E. C. (1953). *Nature, Lond.*, **172**, 975.
 Taylor, T. G. (1953). *Biochem. J.* **54**, 48.
 Turner, C. (1951). *Biochem. J.* **50**, 145.
 Turner, C. (1953). *Biochem. J.* **55**, xxix.
 Turner, C. (1954a). *Biochem. J.* **56**, 471.
 Turner, C. (1954b). *Biochem. J.* **58**, xxxi.
 Weil-Malberbe, H. & Bone, A. B. (1949). *Biochem. J.* **45**, 377.
 Weil-Malberbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.

The Metabolism of Citric Acid in the Mammary Gland

2. THE EFFECT OF *p*-NITROPHENOL AND OF FLUORIDE ON THE SYNTHESIS OF CITRIC ACID IN FLUOROACETATE-BLOCKED HOMOGENATES

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It has been shown in the first paper of this series that relatively large amounts of citric acid accumulated in homogenates of the lactating mammary gland of the guinea pig incubated with pyruvate or acetate in the presence of fumarate, despite the ability of the preparation to oxidize added citrate at rates corresponding to the overall rate of the citric acid cycle. In homogenates metabolizing pyruvate, the addition of *p*-nitrophenol (*p*NP) greatly accelerated the oxygen consumption without more than a small increase in the amounts of pyruvate disappearing and citrate accumulating. When acetate was the substrate, however, *p*NP caused marked inhibition of citrate formation (Turner, 1955).

The effect of *p*NP and other agents has now been studied in mammary homogenates poisoned with fluoroacetate. This substance is known to block the citric cycle by its enzymic conversion into a fluorotricarboxylic acid which specifically inhibits aconitase (Peters, 1952b). In view of the absence of inhibitory effects of fluoroacetate on other reactions of the citric acid cycle (Liébecq & Peters, 1949) and the fact that the equilibrium of the condensing reaction is far in the direction of citrate synthesis (Ochoa, 1954), the rate of accumulation of citric

acid may be expected to provide a measure of the rate of formation of 'active acetate'. In this system *p*NP caused a marked stimulation of respiration and citric acid synthesis, when pyruvate was the substrate, while it inhibited the formation of citrate from acetate. A preliminary account of part of this work has been given to the Biochemical Society (Turner, 1954).

EXPERIMENTAL

Material and methods. The mammary glands of lactating guinea pigs were passed through a Latapie mincer and the minced tissue was washed 3 times with cold 0.154M-KCl. The tissue was then ground in a Potter homogenizer in a medium of 0.154M-KCl containing 0.024M-KHCO₃ and 0.02M nicotinamide. The suspension was further diluted with 0.154M-KCl (isotonic) to give a 1 in 5 homogenate (dry wt. 50–60 mg./ml.).

Suspensions of kidney cortex (guinea pig or rabbit) were prepared essentially as described by Liébecq & Peters (1949). The kidneys were removed, cut in two and cooled in ice. Further operations were carried out in the cold room. The kidneys, after removal of the medulla, were passed through a Latapie mincer and then ground either in a mortar or in a Potter homogenizer. The medium was either 8.55% sucrose or the isotonic KCl containing KHCO₃ and

nicotinamide described above. The suspension was squeezed through muslin and centrifuged at low speed for 1 min.; the supernatant was then centrifuged for 30 min. at 10 000 rev./min. in an angle centrifuge. The sedimented material was resuspended in isotonic KCl and in most experiments centrifuged for a second 30 min. period at high speed. The final suspension of the washed material in KCl solution was then pipetted into the side arms of the Warburg flask. The statements once centrifuged or twice centrifuged will be used to indicate the number of centrifugings at high speed to which the tissue had been subjected.

The term 'homogenate' will be used to designate cell-free preparations which have not been subjected to centrifuging, such as mammary homogenates. The kidney preparations, which were essentially the resuspended insoluble matter isolated by high speed centrifuging, are referred to as 'suspensions'.

The basal medium, analytical methods and reagents were the same as previously employed (see Turner, 1955). The work was made possible by samples of sodium fluoroacetate generously given by Sir Rudolph Peters. α -Oxoglutarate was kindly given by Dr A. L. Greenbaum.

In experiments in which [carboxy- ^{14}C]acetate was used as substrate, the respiratory CO_2 was collected and its radioactivity measured as described by Popják & Tietz (1954).

