MALIC ACID
Hard Copy
Volume One

#18

Hard Copy-Malic Acid-Volume 1 11/11/70

#22

304

Kyushu J. Med. Sci. (1956) 7, 92-100

Studies on the Action of Sodium Succinate on the Heart

Comparison of the Action on Heart Beat between Succinic Acid and Some Other Organic Acids of Krebs TCA Cycle, and Histochemical Examination of the Activity of Succinic Dehydrogenase in the Stimulus-Conducting System of the Heart.

KAZUMA MIYAJI and YOSHIHISA SAEKI*

Department of Internal Medicine III (Director. prof. T. Sawada)
Faculty of Medicine, Kyushu University, Fukuoka, Japan.

In a previous report[1] it was shown that sodium succinate has a remarkable effect on arrhythmia of different kinds.

This action of sodium succinate, an organic acid of Krebs TCA cycle is noteworthy, but the question arises whether or not this antiarrhythmic action is shared by some other substances. We examined and compared the action of succinic acid and other organic acids of the TCA cycle in isolated cat hearts for elucidation of this problem.

The use of sodium succinate in the treatment of heart disease was first undertaken on the assumption that the heart, the part of the body where the activity of succinic dehydrogenase is highest,[2)3)] might have a special interrelation with succinate. The area for the action of sodium succinate was clinically found to be mainly located in the sinus-node and in the stimulus-conducting system. It was, therefore, conceivable that, if sodium succinate acts in conjunction with succinic dehydrogenase, as the author had been supposed, the enzyme must be more active in the sinus-node and stimulus-conducting system than in any other area of the heart. The activity of succinic dehydrogenase in this area was accordingly examined histochemically.

I. The action of succinate, acetate, pyruvate, fumarate, malate, citrate, malonate and lactate on isolated cat hearts.

Method

Six to 8 ml per kg body weight of 20 per cent urethan was given subcutaneously for anesthesia in adult

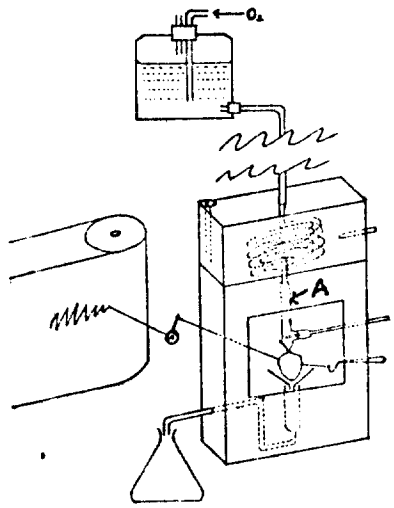


Fig. 1. Langendorff's apparatus for heart perfusion

* 宮地一馬, 佐伯嘉久
Received for publication: May 31, 1956

cats and the heart was removed 30 minutes later, washed in Locke's solution for elimination of the blood contained, and fixed in Langendorff's apparatus (Fig. 1) for heart perfusion. The perfusion fluid was so modulated previously as to stand at 60 to 80 mm Hg in pressure and be 36°C to 37°C in temperature. The cardiac movements were kymographically traced from the right ventricle by means of a lever. The different organic acids to be examined were poured in through a rubber tube A. The perfusion fluid used was Locke's solution prepared anew.

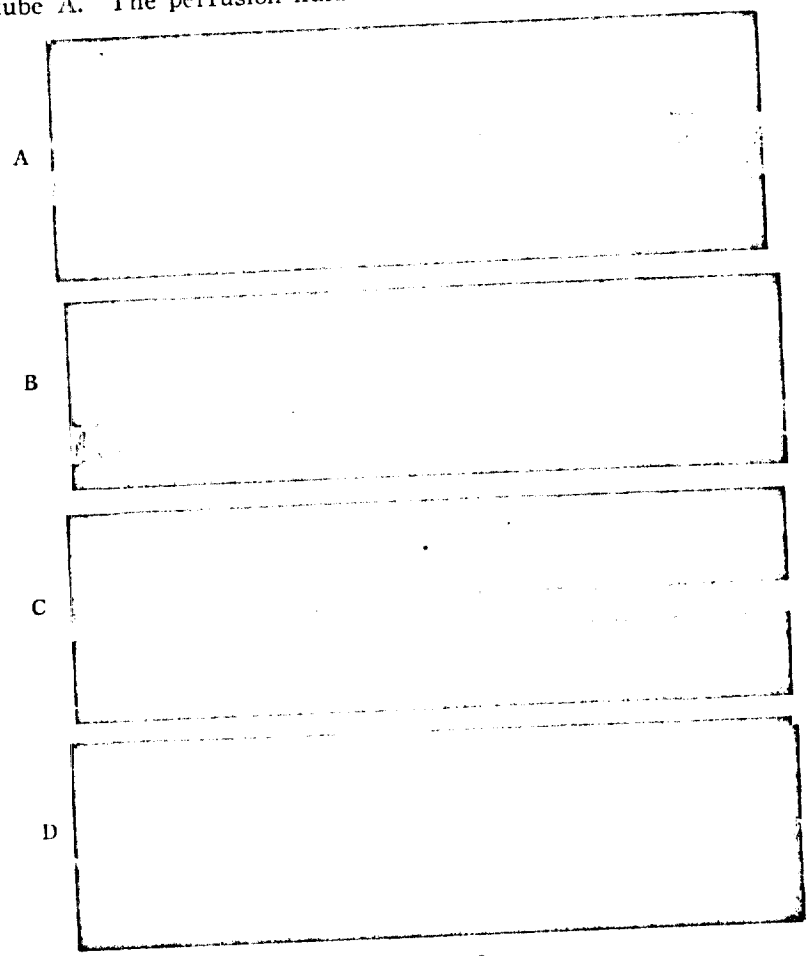


Fig. 2

A. Cardiac movement not affected by sodium succinate when cardiac movement is stabilized.
B. Arrhythmic contraction normalized by sodium succinate when heart is fatigued.
C. Arrhythmic contraction due to myocardial infarction normalized by sodium succinate.
D. Pulsations increased in number by sodium succinate when heart is fatigued, contraction is reduced in amplitude and pulsations in number.

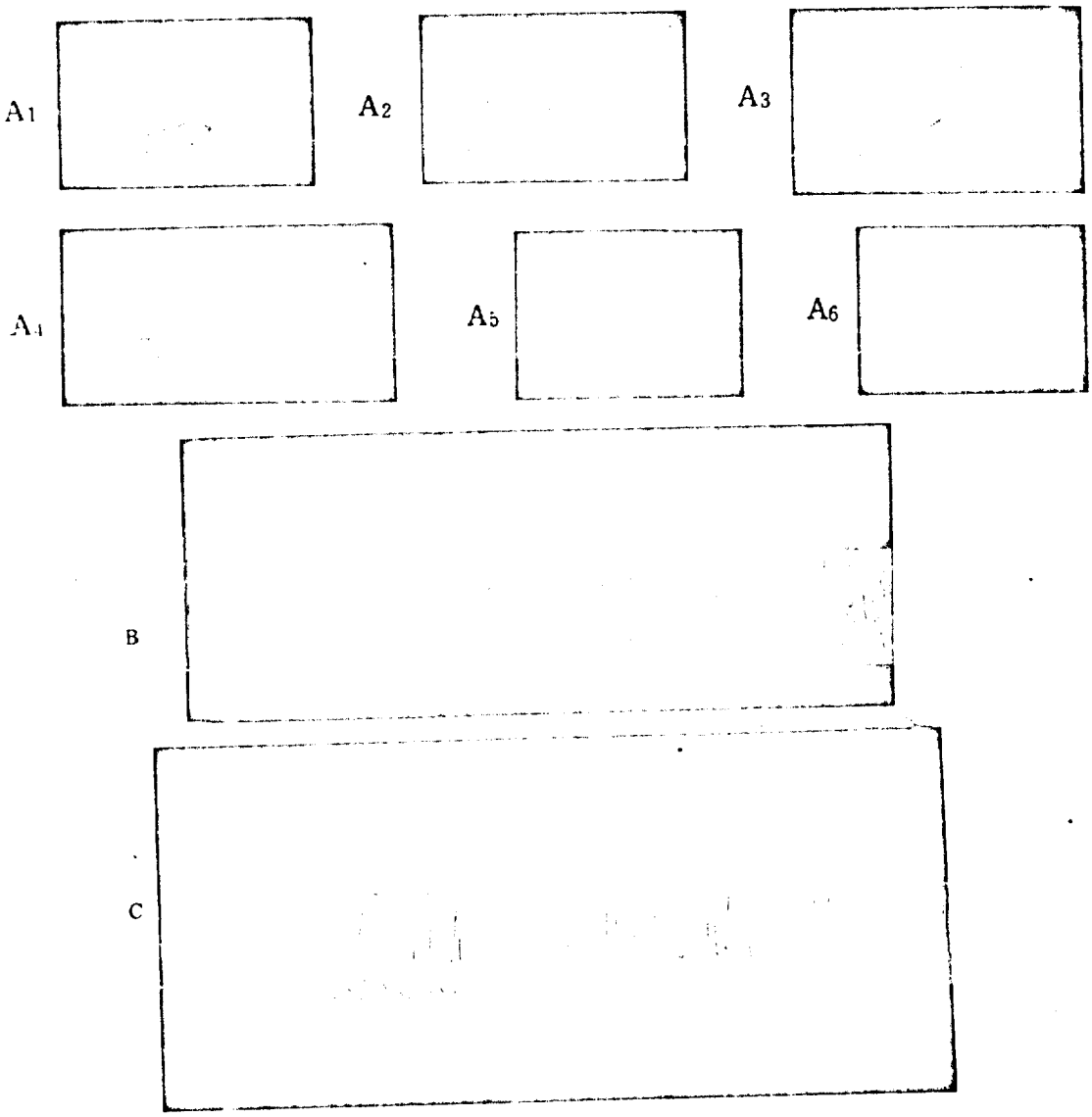


Fig. 3

A. Automatic heart movements brought back by glucose and sodium succinate when heart is in the terminal stage of fatigue and pulsation is stopped. Sodium succinate and glucose acting for longer when used together than when used singly.

B. Arrhythmic contraction produced by single and minimal induction-current stimulation normalized by sodium succinate.

C. Arrhythmic contraction due to repeated induction-current stimulus a trifle above the threshold eliminated by sodium succinate.

The organic acids examined were succinic acid, acetic acid, pyruvic acid, fumaric acid, malic acid, citric acid, malonic acid and lactic acid. Each of their sodium salts (pH 7.0) was injected in a uniform dose of 1 ml of 10^{-1}M solution for comparison of their actions.

Results

1. Sodium succinate

The heart beat was unsteady and arrhythmic systoles occured in rare cases despite of sodium succinate given in the initial stage of the perfusion. it was stabilized and sodium succinate given had no effect in most cases some 30 minutes or more after the beginning of the perfusion (Fig. 2, A); it became more and more arrhythmic and sodium succinate given markedly normalized the arrhythmic systoles some 1 to 2 hours or more after it (Fig. 2, B. C, D, of which C denoting a probable instance of arrhythmic systoles which was ascribable to a thrombus formed in the coronary artery and which became normalized by sodium succinate). The heart beat reduced in frequency and amplitude when the heart was somewhat fatigued was almost invariably improved or normalized by sodium succinate. The

A

B

C

Fig. 4

A. Cardiac movement not affected by sodium acetate when cardiac movement is stabilised.
B. Contraction amplitude reduced by sodium pyruvate.
C. Pulsations reduced in number, contraction amplitude decreased and arrhythmic contraction produced by sodium fumarate when heart is fatigued.

chemical, given together with glucose, increased the amplitude more conspicuously (Fig. 3, A 1~4), but it had no effect when the heart was apparently too tired out to respond to the action of adrenalin (Fig. 3, A5, 6).

This antiarrhythmic action of sodium succinate was tested by giving the chemical in experimental arrhythmic systoles produced by single and minimal induction-current stimulation. The effect was immediate; the arrhythmic condition was soon normalized (Fig. 3, B). The chemical often eliminated arrhythmic systoles even in case an electric stimulus a trifle above the threshold was given continuously (Fig. 3, C).

These experiments demonstrated that sodium succinate increased heart beat (1) and normalized arrhythmic systoles (2). Though it failed to increase the amplitude of pulsation in many cases, it was macroscopically noted that auricular, particularly right auricular, pulsation was made very distinct by the chemical.

2. Sodium acetate

The tracing recorded little or no effect of this chemical (Fig. 4, A). It produced no improvement of arrhythmic systoles in the period of heart fatigue. However, macroscopical observation appeared to show that the pulsation was less vigorous after administration of this chemical in every cases.

Sodium acetate proved noneffective on systoles after all.

3. Sodium pyruvate

Pulsation was unaffected in frequency but manifestly decreased in amplitude after application of this chemical (Fig. 4, B). Distilled water given in an equal quantity as a controle had little or no action on the heart. Arrhythmic systoles in the period of cardiac fatigue was not improved at all, nor was systolic power increased in any way after application of this chemical.

It was thus seen that as a general rule sodium pyruvate had an ill effect on the systolic movements of the heart.

4. Sodium fumarate

This chemical produced a slight reduction in the frequency and amplitude of pulsation when the heart movement was steady and strong; it reduced the frequency and amplitude far more markedly when the heart was moderately fatigued (Fig.4, C). It gave rise to arrhythmic systoles in some cases and never improved them in any case.

Sodium fumarate was thus found to have a fairly strong inhibitory and injurious action on the contractility of the heart.

5. Sodium malate

This decreased the frequency and amplitude of pulsation in many cases when the heart movement was steady and strong, tended to produce arrhythmic systoles and greatly decreased the amplitude when the heart was slightly fatigued (Fig. 5, A). It did not improve arrhythmic systoles at all.

Sodium malate had an inhibitory and injurious effect on the contractility of the heart in all cases.

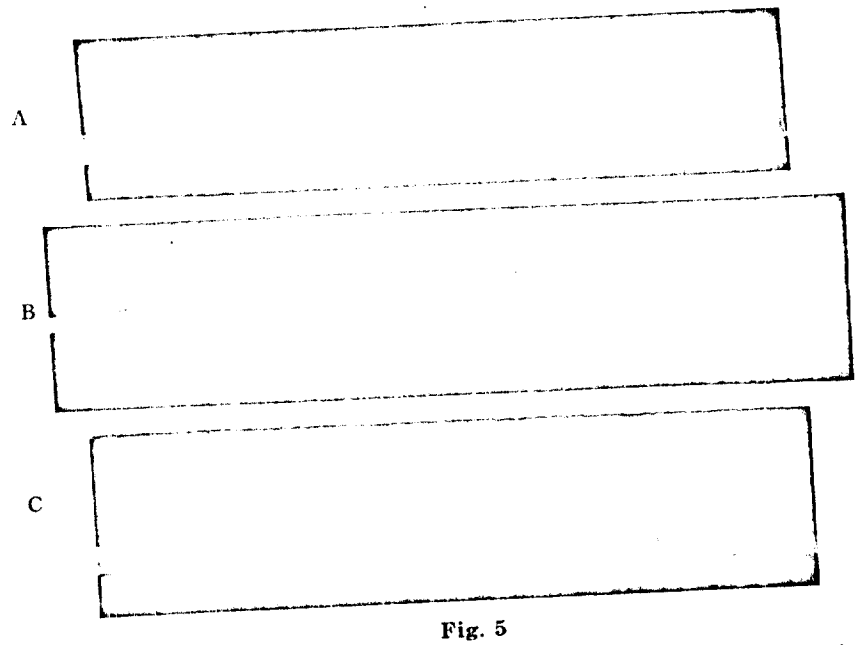


Fig. 5

A. Contraction amplitude reduced and arrhythmic contraction produced by sodium malate when heart is fatigued.
B. Contraction amplitude markedly reduced, pulsation stopped, and arrhythmic contraction produced by sodium citrate when heart is fatigued.
C. Pulsation reduced in number, contraction amplitude decreased, and arrhythmic contraction produced by sodium malonate when heart is fatigued.

6. Sodium citrate

This decreased the amplitude of pulsation in some cases when the heart movement was steady and strong; it gave rise to conspicuous arrhythmic systoles in many cases (Fig. 5, B). This untoward tendency was particularly marked when the heart was fatigued, often resulting in the cessation of pulsation. Needless to add, systolic arrhythmia was never improved by this chemical.

Sodium citrate was found to act very injuriously on the contractility of the heart.

7. Sodium malonate

This proved harmful in any stage of the heart movement, reducing the amplitude of pulsation, producing arrhythmic systole, and arresting pulsation (Fig. 5,C).

8. Sodium lactate

This had no marked effect on the contractility of the heart, bringing about no improvement of its arrhythmic condition.

II. Activity of succinic dehydrogenase in the stimulus-conducting system of the heart.

Method[3)10)6)]

The material used selectively was a fragment of the septums obtained from a fresh ox heart, because the stimulus-conducting system makes its most manifest appearance in this part of the heart. Frozen sections, 20 to 30 μ in thickness, were prepared from this and these were placed in the following mixture of substrates (pH 7.4~7.6), (which previously kept at 37°C, and left for 1 to 2 hours in it. The sections thus treated were washed well in saline solution, fixed in 10 per cent formol water for 30 minutes, and, when dried up, mounted in glycerin for microscopic examination.

The mixture of substrates used was composed of:—

0.2 M sodium succinate; 1
0.1 M phosphate buffer (pH 7.6); 1
0.1 per cent neotetrazolium solution; 1
Distilled water; 1

The control mixture used contained distilled water instead ot sodium succinate and, as inhibitor, sodium malonate in a proportion of 0.2 g per ml.

Result

Succinic dehydrogenase was detectable as purplish-blue-colored particles in the cell. The cells in the stimulus-conducting system (special muscle fibers) reacted in some degree, but far less strongly than the surrounding myocardial tissue (Fig. 6).

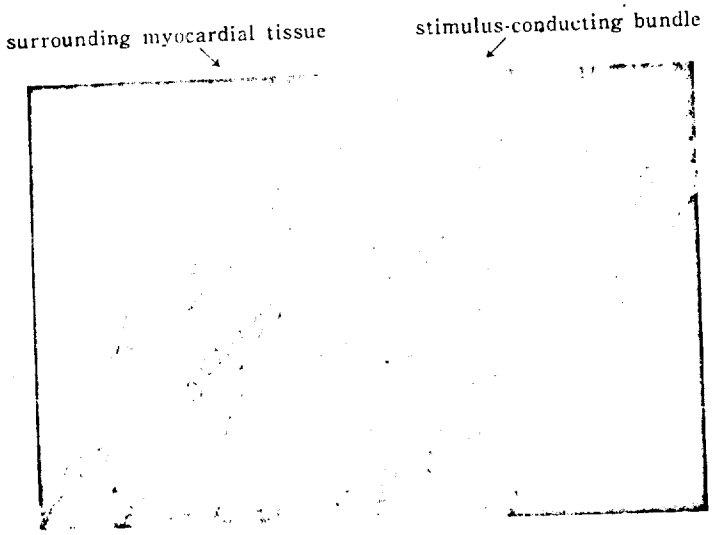


Fig. 6

Histochemical examination of the activity of succinic dehydrogenase in the septums obtained from a fresh ox heart

Frozen section was placed in the mixture of substrates (0.2 M sodium succinate; 1, 0.1 M phosphate buffer (pH 7.6); 1, 0.1 per cent neotetrazolium solution; 1, distilled water; 1).

Succinic dehydrogenase is microscopically detectable as purplish-blue-colored particles in the cells. The cells in the stimulus-conducting bundle react in some degree, but far less strongly than the surrounding myocardial tissue.

III. Discussion

1) The organic acids of Krebs TCA cycle have been studied as to their action on the isolated hearts by a number of investigators, but it is to be noted that these were used in their sodium salts in very low concentrations at 10^{-2} M, 10^{-3}M, 5×10^{-4} M, etc in Forssman's experiments.[5] for instance. Besides, the action of the chemicals was observed only for a short period 10 to 30 minutes after the beginning of a perfusion experiment, when pulsation was invariable in frequency and amplitude and the heart movement was vigorous. It was probably due to such circumstances that the specific properties of the organic acid escaped notice. Accordingly, we used the organic acids in such a high concentration as 10^{-1}M on the expectation that a high concentration would produce a qualitative difference in those acids. We also used the chemicals when the heart was fatigued on the assumption that a drug which showed no action when the heart movement was strong might have some marked action when it was weakened. The result showed that most of the organic acids of the TCA cycle had a inhibitory or injurious effect of varying degrees and that their influence was greater when the heart was more or less fatigued. Malonate, not belonging to the TCA cycle, was especially injurious and so was malate. This action of the two chemicals may be related to their action gaainst the activity of succinic dehydrogenase. Pyruvate and citrate, particularly the latter, had also an injurious action on the heart movement.

On the contrary, succinate and acetate had no ill effect in our case. The former had a notable antiarrhythmic action, especially when the heart was fatigued. This action of succinate, whose mechanism remains unknown, may be ascribable to some specific property of the chemical itself, for, when converted into fumarate on dehydrogenation, it has an injurious action.

2) Arrhythmia results mainly from disturbance of the stimulus-conducting system of the heart, so that this system may reasonably be taken as the area where succinate, the only organic acid that has an antiarrhythmic action, can act.

Histochemical examination of the activity of succinic dehydrogenase in the ordinary and the specific (stimulus-conducting) muscle fibers of myocardial tissue disclosed that the activity of the enzyme was extremely strong in the former muscle fibers and weak in the latter. The reason why succinate acts best in the stimulus-conducting system where succinic dehydrogenase acts weakly is difficult to explain from the enzymological point of view. It is conceivable, though, that the low enzymic activity may promote the action of the substance. It is probable that the antiarrhythmic effect of sodium succinate, though not finally explicable, is due to its stimulating action on the stimulus-conducting system of the heart.

IV. Summary

1. The effects on the heart beat of the sodium salts of succinic acid, acetic acid, pyruvic acid, fumaric acid, malic acid, citric acid, malonic acid and lactic acid—each in 1 ml of its 10^{-1}M solution—was examined and compared each other in isolated cat hearts. Malonate, citrate, malate, fumarate, and pyruvate were found to be injurious roughly in this order, and succinate, acetate and lactate to be harmless. Succinate was confirmed to have an antiarrhythmic action.

2. Histochemical examination of the activity of succinic dehydrogenase in the ordinary and the specific (stimulus conducting) muscle fibers of the ox heart disclosed that the activity of the enzyme was extremely strong in the former fibers and weak in the latter.

3. A series of experiments confirmed the antiarrhythmic action of sodium succinate and the conclusion was that the mechanism of this action of sodium succinate is mainly based on its stimulating action on the sinus-node and the stimulus-conducting system of the heart.

References

1. **K. Miyaji** (1956) *Kyushu J. Med. Sci.* **7**, 14
2. **Elliot, K. A., and Grieg, M. E.** (1938) *Biochem. J.* **32**, 1407
3. **Seligman, A. M.**, and **Rutenburg, A. M.** (1951) *Science* **113**, 817
4. **Rutenburg, A. M., Wolman, M.**, and **Seligman, A. M.** (1953) *J. Histochem. Cytochem.* **1**, 66
5. **Forssman and Lindsten** (1946) *Acta pharm. et Toxicol.* **2**, 329
6. **Rutenburg, A. M., Gofstein R.**, and **Seligman, A. M.** (1950) *Cancer-Research* **10**, 113

Kyushu J. Med. Sci.

Studies
Pre

TANDO
RYOKICHI
TAKESI

Dep

Our clinical, e
western Japan hav
From January
Japan. This epider
During this pe
lized outbreaks of
the fact that they
our studies on this

In Fukuoka a
Yoshii and Chitose
Fukuoka city (Fig.
We went to thes
and examined the
cases clinically an
materials from th
for laboratory inve
The cases obser
usually mild. The c
tures consisted of
tional symptoms, s
den onset, fever
duration, malaise,
muscular pains, et
spiratory sympto
appeared after th
tional symptoms h
such as nasal c
nasal discharge, s

*操 和道, 金久[illegible]
Recived for publi

Pathologia

THE EFFECT OF SUBSTANCES INFLUENCING OXIDO-REDUCTION ON THE GROWTH OF MOUSE CANCER

By

I. BANGA, J. BALÓ and P. REIMAN

I. DEPARTMENT OF PATHOLOGICAL ANATOMY AND EXPERIMENTAL CANCER RESEARCH, MEDICAL UNIVERSITY, BUDAPEST

(Received January 9, 1953)

Accumulating experimental evidence relating to the mechanism of oxidation, or, more precisely, to oxidative enzymes in tumours, appear to confirm *Warburg*'s theory, according to which tumour tissue possesses a respiratory system of low activity. Although »resting Q_{O_2}« values of tumours are comparable to those of normal tissue, the accumulation in the neoplasm of lactic acid in the presence of oxygen suggests an insufficiency of the respiratory system. A similarly low activity of tissue respiration can be found in experiments with the cytochrome system of tumours. A great number of experimental data (1, 2, 3, 4) agree in the respect that in tumour tissue the concentration of cytochrome c is low. The accumulation of lactic acid in the tumour tissue may be ascribed also to a reduction in activity of one or another of the reactions of the Krebs cycle. Pertaining evidence appears to indicate that several steps in the Krebs cycle are considerably less developed in certain tumours than in normal tissue. *Elliot* and *Greig* (5) have found lower rates of succinate oxidation in four experimental tumours. Similarly decreased succinic dehydrogenase activity has been observed in other kinds of experimental tumours, too. [*J. Shack* (6), *C. Hoch—Ligeti* (8), *F. L. Breusch* (7)]. Tumours do not form or reduce oxaloacetic acid, neither is the oxidative utilisation of oxaloacetic acid in the presence of pyruvate carried out. Both aconitase and α-ketoglutaric dehydrogenase show a lower activity (9). The conclusion may therefore be drawn that in tumours the activation of hydrogen is also disturbed. *V. J. Palladin* was the first to point out that alcoholic fermentation and respiration differ only in their final phases, inasmuch as »the hydrogen of glucose appears not as water, but in the form of alcohol«. The most important feature of tissue respiration consists just in the oxidation of hydrogen to water, as emphasized by *Palladin*.

It was assumed that if we could catalyse the autoxidation of H with the aid of certain metal complexes introduced from without, this procedure would affect the metabolism of the tumour-bearing animal so as to increase the rate of oxidation of accumulated substrates — hydrogen carriers — such as e. g. lactic acid, thereby relieving the insufficiency of tissue respiration and inhibiting

28

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

the growth of tumours. The question is, which metals and which substances forming complexes with the metals should be taken into consideration.

It was suggested that complexes of all metals known to play some role in the living organism should be tested. In the first place, Fe, Cu, Mn, Zn and Cd had to be considered. Substances used for preparing complexes with the above metals were selected from among compounds that in an earlier work of one of us had proved to be capable of forming autoxidable complexes with iron and copper (10). The most thoroughly studied of these compounds is the ferro-ascorbic acid complex (10, 11) that is converted into a blue complex when exposed to the oxygen of air. In the presence of $Na_2S_2O_4$ the complex is reduced and becomes colourless, only to turn blue again when shaken with air. This reaction can be repeated at will, indicating that we deal with a reversible oxidation-reduction system. Pyrocatechin-Fe is another well known complex. In addition to these, several organic acids — e. g. tartronic acid, malic acid, tartaric acid, citric acid, oxalic acid, mesoxalic acid and dioxymaleinic acid — also form autoxidable iron complexes (10). In the present experiments complexes formed by the metals Fe, Cu, Mn and Cd with ascorbic and other organic acids were produced and the action of the complexes on the growth of transplantable mouse cancer was studied. The report of *Baló, Vándor* and *Reiman* (12) on the stimulative effect of hydroquinone on the growth of tumours confirmed our theory concerning an eventual correlation between action on tissue oxidation and growth of tumours and added further stimulus to the initiation of the experimental work.

Experimental

Methods

Preparation of metal complexes. The guiding principle in metal complex preparation was to add at neutral reaction to a molar solution of some metal-salt an amount of the molar solution of the complex-forming substance until the particular metal ceased to give the ion reaction indicating that all the metal in the solution entered into complex linkage. Thus, the metal salt solution was, so to say, titrated with the complex-forming substance. The resulting complex was precipitated with 10 to 20 volumes of alcohol, washed with absolute alcohol and dried in a vacuum exsiccator.

Cobalt complexes. $CoNO_3$ was used as initial salt. Co-ascorbic acid, Co-acetate, Co-citrate and Co-histidine complexes were prepared. $CoNO_3$ is a reddish-mauve solution, yielding with alkali a blue precipitate. The blue precipitate is not formed when there are no more free Co ions in the solution, i. e. when they have been bound in the complex. These complexes were used without alcoholic precipitation.

Ascorbic acid-metal complexes. Cu, Mn, Cr ascorbic acid complexes, as well as Fe and Cd ascorbic acid complexes were produced. The Cu-ascorbic acid complex was prepared from Cu acetate. To 1 ml of a saturated Cu acetate solution 10 mg of ascorbic acid were added and the pH was adjusted to 7,0 with N NaOH. The Mn-ascorbic acid complex was prepared from $MnNO_3$, produced from MnO_2, concentrated HNO_3 and H_2O_2. To 1 mg of $MnNO_3$ 1 g of ascorbic acid was added and the mixture neutralized. In the case of Cr-ascorbic acid complex, 1 g of ascorbic acid was added to 1 g of K-bichromate in a neutral solution. The Cu-, Mn- and Cr-ascorbic acid complexes were precipitated with alcohol, the precipitate was washed with absolute alcohol and was dried.

