content. In the comparison of the liver-glycogen results from untreated and salicylate-treated rats no value of P less than 0.3 was obtained.

The effect of salicylate on the blood glucose and liver glycogen of normal rats. Table 3 shows that there was no significant change of blood glucose in the salicylate-treated rats, but that a marked fall in liver glycogen occurred 4 and 7 hr. after the salicylate injection. This depletion appeared to be temporary and partial replacement of the glycogen had occurred in 24 hr. Our results are in agreement with those reported by Lutwak-Mann (1942), except that this worker found in some experiments that 24 hr. after treatment, glycogen in the livers of salicylate-treated rats was greater than that of the controls.

DISCUSSION

The present work shows that in diabetic rats salicylate reduces the glycosuria, lowers the blood glucose, but causes no change in liver-glycogen content. Cortisone exacerbated the glycosuria in these animals and this is of interest because of reports that the therapeutic effects of salicylate are primarily mediated through the pituitary and adrenal glands, a hypothesis which has been criticized (Meade & Smith, 1951). It must be concluded that in diabetic rats depression in the urinary and blood glucose by salicylate is not due to its deposition as liver glycogen. Other possible mechanisms to account for the disappearance of this glucose are: first, inhibition of gluconeogenesis;

second, increased deposition of muscle glycogen; third, increased glucose use in the tissues; and fourth, conversion to fat.

In the non-diabetic rat salicylate caused no change in blood glucose, but a significant reduction of liver glycogen and similar mechanisms may be involved. The possibility that the glucose may be converted to glucuronic acid which is used for conjugation with salicylate is unlikely because of Lutwak-Mann's (1942) conclusion that the ability of rat liver to form a conjugated glucuronide from salicylate was negligible.

It is proposed to extend this work to investigate the possibilities discussed above.

SUMMARY

1. The effect of salicylate on the glycosuria, blood glucose and liver-glycogen content of the alloxan-diabetic rat and on the blood glucose and liver glycogen of the normal rat have been studied.

2. Salicylate reduces the glycosuria and blood glucose in the diabetic rat, but causes no change in the liver-glycogen content. In the normal rat salicylate causes no alteration in the blood glucose, but a depression of the liver-glycogen content.

3. Possible mechanisms of these changes are discussed.

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The Inhibition of Aconitase by 'Inhibitor Fractions' Isolated from Tissues Poisoned with Fluoroacetate

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Fluoroacetate has been shown to inhibit the oxidation of citrate in animal tissues *in vivo* (Liébecq & Peters, 1948; Martius, 1949). Furthermore, Buffa & Peters (1949) have shown that animals poisoned with fluoroacetate show large accumulations of citrate in many of their tissues (their paper may be consulted for references to the earlier literature). This observation has been confirmed by Potter &

Busch (1950), Lindenbaum, White & Schubert (1951) and Kandel, Johnson & Chenoweth (1951). Despite many attempts, no isolated enzyme has been shown to be inhibited by fluoroacetate. This and other work has led to the hypothesis, originally proposed to explain *in vitro* experiments by Liébecq & Peters (1948) and Martius (1949), that fluoroacetate is metabolized in a similar manner to

ordinary acetate via the tricarboxylic acid cycle to a fluorotricarboxylic acid which then inhibits one of the enzymes necessary for citrate oxidation. In a test of this hypothesis by Buffa, Peters & Wakelin (1951), tricarboxylic acid fractions containing fluorine have been isolated from poisoned kidney tissue. These fractions are potent inhibitors of citrate metabolism in kidney homogenates. Other evidence has also been presented which has been interpreted by Elliott & Kalnitsky (1950) as support for the formation of fluorocitric acid.

Since the accumulation of citrate in poisoned tissues is not accompanied by a similar accumulation of α -ketoglutarate (Buffa & Peters, 1949), it is reasonable to assume that the block occurs at the aconitase or isocitric dehydrogenase step of the tricarboxylic acid cycle. Previous work in this laboratory (Buffa, Lotspeich, Peters & Wakelin, 1950) failed to find an inhibition of aconitase, isocitric dehydrogenase, or oxalosuccinic decarboxylase by the fluorotricarboxylic acid fractions ('inhibitor fractions') isolated from poisoned tissue. Reinvestigation using improved methods has shown that aconitase is indeed inhibited by the 'inhibitor fractions', though larger concentrations of these have been found necessary to inhibit the soluble aconitase than the citrate disappearance in kidney homogenate.