Units. Metabolic quotients are expressed in the Q notation ($\mu\text{l./mg. dry wt./hr.}$). Dry weights were determined by evaporation in the steam oven of portions of the mammary homogenates or kidney suspensions and were corrected for the salt content of the diluting fluids.

RESULTS

Effect of fluoroacetate and pNP on the metabolism of pyruvate. Liébecq & Peters (1949) have shown that the oxygen consumption of pigeon brain suspensions oxidizing pyruvate in the presence of fumarate is

Table 1. *Effect of fluoroacetate on metabolism of pyruvate and of acetate*

Mammary homogenate, dry wt., 53 mg. per vessel. Basal medium and fumarate (0.005 M) in all vessels. Additions: pyruvate, 0.005 M; acetate, 0.005 M; fluoroacetate, 0.01 M; pNP, 2×10^{-4} M. Incubation period, 40 min.

Additions	$-Q_{\text{O}_2}$	$-Q_{\text{pyruvate}}$	Q_{citrate}
Pyruvate	9.7	9.6	2.6
Pyruvate, pNP	19.3	10.6	3.2
Pyruvate, fluoroacetate	9.4	9.9	9.0
Pyruvate, fluoroacetate, pNP	14.1	12.0	12.0
Acetate	11.5	—	3.4
Acetate, pNP	14.9	—	1.9
Acetate, fluoroacetate	8.2	—	5.4
Acetate, fluoroacetate, pNP	7.4	—	2.8

not inhibited by fluoroacetate, whereas it is inhibited by fluoride. These findings have been confirmed in the present study using mammary homogenates. As can be seen in Table 1, the addition of fluoroacetate (0.005 M) to mammary homogenates

metabolizing pyruvate did not diminish the amount of oxygen or of pyruvate taken up; the latter could be almost wholly accounted for as citric acid. It was further found that pNP (2×10^{-4} M), when added to the fluoroacetate-blocked system, increased the rates of respiration, pyruvate disappearance and citric acid formation to approximately the same extent.

The inhibitory effect of fluoride on the metabolism of pyruvate in mammary suspensions and its reversal by pNP have been described in the preceding paper (Turner, 1955). Table 2 shows that, in the presence of fluoroacetate, fluoride (0.01 M) retarded the formation of citrate from pyruvate and that in this system also, pNP reversed the inhibition caused by fluoride.

Table 2. *Effect of inhibitors on synthesis of citric acid*

Mammary homogenate, dry wt. 64.6 mg. per vessel. Basal medium; fumarate (0.01 M) and fluoroacetate (0.005 M) in all vessels. Additions: acetate, 0.01 M; pyruvate 0.01 M; fluoride, 0.01 M; arsenate, 0.005 M; pNP, 2×10^{-4} M. Incubation period, 35 min.

Additions	$-Q_{\text{O}_2}$	Q_{citrate}
Acetate	10.6	4.2
Acetate, pNP	9.0	0.5
Acetate, fluoride	6.0	0.1
Acetate, arsenate	8.8	0.4
Pyruvate	14.2	8.2
Pyruvate, pNP	17.8	13.7
Pyruvate, fluoride	8.5	4.4
Pyruvate, fluoride, pNP	11.8	9.0
Pyruvate, arsenate	10.6	3.5

Table 3. *Effect of inhibitors on synthesis of citric acid*

Mammary homogenate, dry wt. 60.6 mg. per vessel. Basal medium; fumarate (0.01 M) and fluoroacetate (0.005 M) in all flasks. Additions: acetate, 0.01 M; pyruvate, 0.01 M; fluoride, 0.01 M; pNP, 2×10^{-4} M; insulin, 2.5 mg./100 ml. Incubation period, 40 min.