Fe-ascorbic acid complex. 270 mg of Fe-sesquichloride were dissolved in 2 ml of water. 528 mg of ascorbic acid and by drops such an amount of water were added that complete dissolution was obtained. A yellowish substance resulted to which NH_4OH was added by drops. At first a brown fluid appeared that turned dark mauve on more NH_4OH. When the smell of NH_4OH persisted, no more of it was added to the solution. The mixture was precipitated with 60 ml of alcohol, centrifuged, the precipitate washed in absolute alcohol and dried in a vacuum exsiccator. Yield : 375 mg of a dark mauve powder.

Cd-ascorbic acid$_3$ complex. At neutral reaction 3 complexes can be prepared with $CdCl_2$ and ascorbic acid : 1. to 1 M of $CdCl_2$ 1 M of ascorbic acid, 2. to 1 M of $CdCl_2$ 2 M of ascorbic acid and 3. to 1 M of $CdCl_2$ 4 M of ascorbic acid is added. In the preliminary tests complex No 3 proved the most effective. The complex was prepared as follows. To 1 ml of M $CdCl_2$ 4 ml of M ascorbic acid were added and the mixture was neutralized with 4,7 ml of N NaOH. With continuous stirring at 0^0 C, 50 ml of alcohol were added to the colourless fluid and after allowing to stand for 2 hours it was centrifuged. The precipitate, separating at first voluminously, then in an oily form, was washed with absolute alcohol and dried in a vacuum exsiccator. Yield : 405 mg of white powder.

Other iron complexes. (Fe-malate, Fe-citrate and Fe-oxaloacetate complexes) were produced from Ferrum sesquichloratum, by the method described above. To 1 ml of M $FeCl_3$, 3 ml of M organic acid were added, the mixture was neutralized and precipitated with alcohol.

Mn complexes. These were prepared from $MnCl_2$. In the case of Mn malate and Mn citrate complexes, to 1 M of $MnCl_2$, 3 M of organic acid was given in concentrated solution, the mixture was neutralized, precipitated with alcohol, washed and dried.

Pyrocatechin complexes. Fe-, Cu-, Mn- and Co-pyrocatechin complexes were prepared, at pH 6, in acetate. Beside the well-known mauve ironpyrocatechin, green complexes were obtained with copper and manganese. The complexes were tested without isolation, in solution.

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

Animal experiments

Prior to the experiments, preliminary toxicity tests were performed in order to determine the dose the mice would tolerate during the experimental period of 10 to 13 days. Male and female white mice of 20 g body weight were used. The lethal dose was determined and was progressively reduced so as to arrive at a dose causing no marked lesions to parenchymal organs. In general, daily doses of 50 to 200 μg (0,0025—0,01 g/kg) were given in 0,1 to 0,2 ml of sterile saline, the 5 nitro-furfural-malononitril suspended in oleum helianthi. The solutions were injected subcutaneously at the beginning of the experiments, but recently preference has been given to the intraperitoneal route of administration, since this mode has brought more satisfactory results. Usually one injection was given daily; in the case of the highly toxic Cd-ascorbic acid complex, however, two injections were given, one in the morning and the other in the evening, in order to reduce the dose of single injections and to maintain a higher level in the blood.

Following the toxicity tests, preliminary assays were carried out with groups consisting of 5 to 10 mice in order to determine whether the complex tested possessed any stimulative or inhibitory effect on tumour growth. In the absence of satisfactory results the tests were discontinued, while successful assays were repeated several times. So, for instance, with the Fe-ascorbic acid complex five, with Mn-complexes nine and with the Cd-ascorbic acid complexes twelve series were set up. In the present experiments, a total of 989 mice was used.

Ehrlich's mouse cancer was the test neoplasm. The tumours were transplanted under sterile conditions either through a cannula or by inoculating a suspension. Treatment was begun on the day following inoculation. In a few cases we started to treat the animals before transplantation (preliminary treatment), but no significant differences were seen from this procedure. Usually, one group consisted of 10 mice and an equal number of controls. The latter received no treatment at all. The animals were fed oats, milk and bread. The duration of treatment usually varied between 10 and 13 days. The mice were sacrificed before the tumours ulcerated. At necropsy, the tumour was carefully removed and its weight was measured with 10 mg accuracy. From the weights of tumours the mean and deviation from the mean were calculated and it was determined whether a significant difference occurred between the treated group and the controls.

The following calculations were made (13).

The mean was obtained (M) by dividing the sum of the weights of individual tumours by the number of animals. The sum of the squares of the individual deviations from the mean divided by one less than the number of animals is the variance (σ), equal to the standard deviation. The square root of the sum of the squares of the means of the two groups is the error of the difference

(m_{diff}). When the difference of the two means was three times or more greater than the error of the difference, the difference between the two groups was considered significant.

In equations : $M = \frac{\Sigma x}{n}$

x = weight of individual tumours, n = number of animals.

$$\sigma = \pm \sqrt{\frac{\Sigma a^2}{n-1}} \qquad a = M - x$$

$$m = \frac{\sigma}{\sqrt{n}} \qquad m_{diff} = \pm \sqrt{m_1^2 + m_2^2}$$

When $\frac{M_1 - M_2}{m_{diff}} \geqq 3$ the difference is significant.

Rényi's method, based on the calculation of the Student's deviation, was also used.

In equations :

t = Student's deviation.

$$t = \frac{(M_1 - M_2\sqrt{n_1 \cdot n_2 (n_1 + n_2 - 2)}}{\sqrt{(n_1 + n_2)(n_1 \sigma_1^2 + n_2 \sigma_2^2)}}$$

If $n_1 = n_2$, i. e. if the number of animals in both groups is equal, the equation is the following :

$$t = \frac{(M_1 - M_2)\sqrt{n-1}}{\sqrt{\sigma_1^2 + \sigma_2^2}}$$

The value of t was looked up in Student's table of (reduced) deviation and the difference was considered significant when the probability that the result was not a product of chance was 99,75% or higher. The values obtained by the two methods were reasonably identical.

Experimental results

26 substances were tested as to their effect upon the growth of transplantable mouse tumour. In this number the initial inorganic salts are included since they were also tested as controls. As mentioned under »Methods«, daily doses of 50 to 200 μg were given, in the manner described. Several series of experiments were performed to study the effects of Fe-ascorbic acid and Cd-ascorbic acid complexes. The results and data in the Tables relate only to complexes that proved effective in the preliminary experiments. The results are

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

discussed in the order of the experiments, since the methods were modified in view of the fact that some of the substances scarcely effective when subcutaneously injected were found to exert a significant action on the growth of mouse tumours when administered through the intraperitoneal route.

TABLE I

Effect of cobalt complexes on the growth of mouse cancer. The mice were treated for a period of 10 days by subcutaneous injections

Compound	Cobalt					Control
	ascorbic ac.	citric ac.	nitrate	histidine	acetate	
	complexes			complexes		
Daily dose in μg	50	50	50	50	50	—
No of mice	10	9	10	10	10	10
Mean of weights of tumours	1,98	2,42	2,10	1,61	1,71	1,41
Standard deviation	1,11	1,13	1,07	0,48	0,64	0,36
Student's variable	1,48	3,2	2,35	0,87	1,03	—
Significant difference	1,56	3,04	1,1	0,92	1,27	—

In *Table I* the growth of tumours under the influence of Co complexes is shown. Of these complexes, Co-citric acid significantly increased the growth of transplantable tumours.

TABLE II

Effect of ascorbic acid complexes on mouse cancer. The mice were treated for 13 days by subcutaneous injections

Compound	Manganese-	Copper-	Cadmium-	Chrome-	Control
	ascorbic acid complexes				
Daily dose in μg	200	200	200	200	—
No of mice	9	13	8	9	10
Mean of weights of tumours	1,42	1,87	0,55	2,93	2,82
Standard deviation	0,91	0,81	0,34	0,74	0,77
Student's variable	3,4	2,56	2.29	0.301	—
Significant difference	3,61	2,85	8,33	0,31	—

In *Table II*, the effects of the ascorbic acid complexes of four different metals (Mn, Cu, Cd and Cr) are summarized. Mn-ascorbic acid and Cd-ascorbic acid significantly inhibited the growth of mouse cancer, while Cr-ascorbic acid and Cu-ascorbic acid were ineffective.

The effect of Fe-ascorbic acid and Cd-ascorbic acid complexes as oxidation-reduction systems on the mouse cancer and investigation of their mode of action

In the experiments, two substances attracted special interest in respect of their action on the growth of neoplasm and their biochemical properties. One of them was the Fe-ascorbic acid complex (Fe-compl.), the other the Cd-ascorbic acid complex (Cd-compl.). They exert opposite actions on the growth of the neoplasm. When given intraperitoneally, the daily freshly prepared solution of Fe-compl. strikingly increased the rate of cancer growth. (*Table III.*)

TABLE III.

Tumour growth stimulating effect of Fe-ascorbic acid complex

Daily dose	200 μg	Control
Weight of individual tumours	3,10	1,55
	2,55	0,75
	2,50	0,52
	2,45	0,25
	2,30	0,20
	2,05	0,18
	1,70	0,15
	1,60	0,15
	0,95	0,05
	0,70	—
	0,60	—
	0,60	—
Mean	1,75	0,42
Standard deviation	0,77	0,44
Student's variable	4,39	
Significant difference	3,89	

In terms of average weights, in animals treated with Fe-compl. the average weight of tumours was four times that of the controls. Cd-compl., on the other hand, exerted a marked inhibitory action on the growth of neoplasms. The average weight of the tumours of animals treated with it was one third of that of the controls. (*Table IV.*) In order to establish whether the growth of tumours was not apparent only and simply due to water uptake, the dry weights were determined. They were found to amount to 18 to 20 per cent in both treated and untreated groups.

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

TABLE IV.

Effect of Cd-ascorbic acid complex on mouse cancer. The mice were treated twice daily by intraperitoneal injections for 13 days

Daily dose	200 μg	Control
Weight of individual tumours	0,81	2,27
	0,80	2,25
	0,76	1,90
	0,66	1,52
	0,65	1,50
	0,62	1,45
	0,57	1,37
	0,54	1,37
	0,50	1,33
	0,48	1,32
	0,47	1,15
	0,45	1,08
	0,43	1,07
	0,35	1,03
	0,35	0,95
	0,14	0,94
	0,08	0,90
	0,05	0,85
	0,03	0,81
	0:01	0,92
Mean	0,43	1,29
Standard deviation	0,23	0,41
Student's deviation	7,8	
Significant difference	8,0	

Thus, we possessed two substances, one of which was already known (9,8) as an oxidation-reduction system. The freshly prepared ferro-ascorbic acid complex, and also the ferri-ascorbic acid complex produced by us, are oxidized at a high rate in the presence of oxygen, as it was shown by their intensive oxygen uptake in the Warburg apparatus. (*Table V.*) This is indicative of a state of equilibrium existing between the ferro- and ferri-complexes as well as between ascorbic acid and dehydroascorbic acid and this state of equilibrium was found to depend on the concentration of the solution, on the O_2 tension and to some extent on the pH.

TABLE V.

O_2 consumption of Ferro-ascorbic acid complex and of Cadmium-ascorbic acid complex. The Warburg vessels contained in 2 ml of water the given amount of Fe-ascorbic acid resp. cadmium-ascorbic acid complex. For binding CO_2, alkali was used. The experiment was carried out at 37° C, in the presence of air

Test substance		emm O_2			At 90 min. values. Addition	Increase %
Fe-asc.-compl. mg	Cd-asc.-compl. mg	30	60	90		
		minutes				
2,5	—	26	31	32	—	—
5,0	—	58	76	93	—	—
10,0	—	133	184	208	—	—
—	2,5	17	32	43	—	—
—	5,0	25	54	70	—	—
—	10,0	61	125	165	—	—
2,5	2,5	65	112	164	75	118
5,0	5,0	92	156	220	163	35
10,0	10,0	209	363	460	373	23

Cd-ascorbic acid$_3$ complex is also autoxidable, although to a lesser extent than the Fe-compl. The most striking fact was, however, that the Cd-compl. increased the oxidation rate of Fe-compl. *Table V* shows the oxygen consumption as determined in the Warburg apparatus. As seen, the oxidizability of complexes depends on the concentration, particularly in the case of the Fe-compl. which is oxidized more rapidly than the Cd-compl. This is obvious, because in a low concentration, during the initial shaking for 10 minutes, required for equalizing the temperatures, a relatively greater amount of substance is oxidized than in a higher concentration. The increasing action of Cd-compl. is more marked with small amounts than with large ones. — As shown in *Table V*, in the presence of 2,5 mg the increase in oxygen consumption amounts to 118 per cent, in the case of 5 mg to 35 per cent, while with 10 mg only to 23 per cent. This is attributable to the fact that oxygen uptake is limited by the tension and by the solubility of oxygen in the fluid. If these two become limiting factors, it is not purely the rate of catalysis that is measured.

To the question, which complex catalyzes the oxidation of the other, the answer was already given when it was stated that it is the Cd-compl. that catalyzes the oxidation of Fe-compl. The validity of this view was confirmed by the following experiment. In one of the series a fresh ferro-ascorbic acid complex was prepared by adding $FeSO_4$ to ascorbic acid, at neutral reaction. A colourless complex was formed (ferro-ascorbic acid complex) which, when shaken with air, turned brown, then became slowly mauve. The appearance

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

TABLE VI.

Catalyzing action of $CdCl_2$ on the oxidation of Fe-ascorbic acid complex

At neutral reaction ascorbic acid + ferrosulphate forms ferro-asc. acid complex that is converted on shaking with air into a mauve ferri-ascorbic acid complex. When to the mixture of ascorbic acid + $FeSO_4$, $CdCl_2$ is added, the mauve colour appears more rapidly, indicating an accelerated rate of ferri-complex formation

M/10 ascorbic acid ml	10 mg/ml $FeSO_4$ ml	N NaOH ml	M $CdCl_2$ ml	Appearance of mauve colour seconds
0,1	1	0,1	—	15
0,2	1	0,2	—	11
0,2	1	0,2	0,1	7

of the mauve colour was accelerated by Cd-compl., and also, to a lesser degree, by $CdCl_2$. In *Table VI*, data showing the rate how Cd-compl. accelerates the conversion of the colourless ferro-ascorbic acid complex into a coloured ferri-ascorbic acid complex are presented. This shows that the Cd-compl. ($CdCl_2$ may also form a complex with the excess of ascorbic acid) catalyzes the oxidation of ferro-ascorbic acid complex. Another evidence supporting this view is the fact that while every Cd-compl. is colourless and so is the ferro-complex, and only the ferri-complex is mauve, still, in the presence of Cd, when the concentrations of the three substances are varied, the appearance of a red complex can be demonstrated that becomes purple on shaking with oxygen. The experiment is thought to be illustrative of the competition between Fe and Cd for the ascorbic acid molecule. There is evidence that iron has a greater affinity to ascorbic acid than cadmium, since even with a Fe : Cd ratio of 1 : 3 ferro-complex is formed. Thus the red or purple complex mentioned above is indicative of the formation of a mixed complex presumably containing both Fe and Cd, both binding ascorbic acid molecules. The formation of this intermediary complex might be held responsible for the increase in the rate of oxidation.

TABLE VII.

Oxidation stimulating action of ferro-ascorbic acid complex with Cd-ascorbic acid complex, in the presence of dipyridil

Fe-asc. compl. mg	Cd.-asc. compl. mg	Volume ml	Dipyridil mg	Appearance of red colour					
				0	2	10	20	60	120
				minutes					
1	—	1	0,2	++	+++	+++	+++	+++	+++
1	2	1	0,2	++	+++		+++	+++	+++
1	4	1	0,2	0	+	++	++	+++	+++
1	8	1	0,2	0	0	0	+	+	+++

It was assumed that Cd-compl. catalyzes ferro-complex oxidation by means of electron uptake. This was proved by experiments made with $\alpha\,\alpha'$dipyridil. Ferro-ions form a red compound with dipyridil. After the ferro-ferri conversion has been completed, the dipyridil reaction falls out and no red colour appears. This happens also when Cd-compl. is added to the ferro-ascorbic acid complex, prior to the addition of dipyridil. The dipyridil reaction does not occur, indicating the conversion by Cd-compl. of ferro-complex into ferri-complex. Data relating to an experiment of this kind are presented in *Table VII*, which shows that in the presence of Cd, ferro ions cannot react with dipyridil and no red ferro-dipyridil complex is formed. The reaction depends on the quantity of Cd-compl. present. The more the Cd-compl., the longer it is able to keep ferro atoms in the state of ferri. But when the excess of ascorbic acid is oxidized, there is nothing left that would convert ferro into ferri and the stable ferro-ascorbic acid complex is formed. The ferro atom of the latter reacts with dipyridil for which it has more affinity and the red colour appears. Hence the relatively great quantity of Cd-compl. required to obtain a marked reaction. It is known that the ferro-ascorbic acid complex and the ferri-ascorbic acid complex form an oxidation-reduction system, in which in the state of equilibrium the ferro-ascorbic acid complex predominates. All these experiments prove that the redox potential of Cd-compl. is higher than that of Fe-compl., i. e. the former is capable of oxidizing the latter. The question is whether the difference between the redox potentials of the two substances could be held responsible for the fact that while the ferri-complex increases, Cd-compl. decreases the growth of tumours.

TABLE VIII.

Effect of Fe-asc. compl. on the paraphenylenediamine oxidation of mouse cancer

Mouse cancer suspended in N/15 phosphate buffer at a ratio 1 : 4. Warburg vessels contain 1 ml of mouse cancer suspension in a total volume of 2 ml + 5 mg of paraphenylenediamine resp. 5 mg of Fe-ascorbic acid complex. Shaken in air at 37° C

Minutes	cmm O_2 Mouse cancer + p. phen. d.	cmm O_2 Mouse cancer + Fe-asc. compl.	Addition	cmm O_2 Mouse cc. + p. phen. d. + Fe-asc. compl.	Fe-asc. inhibitory action %
20	27	36	63	49	22
40	51	71	122	76	38
60	68	95	163	96	41
80	86	127	213	116	45
100	101	160	261	131	50

To elucidate this point, the effects of both substances on the paraphenylenediamine oxidation of mouse cancer were subjected to studies. Since in respiratory experiments the tissues are usually suspended in a phosphate buffer, the oxidation rate of both Fe- and Cd-complex had to be determined in an

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

N/15 phosphate buffer medium. It was revealed that in the phosphate the oxidation of Fe-compl. was slightly reduced, while the reduction in the oxygen uptake of Cd-compl. was rather marked. Nevertheless, even under such conditions the oxidation of Fe-compl. was increased by Cd-compl. Since, when studying the paraphenylenediamine reaction, control experiments were invariably performed and their results are shown in the Tables, the inhibitory action of the phosphate buffer can be ignored. In *Tables VIII* and *IX* are shown the effects of Fe-compl. and Cd-compl. upon the paraphenylenediamine oxidation of mouse cancer. The results in *Table VIII* and *Table IX* prove that the oxidizing activity of the respiratory ferment — cytochrome oxidase —, responsible for paraphenylenediamine oxidation, is reduced by Fe-compl. and increased by Cd-compl.

TABLE IX.

Effect of Cd-ascorbic acid complex on the paraphenylenediamine oxidation of the mouse cancer
Experimental conditions similar to Table VIII, only instead of the Fe-ascorbic acid complex, Cd-ascorbic acid complex was used

Minutes	cmm O_2 Mouse cc.+ p. phen. d.	cmm O_2 Mouse cc.+ Cd-asc. c.	Addition	cmm O_2 Mouse cc.+ p. phen. d. +Cd-asc. c.	Cd-asc. compl. increasing action %
20	26	5	31	44	42
40	50	12	61	93	52
60	64	16	80	132	65
80	73	19	92	155	68
100	84	23	107	205	91

In the Warburg vessels the following observations were made at the end of the experiment. In the vessels containing paraphenylenediamine alone, a blue colour appeared, because the oxidation product of paraphenylenediamine is a blue quinone.The same colour was seen in the vessels containing paraphenylenediamine and Fe-compl. In the presence of Cd-compl., however, the fluid remained white, indicating that Cd-compl. was capable of reducing the quinone at a higher rate than it was formed. In the presence of a great amount of Fe-compl., the quinone is also reduced, because ascorbic acid itself also reduces quinone.

To gain deeper insight into the mechanism of action of the two substances, experiments were carried out with rat muscle. The paraphenylenediamine oxidase contained in the muscle is powerful and the oxidation to quinone progresses more rapidly and intensely. Into long 2 ml tubes of a small diameter 1 ml=300 mg of fresh rat muscle and an amount of paraphenylenediamine (4 mg) were placed that was oxidized within 5 minutes. Thus the tubes contained a suspension of an intensive blue colour. When the tubes were allowed to stand,

on the bottom the muscle particles sedimented and where no contact with the oxygen of air was possible, the blue colour slowly faded and the muscle parts became colourless. The muscle reduced quinone. On shaking with air, the decolourized part turned blue again, indicating the reversibility of the reaction and offering proof that it is the hydrogen carriers in the muscle that are responsible for the reduction, i. e. decolourization, of the oxidized blue quinone. When Fe-compl. was also added to the mixture, decolourization depending on the amount added ensued, but the reaction was not reversible, since the solution did not become blue when shaken with air. Nor does the blue colour appear on prolonged standing, indicating that the respiratory ferment catalyzing paraphenylenediamine oxidation had lost its activity. When the experiment is carried out with Cd-compl., decolourization results, even with smaller amounts of Cd-compl. than what was required of Fe-compl., but here the substance turns blue again when shaken with air. Also repeated decolourization ensues more rapidly than in the control tube, showing that the reduction of quinone is also increased by the Cd-compl.

The results of all these experiments and the colour reactions obtained are all, and emphasis is laid on this point, dependent on the ratio of concentrations of the substances introduced. In the presence of great amounts of muscle and paraphenylenediamine and small amounts of Fe-compl. the respiratory system is not completely blocked and the blue colour of quinone appears. In the presence of large quantities of Fe-compl. paraphenylenediamine oxidation cannot result and the mixture remains colourless. When Fe-compl. is added to tubes containing quinone, the former reduces the latter, but the oxidation of quinone progresses at a much slower rate. On the other hand, the reduction of quinone is increased by Cd-compl., and thereby respiration is increased.

Since Fe-compl. proved remarkably autoxidable in the experiments and was found to inhibit the action of the respiratory ferment, there are two explanations for its increasing the rate of neoplastic growth. On the one hand, it may absorb oxygen, thereby increasing the oxygen deficiency in the anoxaemic tissues of the tumour-bearing organism. This explanation, however, does not appear probable, since the injected amount of Fe-compl. is too small to absorb significant amounts of oxygen. In spite of this, there still appears to be a difference between the actions of fresh and oxidized Fe-complexes in their influence on the growth of tumours. In one series, 10 tumour-inoculated mice were given freshly prepared Fe-comp. previously oxidized by means of shaking with air. Since there is an equilibrium between the oxidized and reduced complexes, no 100 per cent oxidation can result. By means of experiments carried out in the Warburg apparatus it could be determined that after shaking in air for a period of a few hours the rate of oxidation was reduced. In conformity with this observation were the oxidized complexes produced. The results are shown in *Table X*. It can be seen that the oxidized ferri-complex did not increase as

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

actively the growth of tumours as did the freshly prepared complex, although compared with the controls there still remained some tumour growth increasing effect. The difference between the two substances was significant, while no significant difference existed between the oxidized Fe-compl. and the controls. The other explanation of the tumour growth stimulating action might be that the affinity for the respiratory ferment is primarily a property of the freshly prepared Fe-compl., which contains iron in reduced form and reacts with the respiratory enzyme, thereby altering the catalyzing action of the latter.

TABLE X.

Effect of fresh and oxidized Fe-ascorbic acid complexes on the growth of mouse cancer. The mice were treated for 13 days by intraperitoneal injections

Compound	Fe-ascorbic acid complex		Control
	Oxidized	Fresh	
Daily dose	200 μg	200 μg	
Weight of the single tumours	2,27	3,95	1,27
	1,97	3,30	1,12
	1,55	2,97	1,08
	1,51	2,64	1,05
	1,27	2,02	1,00
	1,04	1,80	0,84
	0,77	1,79	0,65
	0,75	1,77	0,54
	0,59	1,64	0,51
	0,56	1,09	0,48
Mean	1,22	2,29	0,85
Standard deviation	0,56	0,84	0,28
Student's variable	3,17	4,87	
Significant difference	3,36	5,3	

In the case of Cd-compl., the main characteristic of the reaction mechanism consists in the increased rate at which oxidized products are reduced and consequently the process of oxidation is accelerated, as with paraphenylenediamine oxidation. Further investigations are required to justify the assumption that Ca-compl. reacts with the respiratory ferment (cytochrome oxidase) and that in this way a new autoxidable complex is formed. Evidence supporting this view was obtained in the experiments in which the Cd-compl. proved to be capable of catalyzing the oxidation of Fe-compl., and it might be assumed that it may influence the catalysis of the Fe-atom in the respiratory ferment as well.

Experiments with Fe-compl. and Cd-compl. offered proof that on increasing the activity of tissue respiratory systems tumour growth is inhibited, while the decrease in the activity of the oxidation system considerably stimulates the growth of neoplasms.

Due to its high toxicity, Cd-compl. is not suitable for therapeutical use. Therefore, a substance had to be sought for that would similarly to the Cd-compl. exert an inhibitory action on tumour growth, without being toxic. Such substances were found among the manganese complexes.

Mn complexes

From among the Mn complexes, the Mn-ascorbic acid complex *(Table II)*, Mn-malate and Mn-citric acid complexes reduced the growth of tumour in the initial experiments. The effects of Mn-malate and Mn-citric acid were studied in a number of experiments. The results uniformly indicated that both complexes significantly retarded the growth of the tumour, as shown in *Tables XI* and *XII*. Neither of these substances being toxic, they might be used in therapy as well.

TABLE XI.

Effect of manganese-malate and manganese-citrate complexes on the growth of mouse cancer. The mice were treated for 13 days by intraperitoneal injections

Compound	Mn-malate complex	Mn-citrate complex	Control
Daily dose	200 μg	200 μg	
Weight of the single tumours	0,81	1,18	3,20
	0,80	1,13	2,92
	0,76	0,96	2,52
	0,75	0,84	2,36
	0,73	0,74	1,98
	0,70	0,73	1,63
	0,65	0,66	1,53
	0,50	—	1,43
	0,50	—	1,23
	—	—	1,20
Mean	0,68	0,89	2,00
Standard deviation	0,11	0,18	0,67
Student's variable	6,11	2,96	—
Significant diff.	6,13	5,01	—

Attention is drawn to the possibility that this text may be copyrighted. The reader is urged to investigate its copyright status.

TABLE XII.

Effect of manganese-malate complex on the growth of mouse cancer.
The animals received 10 intraperitoneal injections

Daily dose	200 μg	Control
Weight of the single tumours	1,58	3,45
	0,77	2,80
	0,50	2,47
	0,49	2,20
	0,47	1,57
	0,46	1,45
	0,46	1,17
	0,40	1,00
	0,39	1,00
	—	1,00
Mean	0,61	1,83
Standard deviation	0,35	0,83
Student's variable	3,86	
Significant difference	4,25	

TABLE XIII.

Effect of 5-nitro-furfural-malononitril on the growth of mouse cancer.
The animals received 9 intraperitoneal injections in 12 days

Daily dose	50	Control
Weight of the single tumours	1,18	2,80
	0,98	2,02
	0,88	1,75
	0,50	1,72
	0,33	1,32
	0,26	0,90
	0,26	0,60
	0,00	0,45
	0,00	0,28
Mean	0,43	1,31
Standard deviation	0,46	0,77
Student's variable	3,01	
Significant difference	3,01	

Through the courtesy of the Pharmacoindustrial Research Institute (Dr. I. Varga) we have received two substances, (*i*) para-nitrobenzal allononitril and (*ii*) 5-nitro-furfural-malononitril, to test them for action on the growth of transplantable tumours. In our experiments, substance (*ii*) administered intraperitoneally proved to be a growth-inhibiting agent. The results are shown in *Table XIII*. Unfortunately, the substance is not soluble in water and had thus to be administered in an oily suspension. The substance is probably autoxidizable as the originally colourless suspension of the compound in oil becomes orange when allowed to stand for a time. If the substance is really autoxidable, it is likely that the mechanism of its action is similar to that one discussed in connection with the Cd-compl.

TABLE XIV

Comparison of tumour stimulator substances on basis of the significant differences

Fe-ascorbic acid	Cobalt-citrate
5,3	3,04

Comparison of tumour inhibitors on basis of the significant differences

Cd-ascorb. compl.	Mn-malate compl.	Mn-citrate compl.	Mn-ascorbic acid compl.
8,33	6,13	5,01	3,61

A comparative study of tumour inhibitor and stimulator agents based on significant differences is presented in *Table XIV*. According to *Table XIV* the growth of transplantable mouse cancer is significantly increased by two complexes, Fe-ascorbic acid complex and Co-citrate complex while four tumour growth inhibitors were found, namely the Cd-ascorbic acid, Mn-malate, Mn-citrate and Mn-ascorbic acid complexes. Of the latter, Cd-ascorbic acid complex was the most active.

