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# MYOCARDIAL ADENINE NUCLEOTIDES, HEXOSE PHOSPHATES AND INORGANIC PHOSPHATE, AND THE REGULATION OF PHOSPHOFRUCTOKINASE ACTIVITY DURING FLUOROACETATE POISONING IN THE RAT*

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**Abstract**—Myocardial levels of adenine nucleotides, inorganic phosphate (Pi) and hexose phosphates were measured as a function of the time after fluoroacetate (FAc) administration (6 mg/kg i.p.) to the rat. ATP content was progressively depleted, reaching at 6 hr 60 per cent and at 18 hr 45 per cent of the control values. AMP and ADP levels increased during the initial 2 hr and later declined. Intracellular Pi accumulated in great amounts, with a maximum at 4 hr, while serum Pi increased continuously throughout the experimental period. Citrate levels reached a maximum at 6 hr and remained nearly constant thereafter. These results suggest that the energy reserves of the tissue are progressively exhausted during the intoxication, and that the reactivation of the citric acid cycle by the accumulated citrate, postulated by previous authors, does not occur under the present conditions. Fructose diphosphate (FDP) levels were unaltered during the intoxication, while fructose-6-phosphate (F6P) and glucose-6-phosphate only showed transient initial increases. The FDP/F6P ratio, which is indicative of intracellular phosphofructokinase (PFK) activity, was not significantly altered, in spite of the striking changes produced in the levels of PFK effectors. This suggests that the PFK activation usually associated with a depletion of high-energy phosphates and Pi accumulation is blocked in the poisoned tissue. *In vitro* experiments were performed in which PFK activity was evaluated in the presence of concentrations of substrates and metabolites simulating those found *in vivo*. It was observed that in the physiological pH range (6·9–7·1) PFK is strikingly activated in the assays corresponding to intoxicated hearts, regardless of the high citrate levels. This suggests that the activation associated with the fall of ATP is quantitatively more important than the citrate inhibition. However, at lower pH values this activation is not produced, irrespective of the levels of ATP, AMP, Pi or citrate. It is suggested that intracellular acidification, possibly associated with the accumulation of citric acid, might be the main factor responsible for the blockade of PFK activation observed in the intoxicated hearts *in vivo*.

IT IS KNOWN that fluoroacetate (FAc) is metabolized in the tissues to fluorocitrate, which is a potent inhibitor of aconitase.[1] As a consequence of the intoxication, the citric acid cycle is blocked, intracellular citrate levels rise and, if the energy demands of the tissue are high, such as in the working heart, there is a net high-energy phosphates breakdown and an accumulation of inorganic phosphate (Pi).[1–5] It has been mentioned that the accumulated citrate may inhibit the glycolytic pathway in the

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poisoned tissue, through its well-known effect on phosphofructokinase (PFK), the rate-controlling enzyme in glycolysis.[6,7] This effect may be of considerable physiological importance, since glycolytic activity seems to be necessary for the maintenance of the contractile force of the poisoned organ.[8] On the other hand, it is known that citrate-induced PFK inhibition requires the presence of ATP,[9] and that AMP and Pi, as well as other metabolic intermediates, are effective antagonists of the inhibitory effects of ATP and citrate.[6,10,11] These antagonistic effects have been invoked to explain the fact that either inhibition or activation of the glycolytic pathway were observed after FAc poisoning in isolated perfused hearts, although citrate levels were always strikingly elevated.[8,12]

Previous results from this laboratory showed that in the FAc-treated rat *in vivo*, there is a rapid initial depletion of myocardial glycogen, followed by an accumulation several hours later.[13] These findings suggested that the glycolytic pathway is initially activated and later inhibited in the intoxicated tissue. This would be in agreement with the suggestion made by Williamson,[8] who assumed that aconitase can be reactivated by the accumulated citrate, since the FC-induced inhibition was thought to be of a competitive nature.[14] According to this hypothesis PFK would be initially activated by AMP and Pi, together with the fall of ATP, but later the enzyme would become inhibited by citrate and ATP, since reactivation of aconitase would bring about resumption of citric acid cycle activity and improvement of ATP levels.[8] However, further studies on the fluoroacitrate-aconitase interaction suggested that reversal of the inhibition is very difficult,[15] although this subject is still under discussion.[16–18] Therefore, we thought that Williamson's hypothesis deserves a more thorough investigation.

The present experiments were undertaken in order to provide more detailed evidence concerning the changes in energy metabolism and the regulation of PFK activity in the rat heart during FAc poisoning *in vivo*, and to determine if the above mentioned hypothetical mechanisms may actually take place in the intoxicated tissue.