Additions	$-Q_{\text{O}_2}$	Q_{citrate}
None	7.6	3.3
pNP	5.1	1.4
Acetate	8.9	4.7
Acetate, insulin	8.6	4.3
Acetate, pNP	8.3	1.2
Acetate, arsenate, 0.005 M	8.4	0.9
Acetate, arsenate, 0.02 M	6.6	0.4
Pyruvate	10.8	7.8
Pyruvate, insulin	11.2	7.3
Pyruvate, pNP	16.9	11.8
Pyruvate, arsenate, 0.02 M	5.9	2.6
Pyruvate, arsenate, 0.04 M	5.4	2.2

Effect of pNP on the metabolism of acetate in the presence of fluoroacetate. In unpoisoned mammary homogenates acetate appeared to be metabolized more readily than pyruvate, as shown by the

accumulation of larger amounts of citrate when acetate was the substrate (Table 1). In the presence of fluoroacetate, however, the yield of citrate from acetate was considerably smaller than from pyruvate, and did not greatly exceed the amount of citrate produced from fumarate alone, an observation previously made by Liébecq & Peters (1949) in their work with kidney suspensions. In contrast to its stimulating action on the metabolism of pyruvate in fluoroacetate-blocked mammary homogenates, *p*NP was inhibitory in the presence of acetate, causing marked retardation of citric acid formation. Fluoride strongly inhibited the formation of citrate from acetate, an effect which was intensified by *p*NP (Tables 1-3).

Metabolism of acetate in fluoroacetate-blocked kidney suspensions. For comparison with the results of Liébecq & Peters (1949), washed kidney cortex suspensions, prepared in most cases according to their directions, were incubated under the same experimental conditions as the crude mammary preparations. Both tissues behaved in essentially the same manner when metabolizing acetate plus fumarate in the presence of fluoroacetate. As shown in Table 4, the inhibition of citric acid forma-

concluded that the nitrophenol blocked the formation of fluorocitrate. Although these workers stated that the addition of ATP suppressed the protective effect of 2:4-dinitrophenol, it was thought necessary to check the effectiveness of

Table 5. *Effect of inhibitors on synthesis of citric acid from fumarate and acetate*

Kidney cortex suspension (twice centrifuged), dry wt. 12.3 mg. per vessel. Basal medium; fumarate (0.01M), acetate (0.01M) and fluoroacetate (0.005M) in all vessels. Additions: insulin, 2.5 mg./100 ml.; *p*NP, 2×10^{-4} M; arsenate, 0.005M; azide, 0.0025M; iodoacetate, 0.001M; arsenite, 0.0025M. Incubation period, 60 min.

Additions	$-Q_{O_2}$	Q_{citrate}
None	13.6	9.1
Insulin	11.9	8.9
<i>p</i> NP	14.5	5.2
Fluoride, 0.005M	7.4	3.9
Fluoride, 0.005M, <i>p</i> NP	10.0	0.7
Fluoride, 0.01M	7.8	1.4
Fluoride, 0.01M, <i>p</i> NP	9.3	0.6
Arsenate	9.1	0.8
Arsenate, <i>p</i> NP	11.0	0.9
Azide	10.6	5.1
Iodoacetate	4.5	0
Arsenite	1.6	0

Table 4. *Effect of pNP on synthesis of citric acid from fumarate and acetate*

Kidney cortex suspension (once centrifuged), dry wt. 30 mg. per vessel. Basal medium; fumarate (0.01M), acetate (0.01M) and fluoroacetate (0.005M) in all vessels. Initial inorganic P, 925 μ g. Incubation period, 40 min.

Additions	$-Q_{O_2}$	Q_{citrate}	Q_P
None	18.2	14.0	-11.6
<i>p</i> NP, 10^{-4} M	23.9	12.8	-8.1
<i>p</i> NP, 2×10^{-4} M	24.6	8.0	-2.0
<i>p</i> NP, 4×10^{-4} M	16.8	0.2	+3.3

tion by *p*NP increased with increasing concentrations of the inhibitor. Unlike mammary homogenates, kidney cortex suspensions were capable of esterifying appreciable amounts of inorganic phosphate without the addition of phosphate acceptors or fluoride. Thus a close connexion between the inhibitory effects of *p*NP on citric acid synthesis from acetate and on oxidative phosphorylation could be shown to exist (Table 4). In kidney suspensions, as in mammary homogenates, the inhibitory actions of fluoride and *p*NP on the formation of citrate from acetate were cumulative. In both preparations these inhibitors caused little reduction of respiration in comparison to the marked depression of citric acid synthesis (Table 5).