Discussion

The substances prepared and tested for action on the growth of mouse tumours were all autoxidable metal complexes. Some of them increased, while others retarded neoplastic growth. For studying the mechanism of action, two complexes of opposite effect, Fe-complex and Cd-ascorbic acid complex, were used. Action on the respiratory ferment (cytochrome oxidase) was deter-

mined by means of paraphenylenediamine oxidation tests. The oxidation o paraphenylenediamine was inhibited by the tumour stimulator Fe-compl., an increased by the tumour growth inhibitor Cd-compl. In experiments *in vitr* it was shown that the tumour growth inhibitor Cd-compl. increased the autoxi dation of Fe-compl., that hastened tumour growth, and that the two compound together form a more active autoxidable system. It was demonstrated that th quinone-like oxidation product of paraphenylenediamine was strongly reduce by the Cd-compl. and it seems plausible that it is in a similar manner that th Cd-compl. acts in the live organism. It is, however, also possible, that just a it can form a more active autoxidable system with the Fe-compl., in the liv organism it joins the cytochrome oxidase system and thereby intensifies it autoxidation. The tumour growth stimulator Fe-compl. is supposed to exer its action by inhibiting the respiratory ferment. *Hanoniya* (15) has studie the effects of l- and d-ascorbic acid on the Krocker sarcoma and has found tha the growth of the tumour was substantially retarded by d-ascorbic acid. It ma be supposed that in the organism d-ascorbic acid is capable of forming a meta complex catalysing the activity of the oxidative respiratory system. (See Mn ascorbic acid complex.)

All the above point to the great importance of oxidative mechanism i the origin and development of tumours. If tissue oxidation is in some way inhibit ed or its activity decreased, the organism as a whole becomes, so to say, »sus ceptible for carcinogenesis«. Even locally, the neoplasm will probably star at the site of the greatest oxygen deficiency, i. e. in the most anoxaemic tissue Studies with benzpyrene prove that oxygen lack leads to the development o cancer. According to *Berenblum* and *Schoental* (16), and to *Mueller*, *Mille* and *Busch* (17), benzpyrene takes up oxygen and is thereby converted int a non-carcinogenic quinone.

The experiments presented here are, for the time being, of theoretic interest only. The most thoroughly tested Cd-compl. is toxic and cannot b used in therapy. Recently, Mn-malate and Mn-citrate have been found to inhibi the growth of cancer. Their mode of action *in vitro* has not been studied bu since these compounds too form an oxido-reductive system, it is likely tha their mode of action is in all respects similar to that of Cd-compl. These sub stances are far less toxic than Cd-compl. and tests are being carried out to stud their action on other kinds of tumours.

Summary

1. 26 compounds have been tested for their action on the growth of trans plantable mouse tumours. Autoxidable metal complexes either stimulated o inhibited the growth of neoplasms.

2. The most conspicuous tumour stimulator was the Fe-ascorbic acid complex, the most effective inhibitor the Cd-ascorbic acid complex. Experiments *in vitro* have been carried out with these two complexes for studying the mode of action of the oxido-reductive process.

3. The rate of oxidation of the ferroascorbic acid complex can be catalitically increased with Cd-complex. The two complexes form together an autoxidable double complex of increased activity.

4. In *Warburg* experiments paraphenylenediamine oxidation of the mouse cancer is inhibited by Fe-compl. while stimulated by Cd-asc. compl. Experiments performed *in vitro* with muscle suspensions demonstrated that the Fe-complex inhibited the activity of the respiratory ferment (cytochrome oxidase), while the Cd-compl. accelerated the reduction of the quinone formed. The conclusion is drawn that both complexes act on the respiratory ferment, in one case inhibiting, in the other intensifying its action.

5. A tumour growth inhibiting activity similar to that of Cd-complex is displayed by some Mn-complexes. From among the Mn-ascorbic acid, Mn-malate and Mn-citrate complexes, Mn-malate has proved the most active. The toxicity of Mn-malate being considerably lower than that of the Cd-complex, the compound might eventually be used in therapy.

LITERATURE

1. *Holmes, B. E.:* Biochem. J. **20**, 812 (1926).
2. *Euler, H., Gunther, G.* and *Forsman, Z.:* Krebsforsch. **49**, 46 (1939).
3. *Stotz, E.:* J. Biol. Chem. **131**, 555 (1939).
4. *Dubois, K. P.* and *Potter, V. R.:* Cancer Research **2**, 290 (1942).
5. *Elliot, K. A. C.* and *Grieg, M. E.:* Biochem. J. **32**, 1407 (1938).
6. *Shack, J.:* Nat. Cancer. Inst. **3**, 389 (1943).
7. *Breusch, F. L.:* Biochem. Z. **295**, 101 (1938).
8. *Hoch-Ligeti, C.:* Cancer Research **7**, 148 (1947).
9. *Wenner, C. E., Spirtes, M. H.* and *Weinhouse, S.:* Cancer Research **12**, 44 (1952).
10. *Banga, I.:* Z. f. Phys. Chem. **254**, 165 (1938).
11. *Szent-Györgyi, A.:* Z. f. Phys. Chem. **225**, 168 (1934).
12. *Baló, J., Vándor, F.* and *Reiman, P.:* Kisérletes Orvostudomány **2**, 236 (1950).
13. *Fischer, R. A.:* Statistical Methods for Research Workers 5th Ed. London (1934).
14. *Rényi, A.:* Valószínűségszámítás. (Compendium) (1952).
15. Hanoniya, F. S. (Ханония, Ф. С.): Бюл. Научно-исследовательской лаборатории экспериментальной химиотерапии Министерства Здравоохранения СССР, **11**, 1, 61—65 (1949).
16. *Berenblum, I.* and *Schoental, R.:* Cancer Research **3**, 145 (1943).
17. *Mueller, G. C., Miller, J. A.* and *Rusch, H. P.:* Cancer Research **5**, 401 (1945).

From the Private Research Institute Prague II/1482

Effects and fate of acid in the body

I. Determination of hydrogen ion concentration in streaming rabbit blood after intravenous injection of free acids

By Siegwart Hermann, Richard Neiger and Margot Zentner

Text includes 5 figures

(Received on December 15, 1937)

WE had established in earlier studies (1) that after intravenous supply of gluconic acid the hydrogen ion concentration increases. From various effects after oral supply of gluconic acid we could conclude also that an increase of the hydrogen ion concentration takes place in the body. Thus, it was possible with Kombuchal, which contains 30% free gluconic acid, to prevent, and even in part to bring back to normal, phenomena of calcification after Vitasterin (2). Also the variation of the calcium quotient (3), i.e. the increase of dissociated calcium in the serum, may be attributed to variation of the hydrogen ion concentration. Gluconic acid is one of the acids burnable with difficulty, and it is easy to see, therefore, that it can shift the pH in the body toward the acid side. But there are also acids which show the opposite effect, e.g. acetic acid, which for various reasons is grouped with the more easily "burnable" acids. Our studies brought out findings which could not always be reconciled with existing ideas. We therefore decided to examine systematically a number of acids. This paper will deal exclusively with variations of hydrogen ion concentration in the blood after supply of free acids (not salts!). To measure the hydrogen ion concentration we opted to use v. Brehmer's intravital method (4), as we had the impression from publications of Bücheler, Zucker and Capaldi (5), Wünsche (6) and from our own comparative measurements that this method furnishes very useful results for comparative determinations.

Method

Rabbits of an average weight of 2000 g were put on a diet of oats and water before the start of the test. In this experiment it is important that the animals do not come in contact with metal. Clamping devices may have to

be wrapped with insulating tape. The rabbit is strapped on a wooden board, the right front paw passed through an opening in the board and fixed. The paw, from which hair and fat or grease have been removed, is dipped in a vessel containing saturated potassium chloride solution. This vessel is conductively connected with the measuring vessel of the ionometer (Lautenschläger) by a tube filled with potassium chloride agar. The Antimony-Hemoven needle is introduced into the peripheral ear vein of the rabbit and thereby the circuit is closed. The suitability of the respective Hemoven needle is tested on solutions of known pH and also results already from the testing of the rabbit's normal pH values. Sometimes a sudden drop of the pH occurs. If this is due only to the formation of a thrombus, satisfactory values are again obtained after removal of the clot. Otherwise the Hemoven needle must be replaced by another. The rectal temperature of the rabbit was taken into account in the correction of the pH values. We regarded as normal pH values those which remained constant for 1/2 hour before the injection. The acid was injected through a cannula with cock into the Vena jugularis dextra. In the course of 3 minutes, 10 cc of the respective acid were injected, whereupon the pH of the streaming blood was measured for 10 minutes, another injection was made,etc. We report below the results of our measurements after injection of various acids in the form of curves. The observation time was approximately 6-8 hours. Although 4-6 animals were used for each acid, we publish, for the sake of brevity, only one of the curves obtained, where they are substantially coincident.

Results of pH measurements after intravenous supply of free acids in streaming blood.

1. Phosphoric acid

We injected n/2 phosphoric acid of a pH 1.45. It is lipoid-soluble. The lethal dose is 62.30 cc n/2 acid or 1 g H_3PO_4 per kg of rabbit. The average pH lowering is 0.58. Death occurs suddenly with dyspnea and spasms of the extensor muscles. Attempts to save animals immediately after appearance of the first symptoms of intoxication by intravenous injection of sodium bicarbonate failed. The effect of the acid on the streaming blood in the various phases

can be seen from curve 1, Fig. 1.

2. Gluconic acid

By n/2 gluconic acid the pH is lowered on the average by 0.46. The pH of the n/2 acid is 2.05. Gluconic acid is lipoid-insoluble. Very large quantities of gluconic acid were tolerated both orally and parenterally. In our experiments the lethal dose was not reached at all. The animals were given 6 g or 61 cc n/2 gluconic acid per kg intravenously. At these quantities, dyspnea did occur but could be eliminated by injection of 10 cc of a normal sodium bicarbonate solution two tothree times. Also the blood pH returned to normal. After removal of the cannula the animals lived for several more days (Fig. 1, curve 2).

3. Lactic acid

n/2 lactic acid, which has a pH of 1.90, clearly acidifies the blood. It is tolerated by the rabbit in relatively large quantity. It is lipoid-soluble. The toxic dose is 1.76 g per kg of animal, the lethod dose, 2.01 g or 80 cc n/2 acid per kg (Fig. 1, curve 3).

4. Tartaric acid

We injected n/2 acid of pH 1.8. The average lowering of the pH after the intravenous injection is 0.4. Tartaric acid is lipoid-soluble. After oral absorption it is said to be unburnable (7). The lethal dose was 1.1 g or 30 cc n/2 tartaric acid per kg of rabbit. Tonic spasm does not occur until just before death (Fig. 2, curve 1).

5. Hydrochloric acid

We injected n/4 hydrochloric acid. Its pH is less than 1.0. It is lipoid-soluble and unburnable. The maximum quantity that can be injected per kg of rabbit until death occurs is 0.6 g or 54.2 cc n/4 hydrochloric acid. The pH is reduced on the average by 0.35, the hydrogen ion concentration increased accordingly. Death occurs with the same symptoms as for phosphoric acid. Injections of sodium bicarbonate as antipoison are unsuccessful (Fig. 2, curve 2).

6. Mandelic acid

We injected n/4 solutions of a pH 2.05. The average reduction of the pH was 0.25. Mandelic acid is lipoid-soluble and unburnable in the body. Note the high toxicity. The lethal dose is 1.65 g or 15.5 cc of a n/4 solution per kg of rabbit. n/2 solutions could not be injected at all because of the great toxicity. The response of the pH appears in Fig. 2, curve 3.

7. Acetic acid

After intravenous injection of n/4 acetic acid one sees a definite alkalization of the blood. The blood pH is increased on the average by 0.5. This increase is attributable to sodium acetate formation. The pH of a n/2 sodium acetate solution is 7.9. After injection of the 50th cc of n/4 acetic acid the blood pH dropped sharply. The animals then perished rapidly showing dyspnea and spasm. At this point the acetic acid has exhausted the available alkali reserve, so that an excess of free acetic acid causes death very rapidly. The spontaneously discharged urine is bloody. The measurements of the urine pH during the experiment showed a slight increase from 5.3 to 5.45. Acetic acid is lipoid-soluble, the pH of the n/4 acid is 2.70. The lethal dose is 0.5 g CH_3COOH or 33.5 cc of a n/4 solution per kg of animal (Fig. 3, curve 1).

8. Maleic acid

We injected n/2 solutions of a pH 1.50; n/4 acid of a pH 1.60, and n/8 acid of a pH 1.70. The n/2 acid is extremely poisonouse. After the injection of a n/2 acid, the pH drops quickly, in order then to rise a little. At this point death occurs. After injection of the n/4 acid, the pH increases somewhat, then returns to the starting point, and again rises greatly. Only just before death, with the appearance of dyspnea and spasm, there occurs a decrease of the blood pH almost to normal. After the injection of the n/8 acid, the alkalization is quite pronounced. Maleic acid is lipoid-soluble. The lethal dose for the n/8 solution is 0.19 g maleic acid or 26 cc per kg of animal (Fig. 3, curve 2).

9. Fumaric acid

Fumaric acid is isomeric with maleic acid as to structure. Looking at curve 3 of Fig. 3 in the course of the injections with a n/8 acid, one finds a striking difference from the maleic acid curve. As much as 70 cc of fumaric acid can be injected without any pH variation. Later the pH rises rather steeply, and before death drops very rapidly to below normal. n/8 fumaric acid has a pH of 2.25. It is lipoid-soluble. The lethal dose of the n/8 fumaric acid is 0.47 g per kg of animal, that is, more than double that of maleic acid.

10. Malic acid

n/4 malic acid, which has a pH of 2.20, shows a varying shape of the curves during the injection in four different tests. Two tests showed an increase, two a decrease of the pH. Perhaps this is due to the convertibility of this acid in the body. Or it may be that malic acid as a dibasic acid forms acid as well as neutral salts in the blood, so that the reaction is determined by the ones or the others. Malic acid is little lipoid-soluble. The lethal dose of a n/4 acid is 2.40 g or 106 cc per kg of animal (Fig. 4). (LD 100?)

11. Succinic acid

There is a great difference in tolerance depending on whether n/4 or n/2 acid was injected. Of the 4/n acid more than 200 cc were tolerated, while a maximum of 50 cc of the n/2 could be injected. The shape of the curves is similar to that of malic acid (there is a very close chemical kinship) in that we have again a zig-zag course. The pH values fluctuate about the starting value. It is likely that with succinic acid, just as with malic acid, in part the acid salt and in part the neutral salt are formed, so that a clear pH variation cannot develop. Succinic acid is lipoid-insoluble. Of the n/2 acid 0.54 g or 18.1 cc perkg of animal were tolerated, of the n/4, 1.63 g or 88.0 cc per kg (Fig. 5, curve 1).

12. Citric acid

We injected n/4 and n/8 solutions. The pH of the n/4 acid is 2.15, that of the n/8 acid, 2.3. It is little lipoid-soluble. Supplied orally, it is burnable, according to Langecker (8). The lethal dose for the n/4 acid is

0.28 g or 17.10 cc per kg; for the n/8 acid, 0.22 g or 32.90 per kg of animal. From Fig. 5, curve 2 is seen the tendency to alkalize temporarily. The pH is temporarily increased, the hydrogen ion concentration decreased accordingly.

Summary

1. After intravenous injection of various acids, the variation of the hydrogen ion concentration in streaming venous blood was determined.

2. We may distinguish three basic types of acids: Those which acidify the blood, those which alkalize it, and those which give deflections in the acid and alkaline direction.

3. According to their capacity of blood acidification we have the following series: Phosphoric acid > gluconic acid > lactic acid > tartaric acid > hydrochloric acid > mandelic acid.

4. Arranged according to capacity of blood alkalization, we have the following series: Acetic acid > maleic acid > fumaric acid.

5. Malic acid shows acidification as well as alkalization. Succinic acid shows temporary deflections toward the acid, citric acid toward the alkaline side.

---

(1) Siegwart Hermann: Naunyn-Schmiedebergs Arch. 154: 144-192 (1930).

(2) Siegwart Hermann: Klin. Wschr. 1929, No. 38, p. 1752-57.

(3) Siegwart Hermann and Margot Zentner: Naunyn-Schmiedebergs Arch. 175: 500 (1934).

(4) v. Brehmer: Reprint from Paracelsus Inst. Schriftenreihe des Vereins Deutsche Volksheilkunde, 2.

(5) Zucker and Capaldi: Radiologische Rundschau VI, 1937.

(6) Otto Wünsche: Klin. Wschr. 1936, No. 31, p. 1102.

(7) Underhill, Gross and Jalesky: J. of Pharm. 43: 359 (1931)

(8) Hedwig Langecker: Naunyn-Schmiedebergs Arch. 171: 744 (1933).

Translated by Carl Demrick Associates, Inc./LH/db

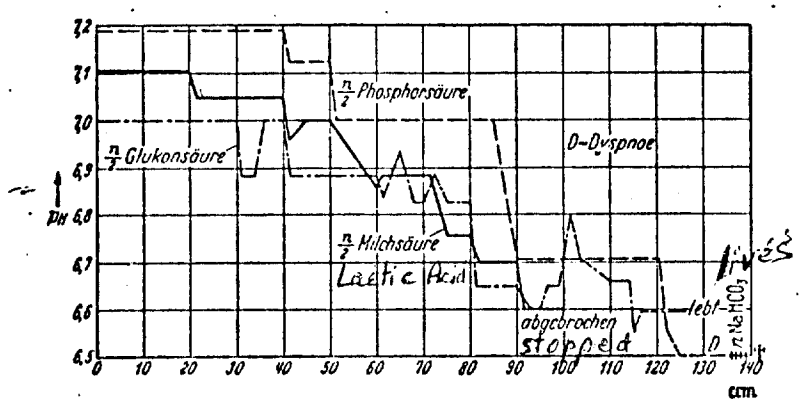


Figure 1

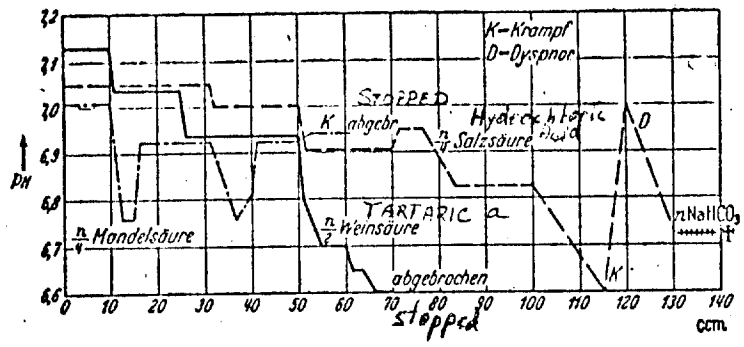


Figure 2

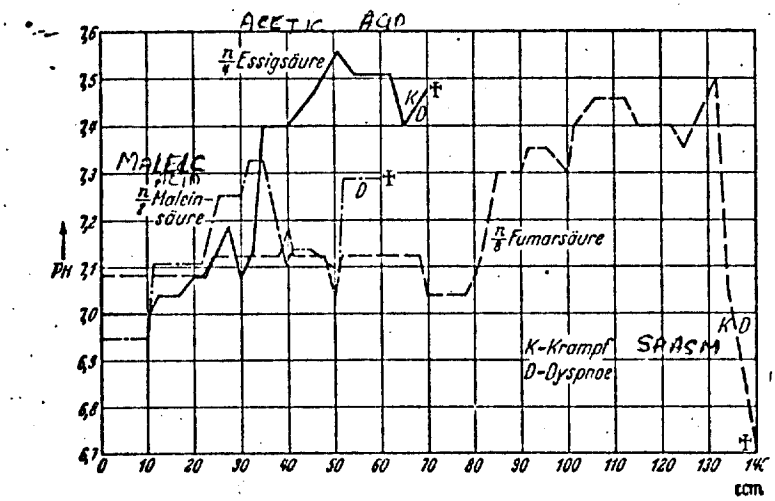


Figure 3

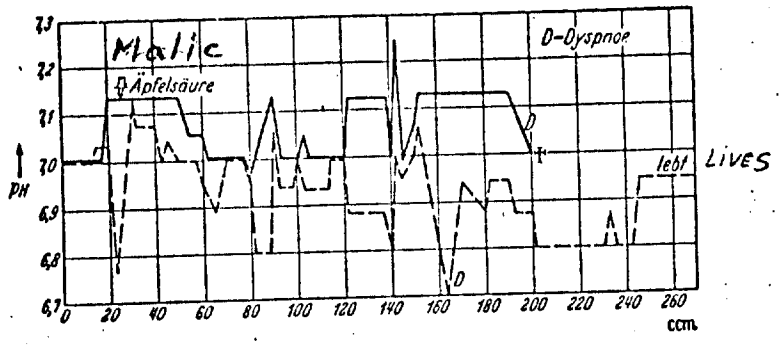


Figure 4

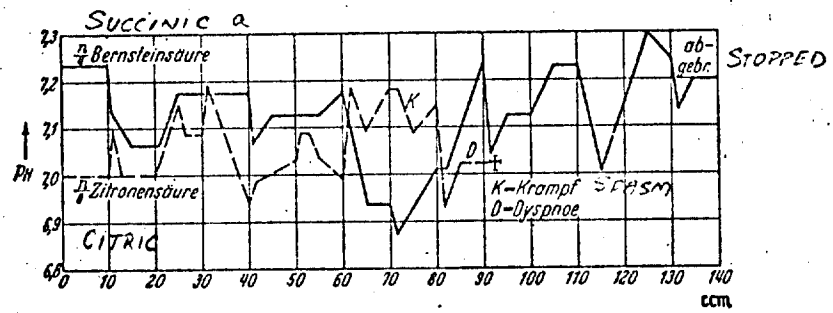


Figure 5

Attention is drawn to the possibility that this text may be copyrighted.
The reader is urged to investigate its copyright status.

Aus dem Privat-Forschungsinstitut Prag II/1482.
(Vorstand: Dr. Siegwart Hermann, Privat-Dozent der Deutschen Universität in Prag.)

Säurewirkungen und Säureschicksal im Organismus.

I. Mitteilung: Bestimmung der Wasserstoffionenkonzentration im strömenden Kaninchenblut nach intravenöser Injektion freier Säuren.

Von

Siegwart Hermann, Richard Neiger und Margot Zentner.

Mit 5 Textabbildungen.

(Eingegangen am 15. Dezember 1937.)

In früheren Untersuchungen[1] haben wir bereits festgestellt, daß sich die Wasserstoffionenkonzentration nach intravenöser Zufuhr von Gluconsäure erhöht. Aus verschiedenen Wirkungen nach peroraler Zufuhr von Gluconsäure konnten wir auch den Schluß ziehen, daß eine Zunahme der Wasserstoffionenkonzentration im Organismus stattfindet. So konnten mit Kombuchal, welches 30% freie Gluconsäure enthält, Verkalkungserscheinungen nach Vitasterin verhindert, ja zum Teil sogar rückgängig gemacht werden[2]. Auch die Veränderung des Calciumquotienten[3], d. h. die Zunahme von dissoziiertem Calcium im Serum kann auf Änderung der Wasserstoffionenkonzentration zurückgeführt werden. Die Gluconsäure gehört zu den schwer verbrennbaren Säuren und es ist infolgedessen leicht einzusehen, daß sie im Organismus das p_H nach der sauren Seite verschieben kann. Es gibt aber auch Säuren, die ein entgegengesetztes Verhalten zeigen, z. B. die Essigsäure, die man aus verschiedenen Gründen zu den leichter „verbrennbaren" Säuren rechnet. Bei unseren Untersuchungen ergaben sich Befunde, die mit den bisherigen Vorstellungen nicht immer in Einklang zu bringen waren. Aus diesem Grunde haben wir uns entschlossen eine größere Zahl von Säuren systematisch zu untersuchen. Die vorliegende Arbeit soll sich ausschließlich mit den Änderungen der Wasserstoffionenkonzentration im Blute nach Zufuhr von freien Säuren (nicht Salzen) befassen. Bei der Auswahl der Methode zur Messung der Wasserstoffionenkonzentration haben wir uns für die intravitale Methode von v. Brehmer[4]

---

[1] Hermann, Siegwart: Naunyn-Schmiedebergs Arch. 154, 144—192 (1930). — [2] Hermann, Siegwart: Klin. Wschr. 1929, Nr. 38, S. 1752—57. — [3] Hermann, Siegwart u. Margot Zentner: Naunyn-Schmiedebergs Arch. 175, 500 (1934). — [4] v. Brehmer: Sonderdruck aus dem Paracelsus Inst. Schriftenreihe des Vereins Deutsche Volksheilkunde, 2.

Attention is drawn to the possibility that this text may be copyrighted.
The reader is urged to investigate its copyright status.

entschieden, da wir sowohl aus den Arbeiten von Bücheler, Zucker und Capaldi[5], Wünsche[6] als auch aus eigenen Vergleichsmessungen den Eindruck gewonnen haben, daß diese Methode für vergleichende Bestimmungen sehr brauchbare Resultate liefert.

Methodik.

Kaninchen im Gewichte von durchschnittlich 2000 g wurden vor Versuchsbeginn auf Hafer-Wasserkost gesetzt. Für die Durchführung des Versuchs ist es wichtig, daß die Tiere nicht mit Metallteilen in Berührung kommen. Erforderlichenfalls sind Klemmvorrichtungen mit Isolierband zu umwickeln. Das Kaninchen wird auf einem Holzbrett aufgespannt, die rechte Vorderpfote durch eine Öffnung im Brett durchgesteckt und fixiert. Die von Haaren und Fett befreite Pfote wird in ein Gefäß mit gesättigter Kaliumchloridlösung getaucht. Dieses Gefäß wird durch eine mit Kaliumchloridagar gefüllte Röhre mit dem Meßgefäß des Ionometers (Lautenschläger) leitend in Verbindung gebracht. Die Antimon-Hämovennadel wird in die Ohrrandvene des Kaninchens eingeführt und dadurch der Stromkreis geschlossen. Die Brauchbarkeit der jeweils verwendeten Hämovennadel wird an Lösungen mit bekanntem p_H geprüft und ergibt sich auch schon aus der Prüfung der normalen p_H-Werte des Kaninchens. Bisweilen kommt es zu einem plötzlichen Abfall des p_H. Ist nur eine Thrombenbildung daran schuld, so erhält man nach Entfernung des Thrombus wieder einwandfreie Werte. Anderenfalls ist die Hämovennadel durch eine andere zu ersetzen. Die rektale Temperatur des Kaninchens wurde bei der Korrektur der p_H-Werte berücksichtigt. Als normale p_H-Werte wurden diejenigen angesehen, die vor der Injektion $^1/_2$ Stunde konstant blieben. Die Injektion der Säure erfolgte durch eine Hahnkanüle in die Vena jugularis dextra. Im Verlauf von 3 Minuten wurden 10 ccm der betreffenden Säure injiziert, hierauf durch 10 Minuten das p_H des strömenden Blutes gemessen, wieder injiziert usw. Im nachstehenden bringen wir die Ergebnisse unserer Messungen nach Injektion verschiedener Säuren an Hand von Kurven. Die Beobachtungsdauer betrug ungefähr 6—8 Stunden. Obwohl für jede Säure 4—6 Tiere verwendet wurden, veröffentlichen wir der Kürze halber nur je eine der erhaltenen Kurven, für den Fall, daß sie im wesentlichen übereinstimmen.

Ergebnisse der p_H-Messungen nach intravenöser Zufuhr freier Säuren im strömenden Blute.

1. Phosphorsäure.

Injiziert wurde n/2 Phosphorsäure von einem p_H 1,45. Sie ist lipoidlöslich. Die tödliche Dosis beträgt 62,30 ccm n/2 Säure bzw. 1 g H_3PO_4 pro kg Kaninchen. Die p_H-Erniedrigung beträgt im Mittel 0,58. Der Tod erfolgt plötzlich unter Auftreten von Dyspnoe und Krämpfen der Streckmuskulatur. Versuche, sofort nach Eintreten der ersten Vergiftungssymptome Tiere durch intravenöse Injektion von Natriumbicarbonat zu retten, schlugen fehl. Der Einfluß der Säure auf das strömende Blut ist in den einzelnen Phasen aus der Kurve 1, Abb. 1 zu ersehen.

---

[5] Zucker u. Capaldi: Radiologische Rundschau VI, 1937. — [6] Wünsche, Otto: Klin. Wschr. 1936, Nr. 31, S. 1102.

Attention is drawn to the possibility that this text may be copyrighted.
The render is urged to investigate its copyright status.