## MATERIALS AND METHODS

*Chemicals.* Monofluoroacetic acid (sodium salt), ADP (disodium), AMP (monosodium), ATP (disodium), D-glucose-6-phosphate (G6P) (disodium), D-fructose-6-phosphate (F6P) (disodium), D-fructose-1,6-diphosphate (FDP) (tetrasodium), phosphoenolpyruvate (PEP) (tricyclohexylammonium), NADH (disodium) and NADP (disodium) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

All other chemicals were of A. R. grade.

*Enzymes.* Commercial preparations of phosphoglucose isomerase (PGI), myokinase (MK), hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were purchased from Sigma. Aldolase (ALD), triosephosphate isomerase (TIM) and glycerophosphate dehydrogenase (GDH) were from Boehringer Mannheim BmbH, Germany. Glucose-6-phosphate dehydrogenase (G6PDH) was obtained from Calbiochem, U.S.A.

*Animals and FAc dosage.* Male albino rats, weighing 240–280 g were used. Food was withdrawn 24 hr before the injection of either FAc (i.p., 6 mg/kg body wt, 0·18 per cent solution in physiological saline) to the experimental group, or an equivalent amount of physiological saline to the control group. Shortly before these injections both groups of animals were anaesthetized with pentobarbital (i.p., 50 mg/kg), and

were kept unconcious throughout the initial 6 hr of experiments by small additional injections of pentobarbital as required. With this procedure no animal developed the characteristic convulsions produced by FAc. In the 12 and 18 hr experiments another injection of pentobarbital was given 30 min before sacrifice.

*Tissue sampling procedure.* For the analysis of labile metabolites, the procedure outlined by Kraupp *et al.*[19] to avoid tissue anoxia during sampling was followed. Under artificial respiration the thorax was opened and the heart was quickly frozen *in situ* by means of tongs pre-cooled in liquid nitrogen.[20] The frozen tissue was crushed in a mortar under liquid nitrogen, weighed, and quickly homogenized with 6% perchloric acid. The resultant suspension was centrifuged in the cold, the supernatant was neutralized with 5 M $K_2CO_3$, and the $KC10_4$ formed was separated by centrifugation in the cold and discarded. The neutral extracts were kept at $-50°$ until chemical analyses were performed (usually not more than 3–4 days). These conditions of storage were shown to be safe enough to assure the stability of the compounds being tested.[21]

*Methods of analysis.* ATP, G6P and F6P were determined sequentially in the same cuvette by means of G6PDH, PHI and HK.[22] Similarly, ADP and AMP were measured with LDH, PK and MK,[23] while FDP was assayed with GDH, TIM and ALD.[24] In every case, the oxidation of NADH or the reduction of NADP was followed at 340 nm, using 1 cm light-path cells in a Gilford 2400 spectrophotometer. Some of the ATP determinations were performed by the luciferase method,[25] using a Beckman DU spectrophotometer to measure the intensity of the emitted light.[26] The results were in excellent agreement with those obtained by the HK method.

Tissue Pi was determined by the method of Martin and Doty.[27] Suitable controls with added creatine phosphate confirmed that no hydrolysis of labile phosphorus is produced under the conditions of the assay. Citrate was measured by the method of Natelson *et al.*[28] Blood samples were obtained from portal vein and serum Pi was assayed by the method of Fiske and Subbarow.[29]

*Experiments with PFK* in vitro. Rat heart PFK was purified with DEAE-cellulose as described by Pogson and Randle,[9] to yield a preparation virtually free of NADH oxidase and ATPase. The enzyme was freshly prepared for each experiment.

Total PFK activity was determined enzymatically by converting FDP into glycerophosphate, using ALD, TIM and GDH as auxiliary enzymes.[30] The reaction mixture contained 50 mM Tris, 5 mM $MgCl_2$, 2 mM ATP, 125 mM KCl, 0·1 mM NADH, 20 μg ALD, 10 μg GDH and 10 μg TIM, final pH 7·5. A suitable amount of PFK was added, and after 2 min of incubation the reaction was initiated by the addition of F6P (final concentration 2 mM). Readings were taken at 15 sec intervals for 2–3 min. The rate always remained linear over this period. The temperature was kept constant at 37°. One unit is defined as the amount of enzyme that catalyzes the formation of 1·0 μmole of FDP per min at 37°. The specific activity of the extracts was about 1 unit/mg protein.