Effectiveness of fluoroacetate inhibition. Judah & Rees (1953) found that 2:4-dinitrophenol allowed the oxidation of citrate by kidney mitochondria to proceed despite the presence of fluoroacetate, and

Table 6. *Effect of fluoroacetate and pNP on metabolism of citric acid*

Mammary homogenate, dry wt. 53.2 mg. per vessel. Basal medium. Additions: fumarate, 0.005M; citrate, 0.005M; fluoroacetate, 0.005M; *p*NP, 2×10^{-4} M. Incubation period, 45 min.

Additions	$-Q_{O_2}$	Q_{citrate}
Fumarate	10.8	+0.6
Fumarate, fluoroacetate	7.1	+2.1
Citrate	11.8	-6.4
Citrate, <i>p</i> NP	18.7	-6.7
Citrate, fluoroacetate	8.6	-0.2
Citrate, fluoroacetate, <i>p</i> NP	9.5	-0.3
Fumarate, citrate, fluoroacetate	7.8	+1.8
Fumarate, citrate, fluoroacetate, <i>p</i> NP	7.9	+3.1

fluoroacetate under the present experimental conditions. Table 6 shows that fluoroacetate almost completely prevented the oxidation of added citrate, only a small part of which disappeared. If the onset of inhibition of citric acid oxidation were delayed until the fluorocitrate inhibitor is formed (see Liébecq & Peters, 1949; Peters, 1952b), some citrate might have 'leaked' in the early stages to be oxidized via the Krebs cycle. Experiments with [14 C]acetate showed that the loss of citrate was due to a small, continuous 'leak' past the barrier set up by the inhibitor. This resulted in the appearance of radioactivity in the respiratory CO_2 . As shown in Fig. 1, the rate of production of $^{14}CO_2$ increased with time throughout the experimental period. This

would be expected if there were competitive inhibition by the fluorotricarboxylic acid of the oxidation of the citric acid (see Judah & Rees, 1954) which was accumulating in the reaction mixture. No diminution of the inhibitory effect of fluoroacetate was observed in the presence of *p*NP.

Fig. 1. Production of $^{14}\text{CO}_2$ from ^{14}C acetate in the presence of fluoroacetate. Mammary homogenate. Basal medium: [carboxy- ^{14}C]acetate, 0.0025 M; fumarate, 0.0025 M; fluoroacetate, 0.005 M. Replicate vessels incubated for 0, 5, 10, 15, 25 and 40 min.

Effect of *p*NP and fluoride on the oxidation of [carboxy- ^{14}C]acetate. Judah & Williams-Ashman (1951) concluded, from respiration measurements, that fluoride diminished the inhibition by 2,4-dinitrophenol of the oxidation of acetate in a kidney cyclophorase. Although in the present work *p*NP has been found to reverse the inhibition by fluoride of the metabolism of pyruvate, the metabolism of acetate differed characteristically from that of pyruvate in being susceptible to inhibition by both inhibitors either singly or combined, as shown by the formation of citrate in mammary and in kidney suspensions. Since, however, the presence of fluoroacetate might have modified the effect of the other inhibitors, their action was investigated in an unblocked mammary homogenate with the aid of radioactive acetate. As shown in Table 7, the amount of radioactivity in the respiratory CO_2 derived from the oxidation of acetate was considerably diminished in the presence of either *p*NP or fluoride, which, when combined, almost abolished the oxidation of acetate.

Table 7. *Effect of pNP and fluoride on oxidation of acetate*

Mammary homogenate, dry wt. 58.6 mg. per vessel. Basal medium; [carboxy- ^{14}C]acetate (0.0025 M) and fumarate (0.0025 M) in all vessels. Additions: *p*NP, 2×10^{-4} M; fluoride, 0.005 M. Incubation period, 40 min.

Additions	$-Q_{\text{O}_2}$	Total activity of respiratory CO_2 (counts/min.)
None	9.0	38 800
<i>p</i> NP	6.0	14 400
Fluoride	5.6	11 600
Fluoride, <i>p</i> NP	4.1	1 620

Effect of other inhibitors. Arsenate (0.005–0.04 M) retarded the formation of citrate both from pyruvate and from acetate in fluoroacetate-blocked mammary homogenates and kidney suspensions, acetate being more sensitive than pyruvate to lower concentrations of arsenate. Azide (0.0025 M) diminished the yield of citrate from acetate, while arsenite (0.0025 M) and iodoacetate (0.001 M) abolished it (Tables 2, 3 and 5).