2. Gluconsäure.

Durch n/2 Gluconsäure wird das p_H im Durchschnitt um 0,46 erniedrigt. Das p_H der n/2 Säure beträgt 2,05. Gluconsäure ist lipoidunlöslich. Von Gluconsäure werden sowohl oral als auch parenteral sehr

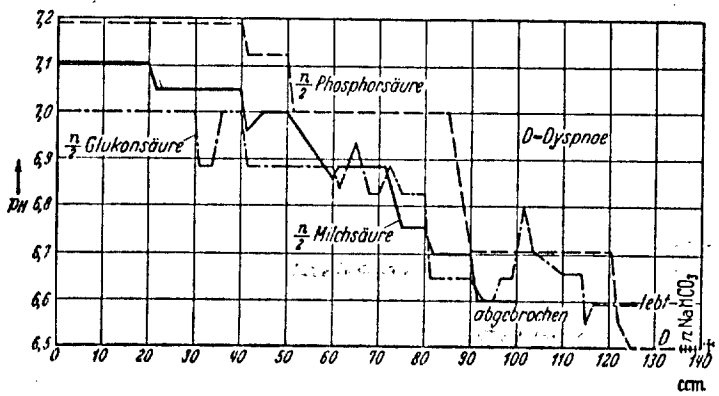


Abb. 1.

große Mengen vertragen. In unseren Versuchen wurde die letale Dosis überhaupt nicht erreicht. Die Tiere erhielten 6 g bzw. 61 ccm n/2 Gluconsäure pro kg intravenös. Bei diesen Mengen trat allerdings bereits Dyspnoe auf, die aber durch Injektion von zwei- bis dreimal 10 ccm einer n Natriumbicarbonatlösung aufgehoben wurde. Auch das Blut-p_H kehrte wieder zur Norm zurück. Die Tiere lebten nach Entfernung der Kanüle noch mehrere Tage (Abb. 1, Kurve 2).

3. Milchsäure.

n/2 Milchsäure, die ein p_H von 1,90 hat, säuert deutlich das Blut. Sie wird in verhältnismäßig großer Menge vom Kaninchen vertragen. Sie ist lipoidlöslich. Die toxische Dosis beträgt 1,76 g pro kg Tier, die letale Dosis 2,01 g bzw. 80 ccm n/2 Säure pro kg (Abb. 1, Kurve 3).

4. Weinsäure.

Injiziert wurde n/2 Säure von p_H 1,8. Die durchschnittliche Erniedrigung des p_H beträgt nach der intravenösen Injektion 0,4. Weinsäure ist lipoidlöslich. Nach oraler Aufnahme soll sie unverbrennbar sein[7]. Die tödliche Dosis betrug 1,1 g bzw. 30 ccm n/2 Weinsäure pro kg Kaninchen. Erst vor dem Tode kommt es zu Streckkrämpfen (Abb. 2, Kurve 1).

5. Salzsäure.

Injiziert wurde n/4 Salzsäure. Ihr p_H ist kleiner als 1,0. Sie ist lipoidlöslich und unverbrennbar. Die größte Menge, die pro kg Kaninchen bis

[7] Underhill, Gross u. Jalesky: J. of Pharm. 43, 359 (1931).

Attention is drawn to the possibility that this text may be copyrighted.
The render is urged to investigate its copyright status.

zum Eintritt des Todes injiziert werden kann, beträgt 0,6 g bzw. 54,2 ccm n/4 Salzsäure. Das p_H wird im Durchschnitt um 0,35 gesenkt, die Wasserstoffionenkonzentration dementsprechend erhöht. Der Tod erfolgt unter den gleichen Symptomen wie bei der Phosphorsäure. Injektionen von Natriumbicarbonat als Gegengift sind ohne Erfolg (Abb. 2, Kurve 2).

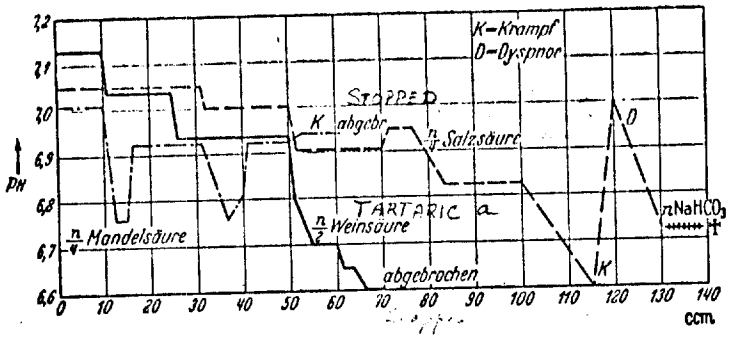


Abb. 2.

6. Mandelsäure.

Injiziert wurden n/4 Lösungen von einem p_H 2,05. Die Durchschnittserniedrigung des p_H betrug 0,25. Mandelsäure ist lipoidlöslich und im Organismus unverbrennbar. Hervorzuheben ist die starke Toxizität. Die tödliche Dosis beträgt 1,65 g bzw. 15,5 ccm einer n/4 Lösung pro kg Kaninchen. n/2 Lösungen konnten wegen der großen Giftigkeit überhaupt nicht mehr injiziert werden. Der Verlauf der p_H-Beeinflussung ist aus der Abb. 2, Kurve 3 zu ersehen.

7. Essigsäure.

Nach intravenöser Injektion von n/4 Essigsäure sieht man eine deutliche Alkalisierung des Blutes. Das Blut-p_H wird durchschnittlich um 0,5 erhöht. Diese Erhöhung ist auf Natriumacetatbildung zurückzuführen. Das p_H einer n/2 Natriumacetatlösung beträgt 7,9. Nach Injektion des 50. ccm von n/4 Essigsäure erfolgte ein steiler Abfall des Blut-p_H. Die Tiere gingen dann unter Auftreten von Dyspnoe und Krämpfen rasch zu Grunde. Die Essigsäure hat in diesem Zeitpunkt die verfügbare Alkalireserve abgebunden, so daß ein Überschuß der freien Essigsäure sehr rasch zum Tode führt. Der spontan entleerte Harn ist blutig. Die Messungen des Harn-p_H während des Versuchs ergaben einen leichten Anstieg von 5,3 auf 5,45. Die Essigsäure ist lipoidlöslich, das p_H der n/4 Säure beträgt 2,70. Die tödliche Dosis ist 0,5 g CH_3COOH bzw. 33,5 ccm einer n/4 Lösung pro kg Tier (Abb. 3, Kurve 1).

8. Maleinsäure.

Injiziert wurden n/2 Lösungen mit einem p_H 1,50; n/4 Säure mit einem p_H 1,60 und n/8 Säure mit einem p_H 1,70. Die n/2 Säure ist außer-

Attention is drawn to the possibility that this text may be copyrighted.
The render is urged to investigate its copyright status.

ordentlich giftig. Nach der Injektion einer n/2 Säure fällt das p_H rasch ab, um alsbald ein wenig wieder anzusteigen. In diesem Zeitpunkt tritt der Tod ein. Nach Injektion der n/4 Säure steigt das p_H ziemlich an, kehrt alsbald zum Ausgangspunkt zurück um wieder stark anzusteigen. Erst kurz vor dem Tode erfolgt unter Auftreten von Dyspnoe und Krämpfen ein Abfall des Blut-p_H bis fast zur Norm. Nach der Injektion der n/8 Säure ist die Alkalisierung ganz deutlich ausgeprägt. Maleinsäure ist lipoidlöslich. Die letale Dosis beträgt für die n/8 Lösung 0,19 g Maleinsäure bzw. 26 ccm pro kg Tier (Abb. 3, Kurve 2).

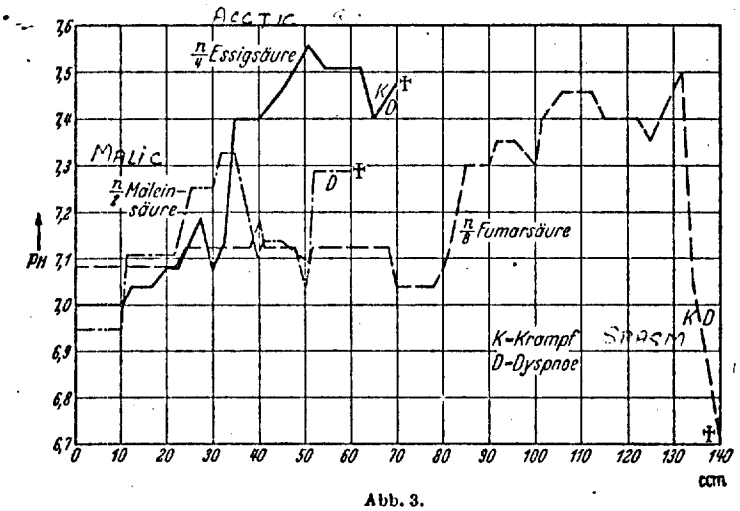


Abb. 3.

9. Fumarsäure.

Die Fumarsäure ist mit der Maleinsäure strukturisomer. Betrachtet man die Kurve 3 der Abb. 3 im Verlauf der Injektionen mit einer n/8 Säure, so fällt der Unterschied gegenüber der Maleinsäurekurve auf. Es können 70 ccm Fumarsäure injiziert werden, ohne daß überhaupt eine p_H-Änderung erfolgt. Erst später steigt das p_H ziemlich steil an, um vor dem Tode sehr rasch unter die Norm abzusinken. n/8 Fumarsäure hat ein p_H von 2,25. Sie ist lipoidlöslich. Die letale Dosis der n/8 Fumarsäure beträgt 0,47 g pro kg Tier, also mehr als das Doppelte der Maleinsäure.

10. Äpfelsäure.

n/4 Äpfelsäure, die ein p_H von 2,20 hat, zeigt in vier verschiedenen Versuchen während der Injektion einen wechselnden Verlauf der Kurven. Zwei Versuche zeigten eine Erhöhung, zwei eine Erniedrigung des p_H. Vielleicht hängt dies mit der Umwandlungsfähigkeit dieser Säure im Organismus zusammen. Es ist auch möglich, daß die Äpfelsäure als zweibasische Säure sowohl saure, als auch neutrale Salze im Blute bildet, so daß

Attention is drawn to the possibility that this text may be copyrighted.
The render is urged to investigate its copyright status.

die einen oder die anderen die Reaktion bestimmen. Äpfelsäure ist wenig lipoidlöslich. Die letale Dosis einer n/4 Säure beträgt 2,40 g bzw. 106 ccm pro kg Tier (Abb. 4).

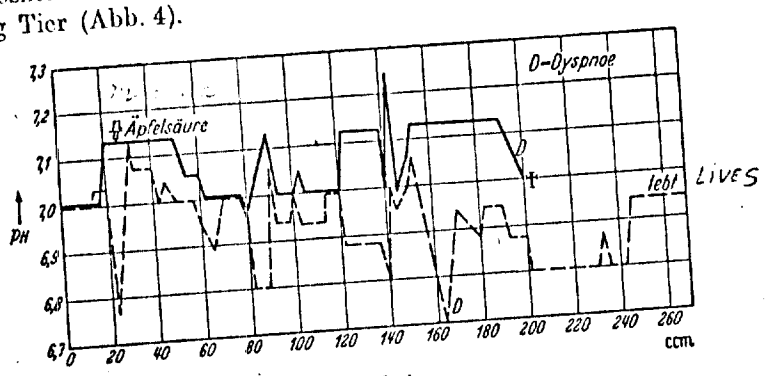


Abb. 4.

11. Bernsteinsäure.

Hervorzuheben ist der große Unterschied in der Verträglichkeit je nachdem ob n/4 oder n/2 Säure injiziert wurde. Von der n/4 Säure wurden über 200 ccm vertragen, während von der n/2 maximal 50 ccm injiziert werden konnten. Der Verlauf der Kurven erinnert insofern an den der Äpfelsäure (die chemische Verwandtschaft ist eine sehr nahe) als auch hier ein Zick-Zack-Verlauf vorliegt. Die p_H-Werte schwanken um den Anfangswert. Bei der Bernsteinsäure dürften sich vielleicht ebenso wie bei der Äpfelsäure teils das saure, teils das neutrale Salz bilden, so daß es zu keiner eindeutigen p_H-Änderung kommen kann. Die Bernsteinsäure ist lipoidunlöslich. Von der n/2 Säure wurden 0,54 g bzw. 18,1 ccm pro kg, von der n/4 1,63 g bzw. 88,0 ccm pro kg Tier vertragen (Abb. 5, Kurve 1).

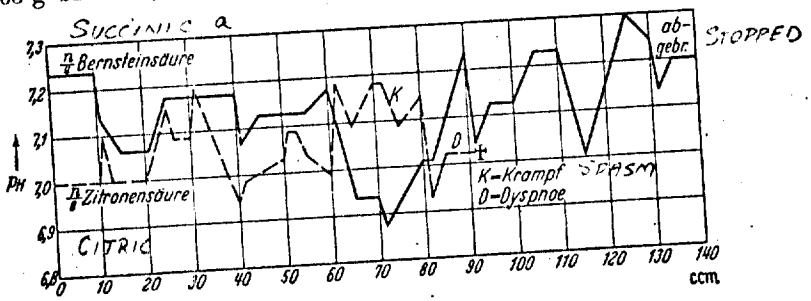


Abb. 5.

12. Citronensäure.

Es wurden n/4 und n/8 Lösungen injiziert. Das p_H der n/4 Säure beträgt 2,15, das der n/8 Säure 2,3. Sie ist wenig lipoidlöslich. Bei oraler

Attention is drawn to the possibility that this text may be copyrighted.
The reader is urged to investigate its copyright status.

Zufuhr ist sie nach Langecker[8] verbrennbar. Die letale Dosis beträgt für die n/4 Säure 0,28 g bzw. 17,10 ccm pro kg; für die n/8 Säure 0,22 g bzw. 32,90 ccm pro kg Tier. Aus Abb. 5, Kurve 2 ersieht man die Tendenz vorübergehend zu alkalisieren. Das p_H wird vorübergehend erhöht, die Wasserstoffionenkonzentration dementsprechend erniedrigt.

Zusammenfassung.

1. Nach intravenöser Injektion verschiedener Säuren wurde die Änderung der Wasserstoffionenkonzentration im strömenden venösen Blute bestimmt.

2. Man kann drei Grundtypen von Säuren unterscheiden: Solche die das Blut säuern, solche die es alkalisieren und schließlich solche die Ausschläge nach der sauren und alkalischen Richtung geben.

3. Nach ihrem Blutsäuerungsvermögen ergibt sich folgende Reihe: Phosphorsäure > Gluconsäure > Milchsäure > Weinsäure > Salzsäure > Mandelsäure.

4. Nach dem Blutalkalisierungsvermögen geordnet ergibt sich folgende Reihe: Essigsäure > Maleinsäure > Fumarsäure.

5. Sowohl Säuerung als auch Alkalisierung zeigt Äpfelsäure; Bernsteinsäure zeigt nach der sauren, Citronensäure nach der alkalischen Seite vorübergehende Ausschläge.

---

[8] Langecker, Hedwig: Naunyn-Schmiedebergs Arch. 171, 744 (1933).

UDK (Universal Decimal Classification): 616.153.922.01 + 616 13-004.6)-002.9

The Effect of Malic Acid and of Carbonates of Alkali and Alkali-Earth Metals on the Involution (Retrograde Development) of Alimentary Hypercholesterolemia and Atherosclerosis in Rabbits

D.A. Kaidin

Laboratory of Biochemistry (Director -- Candidate in Biological Sciences L. I. Grebennik) of the Division of Chemotherapy (Director -- Corresponding Member of the AMN SSSR (Academy of Medical Sciences of the U.S.S.R.) Prof. G.N. Pershin) of the S. Ordzhenikidze All-Union Scientific-Research Chemico-Pharmaceutical Institute, Moscow

Experiments were carried out on 31 male rabbits having a weight of 2.4 - 2.6 kilograms. In the course of 100 days, all the animals received a diet of 50 grams of hay, 80 grams of oats, 100 grams of vegetables, and cholesterol, calculated on 200 milligrams/kilogram in the form of a suspension on a starchy jelly mixed with the feed. The cholesterol content in the blood was determined according to Chiamori and Henry (3), in the organs -- according to Abell (2), the overall lipids -- by the gravimetric method, and the activity of the enzymes -- in accordance with the Pokrovskii method (1). The addition of cholesterol to the diet of the rabbits in all 4 groups, was discontinued after 100 days. From the following day, the animals of the 1st group (control) received a basic diet without the addition of cholesterol, the animals of the 2nd group received with their feed an additional mixture of carbonates in a dose of 121 milligrams/kilogram, and the 3rd group -- a mixture of carbonates (121 milligrams/kilogram) and malic acid (940 milligrams/kilogram). The animals of the 4th group were put to death by means of an aeroembolism for the purpose of studying the organs. The ratio between the alkaline elements was taken by proceeding from the amount of these substances in the natural products of a daily diet ($NaHCO_3$ -- 12 milligrams; K_2CO_3 -- 51 milligrams; $CaCO_3$ -- 49 milligrams; $MgCO_3$ -- 9 milligrams).

The results of the experiments are set forth in a table, from it may be seen that the discontinuance of administering cholesterol brought about, in the animals of all the groups, even over the course of the first 10 days, a statistically reliable (P is less than 0.05) reduction in the cholesterol content in the blood. The addition of malic acid to the diet accelerated this process. Under its influence, cholesterolemia gradually diminished, and by the 60 th day, the cholesterol content in the blood plasma became normalized. The addition, to the diet, of carbonates of alkali and alkali-earth metals, inhibited the involution of the changes.

((Table)

Effect of Malic Acid and of Carbonates of Alkali and Alkali-Earth Metals on the Cholesterol Content and on the Amount of Overall Lipids in the Blood and in Some Organs (M ± m) Following the Discontinuance of a Cholesterol Addition to the Diet of the Animals

Group of Animals	Preparation Being Tested	Number of Animals	Cholesterol Level in the Blood (in milligram %) after 100 days	Change in the Cholesterol Content in the Blood Plasma Following the Discontinuance of Feeding Cholesterol to the Rabbits (in milligram %); 2nd period of the experiment			
				10 days	30 days	50 days	60 days
1st	Cholesterol (200 milligrams/kilogram)	8	1st period of the experiment	444.4 ± 72.1	206.8 ±47.3	119.8 ±13.7	72.0 ±6.4
2nd	Cholesterol (200 milligrams/kilogram) + a mixture of carbonates of alkali-earth metals (121 milligrams/kilogram)	8	787±87.6	461.8 ±66.5	215.0 ±49.2	125.1 ±15.2	96.7 ±11.4
3rd	Cholesterol + a mixture of carbonates of alkali and alkali-earth metals + malic acid (940 milligrams/kilogram)	8	732.0 ±134.0	322.0 ±85.2	165.3 ±44.5	87.8 ±18.4	60.3 ±7.2
4th	Cholesterol 200 milligrams/kilogram	7	-	-	-	-	-

Group of Animals	Preparation being Tested	Cholesterol Content: Liver (in milligram %)	Cholesterol Content: Suprarenal glands (in %)	Cholesterol Content: Aorta (in milligrams in 1 gram of raw tissue)	Amount of Overall Lipids: Liver (in %)	Amount of Overall Lipids: Suprarenal Glands (in %)	Amount of Overall Lipids: Aorta (In milligrams in 1 gram of raw tissue)
1st	Cholesterol (200 milligrams/kilogram)	629.1 ±110	13.5 ±2.2	24.8 ±5.7	6.6 ±0.6	57.6 ±2.7	74.6 ±6.0
2nd	Cholesterol (200 milligrams/kilogram) + a mixture of carbonates of alkali and alkali-earth metals (121 milligrams/kilogram)	1281.9 ±162.0	14.4 ±2.1	38.2 ±9.5	9.0 ±0.3	60.0 ±5.7	81.0 ±6.0
3rd	Cholesterol + a mixture of carbonates of alkali and alkali-earth metals + malic acid (940 milligrams/kilogram)	606.4 ±136.8	10.0 ±2.1	22.0 ±4.4	5.2 ±1.0	44.8 ±4.2	72.2 ±7.5
4th	Cholesterol 200 milligrams/kilogram	1010 ±233.7	17.9 ±0.5	31.2 ±4.1	7.9 ±0.82	55.2 ±7.7	75.4 ±6.1

	Manifestation of Morphological Changes in the Aorta
1st	From +++ to ++++
2nd	From +++ to +++++
3rd	From ++ to ++++
4th	From + to +++++

Note The rabbits from all the groups received cholesterol over a period of 100 days. The animals of the 4th group were put to death, 100 days following feeding with cholesterol, for the purpose of determining the cholesterol, the overall lipids and the enzymes -- esterases; all the remaining rabbits were put to death 160 days following the beginning of the experiment.

In the animals of all the experimental groups there was observed a pronounced increase in the cholesterol content and in the overall lipids in the organs. However, in the animals that had received malic acid with their feed, there were 2 times less of them than in rabbits that had received a mixture of carbonates.

The overall content of lipids in the aorta, counting on 1 gram of weight of the tissue under examination, reflected the degree of affection of the aorta in the animals. Atherosclerotic changes in the aorta of the animals in all the groups were pronounced in varying degrees and could be evaluated macroscopically from + to +++++. In the majority of animals in all the groups, the intima of the aorta was rought over its entire extent, and contained a large amount of yellowish-white platelets of varying sizes, which were confluent over large spaces. In addition to this, the liver and suprarenal glands were markedly enlarged in dimensions and in weight.

In rabbits that had received malic acid (the 3rd group), the activity of the enzymes of the esterase group was considerably greater than in the aortas of animals belonging to the 1st, 2nd and 4th groups. In the rabbits of the 4th group, which had received cholesterol along with their basic diet, over the course of 100 days, there was observed a sharp reduction in the activity of the enzymes in the aorta wall, particularly in the animals with a pronounced atheromatosis of the aorta and with a high cholesterol content and overall lipids in the organs.

Conclusion

There was established a favorable effect of malic acid on the involution of cholesterolemia and on the restoration of activity in enzymes belonging to the esterase group. The feeding of malic acid did not produce any visible negative side effects in the rabbits.

BIBLIOGRAPHY

1. Pokrovskii, A. A., Shchevletsev, V. L. , Byull. eksper. biol. (Bulletin of Experimental Biology), 1966, No. 3, p. 52.

2. Abell, L. L., et al., In the book: Standard Methods of Clinical Chemistry, New York, 1958, v. 2, p. 26.

3. Chiameri, B. S., Henry, M.D., Amer. J. Clin. Path., 1959, vo. 31, p.305.

(Translated by Carl Demrick Associates,Inc/LB/t)

ризация основного вещества соединительной ткани осуществляется с участием гормонов коры надпочечников.

Резкое снижение дифениламиновой реакции в печени и сыворотке у адреналэктомированных животных при воздействии водяных ванн, сопровождавшееся в ряде опытов гибелью животных, свидетельствует в пользу концепции, рассматривающей повышение показателя дифениламиновой реакции как отражение неспецифической защитно-приспособительной реакции организма.

ЛИТЕРАТУРА

1. Блюгер А. Ф. Структура и функция печени при эпидемическом гепатите. Рига, 1964. — 2. Ларский Э. Г. Лабор. дело, 1957, № 4, с. 13. — 3. Andersson A. I., Lack C. H., Alis Y., Nature, 1963, v. 197, p. 707. — 4. Greenspan E. M., Advanc. intern. Med., 1955, v. 7, p. 101. — 5. Miller L. L., Bale W. F., J. exp. Med., 1954, v. 99, p. 125. — 6. Siebert T. B. et al. J. clin. invest., 1947, v. 26, p. 90. — 7. Pirani C. L., Catchapole H. R., Arch. Phat., 1951, v. 51, p. 597.

Поступила 19/VI 1967 г.

THE ROLE PLAYED BY THE LIVER AND ANDRENAL GLANDS IN THE MECHANISM OF DIPHENYLAMINE REACTION

E. L. Pidemsky, A. M. Mulmenko

Changes of diphenylamine reaction index were studied in the blood serum and liver homogenates of intact, adrenalectomized and poisoned (with carbon tetrachloride) rats. A relationship was established between the index of diphenylamine reaction and the functional state of the liver and adrenal glands. The data obtained are interpreted from the viewpoint of the concept regarding the diphenylamine reaction as a reflection of the nonspecific protective-adaptive body reaction to the external environmental effect.

УДК 616.153.922.01+616.13-004.6]-092.9

ВЛИЯНИЕ ЯБЛОЧНОЙ КИСЛОТЫ И КАРБОНАТОВ ЩЕЛОЧНЫХ И ЩЕЛОЧНО-ЗЕМЕЛЬНЫХ МЕТАЛЛОВ НА ОБРАТНОЕ РАЗВИТИЕ АЛИМЕНТАРНОЙ ГИПЕРХОЛЕСТЕРИНЕМИИ И АТЕРОСКЛЕРОЗА У КРОЛИКОВ

Д. А. Кайдин

Лаборатория биохимии (зав. — канд. биол. наук Л. И. Гребенник)
отдела химиотерапии (зав. — член-корр. АМН СССР проф. Г. Н. Першин)
Всесоюзного научно-исследовательского химико-фармацевтического
института им. С. Орджоникидзе, Москва

Опыты проведены на 31 кролике-самце весом 2,4—2,6 *кг*. В течение 100 дней все животные получали рацион из 50 *г* сена, 80 *г* овса, 100 *г* овощей и холестерина из расчета 200 *мг/кг* в виде суспензии на крахмальном киселе, перемешанной с кормом. Содержание холестерина в крови определяли по Chiamori и Henry [3], в органах — по Abell [2], общие липиды — весовым способом и активность ферментов — по методу Покровского [1]. Добавление холестерина к рациону кроликов всех 4 групп было прекращено через 100 дней. Со следующего дня животные 1-й группы (контроль) получали основной рацион без добавления холестерина, животные 2-й группы получали с кормом дополнительно смесь карбонатов в дозе 121 *мг/кг*, 3-й группы — смесь карбонатов (121 *мг/кг*) и яблочной кислоты (940 *мг/кг*). Животных 4-й группы забивали воздушной эмболией для исследования органов. Соотношение между щелочными элементами брали, исходя из количества этих веществ в естественных продуктах суточного рациона ($NaHCO_3$ — 12 *мг*, K_2CO_3 — 51 *мг*, $CaCO_3$ — 49 *мг*, $MgCO_3$ — 9 *мг*).

Результаты опытов представлены в таблице, из которой видно, что прекращение введения холестерина вызывало у животных всех групп уже в течение первых 10 дней статистически достоверное ($P<0,05$) снижение содержания холестерина в крови. Добавление яблочной кислоты к рациону ускоряло этот процесс. Под ее влиянием холестеринемия постепенно уменьшалась и к 60-му дню содержание холестерина в плазме крови нормализовалось. Добавление к рациону карбонатов щелочных и щелочно-земельных металлов задерживало обратное развитие изменений.

Влияние яблочной кислоты и карбонатов щелочных и щелочно-земельных металлов на содержание холестерина и количество общих липидов в крови и некоторых органах ($M \pm m$) после прекращения добавления холестерина к рациону животных

Группа животных	Испытуемый препарат	Уровень холестерина в крови (в *мг*%)	Изменение содержания холестерина в плазме крови после прекращения скармливания холестерином кроликов (в *мг*%) 2-й период опыта
		до опыта	

Влияние яблочной кислоты и карбонатов щелочных и щелочно-земельных металлов на содержание холестерина и количество общих липидов в крови и некоторых органах ($M \pm m$) после прекращения добавления холестерина к рациону животных

Группа животных	Испытуемый препарат	Число животных	Уровень холестерина в крови (в *мг*%) через 100 дней	Изменение содержания холестерина в плазме крови после прекращения кормления холестерином кроликов (в *мг*%) 2-й период опыта			
				10 дней	30 дней	50 дней	60 дней
1-я	Холестерин (200 *мг/кг*)	8	1-й период опыта 740±16,3	444,4±72,1	206,8±47,3	119,8±13,7	72,0±6,4
2-я	Холестерин (200 *мг/кг*)+смесь карбонатов щелочных и щелочно-земельных металлов (121 *мг/кг*)	8	787±87,6	461,8±66,5	215,0±49,2	125,1±15,2	96,7±11,4
3-я	Холестерин+смесь карбонатов щелочных и щелочно-земельных металлов+яблочная кислота (940 *мг/кг*)	8	732,0±134,0	322,0±85,2	165,3±44,5	87,8±18,4	60,3±7,2
4-я	Холестерин 200 *мг/кг*	7	761,0±144,0	—	—	—	—

Группа животных	Испытуемый препарат	Содержание холестерина			Количество общих липидов			Выраженность морфологических изменений в аорте
		печень (в *мг*%)	надпочечники (в %)	аорта (в *мг* в 1 *г* сырой ткани)	печень (в %)	надпочечники (в %)	аорта (в *мг* в 1 *г* сырой ткани)	
1-я	Холестерин (200 *мг/кг*)	629,1±110	13,5±2,2	24,8±5,7	6,6±0,6	57,6±2,7	74,6±6,0	От +++ до ++++
2-я	Холестерин (200 *мг/кг*) + смесь карбонатов щелочных и щелочно-земельных металлов (121 *мк/кг*)	1281,9±162,0	14,4±2,1	38,2±9,5	9,0±0,3	60,0±5,7	81,0±6,0	От +++ до +++++
3-я	Холестерин + смесь карбонатов щелочных и щелочно-земельных металлов + яблочная кислота (940 *мг/кг*)	606,4±136,8	10,1±2,1	22,0±4,4	5,2±1,0	44,8±4,2	72,2±7,5	От ++ до ++++
4-я	Холестерин 200 *мг/кг*	1010±233,7	17,9±0,5	31,2±4,1	7,9±0,82	55,2±7,7	75,4±6,1	От + до +++++

Примечание. Кролики всех групп получали холестерин в течение 100 дней. Животные 4-й группы были забиты через 100 дней после скармливания холестерина для определения холестерина, общих липидов и ферментов — эстераз, всех остальных кроликов забивали через 160 дней после начала опыта.