In the experiments simulating conditions found *in vivo*, it was necessary to dialyze the auxiliary enzymes, since the ammonium sulphate present in the commercial preparations can give rise to erroneous results.[9] A mixture of 2 mg of ALD, 0·4 mg of TIM and 0·4 mg of GDH was dialyzed against 4 changes of EDTA $10^{-3}$ M, pH 6·0, over 4 hr. Following this procedure, most of the unwanted ammonium sulphate is eliminated.[31]

Reaction mixtures were made up by the addition of appropriate volumes of the following solutions (final concentrations as detailed in Table 3): 30 mM ATP, 10 mM ADP, 10 mM AMP, 125 mM sodium citrate, 0·2 M potassium phosphate and 25 mM G6P to a medium with final concentrations of 150 mM Tris, 5 mM $MgCl_2$ and 125 mM KCl. The total volume was 1·0 ml. The pH of the reaction mixtures was altered by varying the pH of the buffer. Dialyzed auxiliary enzymes (20–40 $\mu$l) and PFK extract (200–400 $\mu$l) were added, and after temperature equilibration (37°) the reaction was initiated by the addition of 25 mM F6P. Readings were taken every 10 sec over 2–3 min, after which an excess of FDP was added to test the proper functioning of the system. Adequacy of the amount of auxiliary enzymes was tested additionally by changing their concentrations 2–3 fold in separate experiments, and checking that the reaction rate was not affected by this alteration.[32] At the end of the reaction the pH was accurately measured to the nearest 0·01 unit. Results were expressed in terms of total PFK activity added to the mixture, i.e. as $\mu$moles of FDP formed $min^{-1}$ unit of $PFK^{-1}$. The actual final concentrations of substrates and effectors in the reaction mixtures were checked as described above under Methods of Analysis.

## RESULTS

The myocardial contents of the metabolites assayed during the intoxication are shown in Tables 1 and 2. Percentage variations with respect to the controls are graphically expressed in Figs. 1 and 2. There was a fairly good correlation between the control levels of adenine nucleotides, Pi and hexose phosphates reported here and those obtained by previous authors in rat hearts under comparable experimental conditions.[7,33,34] This suggests that the technique of cardiac removal was adequate, since it has been shown that the FDP concentration, and especially the FDP/F6P ratio are very sensitive indicators of even minimal tissue anoxia.[19] No variations were observed in any of the control data throughout the experimental period, thus for simplicity only initial (O hr) values were included in the Tables.

As shown in Fig. 1, the effects of FAc on the myocardial adenylic acid system are striking. There was an initial rapid fall of ATP, but between 0·5 and 6 hr the levels were relatively stable, indicating that a transient steady state is reached. At 12 and

TABLE 1. MYOCARDIAL LEVELS OF ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATE AND SERUM INORGANIC PHOSPHATE AFTER FAc POISONING

| Time (hr) | N | $\mu$moles/g wet tissue ATP | ADP | AMP | Tissue Pi | Serum Pi $\mu$mole ml |
|---|---|---|---|---|---|---|
| 0 | 10 | 4·25 ± 0·22 | 0·69 ± 0·040 | 0·139 ± 0·011 | 2·81 ± 0·17 | 1·82 ± 0·17 |
| 0·5 | 9 | 2·86 ± 0·13† | 0·81 ± 0·067 | 0·236 ± 0·019‡ | 4·99 ± 0·70‡ | 2·20 ± 0·48 |
| 1 | 10 | 2·27 ± 0·21‡ | 0·90 ± 0·058† | 0·230 ± 0·025‡ | 7·16 ± 0·36‡ | 2·16 ± 0·17 |
| 2 | 7 | 2·42 ± 0·28† | 0·63 ± 0·034 | 0·220 ± 0·067 | 7·21 ± 0·41‡ | 2·34 ± 0·10* |
| 3 | 5 | 2·58 ± 0·21† | 0·65 ± 0·077 | 0·176 ± 0·065 | 8·66 ± 0·28‡ | 3·54 ± 0·33‡ |
| 4 | 7 | 2·70 ± 0·43* | 0·56 ± 0·058 | 0·125 ± 0·020 | 10·98 ± 1·25‡ | 3·71 ± 0·31‡ |
| 6 | 8 | 2·74 ± 0·58† | 0·54 ± 0·045* | 0·117 ± 0·024 | 9·49 ± 0·37‡ | 3·03 ± 0·37† |
| 12 | 9 | 1·56 ± 0·11‡ | 0·49 ± 0·063† | 0·102 ± 0·016 | 7·50 ± 0·60‡ | 4·18 ± 0·19‡ |
| 18 | 10 | 1·97 ± 0·12† | 0·33 ± 0·053‡ | 0·113 ± 0·011 | 5·71 ± 0·29‡ | 5·04 ± 0·62‡ |

Values are expressed as mean ± S.E.M. N indicates the number of animals in each group.
Significantly different from controls (0 hr): *$P < 0·05$. †$P < 0·01$. ‡$P < 0·001$.