Effect of insulin. This hormone, which has been found to stimulate the synthesis of fatty acids from acetate in mammary gland slices (Balmain, Folley & Glascock, 1952), had no effect on the rate of formation of citric acid from pyruvate or acetate in mammary homogenates and in kidney suspensions when present in the concentration of 2.5 mg./100 ml. (Tables 3 and 5).

Effect of fluoroacetate on the metabolism of acetoacetate in mammary suspensions. As reported in the preceding paper (Turner, 1955), mammary homogenates metabolize acetoacetate. Fluoroacetate, either alone or in combination with *p*NP, had an inhibitory effect on the breakdown of acetoacetate (Table 8). As in the case of acetate, the amount of

Table 8. *Effect of fluoroacetate on oxidation of acetoacetate*

Mammary homogenate, dry wt. 58.8 mg. per vessel. Basal medium; fumarate (0.01 M) and acetoacetate (0.005 M) in all vessels. Additions: fluoroacetate, 0.005 M; *p*NP, 2×10^{-4} M. Incubation period, 50 min.

Additions	$-Q_{\text{O}_2}$	$-Q_{\text{ac-ac}}$
None	8.5	1.3
<i>p</i> NP	5.2	0.3
Fluoroacetate	4.3	0.4
Fluoroacetate, <i>p</i> NP	3.1	0.2

citric acid accumulating in the presence of fluoroacetate did not greatly exceed the amount of citrate formed from fumarate alone; fluoride caused some inhibition (Table 9).

Table 9. *Citric acid formation from acetoacetate*

Mammary homogenate, dry wt. 46.5 mg. per vessel. Basal medium; fumarate (0.005M) and fluoroacetate (0.005M) in all vessels. Additions: acetoacetate, 0.0025M; fluoride, 0.01M. Incubation period, 40 min.

Additions	$-Q_{O_2}$	Q_{citrate}
None	5.6	2.7
Fluoride	4.2	1.3
Acetoacetate	5.9	3.5
Acetoacetate, fluoride	4.5	2.1

Effect of α -oxoglutarate on the metabolism of pyruvate and acetate in fluoroacetate-blocked mammary homogenates. Since the inhibition of the citric acid cycle in the poisoned tissue results in the loss of a large amount of phosphate bond energy, which may be expected to have a profound influence on the reactions leading to the formation of citric acid, an attempt was made to restore to some extent the normal balance of the Krebs cycle reactions by the addition of α -oxoglutarate. It was found that the addition of α -oxoglutarate retarded the dis-

appearance of pyruvate and at the same time checked the formation of citric acid; *p*NP reversed the restraint imposed by the oxidation of α -oxoglutarate, and caused stimulation of citric acid synthesis in the manner characteristic of pyruvate. When acetate was the substrate, the concurrent oxidation of α -oxoglutarate did not result in the expected acceleration of the formation of citric acid. However, as shown in Tables 10 and 11, α -oxoglutarate appeared to protect the metabolism of acetate against the inhibitory action of *p*NP and this reagent had to be added in higher concentration in order to suppress the formation of citric acid.

DISCUSSION

Effect of pNP on the oxidation of pyruvate in fluoroacetate-blocked suspensions. It has been shown in the preceding paper (Turner, 1955) that the oxygen consumption of mammary homogenates metabolising pyruvate in the presence of fumarate was inadequate to account for the complete oxidation of the amount of pyruvate disappearing. The incomplete oxidation of pyruvate could only be

Table 10. *Effect of α -oxoglutarate on synthesis of citric acid from acetate and pyruvate*

Mammary homogenate, dry wt. 51.6 mg. per vessel. Basal medium; fluoroacetate (0.005M) in all vessels. Additions: fumarate, 0.01M; acetate, 0.01M; pyruvate, 0.01M; α -oxoglutarate, 0.005M; *p*NP, 2×10^{-4} M. Incubation period, 35 min.