У животных всех опытных групп наблюдалось резкое увеличение содержания холестерина и общих липидов в органах. Однако у животных, получавших с кормом яблочную кислоту, их было в 2 раза меньше, чем у кроликов, получавших смесь карбонатов.

Общее содержание липидов в аорте при расчете на 1 *г* веса исследуемой ткани отражало степень поражения аорты животных. Атеросклеротические изменения в аорте животных всех групп были выражены различно и могли быть оценены макроскопически от + до ++++. У большинства животных всех групп интима аорты была на всем протяжении шероховатой, содержала большое количество желтовато-белых бляшек различной величины, сливающихся между собой на больших пространствах. Кроме того, печень и надпочечники были резко увеличены в размере и в весе.

У кроликов, получавших яблочную кислоту (3-я группа), активность ферментов группы эстеразы была значительно выше, чем в аортах животных 1-й, 2-й и 4-й групп. У кроликов 4-й группы, получавших с основным рационом холестерин в течение 100 дней, отмечено резкое снижение активности ферментов в стенке аорты, особенно у животных с выраженным атероматозом аорты и высоким содержанием холестерина и общих липидов в органах.

Вывод

Было установлено благоприятное влияние яблочной кислоты на обратное развитие холестеринемии и на восстановление активности ферментов группы эстеразы. Скармливание яблочной кислоты не вызывало видимых отрицательных побочных реакций у кроликов.

ЛИТЕРАТУРА

1. Покровский А. А., Щеблецов В. Л. Бюлл. экспер. биол., 1966, № 3, с. 52. — 2. Abell L. L. et al. Вкн.: Standard Methods of Clinical chemistry. New York, 1958, v. 2, p. 26. — 3. Chiamori B. S., Henry M. D., Amer. J. clin. Path., 1959, v. 31, p. 305.

Поступила 5/VIII 1966 г.

THE EFFECT OF MALIC ACID AND OF THE CARBONATES OF ALKALINE AND ALKALINE-EARTH METALS ON THE RETROGRADE DEVELOPMENT OF ALIMENTARY HYPERCHOLESTEROLEMIA AND ATHEROSCLEROSIS IN RABBITS FOLLOWING SUSPENSION OF CHOLESTEROL FEEDING

D. A. Kaidin

Addition to the animal diet of a mixture of carbonates of alkaline and alkaline-earth metals in a dose of 121 mg/kg of body weight for 60 days after suspension of cholesterol feeding the degree of aortic atheromatosis proved to enhance somewhat, reduction of blood cholesterolemia and of total lipids in the organs decoming somewhat delayed. Addition of malic acid into the animal diet was followed within 30 to 50 days after discontinuance of cholesterol feeding by a distinct fall of blood plasma cholesterol to the normal level: correspondingly, there was a reduction of total cholesterol and total lipid content in the organs.

УДК 616.8-009.24-07:616.831-008.931:577.154.4-074

АКТИВНОСТЬ КАРБОАНГИДРАЗЫ ГОЛОВНОГО МОЗГА ВО ВРЕМЯ СУДОРОГ И ПРИ ИЗМЕНЕННОЙ СУДОРОЖНОЙ ГОТОВНОСТИ

Е. А. Захария

Кафедра патофизиологии и клинической лабораторной диагностики (зав. — проф. И. И. Федоров) Киевского института усовершенствования врачей и кафедра патофизиологии (зав. — доц. В. П. Безуглов) Львовского медицинского института

Внутриклеточный фермент карбоангидраза (угольная ангидраза, карбонат-гидролиза, карбонат-дегидратаза) активирует процессы гидратации и дегидратации CO_2 в клетках различных органов, в том числе и в центральной нервной системе.

Отмечено повышение активности карбоангидразы крови перед эпилептическими припадками [9]. Эти данные согласуются с тем, что состояние гипокапнии, вызываемое гипервентиляцией, провоцирует у больных эпилепсией судорожные припадки [6], а при накоплении в организме углекислоты [3, 11] и солей молочной кислоты [10] понижается чувствительность организма к действию эпилептогенных раздражителей. Роль карбоангидразы в пред-

UDK (Universal Decimal Classification): 616.132.003.826.092.02.616.008.
934.5

The Effect of Carbohydrate Metabolism Disturbances on the Wall of the Aorta and on the Development, In It, of Alimentary Lipoidosis, in Rabbits

M. V. Bavina, G. A. Ivanova, N. M. Poxdyunina, A. M. Vikhert (Moscow)
Laboratory of Biochemistry (Director -- Prof. M.G. Kritsman) and Laboratory of Pathological Anatomy (Director -- Prof. A. M. Vikhert) of the Institute of Therapy (Director-Acting Member of the AMN SSSR (Academy of Medical Sciences of the U.S.S.R.), Prof. A. L. Myasnikov) of the AMN SSSR (Academy of Medical Sciences of the U.S.S.R.).

(Received for Publication 14/VI 1965 (June 14, 1965)

The significance of disturbances of metabilic processes in the pathogenesis of atheresclerosis has been represented clearly only in relation to lipids. Comparatively little study has been devoted to the effect of changes in other aspects of metabolism on the state of the vascular wall and on the development of atherosclerosis.

It is of definite interest to make a study of the state of the structural elements in the aorta in disturbances of the Sent-Dierdi-Krebs oxidative cycle, which represents the fundamental biochemical process in all tissues of the animal organism. At the present time, there are in existence only several papers of Japanese authors that pertain directly to the given problem (Arai and Jao, 1957; Jshigami, 1956, Takenaka, 1956; Arai and coauthers, 1954; Jao, 1956; Arai and coauthors, 1955). These researchers disturbed the normal flow of oxidative processes by means of administering to animals (rabbits) some components of the Sent-Dierdi-Krebs cycle -- citric and fumaric acids. To such an influence, the vascular wall, according to the data of the indicated authors, responded with singular changes; the intime partially thickened, and on the boundary of the media and intima, there were disclosed sections of degeneration. The elastic fibers in these places were poorly stained, were thickened, fragmented, incorrectly oriented,

and their amount was diminished. The mucopolysaccharides, in particular, hyalurenic acid, disclosed a tendency towards accumulation. Fat was encountered in small amounts in the individual cells of the endothelium.

We posed the problem for ourselves of not only disclosing the characteristic morphological and histochemical changes that develop in the vascular wall under the influence of other components of the Sent-Dierdi-Krebs cycle, but also of establishing to what extent the disturbances, in the indicated oxidative cycle, have an effect on the development of alimentary hypercholesterelemia and lipoidosis of the aorta in rabbits, upon their being fed small amounts of cholesterol, which, in and of themselves, produce a moderate increase in the cholesterol level in the blood and only negligible manifestations of lipoidosis in the aorta.

In the experiments were 30 male rabbits of the Chinchilla breed, having a weight of 2.7 - 3 kilograms. All the animals were divided into 5 groups. To the rabbits of the 1st group (6) were administered, intraperitoneally, 200 milligrams of oxaloacetic acid 2 times a week. The animals of the 2nd group (5), in addition to oxaloacetic acid, received, daily, 0.07 gram-kilogram of cholesterol. To the rabbits of the 3rd group (6) were administered, intraperitoneally, 300 milligrams of malic acid 2 times a week. The animals of the 4th group (5) received malic acid and cholesterol in the above-indicated amounts; the rabbits of the 5th (5) -- only cholesterol, in the same amounts, as in the case of the animals of the 2nd and 4th groups. Three rabbits served as a control.

The duration of the experiment was 5 months. In the course of this period, in all the animals, a study was made of the cholesterol content in the blood, 1 time per month, in accordance with the Engelhardt-Smirnova method.

The aortas were fixed according to Lizon, and were poured into paraffin. Sections having a thickness of 5 microns were stained with hematoxylin-easin, azan, according to Haidenhain, orcein, and toluidine blue. The lipids were

stained according to Goldman (formalin, frozen sections).

The data obtained indicated that on feeding cholesterol to the rabbits, in the indicated doses, there gradually develops a moderately pronounced hypercholesterolemia that reached, by the end of the experiment, on an average, 371 milligram %. In the group of animals that had been administered, in addition to cholesterol, malic acid, there took place a more significant accretion of cholesterol -- 604 milligram %. Under the influence of oxaloacetic acid, the development of hypercholesterolemia was marked to the greatest extent. In the animals of this group, the level of the cholesterol content in the blood, at the end of the experiment, comprised 738 milligram %. The administration, in the rabbits, of only malic and oxaloacetic acids did not change the cholesterol level in the blood (see table)

Table

The Effect of Malic and Oxaloacetic Acids on the Development of Alimentary Hypercholesterolemia in Rabbits

Group of Animals	Cholesterol Content (in milligram %) on Various Days of the Experiment					
	prior to the beginning of the experiment	30th	60th	90th	120th	150th
5th	67± 7.4	142±6.7	208±25.4	266±31.4	315±13.4	371±30.0
4th	73± 7.4	257±14.8	380±30.0	369±17.0	435±46.0	604±73.3
2nd	70± 5.5	168±22.2	325±45.8	429±65	644±68	738±34.4

The vessels of the rabbits that had received, for a prolonged time, oxaloacetic and malic acids, and also, these acids jointly with cholesterol, underwent considerable and singular morphological and histochemical changes. ((Illustration on pg. 22 of text)) Fig. 1 Platelet in the aorta of the rabbit that had received oxaloacetic acid.

Changes in elasticity

a -- staining with hematoxylin-eosin. X 112; b -- staining with orcein. X 112.

In the vessels of rabbits that had been administered oxaloacetic acid, the greatest changes consisted in the formation of platelets of varying sizes. The largest platelets develop as a result of a considerable thickening of the intima, which bulges in the lumen of the vessel, and of the inner layers of the media. In the area of the platelet, the structure of the vessel wall is markedly impaired: there are evident breaks in the elasticity, which becomes basophilic and begins to be stained with hematoxylin-eosin. It may be observed that the amount of elastic fibers has considerably diminished (Fig. 1, a and 1, b). Some of the fibers thin out, tear, lose their configuration, scatter and are hardly stained with orcein; others thicken considerably, and on high magnification, light vacuoles are in them (Fig.2). Third elastic fibers have claviform excrescences.

In addition to the degenerating and disintegrating elastic fibers, there are disclosed, in the platelet, in a considerable amount, fibrous structures that have been stained, according to Haidenhain, a blue color; this indicates the cellagenic nature of these structures. In these places where there are no platelets, the elastic fibers are also considerably changed: there are observed breaks in the elastic fibers and their fragmentation. On staining these vessels with toluidine blue, it was established that, in the thickness of the large, markedly developed platelets, the amount of acid mucopolysaccharides was not increased. However, in the spaces between thefibers in small platelets, and on the boundary of platelets and visibly normal tissue, directly beneath the endothelium, the gamma-metachromasia is intensified; this testifies to an increase in the amount of acid mucopolysaccharides in these places. In the case of less marked changes, the endothelium cells, in some places, swell, are arranged in 2-3 layers, and beneath them, in the intima,and in the inner layers of the media, there are formed free non-stained spaces, which, on being stained fibers in these places move apart and become fragmented. Apparently, these changes may be considered to be the earliest ones.

((Two illustrations on pg. 23 of text)

Fig. 2 Changes in the elastic fibers. Light vacuoles are evident. Staining with orcein. X 1000.

Fig. 3 Formation of foamy cells in the aorta of a rabbit that has received malic acid in cholesterol. Staining with hematoxylin-eosin. X 112

The changes in the walls of the vessels in the rabbits that had received malic acid are reminiscent, to a large extent, of those that had been brought about by oxaloacetic acid. In all cases, there is observed a focal swelling of the endothelium. The elastic fibers, in the internal layers of the media, are likewise partially destroyed, fragmented, distended, and are poorly stained with orcein. The inner,elastic membrane, in places, is swollen, tears, and sometimes, separates into fibers. In the inner layers of the intima, between the fibers, there is noted a rather bright gamma-metachromasia, which attests to the accumulation of acid mucopolysaccharides. Platelets are encountered, which, by their structure, are reminiscent of the platelets in the vessels of those rabbits that had received oxaloacetic acid.

The character of the changes in the vessel wall of the rabbits that had received oxaloacetic and malic acids, along with a small amount of cholesterol, is analogous to the one described above: the endothelium cells are swollen, it is true, to a much greater extent than in the case of rabbits not fed with cholesterol, sometimes forming platelets in the form of pillows (cushions) that consist of several layers of endothelial cells (fig.3). Platelets are formed in which the leasticity has been markedly destroyed and changed; they are similar, in their structure, to the platelets shown in Fig.1. In addition to this, almost over the entire extent of the aorta, where there are no platelets, the elastic fibers are markedly changed, fragmented, and degenerated (Fig.4).

((two illustrations on pg. 24 of text))

Fig. 4 Breakdown in elasticity in the aorta of a rabbit that had received malic acid and cholesterol. Staining with orcein. X 280.

Fig. 5 Adiposis in the aorta wall of a rabbit that had received oxaloacetic acid and cholesterol. Staining according to Goldmin X 112

In studying the degree of lipoidosis of the vessels in animals of all the groups, it was disclosed that the vessels of those rabbits that had received only oxaloacetic and malic acids, in the majority of cases, contain fat, it is true, sometimes in very small amounts in individual cells of the endothelium. In the aorta wall of rabbits that had received, for a prolonged time, small doses of cholesterol, lipoidosis did not develop, and fat could be encountered only in individual cells of the endotherlium. However, in the vessels of rabbits that had received cholesterol together with malic and oxaloacetic acids, there developed a considerable lipoidosis of the aorta; at the same time, fat penetrated the media to a considerable depth (Fig.5).

The results that we obtained upon administering to rabbits malic and oxaloacetic acids, which also participate in the Sent-Dierdi-Krebs cycle, confirm the changes that had been observed by the authors indicated above. Under the influence of these acids, we disclosed similar morphological and histochemical changes in the wall of veseels where there is observed the most marked affection of the elasticity. It is possible to be in agreement with the Japanese authors who believe that the principal reason for the arteriosclerotic changes in the vessel wall of the rabbits, which develop under the influence of the acids being administered, is the impairment of the carbohydrate metabolism in it. The result of this, in the first place, is the degeneration of the elastic fibers and the accumulation of acid mucopolysaccharides. After that, in the region of the affection, there takes an accumulation of the disintegration products and the neoplasm of collagen; and it is this that brings about the arteriosclerotic changes in the vessel wall.

As has been shown by the results of our experiments, feeding the rabbits small amounts of cholesterol, which, in themselves, do not bring about the deposition of lipids in the aorta, against a background of administering acids of the tricarboxylic cycle, leads to a considerable lipoidosis of the vascular wall. This, apparently may be explained in the following manner: a prolonged administration to the rabbits of acids that impair the Krebs cycle, brings about the above-described changes in the vessel wall; and this, in turn, markedly changes the permeability of the vascular wall. The accumulation of acid mucopolysaccharides, which is expressed in an intensification of the gamma-metachromasia, may be determined both by the direct impairment of the Krebs cycle, as well as by their liberation during the disintegration of the elasticity.

It is highly probable that the lipids readily penetrate, from the blood plasma, such a changed vessel wall and are deposited in it. In addition to this, the pronounced accumulation of lipids in the aorta of rabbits that had received, along with cholesterol, malic and exaloacetic acids, may be associated, also, with the development and higher cholesterolemia that was observed in these animals; at the same time, the administration, by itself, to the rabbits, both of malic, as well as oxaloacetic acids, did not manifest any influence on the concentration of cholesterol in the blood. Consequently, these acids, which represent, in normal concentrations, and under normal conditions, catalysts of tissue respiration, particularly, of the oxidation of pyruvic acid, in the case of a surplus administration of them to the rabbits, which had received cholesterol, are instrumental in bringing about an intensified development of alimentary hypercholesterolemia. It is highly probable that this is associated with a change in the acid-alkaline equilibrium which brings about an excess admission of the indicated acids; against this background, there is a reduction in the metabolism of exogenous cholesterol.

Conclusion

A prolonged administration to rabbits of acids from the Sent-Dierdi-Krebs cycle, which disturb the carbohydrate metabolism of the vessel wall, leads to marked changes in the morphology and biochemistry of the vascular wall. The considerable lipoidosis of the aorta, which develops in the case of a simultaneous feeding of these rabbits with small doses of cholesterol, is determined, apparently, both by a more pronounced hypercholesterolemia in these animals, as well as by the fact that, in such an affected vessel wall, there is a change in the premeability, a marked reduction in the metabolic processes, and a more ready deposition of blood lipids.

Bibliography

1. Arai, T., Jao, K., Acta path. jap., 1957,v.7,p.237
2. Arai, T., Takenaka, J., Jshigami,A.et al., Trans. Soc.path.jap., 1954,v.43,p.231.
3. Arai, T., Takenaka, J., Kinoshita, J.et al.,Ibid.v.44,p203.
4. Jao,K.,J.Wakayama med.Soc.,1956, v.7, p.385.
5. Jshigami, A.,Trans.,Soc.path. jap.1 1956, v.45,p.538.
6. Takenaka, J.,J.Wakayama med Soc., 1956, v. 7, p.25.

(Translated by Carl Demrick Associates,Inc/LB/t)

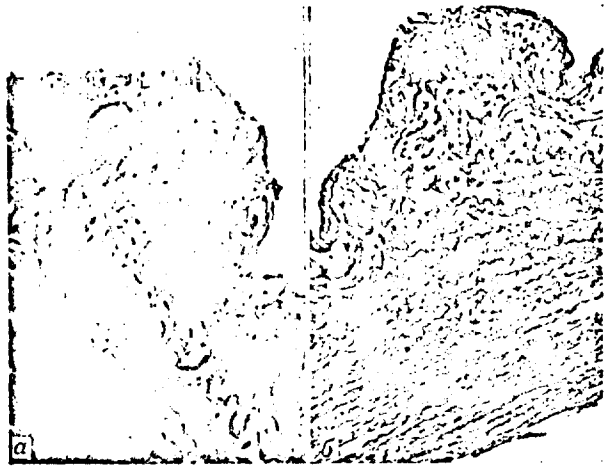
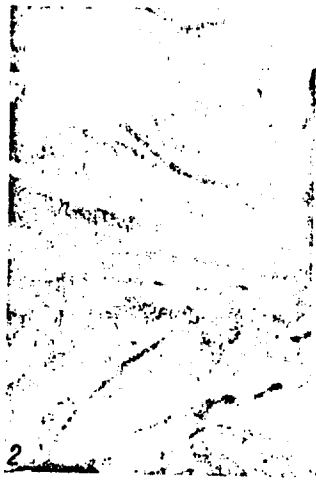


Fig. 1.



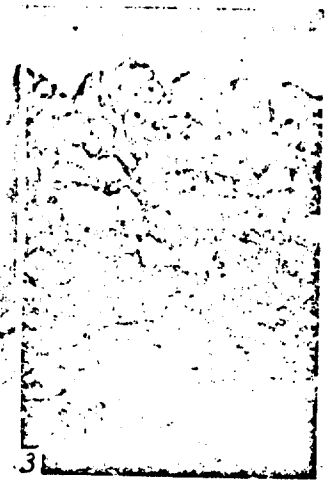


Fig. 2. Fig. 3.

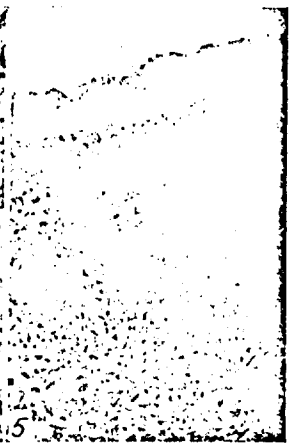


Fig. 4. Fig. 5.

Выводы

1. Внутристеночные артериальные геморрагии — одно из частых проявлений повышенной проницаемости при атеросклерозе возникают уже в самых ранних стадиях развития заболевания.

2. Эти интрамуральные геморрагии — важный фактор для формирования атеросклеротической бляшки, и они отражают высшую степень повышения проницаемости сосудистой стенки.

3. Прогрессирующие формы атеросклероза по сравнению с латентно текущими показывают наиболее интенсивную степень свежих и старых геморрагий в различных отрезках артериальной системы независимо от причины смерти.

4. Внутристеночные геморрагии, как и другие проявления повышенной проницаемости артериальной стенки, предшествуют анатомическим изменениям соответствующего органа.

5. В сердце и головном мозгу можно установить прямые положительные корреляции между наличием, частотой, интенсивностью интрамуральных артериальных геморрагий, клинической картиной заболевания и анатомическими изменениями самого органа.

6. По-видимому, внутристеночные кровоизлияния являются микромоделями диссоциирующих аневризм аорты, реже венечных и мозговых артерий.

ЛИТЕРАТУРА

Анестиади В. Х. В кн.: Материалы 2-й конференции патологоанатомов Латвии. Рига, 1962, с. 92. — Аничков Н. Н., Вальтер А. В., Волкова К. Г. и др. Тезисы докл. Всесоюзн. конференции патологоанатомов. М., 1954, с. 106. — Вихерт А. М., Ушкалов А. Ф. Кардиология, 1963, № 1, с. 8. — Горнак К. А. В кн.: Атеросклероз. Куйбышев, 1960, с. 56. — Гротель Д. М. Клин. мед., 1945, № 3, с. 3. — Золотова-Костомарова М. П. Клиника и патология инфаркта миокарда. Автореф. дисс. докт. М., 1951. — Козачук Ю. С. Патоморфологические изменения в миокарде и коронарных сосудах сердца при гипертонической болезни. Автореф. дисс. канд. Киев, 1953. — Лямпев В. Т. Арх. пат., 1957, в. 12, с. 40. — Струков А. И. (ред.) Вопросы морфологии и патогенеза инфаркта. М., 1959. — Хавкин Т. Н. В кн.: Атеросклероз. М., 1953, с. 88. — Хамитов С. Х. Сов. здравоохр. Киргизии, 1962, № 3, с. 22. — Юстер Ф. И. Труды Всесоюзн. конференции патологоанатомов. М., 1956, с. 286. — Drury R. A., J. Path. Bact., 1954, v. 67, p. 207. — Geiringer E., Ibid., 1951, v. 63, p. 201. — Higgenbotham A. C., Higgenbotham F. H., J. Atheroscler. Res., 1961, v. 1, p. 283. — Horn H., Finkelstein L. E., Am. Heart J., 1940, v. 19, p. 655. — Leary T., New Engl. J. Med., 1931, v. 205, p. 231. — Idem. Arch. Path., 1941, v. 32, p. 507. — Idem. Ibid., 1944, v. 37, p. 16. — Morgan A. D., The Pathogenesis of Coronary Occlusion. Oxford, 1956. — Nelson M. G., J. Path. Bact., 1941, v. 53, p. 105. — Paterson J. C., Arch. Path., 1936, v. 22, N 3, p. 313. — Paterson J. C., Arch. Path., 1938, v. 25, p. 474. — Idem. Am. Heart J., 1939, v. 18, p. 451. — Paterson J. C., Moffatt T., Mills J., Arch. Path., 1956, v. 61, p. 496. — Idem. Ibid., 1957, v. 64, p. 129. — Wartman W. B., Am. Heart J., 1950, v. 39, p. 79. — Winternitz M. C. et al., The Biology of Arteriosclerosis. Baltimore, 1938. — Papacharalampous N., Zollinger H. V., Schweiz. med. Wschr., 1953, Bd. 83, S. 859.

ON INTRAMURAL HEMORRHAGES IN THE ARTERIAL SYSTEM

L. A. Gulina, I. V. Davydovsky, A. I. Ozarai

Summary

Coronary arteries of the heart, arteries of the base of the brain, mesenteric and renal arteries were examined macro- and microscopically; those were obtained from 40 persons who died at the age of 35–80 years of various diseases. As detected, intramural arterial hemorrhages — one of the frequent manifestations of increased permeability in atherosclerosis, occurred at the earliest stages of the disease proving to be an important factor in the formation of atherosclerotic patches. In the heart and the brain there were established direct positive correlations between the presence, frequency, intensity of intramural arterial hemorrhages, the clinical picture of the disease and anatomical changes of the organs. Progressive forms of atherosclerosis, as compared to latent ones, indicate the most intensive degree of fresh and old hemorrhages in various portions of the arterial system, irrespective of the cause of death.

УДК 616.132-003.826-092.9-02:616-008.934.5

ВЛИЯНИЕ НАРУШЕНИЯ УГЛЕВОДНОГО ОБМЕНА НА СТЕНКУ АОРТЫ И РАЗВИТИЕ В НЕЙ АЛИМЕНТАРНОГО ЛИПОИДОЗА У КРОЛИКОВ

М. В. Бавина, Г. А. Иванова, Н. М. Поздюнина, А. М. Вихерт (Москва)

Лаборатория биохимии (зав. — проф. М. Г. Крицман) и лаборатория патологической анатомии (зав. — проф. А. М. Вихерт) Института терапии (дир. — действ. член АМН СССР проф. А. Л. Мясников) АМН СССР

(Поступила в редакцию 14.VI 1965 г.)

Значение нарушений метаболических процессов в патогенезе атеросклероза довольно четко представлено лишь в отношении липидов. Влияние изменений других сторон обмена веществ на состояние сосудистой стенки и развитие атеросклероза изучено сравнительно мало.

Представляет определенный интерес исследовать состояние структурных элементов аорты при нарушении окислительного цикла Сент—Дьерди - Кребса, являющегося основным биохимическим процессом во всех тканях животного организма. В настоящее время имеется лишь несколько работ японских авторов, непосредственно касающихся данного вопроса (Arai и Jao, 1957; Jshigami, 1956; Takenaka, 1956; Arai с соавторами, 1954; Jao, 1956; Arai с соавторами, 1955). Эти исследователи нарушали нормальное течение окислительных процессов путем введения животным (кроликам) некоторых компонентов цикла Сент—Дьерди - Кребса — лимонной и фумаровой кислот. На такое воздействие сосудистая стенка, по данным указанных авторов, отвечала своеобразными изменениями: интима частично утолщалась, а на границе медии и интимы обнаруживались участки дегенерации. Эластические волокна в этих местах плохо окрашивались, были утолщены, фрагментированы, неправильно ориентированы, количество их уменьшено. Мукополисахариды, в частности гиалуроновая кислота, обнаруживали тенденцию к накоплению. Жир встречался в небольших количествах в отдельных клетках эндотелия.

Мы поставили перед собой задачу не только выявить характерные морфологические и гистохимические изменения, развивающиеся в сосудистой стенке под влиянием других компонентов цикла Сент—Дьерди — Кребса, но и установить, в какой мере нарушения в указанном окислительном цикле сказываются на развитии алиментарной гиперхолестеринемии и липоидоза аорты у кроликов при скармливании им малых количеств холестерина, которые сами по себе вызывают умеренное повышение уровня холестерина в крови и лишь незначительные проявления липоидоза в аорте.

Под опытом находилось 30 кроликов-самцов породы шиншилла, весом 2,7—3 кг. Все животные были разделены на 5 групп. Кроликам 1-й группы (6) вводили внутрибрюшинно по 200 мг щавелевоуксусной кислоты 2 раза в неделю. Животные 2-й группы (5), кроме щавелевоуксусной кислоты, получали ежедневно по 0,07 г/кг холестерина. Кроликам 3-й группы (6) вводили внутрибрюшинно по 300 мг яблочной кислоты 2 раза в неделю. Животные 4-й группы (5) получали яблочную кислоту и холестерин в указанных выше количествах, кролики 5-й (5) — только холестерин в тех же количествах, что и животные 2-й и 4-й группы. Три кролика служили контролем.

Продолжительность эксперимента 5 месяцев. В течение этого срока у всех животных исследовали содержание холестерина в крови 1 раз в месяц по способу Энгельгардта — Смирновой.

Аорты фиксировали по Лизону, заливали в парафин. Срезы толщиной 5 мк окрашивали гематоксилин-эозином, азаном по Ганденгайну, орсеином, толуидиновым синим. Липиды окрашивали по Гольдману (формалин, замороженные срезы).

Полученные данные показали, что при скармливании кроликам холестерина в указанных дозах постепенно развивается умеренно выра-

женная гиперхолестеринемия, достигавшая к концу опыта в среднем 371 мг%. В группе животных, которым, кроме холестерина, вводили яблочную кислоту, происходило более значительное нарастание холестерина — 604 мг%. Под влиянием щавелевоуксусной кислоты развитие гиперхолестеринемии было выражено в наибольшей степени. У животных этой группы уровень содержания холестерина в крови в конце опыта составлял 738 мг%. Введение кроликам только яблочной и щавелевоуксусной кислот не изменяло уровня холестерина в крови (см. таблицу).

Влияние яблочной и щавелевоуксусной кислот на развитие алиментарной гиперхолестеринемии у кроликов

Группа животных	Содержание холестерина (в мг%) в различные дни опыта					
	до начала опыта	30-й	60-й	90-й	120-й	150-й
5-я	67±7,4	142±6,7	208±25,4	266±31,4	315±13,4	371±30,0
4-я	73±7,4	257±14,8	380±30,0	369±17,0	435±46,0	604±73,3
2-я	70±5,5	168±22,2	325±45,8	429±65	644±68	738±34,4

Сосуды кроликов, длительное время получавших щавелевоуксусную и яблочную кислоты, а также эти кислоты совместно с холестерином, претерпели значительные и своеобразные морфологические и гистохимические изменения.

Рис. 1. Бляшка в аорте кролика, получавшего щавелевоуксусную кислоту. Изменения эластики.

а — окраска гематоксилин-эозином. × 112, *б* — окраска орсеином. × 112.