A preliminary report of this work was presented before the Physiological Society in May 1951 (Lotspeich, Peters & Wilson, 1951).

EXPERIMENTAL

Aconitase preparation. Pigeon-breast muscle was ground in a mortar with 5 vol. of glass-distilled water. It was then centrifuged for 30 min. at 5000 rev./min. (3500–4700 g). To the supernatant fluid there was added neutralized cysteine hydrochloride and unneutralized ferrous sulphate to give final concentrations of 0.01 and 0.005 M respectively, as suggested by Dickman & Cloutier (1950, 1951). The mixture was left standing at room temperature for 10 min., neutralized to pH 7.2 and stored under oil in the refrigerator. For each experiment a sample of the stock solution was removed and diluted with a solution containing freshly prepared 0.005 M-ferrous sulphate, 0.01 M-cysteine and 0.05 M-phosphate buffer, pH 7.2. An extract equivalent to 10–15 mg. of the original fresh muscle per 3 ml. was used in most experiments. It has been found difficult to obtain reproducible activities from similar quantities of enzyme on different occasions, so that repeated trial runs were often necessary prior to the actual experiment.

isocitric dehydrogenase-aconitase preparation. When a mixture of these enzymes was required, fresh kidney, heart or skeletal muscle was ground in a mortar with 5 vol. of 1% (w/v) KCl and centrifuged at 0° at 5000 rev./min. for 15–20 min. The supernatant fluid contained both aconitase and isocitric dehydrogenase. A few experiments were carried out using the stored aconitase preparation described above, in which both of the enzymes were also present. The isocitrate formed from citrate was measured by the reduction of added coenzyme II (CoII) in a Beckman photoelectric

colorimeter. The procedure was as follows: a 3 ml. glass Beckman cell was filled with 0.3 ml. of 0.5 M-phosphate buffer, pH 7.2, 0.1 ml. of 0.2% (w/v) MnCl_2 , 0.2 ml. of tissue extract, substrate, inhibitor, and the tube made up to 2.8 ml. with water. After mixing the contents, the tube was placed in the Beckman apparatus and a reading taken at 340 m μ . CoII was then added and the increase in absorption was followed.

isocitrate estimation. The method of Ochoa (1948) using isocitric dehydrogenase and CoII was used with slight modification. The very small amounts of aconitase still present in the dialysed pig-heart powder preparation (Lotspeich & Peters, 1951) were eliminated with 0.005 M-KCN (Krebs & Eggleston, 1944). This was found necessary because, in these experiments, very small amounts of isocitrate were estimated in the presence of large amounts of citrate. The cyanide was also helpful in preventing reoxidation of CoII which often occurred in its absence. Since large amounts of trichloroacetic acid interfered with the isocitrate estimation, the aconitase reaction was stopped with sufficient HCl to bring the pH to 3.0 approx. followed by a small amount of trichloroacetic acid to make the final concentration 1% (v/v). Though this did not completely precipitate the protein, after neutralizing there was no interference with the estimation. Aconitase was completely inactivated under these conditions. The neutralized solution was centrifuged and samples containing between 2 and 15 μ g. isocitrate were placed in Beckman tubes and estimated using 80 μ g. CoII/tube. The accuracy was of the order of $\pm 0.3 \mu$ g.

Citrate estimation. The method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949) was used.

Reagents. Sodium citrate $3\text{H}_2\text{O}$ (A.R.) was used throughout, DL-isocitrate (m.p. 136°) and cis-aconitate prepared from the anhydride (m.p. 77°) were prepared by Mr R. W. Wakelin. All substrates were neutralized to pH 7.2 before use. CoII was prepared by the method of Lepage & Mueller (1949) using a Nuchar C charcoal column.