Additions	$-Q_{O_2}$	$-Q_{\text{pyruvate}}$	$-Q_{\alpha\text{-oxoglut.}}$	Q_{citrate}
Fumarate	5.4	—	—	2.2
α -Oxoglutarate	5.3	—	5.9	0.7
α -Oxoglutarate, <i>p</i> NP	14.6	—	12.2	0.6
Fumarate, acetate	7.0	—	—	4.4
Fumarate, acetate, <i>p</i> NP	4.2	—	—	0.8
Fumarate, acetate, α -oxoglutarate	9.2	—	5.4	3.4
Fumarate, acetate, α -oxoglutarate, <i>p</i> NP	14.6	—	10.2	3.1
Fumarate, pyruvate	7.9	8.7	—	6.7
Fumarate, pyruvate, <i>p</i> NP	18.5	12.3	—	11.9
Fumarate, pyruvate, α -oxoglutarate	7.4	6.6	5.5	4.0
Fumarate, pyruvate, α -oxoglutarate, <i>p</i> NP	23.5	10.4	7.6	9.1

Table 11. *Effect of pNP and α -oxoglutarate on citric acid formation*

Mammary homogenate, dry wt. 42.5 mg. per vessel. Basal medium; fumarate (0.01M) and fluoroacetate (0.005M) in all flasks. Additions: acetate, 0.01M; pyruvate, 0.01M; α -oxoglutarate, 0.005M. Incubation period, 45 min.

Additions	$-Q_{O_2}$	Q_{citrate}
Acetate	7.7	4.3
Acetate; <i>p</i> NP, 2×10^{-4} M	4.2	0.8
Acetate; α -oxoglutarate	12.5	5.4
Acetate; α -oxoglutarate; <i>p</i> NP, 2×10^{-4} M	16.7	4.5
Acetate; α -oxoglutarate; <i>p</i> NP, 4×10^{-4} M	8.9	1.8
Pyruvate	10.4	7.7
Pyruvate; <i>p</i> NP, 2×10^{-4} M	18.4	13.0
Pyruvate; α -oxoglutarate	11.2	5.6
Pyruvate; α -oxoglutarate; <i>p</i> NP, 2×10^{-4} M	19.3	12.1

explained in part by the accumulation of citric acid, and it was concluded that other products were formed at the expense of energy derived from oxidative phosphorylation, since in the presence of *p*NP the substrate appeared to be oxidized to carbon dioxide and water. In the presence of fluoroacetate, however, almost all the pyruvate disappearing could be accounted for as citric acid and the amount of oxygen taken up corresponded to the theoretical amount expected for the conversion of pyruvate plus fumarate to citrate. While in the absence of fluoroacetate the addition of *p*NP resulted in a marked stimulation of respiration without a corresponding increase in the rate of pyruvate utilization, the fluoroacetate-blocked system responded to *p*NP with acceleration of oxygen consumption, pyruvate disappearance and citric acid accumulation to about the same extent.

It is generally recognized that the generation of energy derived from oxidative phosphorylation plays an important part in the regulation of metabolic rates (see Lardy & Wellman, 1952; Siekevitz & Potter, 1953). The susceptibility to the stimulating action of *p*NP of the conversion of pyruvate into citrate in the fluoroacetate-blocked system suggests that high-energy phosphate bond intermediates, generated in the course of this reaction, exert a restraining influence on the rate of its progress. This is supported by the observation that the addition of α -oxoglutarate, which largely restored to the fluoroacetate-blocked system the energy-producing reactions of the Krebs cycle, retarded the rate of conversion of pyruvate into citrate, and that *p*NP fully reversed this inhibition. Thus it may be concluded that, while the oxidation of pyruvate in the unpoisoned system is restrained by the phosphorylations accompanying the oxidation of the various intermediates of the citric acid cycle, the increased rate of citrate synthesis in the fluoroacetate-blocked system treated with *p*NP provides a measure of the capacity of the preparation to oxidize pyruvate when freed from rate-restricting influences.

Effect of pNP on the formation of citrate from acetate. Liébecq & Peters (1949) found that in unpoisoned washed kidney suspensions the amount of citrate formed from fumarate plus acetate did not greatly exceed the amount of citrate formed from fumarate alone, and concluded that the factor responsible for bringing acetate into the cycle was not very active in that preparation. In unpoisoned homogenates of the mammary gland of the guinea pig, however, acetate appeared to be metabolized more readily than pyruvate, since larger amounts of citric acid accumulated during the metabolism of acetate (see Table 1). In the presence of fluoroacetate, on the other hand, while the yield of citrate from pyruvate was greatly increased, the amount of citrate derived from acetate was relatively small; furthermore, *p*NP, which accelerated the conversion of pyruvate to citrate, was inhibitory when acetate was the substrate.