TABLE 2. MYOCARDIAL LEVELS OF HEXOSE PHOSPHATES AND CITRATE AFTER FAc POISONING

| Time (hr) | N | μmoles/g wet tissue G6P | F6P | FDP | FDP/F6P | Citrate |
|---|---|---|---|---|---|---|
| 0 | 6 | 0·326 ± 0·041 | 0·091 ± 0·008 | 0·013 ± 0·002 | 0·127 | 0·96 ± 0·10 |
| 0·5 | 5 | 0·538 ± 0·058* | 0·109 ± 0·035 | 0·011 ± 0·002 | 0·109 | 2·66 ± 0·32‡ |
| 1 | 9 | 0·495 ± 0·029† | 0·120 ± 0·009* | 0·013 ± 0·002 | 0·098 | 5·33 ± 0·27‡ |
| 2 | 7 | 0·384 ± 0·073 | 0·102 ± 0·016 | 0·013 ± 0·002 | 0·122 | 6·32 ± 0·38‡ |
| 3 | 8 | 0·423 ± 0·025 | 0·087 ± 0·006 | 0·015 ± 0·002 | 0·169 | 9·77 ± 1·10‡ |
| 4 | 5 | 0·438 ± 0·044 | 0·106 ± 0·019 | 0·012 ± 0·002 | 0·113 | 9·45 ± 1·28‡ |
| 6 | 7 | 0·497 ± 0·063 | 0·097 ± 0·016 | 0·011 ± 0·004 | 0·126 | 10·23 ± 0·60‡ |
| 12 | .4 | 0·298 ± 0·012 | 0·087 ± 0·012 | 0·010 ± 0·001 | 0·114 | 11·45 ± 0·45‡ |
| 18 | 9 | 0·403 ± 0·045 | 0·100 ± 0·010 | 0·012 ± 0·004 | 0·121 | 12·54 ± 0·29‡ |

Values are expressed as means ± S.E.M. N indicates the number of animals in each group.
FDP/F6P values are geometrical means $\sqrt[n]{x_1 x_2 x_3 \ldots x_n}$. The significance of the differences was calculated on the basis of a *t*-test performed on the logarithm of the individual ratio values.[33]
Significantly different from controls (0 hr): * $P < 0{\cdot}05$, † $P < 0{\cdot}01$, ‡ $P < 0{\cdot}001$.

18 hr, however, further ATP depletion occurred. ADP and AMP, on the other hand, both showed a transient initial increase followed by a later decrease to below control values. Since this decrease was not accompanied by a concomitant rise of ATP levels, it cannot be atrributed to rephosphorylation of the nucleotides. There was a net decrease of total adenine nucleotides during the intoxication process.

Myocardial Pi rose during the initial 4 hr of the poisoning to almost 400 per cent of the normal values. Later on there was a relative decrease, but there was always

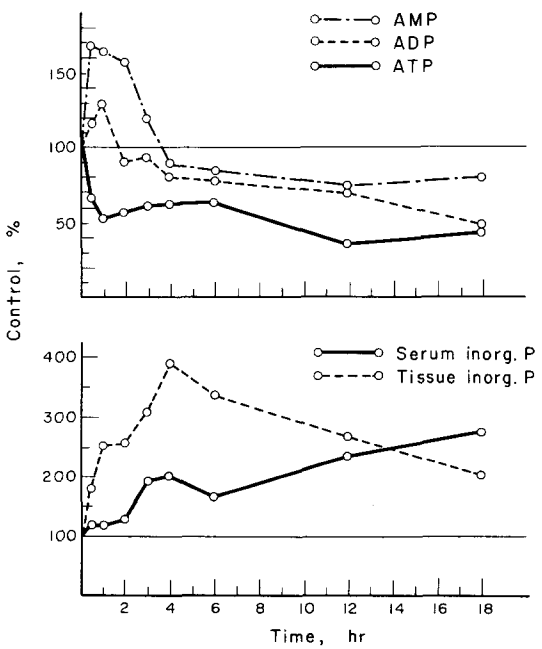


FIG. 1. Myocardial levels of adenine nucleotides and inorganic phosphate, and serum inorganic phosphate after FAc poisoning in the rat. Results are expressed as a percentage of the respective control values. Standard errors and significance of the differences are shown in Table 1.