В сосудах кроликов, которым вводили щавелевоуксусную кислоту, наибольшие изменения заключались в образовании бляшек разной величины. Наиболее крупные бляшки возникают за счет значительного утолщения интимы, которая выбухает в просвет сосуда, и внутренних слоев медии. В области бляшки структура стенки сосуда резко нарушена: видны обрывки эластики, которая становится базофильной и начинает окрашиваться гематоксилин-эозином. Можно отметить, что количество эластических волокон значительно уменьшено (рис. 1, *а* и 1, *б*). Одни волокна истончаются, рвутся, теряют очертания, расплываются и почти не окрашиваются орсеином; другие значительно утолщаются, при большом увеличении в них видны светлые вакуоли (рис. 2). Третьи эластические волокна имеют своеобразные булавовидные наплывы.

Помимо дегенерирующих и распадающихся эластических волокон, в бляшке в значительном количестве обнаруживаются волокнистые структуры, окрашивающиеся по Гайденгайну в синий цвет, что говорит о коллагеновой природе этих структур. В тех местах, где нет бляшек, эластические волокна также значительно изменены: наблюдаются разрывы эластических волокон и их фрагментация. При окрашивании этих сосудов толуидиновым синим установлено, что в толще больших, сильно развитых бляшек количество кислых мукополисахаридов не увеличено. Однако в промежутках между волокнами в небольших бляшках и на границе бляшек и видимо нормальной ткани, непосредственно под эндотелием, γ-метахромазия усиливается, что свидетельствует об увеличении количества кислых мукополисахаридов в этих местах. При менее выраженных изменениях клетки эндотелия в некоторых местах набухают, располагаются в 2—3 слоя, а под ними в интиме и во внутренних слоях медии образуются свободные неокрашенные пространства, которые при окраске толуидиновым синим дают довольно яркую γ-метахромазию. Эластические волокна в этих местах раздвигаются, фрагменти-

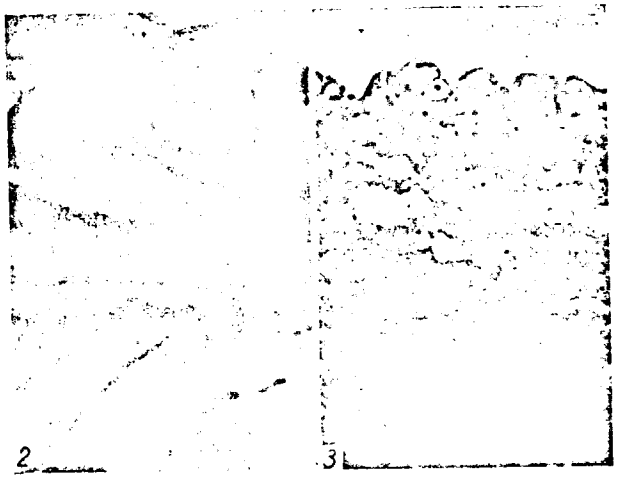


Рис. 2. Изменения эластических волокон. Видны светлые вакуоли. Окраска орсеином. × 1000.

Рис. 3. Образование пенистых клеток в аорте кролика, получавшего яблочную кислоту и холестерин. Окраска гематоксилин-эозином. × 112.

руются. По-видимому, эти изменения можно рассматривать как наиболее ранние.

Изменения в стенках сосудов у кроликов, получавших яблочную кислоту, во многом напоминают таковые, вызванные щавелевоуксусной кислотой. Во всех случаях наблюдается очаговое набухание эндотелия. Эластические волокна во внутренних слоях медии также частично разрушаются, фрагментируются, разбухают, плохо окрашиваются орсеином. Внутренняя эластическая мембрана местами набухает, рвется, а

иногда и разволокняется. Во внутренних слоях интимы между волокнами отмечается довольно яркая γ-метахромазия, свидетельствующая о накоплении кислых мукополисахаридов. Встречаются бляшки, по строению напоминающие бляшки в сосудах кроликов, получавших щавелевоуксусную кислоту.

Характер изменений стенки сосудов кроликов, получавших щавелевоуксусную и яблочную кислоты вместе с небольшим количеством холестерина, аналогичен описанным выше: клетки эндотелия набухают, правда, гораздо больше, чем у кроликов, не кормленных холестерином, образуя иногда бляшки в виде подушек, состоящих из нескольких слоев эндотелиальных клеток (рис. 3). Образуются бляшки, в которых сильно разрушена и изменена эластика, они сходны по строению с бляшками, показанными на рис. 1. Кроме того, почти на всем протяжении аорты, там, где нет бляшек, эластические волокна сильно изменены, фрагментированы, дегенерированы (рис. 4).

Рис. 4. Разрушение эластики в аорте кролика, получавшего яблочную кислоту и холестерин.
Окраска орсеином. × 280.

Рис. 5. Отложение жира в стенке аорты кролика, получавшего щавелевоуксусную кислоту и холестерин.
Окраска по Гольдшиду. × 112.

При изучении степени липоидоза сосудов у животных всех групп было обнаружено, что сосуды кроликов, получавших только щавелевоуксусную и яблочную кислоты, в большинстве случаев содержат жир, правда, иногда в очень небольших количествах в единичных клетках эндотелия. В стенке аорты кроликов, получавших длительное время лишь небольшие дозы холестерина, липоидоз не развивался и жир можно было встретить только в отдельных клетках эндотелия. Однако в сосудах кроликов, получавших холестерин с яблочной и щавелевоуксусной кислотами, возникал значительный липоидоз аорты, причем жир проникал в медию на значительную глубину (рис. 5).

Результаты, полученные нами при введении кроликам яблочной и щавелевоуксусной кислот, которые также участвуют в цикле Сент-Дьерди—Кребса, подтверждают изменения, наблюдаемые указанными выше авторами. Под воздействием этих кислот мы обнаружили сход-

ные морфологические и гистохимические изменения в стенке сосудов, где отмечается наиболее сильное поражение эластики. Можно согласиться с японскими авторами, считающими, что основной причиной артериосклеротических изменений в стенке сосудов кроликов, развивающихся под влиянием вводимых кислот, является нарушение углеводного обмена в ней. Результатом этого прежде всего является дегенерация эластических волокон и накопление кислых мукополисахаридов. Затем в области поражения происходит накопление продуктов распада и новообразование коллагена, что и приводит к артериосклеротическим изменениям стенки сосудов.

Как показали результаты наших опытов, скармливание кроликам небольших количеств холестерина, которые сами по себе не вызывают отложения липидов в аорте, на фоне введения кислот трикарбонового цикла приводит к значительному липоидозу сосудистой стенки. Это можно объяснить, по-видимому, так: длительное введение кроликам кислот, нарушающих цикл Кребса, приводит к описанным выше изменениям стенки сосудов, что в свою очередь резко меняет проницаемость сосудистой стенки. Накопление кислых мукополисахаридов, выражающееся в усилении γ-метахромазии может быть обусловлено как прямым нарушением цикла Кребса, так и освобождением их при распаде эластики.

Весьма вероятно, что липиды легко проникают из плазмы крови в такую измененную стенку сосуда и откладываются в ней. Кроме того, выраженное накопление липидов в аорте кроликов, получавших вместе с холестерином яблочную и щавелевоуксусную кислоты, может быть связано также с развитием и более высокой холестеринемии, наблюдавшейся у этих животных, причем само по себе введение кроликам как яблочной, так и щавелевоуксусной кислот не оказывало влияния на концентрацию холестерина в крови. Следовательно, эти кислоты, являющиеся в нормальных концентрациях и при нормальных условиях катализаторами тканевого дыхания, особенно окисления пировиноградной кислоты, при избыточном введении их кроликам, получающим холестерин, способствуют усиленному развитию алиментарной гиперхолестеринемии. Весьма вероятно, что это связано с изменением кислотно-щелочного равновесия, вызываемого избыточным поступлением указанных кислот, на фоне которого снижается метаболизм экзогенного холестерина.

Заключение

Длительное введение кроликам кислот из цикла Сент-Дьерди—Кребса, нарушающих углеводный обмен стенки сосудов, приводит к резким изменениям в морфологии и биохимии сосудистой стенки. Значительный липоидоз аорты, развивающийся при одновременном кормлении этих кроликов малыми дозами холестерина, обусловлен, по-видимому, как более выраженной гиперхолестеринемией у этих животных, так и тем, что в такой пораженной стенке сосуда изменяется проницаемость, резко снижаются метаболические процессы и легче откладываются липиды крови.

ЛИТЕРАТУРА

Arai T., Jao K., Acta path. jap., 1957, v. 7, p. 237. — Arai T., Takenaka J., Jshigami A. et al., Trans. Soc. path. jap., 1954, v. 43, p. 231. — Arai T., Takenaka J., Kinoshita J. et al., Ibid., v. 44, p. 203. — Jao K., J. Wakayama med. Soc., 1956, v. 7, p. 385. — Jshigami A., Trans. Soc. path. jap., 1956, v. 45, p. 538. — Takenaka J., J. Wakayama med. Soc., 1956, v. 7, p. 25.

THE EFFECT OF CARBOHYDRATE METABOLISM DISTURBANCES ON THE WALL OF THE AORTA AND ON THE DEVELOPMENT IN IT OF ALIMENTARY LIPOIDOSIS IN RABBITS

M. V. Bavina, G. A. Ivanova, N. M. Pozdyunina, A. M. Vikhert

Summary

Prolonged administration to rabbits of acids of the Sent-Dierdi-Krebs cycle, disturbing the carbohydrate metabolism of the vascular wall leads to marked changes of the morphology and biochemistry in it. Simultaneous feeding of these animals with low cholesterol doses, which by themselves caused no lipid deposition in the vascular wall, provoked considerable lipoidosis of the aorta, evidently caused both by more marked cholesterolemia and also by the fact that the vascular wall affected in such a way exhibited changes of permeability, and marked reduction of metabolic processes, facilitating blood lipid deposition in it.

УДК 612.014.1:611.13+616.13.004.6+008.935.87:12.002.18

ГИСТОХИМИЧЕСКАЯ ХАРАКТЕРИСТИКА ТИРОЗИНА В АРТЕРИЯХ ЭЛАСТИЧЕСКОГО И МЫШЕЧНОГО ТИПОВ В НОРМЕ И ПРИ АТЕРОСКЛЕРОЗЕ

М. М. Багомедов (Махачкала)

Кафедра патологической анатомии (зав. — проф. С. С. Касабьян) Дагестанского медицинского института

(Поступила в редакцию 25.I.1965 г.)

Вопрос о роли биохимических и физико-химических изменений артериальной стенки в патогенезе атеросклероза до сих пор не разрешен и является предметом дискуссии.

Среди исследований, предпринятых в этом направлении (С. С. Касабьян, 1961, 1962, 1963; Banga и Balo; Bredt; Bunting C. H., Bunting H.; Lindner, 1958, 1961; Sulkin), значительное место отводится изменениям основного, промежуточного вещества и, в частности, важному его компоненту — мукополисахаридам. Считается, что количественные (А. П. Авцын с соавторами; Holle; Rinehart и Greenberg; Taylor) и качественные (К. Великан и Д. Великан; С. С. Касабьян) сдвиги мукополисахаридов, изменения их дисперсности (Л. В. Касаткина, 1961, 1962, 1963) создают условия для проникновения и накопления липидов в стенке артерий. Что касается других элементов основного вещества сосудистой стенки — структурных белков — они исследованы недостаточно. В этой связи определенное значение имеют исследования М. Г. Крицмач и М. В. Бавиной, которые при экспериментальном атеросклерозе у кроликов обнаружили ослабление синтеза и резкое усиление распада белков аорты. По мнению Bjorksten, Lindner, Sulkin, Taylor, накопление мукополисахаридов в сосудистой стенке в начале атеросклероза связано с нарушением протеидных свойств коллагена и эластина, вследствие чего мукополисахариды освобождаются от связей с белком. Результаты исследований Н. В. Окунева, А. Т. Никулева, Banga и Balo, Lansing свидетельствуют о возможности отщепления холестерина от эластина. Н. М. Путкарадзе отметил изменение изоэлектрической точки выявления РИП и ДИП еще задолго до отложения липидов в стенке аорты кроликов, получавших холестерин.

Цель данного исследования — изучение содержания и распределения тирозина в нормальных артериях в различные возрастные периоды и при атеросклерозе при помощи гистохимических методов.

Тирозин изучали в аорте и внутренней подвздошной артерии 20 человеческих плодов (от 12 до 32 недель утробной жизни), 22 детей (от 3 месяцев до 14 лет) и 40 взрослых (от 18 до 85 лет), умерших от различных заболеваний, не связанных с поражением сосудов, и от травм. При атеросклерозе тирозин изучали в тех же сосудах у 21 взрослого с начальными изменениями (липоидоз) и у 56 с выраженным атеросклерозом (от 18 до 87 лет.)

Исследовали нисходящую часть аорты и левую внутреннюю подвздошную артерию в первые 3—15 часов после смерти. Кусочки включали бляшку с неизмененной стенкой сосуда; материал фиксировали в 10% растворе формалина и в жидкости Карнуа и заливали в парафин.

Тирозин определяли гистохимическим методом по Миллону в модификации Bensley и Gersch, а также Baker. Методика основана на выявлении оксифенильной группы тирозина, входящего в молекулу белка, и строго специфична (Э. Пирс). Для приближенной количественной характеристики содержания тирозина в гистологических структурах придерживались трех градаций: большое, среднее и малое количество. Препараты также окрашивали гематоксилин-эозином, по ван Гизону и суданом III, IV и черным В.

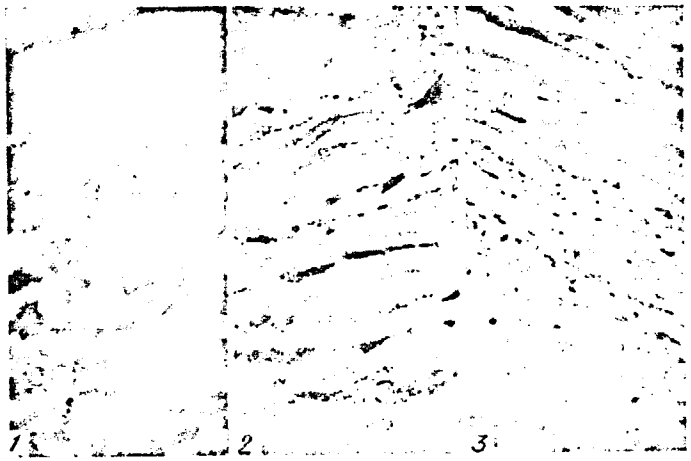


Рис. 1. Значительное содержание тирозина в эластических элементах медиа аорты 26-недельного плода.

Окраска по Миллону в модификации Bensley и Gersch. × 700.

Рис. 2. Уменьшение содержания тирозина в местах небольших деструктивных изменений в интиме при одновременном большом содержании его в медиа (внутренняя подвздошная артерия взрослого).

Окраска по Миллону в модификации Bensley и Gersch. × 600.

Рис. 3. Значительное содержание тирозина в фиброзной покрышке бляшки по ходу пучков и отсутствие его в зоне клеточного распада

Окраска по Миллону в модификации Bensley и Gersch. × 290.

Результаты изучения тирозина в стенке нормальных артерий показали его значительное возрастное колебание.

В **н а т а л ь н ы й п е р и о д** в стенке аорты и внутренней подвздошной артерии тирозин определялся в среднем количестве, распределялся неравномерно. В интиме он выявлялся в эндотелии в малом количестве в виде отдельных мельчайших красных гранул, чаще в ядре, реже в цитоплазме. Внутренняя эластическая мембрана окрашивалась более интенсивно, но гранулы были видны не всегда. В медиа тирозина было несколько больше — мелкие зерна по ходу эластических мембран и мышечных волокон, главным образом в ядрах (рис. 1.). В адвентиции тирозин обнаруживался главным образом в ядрах соединительнотканных

UDK (Universal Decimal Classification) 616.132-004.

6-092.9-092

Effect of Malic Acid and Alkaline and Alkali-Earth Metal Carbonates on the Development of Alimentary Hypercholesterolemia and Lipoidosis of the Aorta in Rabbits

D.A. Kaidin

Laboratory of Biochemistry (Director -- Candidate in Biological Sciences, L. I. Grebennik) of the Division of Chemotherapy (Director -- Corresponding Member of the AMN SSSR (Academy of Medical Sciences of the U.S.S.R.), Prof. G. N. Pershin) of the All-Union Scientific-Research Chemico-Pharmaceutical Institute Named After S. Ordzhonikidze, Moscow

(Received for Publication on 27/X 1967 (October 27, 1967), Presented by Active Member of the Academy of Medical Sciences of the U.S.S.R., V. D. Gorizontov).

Malic acid, against a backgrounf of an atherogenous diet, reduces the level of cholesterol and general lipids in the liver, in the suprarenal glands and in the aorta of rabbits, while alkaline and alkali-earth metal cabonates yield the opposite effect (Byull. eksper. biol. (Bulletin of Experimental Biology), 1968, No. 12, p. 29).

An impairment in the oxidation-reduction reactions in the Krebs cycle leads to an accumulation of ketone bodies (5). This does not take place in the normalization or operation of the Krebs cycle by means of administering citric acid (1).

It was snteresting to clarify the effect, on the metabolism of cholesterol and other intermediates of the Krebs cycle, of malic acid that was employed along with food in large amounts (4).

Experimental Procedure

The experiments were carried out in the summer on 24 male rabbits having a weight of 2.7 - 2.8 kilograms. In the course of 100 days, the animals received a basic diet: 50 grams of hay, 80 grams of oats, 100 grams of vegetables and cholesterol, figuring on 200 milligrams/kilogram, in the form of a suspension on a starchy jelly (semi-liquid) mixed with the feed (3). A determination was made of the cholesterol content in the blood (7) and in several organs (6). The general lipids were determined by the gravimetric method. The control animals (the 1st group) received only the basic diet; for the experimental ones (the 2nd group), there was added to the basic diet malic acid in a dose of 1 gram/kilogram, or a mixture of alkaline and alkali-earth metal carbonates (the 3rd group) in a dose of 500 milligrams/kilogram ($NaHCO_3$ -- 83 milligrams; K_2CO_3 -- 218 milligrams; $CaCO_3$ -- 91 milligrams; $MgCO_3$ -- 108 milligrams). The ratio between the salts approximately corresponded to the same one in the natural products in the daily diet of the animals (2).

Results of the Experiments

The results of the investigations (see table) indicated that, by the end of the experiment, in the rabbits of the 3rd group, there was observed a reliably (P is less than 0.05) greater accretion in the concentration of cholesterol in the blood than was the case in the controls. In the rabbits of the 2nd group, the cholesterol content in the blood was the least (P is less than 0.002). There was a marked increase in the cholesterol content and in the general lipids in the organs of the animals in all the groups. However, in the rabbits of the 2nd group, they were determined to be in a lesser concentration. The cholesterol content and that of the general lipids, in the aorta of the animals in all the groups, corresponded to the manifestation of a macroscopic picture of atheromatosis. Atherosclerotic changes in the aorta in the rabbits of the 3rd group ranged from 3 to 5 pulses, and in the rabbits of the 2nd group -- from 1 to 4 pluses.

In a macroscopic investigation of the aorta in the control animals, there was noted a large amount of fine and large platelets that were partially merged with one another. A histological examination of small pieces of the aorta indicated that the tissue of atheromatose platelets, bascially, consists of xanthomatous cells. A diffuse infiltration by lipoids was disclosed in the wall of the large vessels that leave the aorta. In the rabbits of the 2nd group, changes in the aorta were pronounced to a considerably weaker degree: on the inner surface of the aorta were observed single, very fine atherosclerotic platelets with the presence of large sections free of pathological changes. In the rabbits of the 3rd group, atheromatosis of the aorta was considerably more markedly pronounced than was the case in the controls. In the majority of animals in the 3rd group, on the inner surface of the aorta (from the arch to the bifurcation), there were found numerous large platelets of an irregular shape, which merged with one another. A histological study indicated that the platelets consist of cells that are intensively stained with hematoxylin, and of xanthomatous cells containing lipoids. There were noted a diminution of the amount of elastic fibers, their breakages and fragmentation (on staining with orcein). In a macroscopic examination of the liver in animals of the 1st and 2nd groups, no changes whatsoever were disclosed. In the animals of the 3rd group, the liver was flaccid, of a clayey color, and enlarged in size and weight by 2 - 3 times (in comparison with the normal one). Microscopy revealed, in the liver of animals belonging to the 1st and 2nd group, drops of neutral fat located in the individual polygonal cells of the peripheral sections of the liver lobules. In the animals of the 3rd group, there was observed a diffuse infiltration of the liver polygonal cells with fat.

((Table on pg. 30 of text))

Effect of Malic Acid and of Aakaline and Alkali-Earth Metal Carbonates on the Development of Alimentary Hypercholesterolemia and Lipoidosis in Rabbits

(M plus or minus m)

Group and Number of Animals	Weight of the Animals (in grams)		Cholesterol Content in the Blood Plasma (in milligram %)		Amount of Cholesterol			Content of General Lipids		
1st (8)	2 755	2 800	45.4±3.6	661.3±33.0	969±165.9	16.7±0.53	19.1±2.5	8.9±0.91	37±3.2	50.5±4.1
2nd (8)	2 766	2 818	59.9±3.1	P<0.002	P<0.05	P<0.05	P<0.001	P<0.001	P<0.2	P<0.01
				450.3±45.0	682±143.6	13.6±1.5	12.2±1.8	7.2±0.61	33.2±4.1	384.4±5.5
3rd (8)	2 708	2 923	61.8±5.1	P<0.05	P<0.05	P<0.02	P<0.05	P<0.05	P<0.2	P<0.02
				769±69.1	1164±134.5	17.6±1.17	21.7±2.3	11.2±6.5	38±2.1	65.8±3.6

((text continued)): In the rabbits of the 1st and 3rd groups, the suprarenal glands, the aorta, and the thyroid gland were enlarged by $1\frac{1}{2}$ - 2 times in comparison with the norm. In the animals of the 2nd group, these organs scarcely varied, in weight, from the organs of the healthy rabbits. In this manner, malic acid, in a dose of 1 gram/kilogram, on being added to the atherogenous diet, manifests a favorable effect, reducing the level of cholesterol and of the general lipids in the blood and in the organs, whereas the alkaline and alkali-earth metal carbonates intensify cholesterolemia, starting with the 40th day from the beginning of the experiment.

We should like to express our deep appreciation to Doctor of Medical Sciences A. I. Yakovleva for her assistance in describing the pethomorphological preparations.

BIBLIOGRAPHY

1. Gulyi, M. F., Biochemistry of Fatty Metabolism, Kiev, 1961, p. 136.

2. Kaidin, D. A., Grebennik, L. I., Cardiology (Kardiologiya), 1967, No. 4, p. 123.

3. Kaidin, D. A., Byull. eksper. biol. (Bulletin of Experimental Biology), 1968, No, 3, p. 36.

4. Same author, Pat. fiziol. (Pathological Physiology), 1968, No. 2, p. 72.

5. Annau, E., Hoppe-Seylers, Z. physiol. chem., 1939, Bd 61, S. 58.

6. Abell, L. L. et al., In the book: Standard Methods of Clinical Chemistry, New York, 1958, v. 2, p. 26.

7. Chiamori, N., Henry, R. J., Am. J. Clin. Path., 1959, v. 31, p. 305.

Translated by Carl Demrick Associates, Inc./LB/db

Malic acid 224

УДК 616.132-004.6-092.9-092

ВЛИЯНИЕ ЯБЛОЧНОЙ КИСЛОТЫ И КАРБОНАТОВ ЩЕЛОЧНЫХ И ЩЕЛОЧНОЗЕМЕЛЬНЫХ МЕТАЛЛОВ НА РАЗВИТИЕ АЛИМЕНТАРНОЙ ГИПЕРХОЛЕСТЕРИНЕМИИ И ЛИПОИДОЗА АОРТЫ У КРОЛИКОВ

Д. А. Кайдин

Лаборатория биохимии (зав.— канд. биол. наук Л. И. Гребенник) отдела химиотерапии (зав.— член-корр. АМН СССР проф. Г. Н. Першин) Всесоюзного научно-исследовательского химико-фармацевтического института имени С. Орджоникидзе, Москва

(Поступила в редакцию 27/X 1967 г.
Представлена действ. членом АМН СССР В. Д. Горизонтовым)

Яблочная кислота на фоне атерогенной диеты снижает уровень холестерина и общих липидов в печени, надпочечниках и в аорте у кроликов, а карбонаты щелочных и щелочноземельных металлов дают противоположный эффект (Бюлл. экспер. биол., 1968, № 12, с. 29).

Нарушение окислительно-восстановительных реакций в цикле Кребса приводит к накоплению кетоновых тел [5]. Этого не происходит при нормализации функционирования цикла Кребса путем введения лимонной кислоты [1].

Представляло интерес выяснить влияние на обмен холестерина и другого интермедиата цикла Кребса — яблочной кислоты, потребляемой с пищей в больших количествах [4].

Методика опытов

Опыты проводили летом на 24 кроликах-самцах весом 2,7—2,8 *кг*. В течение 100 дней животные получали основной рацион: 50 *г* сена, 80 *г* овса, 100 *г* овощей и холестерин из расчета 200 *мг/кг* в виде суспензии на крахмальном киселе, перемешанной с кормом [3]. Определяли содержание холестерина в крови [7] и некоторых органах [6]. Общие липиды определяли весовым способом. Контрольные животные (1-я группа) получали только основной рацион; подопытным (2-я группа) к основному рациону добавляли яблочную кислоту в дозе 1 *г/кг* или смесь карбонатов щелочных и щелочноземельных металлов (3-я группа) в дозе 500 *мг/кг* ($NaHCO_3$—83 *мг*; K_2CO_3—218 *мг*; $CaCO_3$—91 *мг*; $MgCO_3$—108 *мг*). Соотношение между солями примерно соответствовало таковому в естественных продуктах суточного рациона животных [2].

Результаты опытов

Результаты исследований (см. таблицу) показали, что к концу опыта у кроликов 3-й группы наблюдался достоверно ($P<0,05$) более высокий прирост концентрации холестерина в крови, чем у контрольных. У кроликов 2-й группы содержание холестерина в крови было наименьшим ($P<0,002$). Резко увеличилось содержание холестерина и общих липидов в органах животных всех групп. Однако у кроликов 2-й группы они определялись в меньшей концентрации. Содержание холестерина и общих липидов в аорте животных всех групп соответствовало выраженности макроскопической картины атероматоза. Атеросклеротические изменения в аорте у кроликов 3-й группы были от 3 до 5 плюсов, а у кроликов 2-й группы — от 1 до 4 плюсов.

При макроскопическом исследовании аорты контрольных животных было отмечено большое количество мелких и крупных, частью слившихся между собой бляшек. Гистологическое исследование кусочков аорты показало, что ткань атероматозных бляшек в основном состоит из ксантомных клеток. Диффузная инфильтрация липоидами была обнаружена в стенке крупных

Влияние яблочной кислоты и карбонатов щелочных и щелочноземельных металлов на развитие алиментарной гиперхолестеринемии и липоидоза аорты у кроликов ($M \pm m$)

Группа и число животных	Вес животных (в г)		Содержание холестерина в плазме крови (в мг %)		Количество холестерина			Содержание общих липидов		
	начало опыта	конец опыта	1-й день опыта	100-й день опыта	в печени (в мг %)	в надпочечниках (в %)	в аорте (в мг в 1 г сырой ткани)	в печени (в %)	в надпочечниках (в %)	в аорте (в мг в 1 г сырой ткани)
1-я (8)	2 755	2 800	45,4±3,6	661,3±33,0	969±165,9	16,7±0,53	19,1±2,5	8,9±0,91	37±3,2	50,5±4,1
2-я (8)	2 766	2 818	59,9±3,1	$P<0,002$	$P<0,05$	$P<0,05$	$P<0,001$	$P<0,001$	$P<0,2$	$P<0,01$
				450,3±45,0	682±143,6	13,6±1,5	12,2±1,8	7,2±0,61	33,2±4,1	384,4±5,5
3-я (8)	2 708	2 923	61,8±5,1	$P<0,05$	$P<0,05$	$P<0,02$	$P<0,05$	$P<0,05$	$P<0,2$	$P<0,02$
				769±69,1	1164±134,5	17,6±1,17	21,7±2,3	11,2±6,5	38±2,1	65,8±3,6

сосудов, отходящих от аорты. У кроликов 2-й группы изменения в аорте были выражены значительно слабее: на внутренней поверхности аорты наблюдались единичные, очень мелкие атеросклеротические бляшки с наличием больших участков, свободных от патологических изменений. У кроликов 3-й группы атероматоз аорты был выражен значительно сильнее, чем у контрольных. У большинства животных 3-й группы на внутренней поверхности аорты (от дуги до бифуркации) были найдены множественные крупные бляшки, неправильной формы, сливающиеся одна с другой. Гистологическое исследование показало, что бляшки состоят из клеток, интенсивно окрашивающихся гематоксилином, и из ксантомных клеток, содержащих липоиды. Отмечены уменьшение количества эластических волокон, их разрывы и фрагментация (при окрашивании орсеином). При макроскопическом исследовании печени животных 1-й и 2-й группы каких-либо изменений не обнаружено. У животных 3-й группы печень была дряблая, глинистого цвета и в 2—3 раза увеличена в размере и весе (по сравнению с нормой). Микроскопия выявила в печени животных 1-й и 2-й группы капли нейтрального жира, расположенного в отдельных полигональных клетках периферических участков печеночных долек. У животных 3-й группы наблюдалась диффузная инфильтрация полигональных клеток печени жиром.