Acetate, as is well known, requires activation by ATP (see Lipmann, Jones & Black, 1952) while pyruvate does not (see Ochoa, 1952). The condensing reaction between acetyl CoA (derived from acetate or from pyruvate) and oxaloacetate, being common to both substances, only their activation need be examined. It is to be expected that the activation of acetate, which depends on a continuous supply of phosphate bond energy, should be inhibited by agents such as nitrophenols which interfere with the utilization of this energy. In the presence of fluoroacetate, the inhibition of many of the energy-giving reactions of the cycle would explain the slow rate of activation of acetate, which, under these conditions must depend on the oxidation of fumar-

ate as its only source of activating energy. However, the failure of attempts to restore a high rate of citrate synthesis from acetate by the addition of α -oxoglutarate to the fluoroacetate-blocked suspension, leaves open the question whether fluoroacetate also has an inhibitory effect specific for acetate. Bartlett & Barron (1947) and Liébecq & Peters (1949) have considered the possibility of a competitive action between acetate and fluoroacetate, which may also explain the findings discussed above. If this were the case, it should be possible to observe an activating effect of α -oxoglutarate on acetate in a system in which the oxidation of citrate is inhibited by the fluorocitrate inhibitor of Peters (1952*b*).

pNP as a reagent in the study of fluoride inhibition. Among metabolic inhibitors fluoride displays great versatility. Apart from its well-defined action on enolase, it is an inhibitor of phosphatases and some transphosphorylases (Meyerhof, 1951) and of reactions of the Krebs cycle (Peters, 1952*a*). Its inhibitory effect on the pyruvate oxidase system of pigeon brain suspensions has been noted by Liébecq & Peters (1949). Since, according to Peters (1952*a, b*), the term pyruvate oxidase includes pyruvate dehydrogenase plus the enzymes of the citric acid cycle, the point of inhibition was not defined. Similarly, fluoride is known to inhibit the α -oxoglutarate oxidase system (Slater & Holton, 1954). In mammary homogenates, as described in the preceding paper (Turner, 1955), fluoride retarded the metabolism of pyruvate, citrate and α -oxoglutarate. The inhibition of the oxidation of these intermediates might be attributed to the well-known and extensively studied inhibition by fluoride of the oxidation of succinate (see Slater & Bonner, 1952). However, it has now been shown that fluoride also acts at the stage at which pyruvate is converted into citrate. It has been shown, further, that *p*NP reversed the inhibitory effect of fluoride on the oxidation of pyruvate in the presence of fluoroacetate, and its effect on the oxidation in the unblocked system of citrate and α -oxoglutarate, but not the inhibition of the oxidation of succinate and of malate (Turner, 1955).

Krebs (1943) classified fluoride as a 'selective' inhibitor whose inhibitory action may be taken as merely suggestive, but not conclusive evidence that phosphorylated intermediates are involved. It may be added that if the effect of fluoride can be shown to be reversible under the influence of nitrophenols, the evidence that the effect of fluoride is due to its interference with phosphorylating reactions is considerably strengthened. On the other hand, the finding that a reaction inhibited by fluoride is not reactivated by *p*NP does not justify the conclusion that the step concerned is not coupled with a phosphorylating mechanism since the inhibitory effect

may include a more specific action of fluoride as, for example, in the case of the succinic oxidase system. Slater (1949) and Slater & Bonner (1952) have suggested that the inhibition of the succinic oxidase system is due to the direct action of fluoride on the succinic dehydrogenase rather than to a reaction between fluoride and cytochrome *c*; the finding that an inhibitory effect of fluoride could be demonstrated in fluoroacetate-blocked suspensions metabolizing pyruvate, while not conflicting with this view regarding the inhibitor's action on the succinic oxidase system, does not support their suggestion that the inhibition of intracellular respiration might be due to the effect of fluoride on succinic dehydrogenase alone. It appears probable that, apart from its effect on the succinic oxidase system, fluoride slows down the oxidation of intermediates of the cycle preceding succinate by retarding the breakdown of high-energy phosphate bond intermediates. The accumulation of the latter may be expected to exert a restraining influence on the metabolic turnover of the Krebs cycle intermediates whose oxidation is coupled with the generation of large amounts of phosphate bond energy. The reversal of this inhibition by nitrophenols may be considered a consequence of their enhancement of the 'latent ATPase' activity (Lardy & Wellman, 1953; Siekevitz & Potter, 1953), or the acceleration of the breakdown of unknown phosphorylated intermediates (see Slater, 1953).