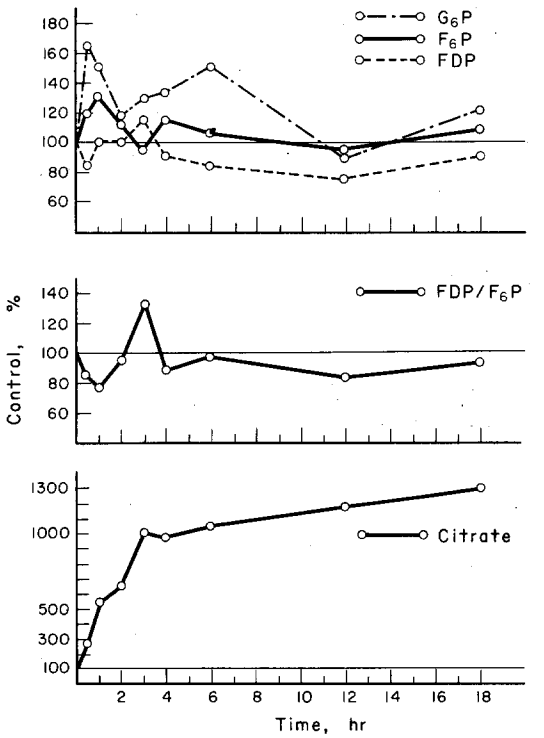


FIG. 2. Myocardial hexose phosphates, the ratio FDP/F6P and citrate levels after FAc poisoning in the rat. Results are expressed as a percentage of the respective control values. Standard errors and significance of the differences are shown in Table 2.

a significantly higher myocardial Pi content in the intoxicated than in the control animals. Serum Pi, conversely, increased continuously during the intoxication.

The effects of FAc on myocardial levels of hexose phosphates and citrate are shown in Fig. 2. Although significant increases of G6P levels were produced during the first 6 hr after FAc, no concomitant changes were observed in F6P levels, which, except for a small increase at 1 hr, were not statistically different from control values. FDP levels, as well as the FDP/F6P ratio, did not significantly differ from control values throughout the period of the experiments. As shown previously,[13] myocardial citrate levels rose abruptly up to the 4th hr after FAc, while later on there were only small additional increases.

*Experiments with PFK* in vitro. In the experiments simulating conditions found *in-vivo*, PFK activity was evaluated in three reaction mixtures, which reproduced the concentrations of metabolites found in control animals, and in intoxicated animals 1 and 18 hr after FAc administration. The composition of these reaction mixtures is described in Table 3. The method used for PFK assay did not allow the inclusion of FDP in the reaction medium. However, it must be remembered that FDP levels were constant during the intoxication (see Fig. 2).

The criterium employed for the selection of these reaction mixtures was based on the fact that at 1 hr after FAc there were maximum PFK activating conditions, since

TABLE 3. COMPOSITION OF THE PFK ASSAY MIXTURES

| Metabolites | Final concns in reaction mixture (mM) Control | FAc 1 hr | FAc 18 hr |
|---|---|---|---|
| G6P | 0·55 | 0·85 | 0·70 |
| F6P | 0·16 | 0·21 | 0·17 |
| AMP | 0·24 | 0·39 | 0·19 |
| ADP | 1·18 | 1·53 | 0·57 |
| ATP | 7·30 | 3·70 | 3·40 |
| Pi | 4·85 | 12·20 | 9·80 |
| Citrate | 1·66 | 9·20 | 21·50 |

All reaction tubes contained 150 mM Tris, 5 mM $MgCl_2$, 125 mM KCl and 0·1 mM NADH, together with the correct amount of dialyzed auxiliary enzymes (20–40 $\mu$l) and PFK extract (200–400 $\mu$l). Final volume was 1·0 ml. Other conditions were as described in the text.

AMP, ADP and Pi levels were high, while citrate was increased only 5-fold. Conversely, at 18 hr citrate was increased 13-fold, while AMP and ADP were below the control values, and therefore maximum inhibitory effects should be expected at this time. A numerical expression of these considerations can be obtained if the ratio of the concentrations of PFK activators (AMP, Pi, ADP, F6P and FDP) to the concentrations of inhibitors (ATP and citrate) in myocardial tissue is calculated for each group of animals. This ratio is 6·85 for controls, 18·9 for intoxicated rats 1 hr after FAc, and 1·05 for the 18-hr-FAc group. At all other times the values were intermediate between these.

