INFLUENCE OF *SALMONELLA PULLORUM* INFECTION ON VARIOUS LIVER TRICARBOXYLIC ACID ENZYMES AND CITRATE LEVELS IN THE CHICK[1]

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It has been shown (Gilfillan *et al.* 1956) that sublethal concentrations of tricarboxylic acid (TCA) cycle inhibitors and intermediates reduce survival time in 1- to 3-day-old chicks infected with *Salmonella pullorum*. A correlation was also indicated between survival time and concentration of the organisms in various tissues of chicks treated with fluoroacetate. Berry *et al.* (1954) demonstrated increased citrate levels in several tissues of mice injected with large numbers of heat-killed *Salmonella typhimurium*. There is no information, however, as to the manner by which the infection influences citrate levels and various enzymes of the TCA cycle of the host as the disease progresses. This report describes the modification of several liver TCA enzymes and citrate levels caused by *S. pullorum* infection in the chick.

MATERIALS AND METHODS

White Leghorn cockerels, 1 to 3 days old, weighing 34 ± 2 g, and obtained from pullorum-free flocks, were injected intraperitoneally with approximately 10^8 viable cells of *S. pullorum* CDC #3562/51. The inoculum was prepared as described in a previous publication (Gilfillan *et al.* 1956).

Inhibitor injections. Two mg per kg of sodium fluoroacetate (90 per cent pure, Monsanto) dissolved in sterile saline was injected IP in 0.5-ml volumes at 0 and 6 hr.

Liver homogenates. Chicks were beheaded and drawn. The livers were removed, blotted dry, weighed on a torsion balance, and placed in cooled homogenizer tubes. The tissue was homogenized and the necessary volumes of diluent added to make a ten per cent homogenate (wt per vol). Homogenates for the assay of hydrases and dehydrogenases were prepared in isotonic KCl with the exception of aconitase, which was prepared in 0.1 M phosphate buffer, pH 7.4. Homogenates for the estimation of succinoxidase and cytochrome c oxidase were prepared in alkaline isotonic KCl. Homogenates were centrifuged in a Servall SP/X angle centrifuge at approximately 3,000 rpm for 10 min, after which the supernatant fluid was removed and used as such or diluted. An examination of the supernatant fluid revealed that practically all of the bacterial cells were removed by centrifugation. No more than three organisms per ml were ever found in aliquots of homogenate used in the assay.

Citrate determinations. Citrate was determined by the method of Ettinger *et al.* (1952). Protein was precipitated with tungstic acid and the precipitate and the supernatant were heated in a water bath at 80 to 90 C for 10 min to insure complete protein precipitation, to remove interfering substances, and to achieve complete extraction of citrate. Pentabromacetone was estimated spectrophotometrically and standard curves were prepared with each experiment. Added citrate could be recovered from fresh tissues in the range of 95 to 105 per cent.

Lactic, malic, and isocitric dehydrogenase. These enzymes were measured spectrophotometrically by a method originally described by Mahler *et al.* (1948), which permits direct assay of the enzymes by measuring the rate of oxidation or reduction of the specific coenzyme at 340 mμ.

Aconitase and fumarase. These hydrases were measured spectrophotometrically. A method described by Racker (1950) was employed.

Succinoxidase and cytochrome c oxidase. These were assayed manometrically by procedures outlined by Umbreit *et al.* (1949). Ascorbate was used as the reductant in cytochrome c oxidase measurements and corrections were made for autoxidation.

From preliminary determinations, all enzyme assays were carried out in excess substrate con-

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centration and with proportionality of enzyme activity and tissue concentration, with the exception of cytochrome c oxidase which was tested in three different tissue concentrations.

RESULTS

Peters *et al.* (1953) have stated that fluoroacetate, through the "lethal synthesis" of fluorocitrate, inhibits aconitase *in vivo* and *in vitro*, and causes an accumulation of citrate in a number of tissues of various animals. No information is available to indicate the specific effect of this compound in the chick and, in particular, in liver tissue.

Liver citrate levels were determined during the course of the disease in normal and infected chicks, and normal and infected chicks treated with fluoroacetate. The results of this experiment are given in table 1. Citrate increased markedly in infected chicks at 48 and 72 hr, although the trend toward higher levels appeared at 24 hr. Fluoroacetate produced accumulations of citrate immediately in normal and infected chicks. At 48 hr, the amount of citrate in infected fluoroacetate-treated chicks was considerably greater than in normal, normal fluoroacetate-treated, or infected chicks at the same interval of time.

Aconitase and fumarase. Increased citrate levels in infected chicks suggested a relationship between the infectious process and an impairment of citrate metabolism. To obtain more direct information on this impairment, several liver

TABLE 1

*Liver citrate levels in normal and infected chicks, and in normal and infected chicks treated with fluoroacetate (10 chicks per group)**

μg citrate per g wet wt tissue†

Time after Infection	Normal	Infected	Normal plus FAc	Infected plus FAc
hr				
1	99	96	139	143
4	99.7	94	191.5	207.1
6	99	95.2	241	248
8	—	—	236.3	230.3
12	95	101	249	258
24	97	107.6	105	119
48	98.4	126.5	103.6	138.8
72	98.4	170.8	—	—

* 2 mg per kg injected at 0 and 6 hr.

† Average values.