Acetate, which requires activation, falls into a different category, as shown by the inhibition of its metabolism by fluoride and by *p*NP when added either separately or in combination.

SUMMARY

1. The synthesis of citric acid in fluoroacetate-blocked homogenates of the mammary gland and in washed suspensions of kidney cortex has been studied.

2. When added in equivalent amounts, pyruvate and fumarate were almost quantitatively converted to citric acid with uptake of an equivalent amount of oxygen. In this system, *p*-nitrophenol (*p*NP, 2×10^{-4} M) accelerated the oxygen consumption, pyruvate disappearance and citric acid formation to approximately the same extent.

3. Fluoride (0.01M) retarded the rates of respiration and of synthesis of citric acid from pyruvate plus fumarate; *p*NP reversed these inhibitory effects.

4. The synthesis of citric acid from acetate plus fumarate was retarded by either fluoride or *p*NP; the latter did not reverse, but intensified the inhibitory effect of fluoride.

5. When fluoroacetate-blocked mammary homogenates metabolizing pyruvate were supplemented with α -oxoglutarate, the rate of synthesis of citric acid was retarded; the addition of *p*NP resulted in the reversal of this inhibition and the usual acceleration of citric acid formation. When acetate was the substrate, the addition of α -oxoglutarate produced no marked effect on the rate of formation of citric acid; the oxidation of α -oxoglutarate appeared, however, to antagonize the inhibitory effect of *p*NP, and higher concentrations of the inhibitor had to be added to suppress the formation of citric acid.

6. Arsenate inhibited the formation of citric acid from both pyruvate and acetate.

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REFERENCES

- Balmain, J. H., Folley, S. J. & Glascock, R. F. (1952). *Biochem. J.* **52**, 301.
- Bartlett, G. R. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 67.
- Judah, J. D. & Rees, K. R. (1953). *Biochem. J.* **55**, 664.
- Judah, J. D. & Rees, K. R. (1954). *Biochem. J.* **57**, 374.
- Judah, J. D. & Williams-Ashman, H. G. (1951). *Biochem. J.* **48**, 33.
- Krebs, H. A. (1943). *Advanc. Enzymol.* **3**, 191.
- Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.
- Lardy, H. A. & Wellman, H. (1953). *J. biol. Chem.* **201**, 357.
- Liébecq, C. & Peters, R. A. (1949). *Biochim. biophys. Acta*, **3**, 215.
- Lipmann, F., Jones, M. E. & Black, S. (1952). *Symp. sur le Cycle Tricarboxylique. 2nd Int. Congr. Biochem., Paris*, p. 55.
- Meyerhof, O. (1951). In Sumner, J. P. & Myrbäck, K. *The Enzymes*, vol. 1, part 2, 1207.
- Ochoa, S. (1952). *Harvey Lect.* 1950-51, Series XLVI, 153.
- Ochoa, S. (1954). *Advanc. Enzymol.* **15**, 183.
- Peters, R. A. (1952a). *Symp. sur le Cycle Tricarboxylique. 2nd Int. Congr. Biochem., Paris*, p. 64.
- Peters, R. A. (1952b). *Proc. Roy. Soc. B*, **139**, 143.
- Popják, G. & Tietz, A. (1954). *Biochem. J.* **56**, 46.
- Siekevitz, P. & Potter, V. R. (1953). *J. biol. Chem.* **201**, 1.
- Slater, E. C. (1949). *Biochem. J.* **44**, 305.
- Slater, E. C. (1953). *Nature, Lond.*, **172**, 975.
- Slater, E. C. & Bonner, W. D. jun. (1952). *Biochem. J.* **52**, 185.
- Slater, E. C. & Holton, F. A. (1954). *Biochem. J.* **56**, 28.
- Terner, C. (1954). *Biochem. J.* **57**, v.
- Terner, C. (1955). *Biochem. J.* **60**, 88.