The results of these experiments are shown in Fig. 3, where the PFK activity found in each reaction mixture is plotted against the pH of the assays. In accordance with previous findings on rabbit, mouse and frog muscle, as well as sheep heart,[32,37,38] it was found here that rat heart PFK is extremely sensitive to even slight pH variations. This can be especially observed in the control mixture, where a shift of pH from 7·10 to 7·20 produced a 25-fold increase in enzyme activity. This pH effect was also produced in the "intoxicated" assay mixtures, but at much lower pH values. Consequently there is a wide pH range (6·6–7·1) where PFK activity is higher in the "intoxicated" than in the "control" mixtures, in spite of the high citrate levels. Conversely, at pH values above 7·30 the enzyme activity was lower in the intoxicated than in the control medium. Below pH 6·60 the activity was low, and was the same in all reaction mixtures.

On the other hand, it was found that PFK activity was always higher in the FAc-1hr than in the FAc-18 hr mixture, as expected.

## DISCUSSION

Previous authors suggested that the citrate accumulated after FAc poisoning may bring about reactivation of aconitase and resumption of the respiration and oxidative phosphorylation of the poisoned myocardium.[8,39] In support of this hypothesis, some evidence was obtained in FAc-treated perfused rat hearts, in which the addition of pyruvate provoked a rise of intracellular citrate levels and also of the other components of the tricarboxylic acid cycle, indicating that the inhibition of aconitase had

been reversed.[39] Moreover, the fact that PFK was sometimes activated and sometimes inhibited in spite of the high citrate levels was taken as an indication of a possible fluctuation of ATP, AMP and Pi levels during the intoxication,[8] which would be in agreement with the above mentioned postulated mechanism. However, direct proof of the actual occurrence of such a fluctuation of the levels of ATP, AMP and Pi was not presented. The results of the present *in vivo* experiments do not support that hypothesis. Our determinations of the contents of adenine nucleotides and Pi in the heart from FAc-poisoned rats show that there is a slow and progressive depletion of myocardial energy reserves, which is not affected by the accumulation of citrate. Although there is a fluctuation of AMP and ADP levels, which after an initial accumulation fall below the control values (see Fig. 1), this phenomenon is probably due to the presence in the tissue of the highly active nucleotide phosphatases.[40] Similarly, although tissue Pi also decreases after the fourth hour, this is perhaps because of a translocation to the extracellular compartment, as suggested by the sustained rise of serum Pi.

The variations in the levels of hexose phosphates, and particularly the FDP/F6P ratio suggest that PFK activity in the intoxicated hearts does not differ significantly from that in the control hearts throughout the process of poisoning. Consequently, neither the activation usually associated with a depletion of high-energy phosphates, nor the inhibitory action of citrate were observed in the present experiments. This suggests that those antagonistic effects might cancel each other in the intoxicated tissue. However, as mentioned previously, there is a striking variation in the ratio of concentrations of PFK activators to inhibitors at different times after FAc administration. The fact that PFK activity is not altered in spite of this variation is surprising, and it suggests that other factors might be present which critically influence the behaviour of the system.

In order to get some insight into this problem, we employed an *in vitro* approach similar to that employed previously by Pogson and Randle[9] and Passonneau and Lowry.[41] Emphasis in these experiments was set on the measurement of the combined action of PFK effectors on enzyme activity, at concentrations simulating those found *in vivo*. Of course, owing to the extraordinary complexity of PFK kinetics, the conclusions obtained from these experiments can only be taken as tentative. Besides, the fact that the enzyme concentration is very different from that existing *in vivo*, and the possibility that metabolites are not uniformly distributed throughout the intracellular water must be taken into consideration. Nevertheless, the results may be of interest to the understanding of the mechanisms of the regulation of myocardial glycolysis during FAc poisoning.

Unlike previous authors, we used the pH of the assay mixtures as an independent variable, because we thought that some degree of variation of intracellular pH during the poisoning seems to be a reasonable expectation.