'Inhibitor fractions'. The inhibitor (referred to as 'inhibitor fraction') was isolated by Peters and Wakelin from homogenates exposed to fluoroacetate by the method of Buffa *et al.* (1951). All three fractions used in this study were free from fluoroacetate: 46 A and 37 contained as impurities citrate and phosphate; 50/51 was purer containing mainly citrate as impurity. In each fraction there were only a few per cent of other tricarboxylic acids. The quantity of inhibitor will be expressed in terms of its inhibition of citrate metabolism in guinea pig kidney homogenates, a test which has previously been used by Buffa *et al.* (1951) in the isolation of the inhibitor. One unit is defined as that amount of 'inhibitor fraction' which inhibits the disappearance of 1 μ mol. of citrate under the conditions of their test system. It can be calculated from the data given by Buffa *et al.* (1951) that one unit is approximately equivalent to 0.065 μ g. fluorine.

RESULTS

The first indication that the 'inhibitor fractions' interfered with aconitase was obtained using the supernatant fluid from kidney, usually discarded in the homogenate tests. The isocitric dehydrogenase present was activated by adding CoII and the isocitrate formed from citrate or cis-aconitate was

measured by the absorption of reduced CoII at $340 \text{ m}\mu$. Since there was less aconitase than *isocitric* dehydrogenase in this preparation, the aconitase step was the rate limiting reaction. Fig. 1 shows the inhibition of *cis*-aconitate oxidation by the 'inhibitor fractions'. In this soluble system from kidney the oxidation of both citrate and *cis*-aconitate was inhibited, but not that of *isocitrate*. As shown in Fig. 1, the CoII added to the inhibitor tube was not completely reduced. Prof. S. Ochoa suggested to us that this might be due to destruction of CoII by a nucleotidase, which proved, indeed, to be the case.

Fig. 1. Inhibition of the soluble guinea pig-kidney aconitase by 'inhibitor fractions'. The *isocitrate* formed from *cis*-aconitate was measured by the reduction of CoII in the presence of aconitase and *isocitric* dehydrogenase. Reduction of CoII was measured in a Beckman spectrophotometer at wavelength $340 \text{ m}\mu$. Glass cells were used, optical path 1 cm. Additions: 0.3 ml. of 0.5 M-phosphate buffer ($\text{pH } 7.2$), $1.1 \mu\text{mol. MnCl}_2$, extract of fresh guinea pig kidney, 17 units of 'inhibitor fraction' 37, $3 \mu\text{mol. cis-aconitate}$, made up with water to a final volume of 3.0 ml. At zero time $0.03 \mu\text{mol. CoII}$ was added.

These experiments were, therefore, repeated using extracts of both rat-heart and pigeon-breast muscle which contain very little nucleotidase. An experiment using an extract of pigeon-breast muscle as a source of aconitase and *isocitric* dehydrogenase is shown in Fig. 2. As with the kidney experiments the oxidation of citrate and *cis*-aconitate was inhibited by the 'inhibitor fractions'. On the other hand, *isocitrate* oxidation was not inhibited by

twice the concentration of inhibitor used for the latter two substrates. This confirmed the earlier work of Buffa *et al.* (1950) showing that *isocitric* dehydrogenase is not inhibited by the 'inhibitor fractions'.

This method of studying aconitase was not ideal, however, partly because of the indirect nature of the measurement and partly because of the reoxidation of the CoII which causes considerable difficulty at low-substrate concentrations. A direct measurement of aconitase was therefore begun. The citrate to *isocitrate* reaction was studied by adding citrate to a suitable muscle extract containing aconitase and then measuring the *isocitrate* produced after various time intervals. Pre-incubation of the inhibitor with the enzyme was found greatly to increase its inhibitory effect. Since the 'inhibitor fractions' contained some citrate as an impurity, it was necessary in these experiments to add equal amounts of citrate to the controls for the pre-incubation period. Before discussing the effects of the inhibitor, it should be emphasized that fluoroacetate itself does not inhibit aconitase; 1 mg. ($10 \mu\text{mol.}$) of sodium fluoroacetate present in 3 ml. of aconitase solution gave no inhibition, confirming earlier work (Buffa & Peters, 1949).