У кроликов 1-й и 3-й группы надпочечники, аорта и щитовидная железа были увеличены в $1^1/_2$—2 раза по сравнению с нормой. У животных 2-й группы эти органы почти не отличались по весу от органов здоровых кроликов. Таким образом, яблочная кислота в дозе 1 *г/кг* при добавлении к атерогенной диете оказывает благоприятное влияние, снижая уровень холестерина и общих липидов в крови и органах, а карбонаты щелочных и щелочноземельных металлов усиливают холестеринемию, начиная с 40-го дня от начала опыта.

Выражаем глубокую признательность доктору мед. наук А. И. Яковлевой за помощь в описании патоморфологических препаратов.

ЛИТЕРАТУРА

1. Гулый М. Ф. Биохимия жирового обмена. Киев, 1961, с. 136. — 2. Кайдин Д. А., Гребенник Л. И. Кардиология, 1967, № 4, с. 123. — 3. Кайдин Д. А. Бюлл. экспер. биол., 1968, № 3, с. 36. — 4. Он же. Пат. физиол., 1968, № 2, с. 72. — 5. Annau E., Hoppe-Seylers, Z. physiol. chem., 1939, Bd 61, S. 58. — 6. Abell L. L. et al. В кн.: Standard Methods of clinical chemistry. New York, 1958, v. 2, p. 26.— 7. Chiamori N., Henry R. J., Am. J. clin. Path., 1959, v. 31, p. 305.

THE EFFECT OF MALIC ACID AND ALKALINE AND ALKALI-EARTH METAL CARBONATES ON THE DEVELOPMENT OF ALIMENTARY HYPERCHOLISTEROLEMIA AND LIPOIDOSIS OF AORTA IN RABBITS

D. A. Kaidin

Pharmacochemical Institute, Moskow

On adding to an atherogenous diet of malic acid in a dose of 1 g/kg body weight the cholesterol and total blood lipids and organs conent goes down distinctly as compared to controls. Alkaline and alkali-earth metal carbonates cause an opposite effect.

УДК 616.155.194.8-07:616.155.1.02-008.927.2-074

ДЕЙСТВИЕ ВИТАМИНА B_6 НА УТИЛИЗАЦИЮ Fe^{59} ПРОНОРМОБЛАСТАМИ И НОРМОБЛАСТАМИ БОЛЬНЫХ ЖЕЛЕЗОДЕФИЦИТНЫМИ АНЕМИЯМИ

С. В. Канаев, Э. И. Щербань, Г. Д. Шостка, В. А. Лапотников

Факультетская терапевтическая клиника (зав.— проф. Т. С. Истаманова) I Медицинского института им. И. П. Павлова, Ленинград

(Поступила в редакцию 6/XII 1967 г.
Представлена действ. членом АМН СССР В. Г. Барановым)

В исследованиях на культурах костного мозга больных различными формами железодефицитного малокровия установлено, что витамин B_6 стимулирует функциональную активность эритроидных элементов: увеличивается захват Fe^{59} эритроидными клетками и уменьшается содержание негемового железа в них (Бюлл. экспер. биол., 1968, № 12, с. 31).

В настоящей работе изучали влияние витамина B_6 на интенсивность включения железа в ядерные эритроидные клетки и динамику изменения сидеробластограммы в культурах костного мозга больных железодефицитной анемией.

Методика исследования

Использованная техника культивирования костного мозга и микроавторадиографии изложена в предыдущей работе [1]. Во всех опытах к 1 *мл* суспензии костного мозга добавляли 1 *мккюри* $Fe^{59}Cl_3$ (специфическая активность 1 *мккюри/мкг*) и 0,01 *мг* пиридоксина. Применение пиридоксина в культуре вполне оправдано, так как доказана способность различных тканей млекопитающих синтезировать фосфопиридоксаль in vitro, если их инкубировать с пиридоксином [3—6]. Все детали, касающиеся особенностей расчета среднего показателя включения (СПВ) и оценки интенсивности мечения клеток Fe^{59} при разных сроках культивации, подробно описаны ранее [2]. Чтобы проследить дальнейшую судьбу железа, захваченного элементами эритропоэза, изучали динамику изменения сидеробластограммы [2]. Всего обследовано 18 больных различными формами железодефицитного малокровия, из них у 5 была хроническая постгеморрагическая анемия, не связанная с эндокринной патологией, 8 страдали эссенциальным железодефицитным малокровием и у 5 диагностирован ювенильный хлороз. При определении действия витамина B_6 на функциональную активность эритроидных клеток все эти лица были объединены в одну группу, так как ранее нами показано, что при упомянутых видах железодефицитных анемий наблюдаются сходная динамика изменения сидеробластограммы и весьма близкие величины поглощения железа клетками в культурах костного мозга [2].

AMERICAN JOURNAL OF PHYSIOLOGY
Vol. 214, No. 1, January 1968. *Printed in U.S.A.*

470

Function, substrate supply, and metabolite content of rabbit heart perfused in situ[1,2]

W. THORN, G. GERCKEN, AND P. HÜRTER[3]
Abteilung für Biochemie, Chemisches Staatsinstitut der Universität Hamburg, Germany

THORN, W., G. GERCKEN, AND P. HÜRTER. *Function, substrate supply, and metabolite content of rabbit heart perfused in situ.* Am. J. Physiol. 214(1): 139–145. 1968.—In order to test the effects of specific substrates on function and metabolite contents of the active myocardium, rabbit hearts were perfused with an artificial blood to which substrates could be added individually. Insufficiency due to lack of substrate was characterized by a 25% reduction of the ATP content, a 75% reduction of the creatine phosphate content, and a 60% reduction in glycogen level. Glucose (even at a concentration of 1 mM) maintained good myocardial function during a 1-hr test period, but a metabolite status resembling that seen in substrate deficiency occurred with the administration of mannose and fructose (5 mM). Among the substrates of mitochondrial metabolism, pyruvate and acetate were utilized well, butyrate and caproate were metabolized less efficiently, and myocardial insufficiency developed when succinate and malate were the substrates.

myocardial insufficiency; substrate deficiency; ATP; creatine phosphate; glycogen; glucose; mannose; fructose; pyruvate; acetate; butyrate; caproate; succinate; malate

THE HEART EXTRACTS carbohydrates and fats from the blood in quantities that depend on their arterial concentrations and on the metabolic condition of the heart. Extraction of substrate by mammalian hearts has been measured by estimation of coronary arteriovenous differences during perfusion with whole blood, which contains a number of substrates (2, 3, 6, 8, 17, 18, 20–22, 24, 33). In order to estimate the utilization of individual substrates without reference to the presence of other substrates, rabbit hearts were perfused with an artificial blood to which substrates could be added singly or omitted altogether. The utilization of a substrate for energy metabolism was judged by its ability to support ventricular pressure-volume work, and by its effect on the metabolite content of the ventricular muscle.

METHODS

Adult rabbits, weighing 2.2–3.0 kg, were anesthetized with butallylonal (Pernocton, 60 mg/kg of body wt) and tracheotomized. After the chest was opened in the sternal line, a mixture of 95% O_2 and 5% CO_2 was given by means of an Engstroem respirator. The pericardium was then opened, and the heart was compressed to a layer thickness of 2.5 mm by means of a forceps previously cooled in liquid air and fixed in liquid air (in vivo control values). In the experimental groups the hearts were perfused by methods described previously (16). The perfusion solution was introduced from a storage vessel at 40 C into the right superior vena cava by means of a Sigma pump T 8. Overdilation of the heart was avoided by opening an overflow placed between the pump and the vessel. Arterial pressure was measured in the left carotid artery, and a tube inserted into the right carotid artery permitted the perfusion solution to flow back into a 300-ml storage vessel. During perfusion, the pressure at the root of the aorta was kept constant at 80 mm Hg by regulation of the resistance in the outflow tube. At the start of the perfusion, the thoracic aorta, right subclavian artery, and both venae cavae were ligated and both vagi were cut. The switchover from natural to artificial cardiopulmonary circulation was carried out without interruption of the flow and without decrease in pressure. Cardiac output was adjusted by the use of a stepless Zero-Max gear. Normal hearts had an output of 134 ml/min. When myocardial insufficiency developed (cardiac dilatation and increased right atrial pressure) the output of the Sigma pump was reduced. The hearts were frozen when there was no further output from the left ventricle, but before the arterial pressure had fallen below 40 mm Hg. Thus, up to the time of freezing, adequate coronary blood flow could be maintained and myocardial hypoxia avoided.

The perfusion solution (pH 7.4, saturated with 95%

Received for publication 27 April 1967.
[1] This work was supported by grants from the Deutsche Forschungsgemeinschaft.
[2] A report was presented to the 29th Congress of the German Physiological Society, Tübingen, 29th September–3rd October, 1964 (15).
[3] Fellow of the Deutsche Forschungsgemeinschaft.

TABLE 1. *Effects of substrate deprivation on metabolite concentrations in the ventricular muscle of rabbit hearts (μmoles/g wet wt)*

	Perfusate	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	CP	P_i	Glyc	Gluc	G-6-P	G-1-P	F-6-P	FDP	DAP	Pyr	Lact	Lact/Pyr
A	Whole Blood, n = 5	4.17	0.794	0.359	5.327	5.5	4.89	4.22	24.1	5.39	0.166	0.016	0.033	0.056	0.006	0.013	1.4	10.9
B	Without Substrate, 10–20 min, n = 2	3.03	0.779	0.349	4.152	4.1	2.42	4.39	8.1	0.26	0.038	0.008		0.064	0.009	0.011	0.43	39.0
C	Without Substrate, 30–35 min, n = 3	3.01	0.872	0.401	4.283	3.5	1.15	5.28	7.3	0.10	0.018	0.010		0.025	0.007	0.005	0.20	54.3

Abbreviations: ATP = adenosine 5′-triphosphate; ADP = adenosine 5′-diphosphate; AMP = adenosine 5′monophosphate, CP = creatine phosphate; P_i = inorganic orthophosphate; Glyc = glycogen; Gluc = glucose; G-6-P = glucose 6-phosphate; G-1-P = glucose 1-phosphate; F-6-P = fructose 6-diphosphate; FDP = fructose 1,6-phosphate; DAP = dihydroxy acetone phosphate, Pyr = pyruvate; Lact = lactate.

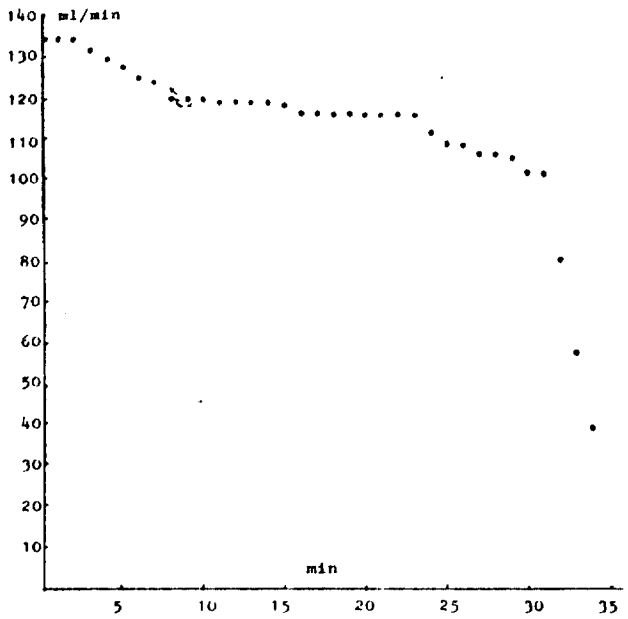


FIG. 1. Flow rate in substrate-free perfusions. Means of *experiments 6, 24,* and *25*; Table 1, line C.

O_2 and 5% CO_2) consisted of seven parts of a phosphate-Ringer solution (NaCl 140.8 mM, KCl 4.37 mM, $CaCl_2$ 5.41 mM, $MgSO_4$ 1.33 mM, Na_2HPO_4 1.58 mM, and NaH_2PO_4 0.42 mM) containing 3% Haemaccel (Behringwerke), a polymer of degradated gelatin, and three parts of washed bovine erythrocytes, prepared by the method of Geiger (13). Haemaccel was added to obtain a normal colloid-osmotic pressure. Diffusible substances were removed from the erythrocytes by washing and dialysis prior to the perfusion experiments. The hearts were perfused with an artificial blood containing 5 mM glucose for 10 min prior to perfusion with other substrates. The following substrates were tested: glucose (1, 5, 10, and 22 mM), mannose (5 mM), fructose (5 mM), pyruvate (5 and 10 mM), acetate (5 mM), butyrate (1 and 2 mM), β-hydroxybutyrate (1 mM), caproate (1 mM), succinate (1 mM), and malate (1 mM). (Substrate concentrations are those of the phosphate-Ringer solution without erythrocytes.) The pH was adjusted after addition of substrate and also measured at the end of the experiment.

To measure metabolite concentrations, ventricular muscle was ground with solid carbon dioxide in a Waring Blendor (Starmix) and homogenized with six volumes of 0.33 M $HClO_4$ in a glass homogenizer. In some experiments the extraction was carried out by pulverizing the tissue with dry ice in a mortar; $HClO_4$ was then introduced directly into the mortar, where it froze immediately. After the extracts were neutralized with 2 N KOH, the concentrations of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), creatine phosphate (CP), inorganic phosphate (P_i), glucose, glucose 6-phosphate (G-6-P), glucose 1-phosphate (G-1-P), fructose, fructose 6-phosphate (F-6-P), fructose 1,6-diphosphate (FDP), dihydroxy acetone phosphate (DAP), pyruvate, and lactate were determined. Glycogen was separately isolated by alkaline tissue digestion and, after precipitation with ethanol and hydrolysis with 1 N HCl, was determined as glucose. Except for the inorganic phosphate content, the metabolite concentrations were determined with the aid of optical enzymatic tests (14, 16, 40, 41). Glucose was analyzed in the coupled test by means of the hexokinase and glucose 6-phosphate dehydrogenase reactions. ATP was measured by the same test to which the creatine kinase reaction was linked in order to determine creatine phosphate. (All concentrations are given as μmoles/g wet weight.)

RESULTS

Table 1 provides a comparison between the control values of metabolite concentrations from blood-perfused hearts (line A) and the metabolite concentrations in hearts with acute insufficiency due to substrate deficiency. Depending on the flow rate involved, the hearts failed to pump blood at a pressure of 80 mm Hg at

different time intervals: after 10–20 min when the input of blood was not reduced (Table 1, line B) and after 30–35 min when the volume per minute was reduced at a rate determined by the decreasing capacity (Fig. 1, Table 1, line C). Hearts with insufficiency contained approximately 1 μmole of ATP/g less than the controls, a decline which amounted to roughly 25% of the initial value. The concentrations of ADP and AMP were only slightly altered, so that the decreased total concentration of free adenine nucleotides paralleled the decline in ATP content. On the other hand, creatine phosphate content was greatly reduced after substrate-free perfusion. A slightly lesser increase of the inorganic phosphate concentration was seen (Table 1).

Myocardial insufficiency due to lack of substrate was accompanied by enhanced consumption of the precursors of carbohydrate metabolism, glycogen, and glucose (Table 1). In addition, the lactate content was reduced during the period of substrate deficiency. Determinations of lactate content in the perfusion solution failed to demonstrate a net efflux of lactate during perfusion with substrate-free solutions. A net efflux of glucose and lactate was seen at the start of the perfusion, but as the experiment progressed, lactate and glucose concentrations in the perfusion solution fell, owing to consumption of these substrates in the heart. With the exhaustion of the carbohydrate supply, the G-6-P level became greatly reduced. The reduction in the concentrations of the glycolytic intermediates which follow G-6-P in the Embden-Meyerhof pathway was less pronounced.

In the presence of glucose alone, the hearts remained compensated (Table 2). A 10-min perfusion with 10 mM glucose led to slight changes in the concentrations of adenine nucleotides, when compared to in vivo controls (Table 1, line A). The ATP and ADP contents were lower by approximately 0.1 μmole/g, so that the ATP/ADP ratio was elevated from 5.5 to 6.2. The creatine phosphate content was markedly increased. Although the concentrations of glycogen, glucose, G-1-P, pyruvate, and lactate agreed closely with control values, those of G-6-P and F-6-P were approximately doubled by the short-term perfusion. There was a decrease in the content of FDP.

After a 60-min perfusion with 22 mM glucose, both cardiac function and metabolite condition were in a favorable steady state (Table 2, line B). The levels of the free adenine nucleotides were scarcely altered from the control values, although the creatine phosphate content was half that seen during the shorter glucose perfusion (Table 2, line A). Whereas the content of glucose had risen to 10.6 μmoles/g, probably reflecting the high concentration in the perfusion solution, no change was found in the glycogen content. The concentration of G-6-P was distinctly reduced, compared with the values of both the in vivo controls and the short glucose perfusion.

During a 120-min perfusion with 5 mM glucose (Table 2, line C) there was no evidence of myocardial insufficiency (normal heart size, normal output of 134 ml/min). However, the glycogen content was reduced by over half and the glucose concentration was one-third that

TABLE 2. *Effects of glucose perfusion on metabolite concentrations in the ventricular muscle of rabbit hearts (μmoles/g wet wt)*

Perfusate	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	CP	P_i	Glyc	Gluc	G-6-P	G-1-P	F-6-P	FDP	DAP	Pyr	Lact	Lact/Pyr
A 10 mM Glucose, 10 min, n = 2	4.04	0.660	0.356	5.051	6.2	7.43	3.08	23.3	4.01	0.373	0.017	0.083	0.019		0.014	1.08	79.1
B 22 mM Glucose, 60 min, n = 3	3.94	0.630	0.378	4.944	7.2	3.58	3.48	24.7	10.61	0.068	0.007		0.053	0.005	0.013	0.77	149.1
C 5 mM Glucose, 120 min, n = 3	3.37	0.744	0.349	4.459	4.5	2.36	3.83	11.5	1.83	0.071	0.010		0.068	0.014	0.008	0.59	95.1
D 1 mM Glucose, 60 min, n = 2	3.64	0.901	0.268	4.799	4.1	2.59	4.74	20.1	0.57	0.037	0.004	0.008	0.045	0.026	0.007	0.30	43.8

For abbreviations, see footnotes, Table 1.

TABLE 3. *Effects of mannose and fructose on metabolite concentrations in the ventricular muscle of rabbit hearts (μmoles/g wet wt)*

Perfusate	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	CP	P_i	Glyc	Gluc	Fruct	G-6-P	G-1-P	FDP	DAP	Pyr	Lact	Lact/Pyr
A 5 mM Mannose, 10 min, n = 2	3.91	0.684	0.251	4.840	5.8	2.53	5.86	15.1	0.35		0.054	0.012	0.030	0.004	0.010	0.30	32.0
B 5 mM Fructose, 12 min, n = 2	3.41	0.612	0.271	4.288	6.6	2.12	5.31	4.9	0.37	1.69	0.072	0.011	0.065	0.006	0.006	0.35	58.7

For abbreviations, see footnotes, Table 1.

of the control hearts. Both ATP and creatine phosphate contents were reduced, as was the ATP/ADP ratio.

To obtain a comparison with the later experiments, in which substrates could not be given at concentrations above 1 mM, we also tested the effects of perfusion with 1 mM glucose (Table 2, line D). The hearts did not fail, pumping the initial volume of 134 ml/min at a pressure of 80 mm Hg until the end of the experiment. However, the metabolite status showed the substrate supply to have been inadequate because both ATP and creatine phosphate contents were lowered. Both glucose and G-6-P contents fell markedly, although this low concentration of glucose prevented a reduction of glycogen content such as was seen following substrate-free perfusion.

Perfusion with mannose (Table 3, line A) and fructose (Table 3, line B) led to severe cardiac insufficiency after 10 and 12 min, respectively, cardiac dilatation, increased right atrial pressure, and failure of the left ventricle to maintain its output at a pressure of 80 mm Hg). Thus, with 5 mM mannose or 5 mM fructose the hearts reacted as they did with lack of substrate. In this short period, the intracellular concentrations of glycogen, glucose, and lactate were greatly reduced. Although the concentration of creatine phosphate fell to half the control value, only slight changes were noted in the concentrations of adenine nucleotides.

The myocardial metabolite concentrations after a 5-min perfusion with 10 mM pyruvate (Table 4, line A) were similar to those after brief perfusion with 10 mM glucose. Glucose concentration was reduced, pyruvate concentration was elevated, and the lactate-to-pyruvate ratio reached the lowest figure (2.1 μmoles/g) seen in these experiments (Table 4, line A). In spite of the low glucose content, G-6-P was markedly elevated. After more prolonged perfusion with pyruvate (5 mM), pyruvate concentration dropped again to extremely low values and glucose content decreased markedly (Table 4,

TABLE 4. *Effects of pyruvate perfusion on metabolite concentrations in the ventricular muscle of rabbit hearts (μmoles/g wet wt)*

Perfusate	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	CP	Pi	Glyc	Gluc	G-6-P	G-1-P	F-6-P	FDP	DAP	Pyr	Lact	Lact/Pyr
A 10 mM Pyruvate, 5 min, n = 2	4.11	0.699	0.210	5.019	5.9	6.54	3.92	22.6	1.29	0.247	0.019	0.041	0.025	0.002	0.473	0.81	2.1
B 5 mM Pyruvate, 30 min, n = 1	3.95	0.783	0.321	5.054	5.0	2.52	5.50	18.9	0.12	0.063	0.022		0.046	0.018	0.003	0.3	117.[illegible]
C 5 mM Pyruvate, 60 min, n = 2	3.22	0.591	0.249	4.060	5.7	1.57	4.75	18.9	0.05	0.028	0.010		0.023	0.017	0.002	0.1	62.[illegible]

For abbreviations, see footnotes, Table 1.

TABLE 5. *Effects of acetate, butyrate, β-hydroxybutyrate, and caproate on metabolite concentrations in the ventricular muscle of rabbit hearts (μmoles/g wet wt)*

Perfusate	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	CP	Pi	Glyc	Gluc	G-6-P	G-1-P	F-6-P	FDP	DAP	Pyr	Lact	Lact/Pyr
A 5 mM Acetate, 30 min, n = 1	3.60	0.481	0.241	4.322	7.5	4.56	5.53	26.2	0.23	0.165		0.021	0.001	0.005	0.005	0.82	164.[illegible]
B 5 mM Acetate, 60 min, n = 1	3.82	0.564	0.259	4.643	6.8	3.39	4.21	18.7	0.13	0.045		0.046	0.006	0.005	0.008	0.59	73.8
C 1 mM Butyrate, 30 min, n = 1	3.07	0.666	0.251	3.987	4.6	1.13	4.45	10.6	0.24	0.036	0.012		0.021	0.011	0	0.29	
D 2 mM Butyrate, 60 min, n = 1	3.65	0.746	0.290	4.686	4.9	1.40	5.31	10.8	0.08	0.022	0.007		0.019	0.012	0.002	0.12	60.0
E 1 mM β-Hydroxybutyrate, 30 min, n = 2	3.85	0.573	0.277	4.695	6.8	5.69	4.69	27.3	0.22	0.094	0	0.015	0.005	0.003	0.007	0.68	97.2
F 1 mM Caproate, 30 min, n = 2	4.20	0.562	0.192	4.949	7.5	5.56	4.00	16.7	0.17	0.168	0.006	0.040	0.012	0.006	0.006	0.53	90.8
G 1 mM Caproate, 60 min, n = 2	3.25	0.796	0.244	4.290	4.1	2.96	4.64	14.2	0.08	0.024	0.002	0.007	0.023	0.008	0.004	0.18	37.5

For abbreviations, see footnotes, Table 1.

TABLE 6. *Effects of succinate and malate on metabolite concentrations in the ventricular muscle of rabbit hearts (μmoles/g wet wt)*

Perfusate	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	CP	P_i	Glyc	Gluc	G-6-P	G-1-P	FDP	DAP	Pyr	Lact	Lact/Pyr
A 1 mM Succinate, 17 min, n = 1	2.94	0.935	0.396	4.271	3.1	1.00	4.76	20.8	0.10	0.083	0.025	0.012	0.040	0.004	0.80	200
B 1 mM Succinate, 34 min, n = 1	3.73	1.023	0.286	5.039	3.6	1.22	5.16	11.4	0.37	0.032	0.014	0.023	0.016	0.002	0.23	115
C 1 mM Malate, 58 min, n = 1	2.78	0.955	0.323	4.058	2.9	0.76	5.44	7.8	0.11	0.014	0.006	0.022	0.010	0.003	0.14	46.7

For abbreviations, see footnotes, Table 1.

lines B and C). The glycogen supply was not significantly reduced after 1-hr perfusion with pyruvate. Cardiac insufficiency did not develop during these studies.

Like perfusion with glucose and pyruvate, perfusion with 5 mM acetate maintained the hearts in a good functional state. Glycogen concentration was normal after 30 min and fell only slightly after 60 min (Table 5, lines A and B). When 1 mM sodium butyrate was offered (Table 5, line C), a 50% reduction of glycogen content was noted after 30 min. Similar findings were obtained after a 60-min perfusion with 2 mM sodium butyrate (Table 5, line D). In both cases the hearts functioned adequately, although the creatine phosphate content was greatly diminished (1.1 μmoles/g and 1.40 μmoles/g). In contrast, 30-min perfusion with 1 mM sodium 3-hydroxybutyrate led to elevation of both creatine phosphate concentration (5.69 μmoles/g) and glycogen content (27.3 μmoles glucosyl residues/g, Table 5, line E). Good cardiac function was maintained under these conditions. Caproic acid (1 mM) did not sustain cardiac function as efficiently as did glucose, pyruvate, or acetate. After 30 min perfusion with caproic acid creatine phosphate remained high although the glycogen content had decreased (Table 5, line F). After 1 hr the glycogen content was 14.2 μmoles/g (glucosyl residues) (Table 5, line G), and incipient insufficiency was noted (98 ml/min left ventricular output at a pressure of 70 mm Hg).

Perfusion with either succinate or malate led to cardiac insufficiency associated with extremely low creatine phosphate contents (Table 6). After 34 min of perfusion with succinate, the glycogen content was reduced by half (Table 6, line B). After 58 min of perfusion with malate, the decreases in levels of ATP, creatine phosphate, and glycogen became even more pronounced (Table 6, line C).

DISCUSSION

It is known from measurement of arteriovenous substrate differences that the heart can take up a variety of substrates. The rates of substrate utilization have been found to depend on both the metabolic condition of the heart and the arterial substrate concentration (2–6, 17, 20, 21, 24, 28). When mixtures of substrates are used, however, the presence of one substrate will affect the utilization of others. The question posed in the present study is to what extent a single substrate can replace all others. Because of the large number of substrates in the plasma, this question can not be investigated in vivo. Furthermore, estimations of the arteriovenous differences in vivo provide no data on the intracellular fate of absorbed substrates, i.e., whether they are catabolized directly or first transferred into storage substances.

The present experiments in which no exogenous substrates were supplied demonstrate the extent to which endogenous substrates can be mobilized to maintain cardiac function. Under these conditions, cardiac insufficiency developed after 30–40 min, at which time the glycogen level was markedly reduced. On the other hand, cardiac insufficiency associated with exhaustion of glycogen stores occurs after only 3–5 min of anoxia (38–40). Under conditions of anoxia, only 3 moles of high-energy phosphate (ATP) are obtained from the breakdown to lactate of one mole of glucosyl residues. Because adequate oxygenation was maintained in the present experiments, glycogen could be converted completely to CO_2 and H_2O, and glucose and lactate, available in the heart at the start of the perfusion, were also available for oxidation. The ratio of the yield of energy-rich phosphate bonds from anaerobic glycogenolysis to that from glycogen oxidation (3/39) is similar to the observed ratio of time to anaerobic or aerobic failure, suggesting that, even under aerobic conditions, other endogenous (noncarbohydrate) substances (11, 34, 35) do not contribute significantly to energy production. In contrast to earlier reports (5, 6), amino acids now appear to have no significance for oxidative energy production (22, 34). These findings preclude the possibility that amino acids, which might be liberated from the Haemaccel used in the experiments contribute to the production of energy.

The finding of myocardial insufficiency with only a slight decrease in ATP content suggests that the adenine nucleotides may be present in several functional compartments. Cardiac insufficiency begins when the ATP associated with the contractile proteins becomes reduced. This seems to be the only ATP fraction which reacts with creatine kinase as indicated by the shifted equilibrium

of the creatine kinase reaction. Although glucose normally contributes only 18% (2) or 31% (20) to the total substrate oxidation in human hearts in vivo, cardiac function can be maintained when glucose is the only substrate. Even at a concentration of 1 mM, glucose can prevent the effects seen with substrate deficiency, i.e., there was neither myocardial insufficiency nor major loss of glycogen for a period of 1 hr. Although the glycogen content was normal after 1-hr perfusion with 22 mM glucose, approximately half the glycogen was lost after 2-hr perfusion with 5 mM glucose. The metabolite contents at the high glucose concentration suggest that the rates of glucose penetration and phosphorylation, as well as the capacity of the Embden-Meyerhof pathway, are adequate to provide for the total consumption of energy. Glucose not only is broken down to lactate and CO_2 or condensed to glycogen, but glucose carbon atoms can appear in glutamate, glutamine, alanine, and aspartate (10). The reduction of G-6-P concentration during perfusion with a high glucose concentration was unexpected (Table 2, line B). This finding suggests that steps prior to G-6-P are rate limiting in the utilization of glucose alone. After the more prolonged perfusions with glucose, creatine phosphate content was reduced although cardiac insufficiency did not occur. It should be remembered, however, that creatine can diffuse into the perfusion solution, thereby leading to an intracellular decrease in the creatine available for phosphorylation (26).