The fact that in the physiological pH range (6·9–7·1)[38] PFK is always strikingly activated in the assay mixtures reproducing conditions found in intoxicated animals, compared with the activity in the "control" mixture, even in the presence of 13-fold increased citrate levels, strongly suggests that the activation associated with the fall of ATP is quantitively much more important than the inhibition produced by citrate. Accordingly, when ATP inhibition is released by slightly raising the pH of the assay mixture,[38] PFK becomes inhibited in the "intoxicated" compared with the "control"

mixtures (see Fig. 3). This is the expected consequence of citrate accumulation, but it can only be observed at a pH where inhibitory effects of ATP are not maximal. On the other hand, at pH values below 6·6 PFK activity is the same in the "intoxicated" as in the "control" mixtures, irrespective of the levels at ATP, AMP, Pi or citrate.

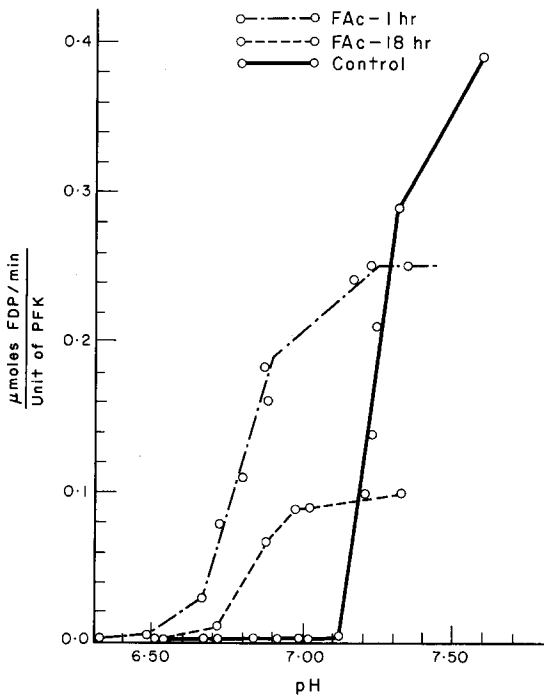


FIG. 3. Effect of metabolite concentrations simulating those found *in vivo* on the *in vitro* activity of rat heart PFK, at different pH values. The composition of the reaction mixtures is shown in Table 3.

These results suggest that the behaviour of PFK observed in the *in vivo* experiments, namely the lack of activation in the presence of a depletion of ATP and an accumulation of AMP and Pi, might be better explained on the basis of an intracellular acidification, rather than by the inhibitory effects of citrate. Of course, this tentative conclusion rests on the assumption that the behaviour of PFK in our *in vitro* system is similar to that in the intracellular environment.

The possibility that FAc poisoning brings about intracellular acidification emerges immediately when a comparison is made between this intoxication and anoxia. In the latter case it is generally accepted that there is a considerable intracellular acidification, probably as a consequence of lactic acid accumulation.[43] For instance, an intracellular pH fall from 7·10 to 6·50 in the rat brain after 3 min of anoxia, during which the tissue lactic acid concentration was increased to about 20 mM/kg of intracellular water has been reported.[44] Similar concentrations of citric acid are readily attained in the rat heart after FAc poisoning, and therefore a similar degree of intracellular acidification might be expected. Besides, in the intoxicated animals there are other physiological alterations, such as respiratory depression[45] and ketonemia,[46]

which may affect the acid-base balance of the tissues. Further investigation will be necessary to test these assumptions, and to evaluate the role played by this hypothetical acidification on the evolution of the symptoms of the poisoning.