Fig. 3a shows the result of an experiment in which the inhibitor was pre-incubated with the enzyme for 30 min. prior to the addition of citrate, added in an amount making $5 \mu\text{mol.}$ of citrate/ ml. Samples (3 ml.) were then removed from the control and the inhibitor bottles at various time intervals, deproteinized with hydrochloric acid and trichloroacetic acid, and analysed for *isocitrate*. In the control tube 30% of the equilibrium amount of *isocitrate* appeared at the end of 30 min. In the presence of the inhibitor a marked decrease in *isocitrate* production was obtained.

Fig. 3b shows a similar experiment measuring the reverse reaction; DL-*isocitrate* was added and citrate measured.

The two steps *cis*-aconitate to citrate and *cis*-aconitate to *isocitrate* were measured simultaneously by adding *cis*-aconitate and determining the other two acids. These two steps were inhibited by the 'inhibitor fractions' as is shown in Fig. 4a, b, but to a lesser degree than the other two reactions. In this experiment the rate of reaction of *cis*-aconitate to *isocitrate* was about 1.5 times the rate of reaction of *cis*-aconitate to citrate, which is in agreement with Martius & Lynen (1950).

DISCUSSION

The 'inhibitor fractions' have been shown to inhibit four of the reactions attributed to the enzyme aconitase, namely: citrate $\rightarrow$ *isocitrate*, *isocitrate* $\rightarrow$ citrate, *cis*-aconitate $\rightarrow$ citrate, and *cis*-aconitate $\rightarrow$ *isocitrate*. The reaction citrate to *isocitrate* is

Fig. 2. The effect of the 'inhibitor fractions' on the oxidation of citrate, *cis*-aconitate, and *isocitrate* using a pigeon-breast muscle extract containing aconitase and *isocitric* dehydrogenase. The reduction of added Co II was measured spectrophotometrically at wavelength 340 m μ . All tubes contained the following: extract of pigeon-breast muscle (stabilized with Fe⁺⁺ and cysteine), 1.1 μ mol. MnCl₂, 0.3 ml. of 0.5 M-phosphate buffer, pH 7.2, made up with water to 3.0 ml. (a) 3 μ mol. citrate, 15 units inhibitor (46 A), (b) 3 μ mol. *cis*-aconitate, 15 units inhibitor (46 A), (c) 3 μ mol. *isocitrate*, 30 units inhibitor (46 A). At zero time 0.1 μ mol. of Co II was added.

Fig. 3. (a) Inhibition of the step citrate $\rightarrow$ *isocitrate* by the 'inhibitor fractions'. An extract of pigeon-breast muscle containing aconitase (stabilized with Fe⁺⁺ and cysteine) was incubated with 'inhibitor fraction' 46 A (final concentration 9.5 units/3 ml.) for 20 min. at 17° prior to the addition of citrate (5 μ mol./ml.). Samples were removed at various time intervals, stopped with HCl and trichloroacetic acid, and *isocitrate* estimated. (b) Inhibition of the step *isocitrate* $\rightarrow$ citrate by 'inhibitor fractions'. A stabilized extract of pigeon-breast muscle was incubated with 'inhibitor fractions' 51/52 (final concentration 8.5 units/3 ml.) for 20 min. at 17° prior to the addition of *isocitrate* (10 μ mol. DL-*isocitrate*/ml.). The protein was precipitated by trichloroacetic acid and supernatant analysed for citrate.

inhibited to the same degree as the reverse reaction; the two steps starting with *cis*-aconitate appear to be less sensitive. It is possible that *cis*-aconitate has greater affinity for the enzyme than does citrate or *isocitrate*, and hence tends to displace

factors seem to be involved in the more organized system present in the kidney homogenate, the inhibition of aconitase is probably the fundamental reason for the accumulations of citrate found in tissues poisoned with fluoroacetate.