TABLE 2

Aconitase and fumarase assays of normal and infected chick liver (units per mg dry wt)

Time after Infection	Aconitase			Fumarase		
	Normal	Infected	% Normal	Normal	Infected	% Normal
hr						
12	49.8	50.2	101	137.2	140	102
24	49	50.6	103	141.4	137.2	96
48	50.4	51.2	102	138.6	134	97
72	50.8	39.8	78	140.4	112.4	80

TABLE 3

Succinoxidase and cytochrome c oxidase assays of normal and infected chick liver (Q_{O_2} per mg dry wt)

Time after Infection	Succinoxidase			Cytochrome Infected Oxidase		
	Normal	Infected	% Normal	Normal	Infected	% Normal
hr						
12	30.2	31.8	101	372.6	372.4	100
24	28.4	27.6	97	376.4	365.2	95
48	31.2	25.8	83	377.6	348	93
72	29	13.8	48	380.2	340	90

TCA enzymes were assayed at intervals as the disease progressed. The influence of infection on the hydrases, aconitase and fumarase, are shown in table 2. From this, it would appear that the activity of both enzymes was impaired at 72 hr, during the terminal phase of the disease. The reduction of aconitase (78 per cent that of normal) at this period may have some particular significance in view of the increased citrate levels and the action of fluoroacetate in reducing survival time. The other hydrase, fumarase, was also considerably reduced, dropping to 80 per cent that of normal at 72 hr.

Succinoxidase and cytochrome c oxidase. Results of assays on these two liver enzymes made during the progress of the disease are presented in table 3. Succinoxidase showed considerable reduction in activity at 48 hr (83 per cent that of normal) and an almost two-fold decrease at 72 hr (48 per cent that of normal). Cytochrome c oxidase was also increasingly depressed with these successive time intervals. However, the reduction was much less than that observed with succinoxidase.

Dehydrogenases. Lactic, malic, and isocitric

TABLE 4

Lactic, malic, and isocitric dehydrogenase assays of normal and infected chick liver (units per mg dry wt)

Time after Infection	Lactic			Malic			Isocitric		
	Normal	Infected	% Normal	Normal	Infected	% Normal	Normal	Infected	% Normal
hr.									
12	126	122	97	150	168	112	30.4	35	116
24	123	117	96	163	182	111	31.1	30.6	97
48	139	123.8	89	174	185	106	33.8	26.6	79
72	136.4	118.4	87	177.8	180.4	102	32.4	23	71

dehydrogenase were measured at indicated intervals during the course of the infection. Lactic dehydrogenase was included in this study because of its relationship to the TCA cycle in providing acetate and the implication of increased concentrations of lactate in various infectious processes described by Dubos (1954). Results of these assays are presented in table 4. It was observed that, of the DPN-dependent enzymes, lactic dehydrogenase was progressively reduced to 86 per cent of normal at 72 hr, while malic dehydrogenase increased at 12 hr to 112 per cent of normal and then progressively decreased to normal at 72 hr. Isocitric dehydrogenase, TPN-dependent, underwent progressive reduction from 79 per cent of normal at 48 hr to 71 per cent of normal at 72 hr.

DISCUSSION

The data demonstrate that sublethal doses of fluoroacetate bring about an increased accumulation of citrate in the livers of normal chicks, thus expanding the spectrum of animals which similarly respond to this compound. The results are comparable in the magnitude of increase and the induction period to those obtained in rats by Lindenbaum *et al.* (1951). However, in the chick, there is a temporary drop in citrate concentration following the final dose of fluoroacetate. Increased citrate levels in infected chicks indicate an impairment in the metabolism of this compound during the course of the disease. These results are of interest when compared with those of Berry *et al.* (1954), who found that injections of large amounts of heat-killed *S. typhimurium* cells elicited marked citrate elevations in various tissues of the mouse. Processes leading up to death in both of these diseases may be closely associated. That is, the elevation of citrate levels may be induced by common effects produced by endotoxin.

The profile of liver TCA enzymes investigated indicates different degrees of impairment. The enzymes specifically inhibited by malonate and fluoroacetate, i. e., succinoxidase and aconitase, respectively, are somewhat reduced at 72 hr. The most marked reduction of the enzymes assayed is observed in the succinoxidase system. Many other induced physiological imbalances, *in vivo* or *in vitro*, are characterized by reduction in succinoxidase with much smaller changes in cytochrome c oxidase activity. It is of interest that Kun *et al.* (1948) found large amounts of endotoxin *in vivo* inhibited succinoxidase in rabbit liver and muscle with little effect on activity of cytochrome c oxidase activity. Infection appears to influence the dehydrogenases somewhat differently. Lactic and isocitric dehydrogenases were found to be continuously depressed, lactic falling much more slowly than isocitric. On the other hand, malic dehydrogenase appears to be increased shortly after infection but falls steadily as the disease progresses. Increased activity of this enzyme may indicate oxalacetate starvation or may be related to increased fat metabolism and ketosis which is frequently associated with bacterial infections localizing in the liver. The chick liver at this period is still actively engaged in transition from embryonic to adult tissue with a concomitant decrease in fat content.

These studies, although primarily exploratory, show that enzymes of the TCA cycle in the liver of the host are progressively affected, and certain systems appear to be influenced by the infection more than others. Further studies will be required to determine how much of this impairment is due to destruction, inactivation, or inhibition.

SUMMARY

Citric acid concentrations were measured in livers of normal chicks, with and without fluoroacetate treatment, and chicks infected with *Salmonella pullorum*, with and without fluoroacetate treatment. Citrate was found to accumulate during the terminal phase of the disease in infected chicks. In non-infected, fluoroacetate-treated chicks, citrate levels rose shortly after introduction of the inhibitor and subsequently declined. In infected, fluoroacetate-treated chicks, the metabolite increased during the first 12 hours, then subsided, and again increased slightly during the terminal phase of infection.

Infection gave evidence of causing varying degrees of impairment of the liver tricarboxylic acid enzymes assayed during the course of the disease.

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