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## REFERENCES

1. R. A. PETERS, *Adv. Enzymol.* **18,** 113 (1957).
2. C. LIEBECQ and R. A. PETERS, *Biochim. biophys. Acta* **3,** 215 (1949).
3. P. BUFFA and R. A. PETERS, *J. Physiol., Lond.* **110,** 488 (1950).
4. G. FAWAZ, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **228,** 377 (1956).
5. P. BUFFA, *Chem. Ind.* 1080 (1952).
6. O. H. LOWRY and J. V. PASSONNEAU, *J. biol. Chem.* **239,** 31 (1964).
7. E. A. NEWSHOLME and P. J. RANDLE, *Biochem. J.* **93,** 641 (1964).
8. J. R. WILLIAMSON, *J. biol. Chem.* **242,** 4476 (1967).
9. C. I. POGSON and P. J. RANDLE, *Biochem. J.* **100,** 683 (1966).
10. T. E. MANSOUR, *Pharmac. Rev.* **18,** 173 (1966).
11. P. B. GARLAND, P. J. RANDLE and E. A. NEWSHOLME, *Nature, Lond.* **200,** 169 (1963).
12. R. H. BOWMAN, *Biochem. J.* **93,** 13C (1964).
13. H. M. GODOV, E. V. CIGNOLI and J. A. CASTRO, *Life Sci.* **7,** 847 (1968).
14. J. F. MORRISON and R. A. PETERS, *Biochem. J.* **58,** 473 (1954).
15. R. A. PETERS, *Biochem. J.* **79,** 261 (1961).
16. D. W. FANSHIER, L. K. GOTWALD and E. KUN, *J. biol. Chem.* **239,** 425 (1964).
17. R. Z. EANES, D. N. SKILLETER and E. KUN, *Biochem. biophys. Res. Commun.* **46,** 1618 (1972).
18. J. J. VILLAFRANCA and E. PLATUS, *Biochem. biophys. Res. Commun.* **55,** 1197 (1973).
19. O. KRAUPP, H. NIESSNER, B. PLOSZCZANSKI, L. ADLER-EASTNER, A. SPRINGER and J. J. CHIRIKDJIAN, *Eur. J. Pharmac.* **1,** 140 (1967).
20. A. WOLLEMBERGER, O. RISTAU and G. SCHOFFA, *Pflüg. Arch.* **270,** 399 (1960).
21. O. H. LOWRY, J. V. PASSONNEAU, F. X. HASSELBERGER and D. W. SCHULZ, *J. biol. Chem.* **239,** 18 (1964).
22. W. LAMPRCHT and I. TRAUSCHOLD, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER) p. 543 Academic Press, New York (1963).
23. H. ADAM, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER) p. 573 Academic Press, New York (1963).
24. T. BUCHER and H. J. HOHORST, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER) p. 246 Academic Press, New York (1963).
25. B. L. STREHLER, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER) p. 559. Academic Press, New York (1963).
26. B. L. STREHLER and J. K. TOTTER, in *Methods of Biochemical Analysis* (Ed. D. GLICK) Vol. I, p. 341. Interscience, New York (1954).
27. I. B. MARTIN and D. M. DOTY, *Analyt. Chem.* **21,** 965 (1949).
28. S. NATELSON, J. B. PINCUS and K. K. LUGOBOY, *J. biol. Chem.* **175,** 745 (1963).
29. C. H. FISKE and Y. SUBBAROW, *J. biol. Chem.* **66,** 375 (1925).
30. T. E. MANSOUR, *J. biol. Chem.* **238,** 2285 (1963).
31. T. E. MANSOUR, in *Methods of Enzymology* (Ed. W. A. WOOD) Vol. IX, p. 430. Academic Press, New York (1966).
32. B. TRIVEDI and W. DANFORTH, *J. biol. Chem.* **241,** 4110 (1966).
33. O. KRAUPP, L. ADDLER-KASTNER, H. NIESSNER and B. PLANK, *Eur. J. Biochem.* **2,** 197 (1967).
34. A. FLECKENSTEIN, J. JANKE and E. GERLACH, *Arch. exp. Path. Pharmak.* **235,** 243 (1959).
35. E. A. NEWSHOLME, P. J. RANDLE and K. L. MANCHESTER, *Nature, Lond.* **193,** 270 (1962).
36. H. E. MORGAN, M. J. HENDERSON, D. M. REGEN and C. R. PARK, *J. biol. Chem.* **236,** 253 (1961).
37. T. E. MANSOUR and CH. E. AHLFORS, *J. biol. Chem.* **243,** 2523 (1968).
38. H. W. HOFER and D. PETTER, *Hoppe-Seyler's physiol. Chem.* **349,** 1368 (1968).
39. J. R. WILLIAMSON, E. A. JONES and G. F. AZZONE, *Biochem. biophys. Res. Commun.* **17,** 696 (1964).
40. H. P. BAER, G. I. DRUMMOND and E. L. DUNCAN, *Molec. Pharmac.* **2,** 67 (1966).
41. J. V. PASSONNEAU and O. H. LOWRY, *Biochem. biophys. Res. Commun.* **7,** 10 (1962).
42. W. J. WADDELL and R. G. BATES, *Physiol. Rev.* **49,** 285 (1969).

43. E. BITTAR, in *Cell pH* (Ed. E. BITTA) p. 16. Butterworth, Washington, D.C. (1964).
44. A. F. KAASIK, L. NILSSON and B. K. SIESJÖ, *Acta physiol. scand.* **78,** 433 (1970).
45. M. B. CHENOWETH, *Pharmac. Rev.* **1,** 383 (1949).
46. F. L. ENGEL, K. HEWSON and B. T. COLE, *Am. J. Physiol.* **179,** 324 (1954).