Fig. 4. Inhibition of the steps *cis*-aconitate $\rightarrow$ citrate (a), and *cis*-aconitate $\rightarrow$ *isocitrate* (b), by the 'inhibitor fractions'. A stabilized aconitase solution was incubated with 'inhibitor fraction' 50/51 (final concn. 17 units/3 ml.) for 20 min. at 17° prior to the addition of *cis*-aconitate (5 µmol./ml.). The reaction was stopped by the addition of HCl and trichloroacetic acid, protein centrifuged, and samples of supernatant analysed for both citrate and *isocitrate*.

the inhibitor more efficiently. Using the inhibitor formed *in situ* by homogenates exposed to fluoroacetate, Elliott & Kalnitsky (1950) found that whereas citrate oxidation was strongly inhibited, *cis*-aconitate oxidation was not. They suggest that either *cis*-aconitate has a greater affinity for the enzyme or that aconitase is two enzymes; they favoured the latter view. The fact that the 'inhibitor fractions' do inhibit the step *cis*-aconitate to *isocitrate* is not decisive in deciding the homogeneity of this enzyme. The theoretical arguments of Ogston (1951), however, are of interest in this regard since they suggest the possibility of one enzyme accomplishing the two types of hydration of *cis*-aconitate.

Assuming that the 'inhibitor fractions' contain fluorocitrate and using the fluorine values given by Buffa *et al.* (1951), it is calculated that in Fig. 3 the concentration of the fluorocitrate would be of the order of 10 µM. Although permeability and other

SUMMARY

1. The fluorotricarboxylic acid fractions isolated from tissues poisoned with fluoroacetate have been shown to inhibit the following reactions of the enzyme aconitase: citrate $\rightarrow$ *isocitrate*, *isocitrate* $\rightarrow$ citrate, *cis*-aconitase $\rightarrow$ citrate, *cis*-aconitase $\rightarrow$ *isocitrate*.

2. The reaction citrate $\rightarrow$ *isocitrate* is inhibited to the same degree as the reverse reaction; the two reactions starting with *cis*-aconitate are less sensitive to the inhibitor.

3. It is considered that this inhibitor of aconitase has a direct relation to the accumulation of citrate in fluoroacetate poisoning.

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Enzymic Transpeptidation Reactions Involving γ -Glutamyl Peptides and α -Amino-acyl Peptides

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In an earlier paper (Hanes, Hird & Isherwood, 1950) glutathione (γ -glutamylcysteinylglycine) was shown to react with various added amino-acids in the presence of an enzyme from kidney and other tissues to form new peptides containing glutamic acid. Circumstantial evidence suggested strongly that the new peptides formed in the system were γ -glutamyl peptides, and that the reaction consisted of the transfer of the γ -glutamyl group from its linkage in glutathione to linkage with the amino group of an 'acceptor' amino-acid.

Observations to be described in the present paper have confirmed this concept and the enzyme will be referred to as γ -glutamyl transpeptidase. The new peptides formed when glutathione reacts with phenylalanine and glycine have now been definitely identified as γ -glutamylphenylalanine and γ -glutamylglycine respectively. Furthermore, it has been shown that glutathione is by no means unique as a 'donor' of the γ -glutamyl group in this system; in fact, various synthetic γ -glutamyl peptides have been found to act readily as donors of the γ -glutamyl group to different acceptor amino-acids and peptides. The same new γ -glutamyl peptide can thus be formed in reactions between different γ -glutamyl donors with the same acceptor.

While the transpeptidation reactions resulting in redistribution of γ -glutamyl groups form a prominent feature in the systems investigated, it should be emphasized that slow, but ultimately complete, breakdown of all γ -glutamyl peptides takes place. Earlier work had established that extracts of kidney and certain other animal tissues contained enzymes which catalysed the breakdown of glutathione and oxidized glutathione in a process presumed to be hydrolytic in character (Woodward & Reinhardt, 1942; Schroeder, Munro & Weil, 1935; Schroeder & Woodward, 1937; Binkley & Nakamura, 1948). It is possible that the liberation of free glutamic acid from γ -glutamyl peptides may be due to hydrolysis catalysed by the same enzyme that catalyses the transfer of γ -glutamyl groups, but this question requires further study. It may be noted, however, that the appearance of such breakdown products may complicate the reaction, since amino-acids originating in this way (e.g. cysteine and glycine from glutathione) may act as acceptors of γ -glutamyl groups to form new dipeptides.

It appears that the γ -glutamyl transpeptidase is specific for γ -glutamyl peptides as distinct from the γ -glutamyl amide (glutamine). No transfer of γ -glutamyl group from glutamine to added amino-