Judging from the early onset of insufficiency after perfusion with fructose or mannose, the heart does not utilize either substrate. Failure to utilize fructose is in accord with earlier studies on myocardial slices (1). Therefore the arteriovenous differences of fructose obtained in the experiments of Danforth et al. (8) may be due only to diffusion. During fructose perfusion, the content of fructose became quite high, 1.69 μmoles/g tissue. This is within the same range as the glucose content in the myocardium perfused with 5 mM glucose (Table 2, line C), so that the failure to utilize fructose is probably due not to blockage of permeability, but to inadequate phosphorylation by hexokinase (42). That the hexokinase reaction is not the only initiating reaction for glucose metabolism has been indicated by recent findings which suggest that G-6-P is not necessarily an intermediate in glycogen synthesis from glucose (37, 41). If glucose is specifically condensed to glycogen via glucose 1-phosphate and uridine diphosphate glucose, the differentiation between glucose and fructose would be greater than that resulting from a less highly specific hexokinase. In this context, Opie et al. (29) found that rat hearts absorb and oxidize much less fructose U-^{14}C than glucose, and Hers (19) has demonstrated on the diaphragm that fructose is not an immediate precursor of glycogen.

Pyruvate and acetate, which are good respiratory substrates for mitochondria (7, 9, 23, 36), like glucose are capable of maintaining myocardial function. The activities of the reactions initiating the mitochondrial breakdown of both substrates (12, 25) (by pyruvate dehydrogenase and acetyl-CoA-synthetase) are adequate to supply substrate for the oxidative production of energy in the heart. Because the glycogen content was only slightly changed after perfusion with pyruvate and acetate, the question arises whether glycogenolysis was inhibited by the mitochondrial metabolism, or whether there was a resynthesis of the glycogen. The increase of the G-6-P concentration in the short-term experiments with pyruvate (Table 4, line A in comparison to Table 1, line A) suggests an inhibition of phosphofructokinase (11, 27, 31, 32) or of glucose phosphate isomerase (30). Later (after 30 and 60 min), the content of G-6-P was greatly reduced and the DAP concentration was increased. Williamson and Jones (44) observed similar time-dependent concentration changes which they interpreted to be the result of an inhibition of pyruvate kinase by high pyruvate concentration. They suggest that, under the special conditions of a high pyruvate concentration, phosphofructokinase is not the major rate-limiting enzyme of the glycolytic pathway.

Butyrate and caproate are less readily utilized than acetate and pyruvate by the intact heart. In isolated mitochondria, on the other hand, Bode and Klingenberg (7) noted an increase in oxidation of both free fatty acids and of fatty acid carnitinic esters as chain length increased up to a length of C_{12}. β-Hydroxybutyrate, which has an in vivo O_2 extraction quotient of only 5–6% (20, 33) produced a favorable metabolite status and cardiac function in the perfusion experiments.

The heart was not maintained in a steady state during perfusion with the metabolites of the citric acid cycle, succinate and malate. Even in isolated mitochondria, succinate has been found to be an inadequate substrate, probably because of the low permeability of the mitochondria for succinate (23).

The ability of the well-oxygenated myocardium to derive energy from various substrates has thus been demonstrated to be highly selective. Glucose, pyruvate, acetate, and β-hydroxybutyrate are well utilized, whereas butyrate and caproate are poorly utilized. Mannose, fructose, succinate, and malate appear to be poorly utilized and are unable to provide for maintained cardiac function.

REFERENCES

1. Berg, W. von. Die Atmung von Herzmuskelschnitten der Katze in Abhängigkeit vom pH und vom Glucose- oder Fructosezusatz. *Arch. Ges. Physiol.* 274: 480–488, 1962.
2. Bernsmeier, A., and W. Rudolph. Neue Ergebnisse über Durchblutung und Substratversorgung des menschlichen Herzens. *Muench. Med. Wochschr.* 104: 46–52 and 75–77, 1962.
3. Bernsmeier, A., and W. Rudolph. Die Fettsäuren im Stoffwechsel des Herzmuskels. *Deut. Med. Wochschr.* 90: 745–749, 1965.
4. Bing, R. J. Der Myocardstoffwechsel. *Klin. Wochschr.* 34: 1–7, 1956.

5. Bing, R. J. Metabolic activity of the intact heart. *Am. J. Med.* 30: 679–691, 1961.
6. Bing, R. J., A. Siegel, I. Ungar, and M. Gilbert. Metabolism of the human heart. II. Studies on fat, ketone and amino acid metabolism. *Am. J. Med.* 16: 504–515, 1954.
7. Bode, C., and M. Klingenberg. Die Veratmung von Fettsäuren in isolierten Mitochondrien. *Biochem. Z.* 341: 271–299, 1965.
8. Danforth, W. H., C. E. Hogangamp, F. B. Ballard, and R. J. Bing. The myocardial metabolism of fructose. *Am. J. Med. Sci.* 238: 477–484, 1959.
9. Davis, E. J. On the oxidation of acetate and pyruvate by guinea-pig heart sarcosomes. *Biochim. Biophys. Acta* 96: 217–230, 1965.
10. Davis, E. J., and J. H. Quastel. The effects of short-chain fatty acids and starvation on the metabolism of glucose and lactate by the perfused guinea pig heart. *Can. J. Biochem.* 42: 1605–1621, 1964.
11. Evans, J. R. Importance of fatty acid in myocardial metabolism. *Circulation Res.* 14 and 15, (Suppl. 2): 96–106, 1964.
12. Evans, J. R., L. H. Opie, and A. E. Renold. Pyruvate metabolism in the perfused rat heart. *Am. J. Physiol.* 205: 971–976, 1963.
13. Geiger, A. Technic of brain perfusion in situ. In: *Methods in Medical Research*, edited by J. H. Quastel. Chicago: Year Book, 1951, vol. 9, p. 248–254.
14. Gercken, G. Stoffwechsel und Funktion des Gehirns und des Herzens in Abhängigkeit von der Durchblutung und Substratversorgung. (Inaugral Dissertation.) Hamburg: *Habilitationsschrift*, 1965.
15. Gercken, G., and P. Hürter. Funktion und Stoffwechsel des Herzens bei Perfusion mit verschiedenen Substraten. *Arch. Ges. Physiol.* 281: 35, 1964.
16. Gercken, G., and P. Hürter. Stationäre Metabolitkonzentrationen im insuffizienten Säugetierherzen nach Monojodacetat- und Natriumfluoridvergiftung. *Arch. Ges. Physiol.* 292: 100–117, 1966.
17. Goodale, W. T., and D. B. Hackel. Myocardial carbohydrate metabolism in normal dogs, with effects of hyperglycemia and starvation. *Circulation Res.* 1: 509–517, 1953.
18. Heck, P., and H. Hochrein. Über den Glucose und Lactatverbrauch des isolierten Warmblüterherzens. *Arch. Ges. Physiol.* 289: R 52, 1966.
19. Hers, H. G. The conversion of fructose-1-C^{14} and sorbitol-1-C^{14} to liver and muscle glycogen in the rat. *J. Biol. Chem.* 214: 373–381, 1955.
20. Keul, J., E. Doll, H. Steim, U. Fleer, and H. Reindell. Über den Stoffwechsel des menschlichen Herzens. III. *Arch. Ges. Physiol.* 282: 43–53, 1965.
21. Keul, J., E. Doll, H. Steim, H. Homburger, H. Kern, and H. Reindell. Über den Stoffwechsel des menschlichen Herzens. I. *Arch. Ges. Physiol.* 282: 1–27, 1965.
22. Keul, J., E. Doll, H. Steim, U. Singer, and H. Reindell. Über den Stoffwechsel des Herzens bei Hochleistungssportlern. *Klin. Wochschr.* 44: 881–887, 1966.
23. Korff, von R. W. Metabolic characteristics of isolated rabbit heart mitochondria. *J. Biol. Chem.* 240: 1351–1358, 1965.
24. Lochner, W. Substratumsatz, Sauerstoffverbrauch und anaerober Energiegewinn des Herzens. *Z. Kreislaufforsch.* 54: 103–113, 1965.
25. Lorber, V., N. Lifson, H. G. Wood, and J. Barcroft. The metabolism of acetate by the completely isolated mammalian heart investigated with carboxyl-labeled acetate. *Am. J. Physiol.* 145: 557–560, 1946.
26. Menne, F., S. Carnier, and M. R. Soliman. Kreatinverluste und Kreatinaufnahme durch isolierte Herzen bei Durchströmung mit kreatinhaltigen Lösungen. *Klin. Wochschr.* 41: 234–239, 1963.
27. Newsholm, E. A., P. J. Randle, and K. L. Manchester. Inhibition of the phosphofructokinase reaction in perfused rat heart by respiration of ketone bodies, fatty acids, and pyruvate. *Nature* 193: 270–271, 1962.
28. Olson, R. E. Effect of pyruvate and acetoacetate on the metabolism of fatty acids by the perfused rat heart. *Nature* 195: 597–599, 1962.
29. Opie, L. H., J. C. Shipp, J. R. Evans, and B. Leboeuf. Metabolism of glucose-U-C^{14} in perfused rat heart. *Am. J. Physiol.* 203: 839–843, 1962.
30. Pedersen, P. L., and J. Sacks. Hexosephosphate formation and the regulation of glycolysis in muscle. *Arch. Biochem. Biophys.* 112: 548–553, 1965.
31. Randle, P. J., and C. I. Pogson. Phosphofructokinase and hexokinase in rat-heart muscle. *Biochem. J.* 97: 5, 1965.
32. Regen, D. M., D. A. B. Young, W. W. Davis, J. Jack, Jr., and C. R. Park. Adjustment of glycolysis to energy utilization in the perfused rat heart. *J. Biol. Chem.* 239: 381–384, 1964.
33. Rudolph, W., D. Maas, J. Richter, F. Hasinger, H. Hofmann, and P. Dohrn. Über die Bedeutung von Acetacetat und β-Hydroxybutyrat im Stoffwechsel des menschlichen Herzens. *Klin. Wochschr.* 43: 445–451, 1965.
34. Shipp, J. C., O. E. Matos, H. Knitzley, and L. E. Crevasse. CO_2 formed from endogenous and exogenous substrates in perfused rat heart. *Am. J. Physiol.* 207: 1231–1236, 1964.
35. Shipp, J. C., J. M. Thomas, and L. Crevasse. Oxidation o carbon-14-labeled endogenous lipids by isolated perfused rat heart. *Science* 143: 371–373, 1964.
36. Slater, E. C., C. Tamblyn-Hague, and W. Davis-Van Thienen. The oxidation of pyruvate by isolated heart sarcosomes. *Biochim. Biophys. Acta* 96: 206–216, 1965.
37. Smith, E. E., P. M. Taylor, and W. J. Whelan. Hypothesis on the mode of conversion of glucose into α-glucose-1-phosphate. *Nature* 213: 733–734, 1967.
38. Thorn, W. Metabolitkonzentrationen im Herzmuskel unter normalen, hypoxischen und anoxischen Bedingungen. *Verh. Deut. Ges. Kreislaufforsch.* 27: 76–90 1961.
39. Thorn, W., J. Heimann, B. Müldener, W. Isselhard, and G. Gercken. Herzstoffwechsel in Abhängigkeit von Versuchsanordnung, Gewebsgewinnung und anoxischer Belastung. *Arch. Ges. Physiol.* 269: 214–231, 1959.
40. Thorn, W., W. Isselhard, and B. Müldener. Glycogen-, Glucose- und Milchsäuregehalt in Warmblüterorganen bei unterschiedlicher Versuchsanordnung und anoxischer Belastung mit Hilfe optischer Fermentteste ermittelt. *Biochem. Z.* 331: 545–562, 1959.
41. Thorn, W., G. Pfleiderer, R. A. Frowein, and I. Ross. Stoffwechselvorgänge im Gehirn bei akuter Anoxie, akuter Ischämie und in der Erholung. *Arch. Ges. Physiol.* 261: 334–360, 1955.
42. Threlfall, C. J. Role of glucose-6-phosphate in the synthesis of glycogen by the rat liver in vivo. *Nature* 211: 1192, 1966.
43. Walker, D. G. The nature and function of hexokinases in animal tissues. *Essays in Biochemistry*, edited by P. N. Campbell and G. D. Greville. London, New York: Academic, 1966, vol. 2, p. 33–67.
44. Williamson, J. R., and E. A. Jones. Inhibition of glycolysis by pyruvate in relation to the accumulation of citric acid cycle intermediates in the perfused rat heart. *Nature* 203: 1171–1173, 1964.

501

Brit. J. Pharmacol. (1950), 5, 87.

THE ACTIONS OF METABOLIC SUBSTRATES AND INHIBITORS ON THE RABBIT AURICLE*

BY

J. LEYDEN WEBB

From the Department of Pharmacology, Oxford

(Received December 7, 1949)

Knowledge of cardiac muscle metabolism has advanced during the past few years to an appreciable extent, but the correlation of metabolic processes with the various functions of the heart has been very little investigated. It is possible that some of the enzyme systems that are highly active in cardiac extracts or homogenates, or even in heart slices, are not intimately involved in the beating of the heart. It is possible that in the intact beating heart there are enzyme systems of great importance, which are either destroyed or altered by any damage to the myocardium. Such systems may be brought to light by a more detailed analysis of the actions of metabolic substrates and inhibitors on the normally contracting cardiac tissue. For the understanding of drug actions on the heart, it is frequently necessary to correlate the metabolism with the functional aspects of impulse discharge, conduction, and contraction. The isolated rabbit auricle was used in the present work because both the amplitude of contraction and the rate of impulse discharge could be more accurately measured with it than with the entire heart where there occur problems of conduction block and complex contractile movement. It is also difficult to maintain normal conditions in the perfused heart, and the tissue soon becomes damaged, whereas auricles will beat regularly for many hours in the proper medium. This work was done in connexion with the problem of the action of acetylcholine on the heart and of how this action is influenced by the metabolic state of the myocardium. The results obtained with acetylcholine will be reported in a subsequent paper.

METHODS

The effects of metabolically important substances on the rabbit auricle were determined by addition of the substances to a bath in which the auricles were beating. Hearts from young rabbits were used, inasmuch as it was found that such preparations beat more regularly and give more reproducible results than those from older rabbits. It is possible that in older animals the auricular walls are so thick that diffusion of oxygen into the tissue, or diffusion of metabolic products from the tissue, is not adequate. It also seemed that better results were obtained when the rabbits were allowed to remain quiet for several minutes before they were killed. Auricles obtained from animals that had been killed immediately after being caught and handled often gave poor results

*This investigation was carried out during a Fellowship granted by the Life Insurance Medical Research Fund, New York, U.S.A.

and would seldom achieve a regular beat. The rabbits were killed by a blow on the head and the hearts were rapidly removed. The hearts were allowed to beat for about half a minute in a dish of physiological medium in order to allow as much blood as possible to be pumped out of the tissues. The ventricles were often massaged in order to rinse the blood from the auricles, care being taken not to touch the auricles. The auricles were then cut from the heart and carefully trimmed so that no ventricular tissue remained. Caution was exercised in the trimming of the arteries and veins that no damage be done to the adjacent regions of the auricle, for it is in these regions that the pacemaker tissue usually lies. The auricles were suspended in 70 ml. of Tyrode solution (0.137 M-NaCl, 0.0027 M-KCl, 0.0018 M-$CaCl_2$, 0.00011 M-$MgCl_2$, 0.012 M-$NaHCO_3$, 0.00042 M-NaH_2PO_4, and 0.0055 M-glucose) which, after saturation with the gas mixture, had a *p*H of 8.1. Substances to be tested were added in 0.7 ml. volumes at a concentration 100 times that required in the final medium. Although this medium is a good buffer, all solutions of these substances were brought to a *p*H close to 8.1 before their addition to the bath. All measurements were made at 30° C. It was found that better results could be obtained at this temperature than at 38° C. At the higher temperature it is likely that the rapid rate often limits the recording of the full amplitude and this would introduce serious errors into the determinations of amplitude effects. The auricles also beat regularly over a much longer period of time at the lower temperature. The medium was continually saturated with a mixture of 5 per cent CO_2 and 95 per cent O_2. The substances used were obtained in as pure a form as possible. The sodium fluoroacetate was kindly supplied by Dr. Saunders, of Cambridge University.

In order to achieve quantitative results the auricular tracings were carefully measured, the amplitude being recorded in millimetres and the rate of beating in contractions per ten seconds. It was necessary to use this small time unit for measuring the rate inasmuch as rapid rate changes could not have been accurately determined if longer intervals had been chosen. The effects produced by the addition of any substance were expressed in terms of the percentage change in the amplitude and rate. Such results might conceivably be expressed as absolute changes, percentage changes, or as final levels of activity to which the auricles were brought by the substances. For most purposes the percentage change is the most significant. In the first place, owing to the recording arrangement, the true amplitude of contraction is not measured; hence, if absolute changes were given, it would not be possible to compare one experiment with another. Furthermore, in considering the effects of substances on the auricular frequency we are usually interested in the changes in the rates of processes occurring within the pacemaker cells. It is likely that the rates of such processes are roughly proportional to the frequencies of beating. In metabolic studies the effect of an inhibitor on an enzymatic system is usually expressed in terms of percentage inhibition, and so here we might expect a more direct relationship to the underlying processes if we also express the frequency changes in similar terms. Justification for this method of expression with regard to both rate and amplitude, was found in the quantitatively comparable results obtained from auricles beating at greatly different rates and amplitudes.

The auricles usually beat somewhat irregularly for 5–30 minutes after placing them in the bath, but eventually became quite constant with respect to both rate and amplitude. Experimental work was always initiated after the auricles had reached this steady state. Such auricles would beat for two to three days, the amplitude gradually decreasing during the second and third days. No auricles were considered to be normal after the first day and all results reported were obtained on fresh auricles. The recorded amplitude was usually adjusted to between 40–60 mm. Normal auricles beat spontaneously at an average rate of 102 contractions per minute, although there was quite a large variation between the auricles from different animals in this respect, the extremes recorded being 72 and 144. At 38° C. the frequency is approximately 80 per cent

gi... ban at 30° C., and thus the rate at body temperature would have been approxi-
m... 84 beats per minute, a figure not far distant from the normal *in situ* rate of the
ra... art.

RESULTS

The effects of metabolic substrates on normal auricles

obvious that if a substance is to alter the beating of the auricles by acting
th... metabolic mechanisms, its quantitative effect will depend on the metabolic
stat... the myocardial cells. If a particular substrate or intermediate, which is
of ... tance in the functional metabolism of the heart, is deficient, we might
expe... is addition to produce an effect. Whether this would be stimulation or
dep... on cannot be predicted until the correlation between the various metabolic
rea... and the functioning of the heart cells is better understood. On the other
han... an enzyme system is saturated with respect to its substrate (that is, if the
sub... is not limiting the rate of the reaction), there may be no response to the
addi... of the substrate, or there may perhaps occur a retardation of the enzyme
syst... hat operate before the particular step in which the substrate is involved.
Fu... ore, the observed actions of substrates on the auricular tissue may be
ind... nt of enzymic mechanisms, the primary effect being upon the cellular
me... potential or the contractile substance itself. Lastly, the possible actions
of ... ubstances on the acetylcholine equilibrium in the cardiac tissue must be
bor... mind. Since there is a slow formation of acetylcholine in the auricles
teiti... hin the heart cells themselves or released from the post-ganglionic nerve
end... it is possible that a substance may stimulate or depress this formation,
or ... e rate at which this endogenous acetylcholine is destroyed by modifying
the ... y of the cholinesterase, or antagonize the action of the acetylcholine on
the ... lar cells.

T... esults obtained on the actions of substrates on normal auricles are
sum... ed in Table I. Changes of less than 5 per cent are probably not significant
unless derived from experiments upon several auricles. An inspection of this Table
will show that stimulation of either rate or amplitude is very seldom seen with any
of the substrates investigated. It is likely that the only really significant stimulation
is that produced by citrate on the rate. These substances, in general, have no effect
on the normal auricle until a certain concentration is reached and then they begin
progressively to depress it. This is seen in the concentration-action curves in Fig. 1.
On the whole, it is probable that the enzymatic systems involved in providing energy
for the beating of the auricles are not limited by any of the substrates tested.

The amplitude is more readily depressed by these substances than the rate.
On the average, for any specific concentration of substrate, the depression of the
amplitude is two to three times as great as the depression of the rate. However,
substrates vary in this respect and it will be seen that acetate at lower concentrations
depresses the rate as strongly as it does the amplitude. The maximum effect, in
most instances, is reached from one to two minutes after the addition of the sub-
strate to the bath. There is then, with the majority of substrates used, a partial
or complete spontaneous recovery from this depression. This was observed with
pyruvate, lactate, fumarate, malate, acetate, oxalacetate, propionate, and butyrate;
it was not seen with citrate or caprylate, the depression produced by these being

TABLE I

THE EFFECTS OF METABOLIC SUBSTRATES ON NORMAL AURICLES

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Succinate	0.0001	1	0	0	1	1
		5	0	+2	1	1
	0.001	1	0	0	1	1
		5	0	+3	1	1
	0.005	0.5	0	+7	1	1
		1	0	+9	2	5
		2	0	+12	2	4
	0.01	0.5	+18	+22	3	3
		1	13	+26	4	4
		2	+10	+27	3	3
Fumarate	0.001	0.5	+6	+4	1	1
		1	+6	+5	1	1
		2	+3	0	1	1
	0.005	0.5	3	+5	2	2
		1	−3	+7	3	3
		2	0	+6	2	2
	0.01	0.5	+9	+8	2	3
		1	+5	+14	3	4
		2	−2	+10	3	3
Malate	0.001	0.5	+3	0	2	2
		1	+3	0	2	2
		2	−6	−1	1	1
	0.005	0.5	+2	+10	3	6
		1	−4	−14		5
		2	0	+15	3	3
	0.01	0.5	+4	+9	2	3
		1	−4	−18	2	3
		2	−4	−20	2	2
		5	0	−15	2	2
		10	0	+8	2	2
		15	0	+5	1	1
	0.02	0.5	−7	+23	1	1
		1	−14	+32	1	1
		2	−7	−27	1	1
		5	0	+22	1	1
		8	0	−12	1	1
		15	0	−8	1	1
Oxalacetate ..	0.002	0.5	0	−2	1	1
		1	0	−2	2	1
		2	0	0	1	1
	0.01	0.5	0	−9	1	1
		1	0	+14	1	1
		2	0	−13	1	1
		3	0	−11	1	1

TABLE 1—*contd.*

	Rate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Pent.	..	0.0001	1	0	0	2	2
			2	0	−2	2	2
		0.001	0.5	0	−2	2	2
			1	0	−5	2	2
			2	0	−6	2	2
			5	0	0	2	2
			10	0	+3	2	2
		0.005	0.5	−1	−3	5	10
			1	−1	−6	7	13
			2	−1	−11	5	10
			3	−2	−9	3	5
			5	−2	−8	4	6
			10	−4	−5	3	3
			15	0	−3	2	2
		0.01	0.5	0	−9	2	2
			1	0	−12	4	4
			2	0	−16	5	5
			3	0	−7	2	2
			15	+5	−6	1	1
		0.02	0.5	0	−8	1	1
			1	−8	−3	1	1
			2	0	−23	1	1
Acetan.	..	0.00002	1	0	0	1	1
			5	0	0	1	1
		0.0001	1	−6	−8	1	1
			2	0	−4	1	1
		0.0005	0.5	9	4	2	3
			1	22	−15	2	3
			2	−17	−10	2	3
		0.001	0.5	5	9	4	5
			1	19	21	3	4
			2	−11	−9	3	3
			5	−3	2	2	3
		0.01	0.5	4	−8	2	2
			1	6	−23	3	4
			2	−4	−16	2	3
			5	0	−7	2	3
			10	+6	−3	2	2
			15	+6	−2	2	2
		0.03	1	6	27	2	2
			2	−4	22	2	2
			5	0	−18	1	1
			10	+6	11	1	1
			15	+6	15	1	1

TABLE 1—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Propionate ..	0.0005	0.5	0	1	2	2
		1	0	−4	2	2
		2	0	−3	2	2
		5	+3	−8	2	4
		10	+7	−3	2	3
	0.005	0.5	−7	10	1	2
		1	−5	−7	1	2
		2	0	−3	1	2
		5	+5	−1	1	2
	0.01	0.5	−7	−4	1	1
		1	−7	−6	1	1
		3	0	−3	1	1
Butyrate	0.0005	1	0	−1	1	1
		5	0	0	1	1
	0.001	1	0	−10	1	2
		2	−3	−8	1	2
	0.005	1	0	−16	1	2
		2	−6	−11	1	2
		5	−3	−8	1	2
	0.01	0.5	0	−7	1	1
		2	0	−6	1	1
3-Hydroxy-butyrate	0.001	0.5	0	−4	1	1
		1	0	−6	1	1
		3	0	−10	1	1
	0.005	1	−7	−11	1	
		3	0	−13	1	
Caprylate	0.00002	0.5	0	0	1	
		1	0	0	1	
		3	0	−4	1	
		5	0	−9	1	
	0.0001	0.5	0	0	1	
		1	0	−5	1	
		3	−6	−16	1	
		5	−6	−26	1	
	0.0005	0.5	−6	0	1	
		1	−17	−11	1	
		5	−6	−24	1	
	0.001	1	−7	−15	1	
		2	−14	−25	1	
	0.01	1	−22	−33	1	
		2	−22	−42	1	

TABLE I—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Glu[illegible]	0.001	1	0	0	1	1
		2	0	0	1	1
	0.005	1	0	0	1	1
		2	0	0	1	1
	0.01	1	0	−3	2	3
		2	0	−1	2	3
	0.03	1	0	−7	1	1
		2	0	−5	1	1
Lact[illegible]	0.001	1	0	−2	1	1
		2	0	−1	1	1
	0.005	1	0	−1	2	2
		2	0	−1	2	2
	0.01	1	0	−6	2	2
		2	0	−2	2	2
		5	0	0	2	2
	0.03	1	0	−9	1	1
		2	0	−3	1	1
		5	0	−2	1	1
Cit[illegible]	0.0001	1	0	0	1	1
		2	0	0	1	1
	0.001	0.5	0	−5	2	3
		1	−2	−11	2	3
		2	−2	−12	2	3
	0.005	0.5	0	−28	2	3
		1	+3	−47	2	3
		2	+6	−60	2	3
		4	+2	−62	2	3
	0.01	0.5	0	−35	2	2
		1	+16	−63	2	2
		2	+25	−74	2	2
		5	+28	−75	2	2
		10	+25	−74	2	2
		15	+20	−72	2	2

irreversible until the substance was washed from the bath. Typical curves illustrating this spontaneous recovery are given in Fig. 2. This recovery is not due to destruction of the substrate, for in most instances the total amount of substrate present in the bath is sufficient to prevent any appreciable decrease in the concentration resulting from amounts destroyed by the tissue in the short time intervals involved. This type of spontaneous recovery is seen in cardiac tissue under the influence of a variety of substances, and is probably to be interpreted in the light of the ability of the cardiac functional systems to adjust themselves to abnormal conditions.

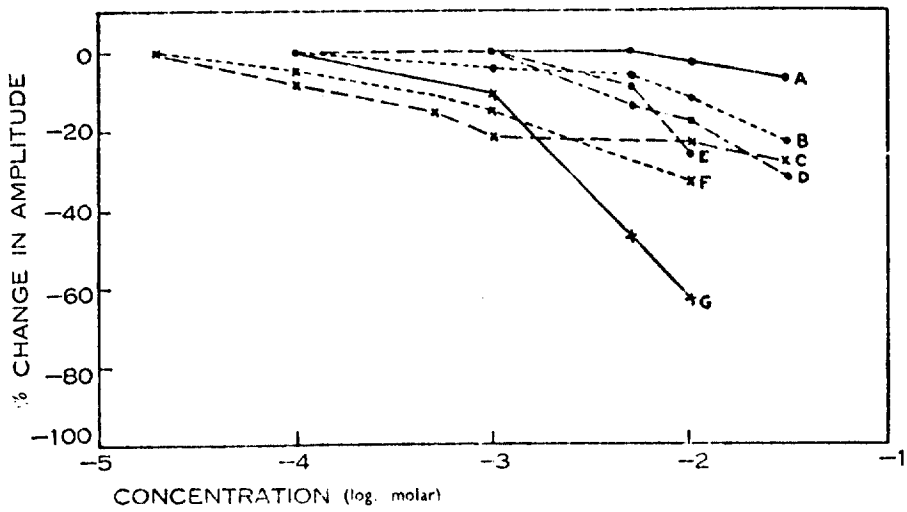


FIG. 1.—The action of substrates on the normal rabbit auricle. (A) glutamate, (B) pyruvate, (C) acetate, (D) malate, (E) succinate, (F) caprylate, (G) citrate. Time of action: 1 min.

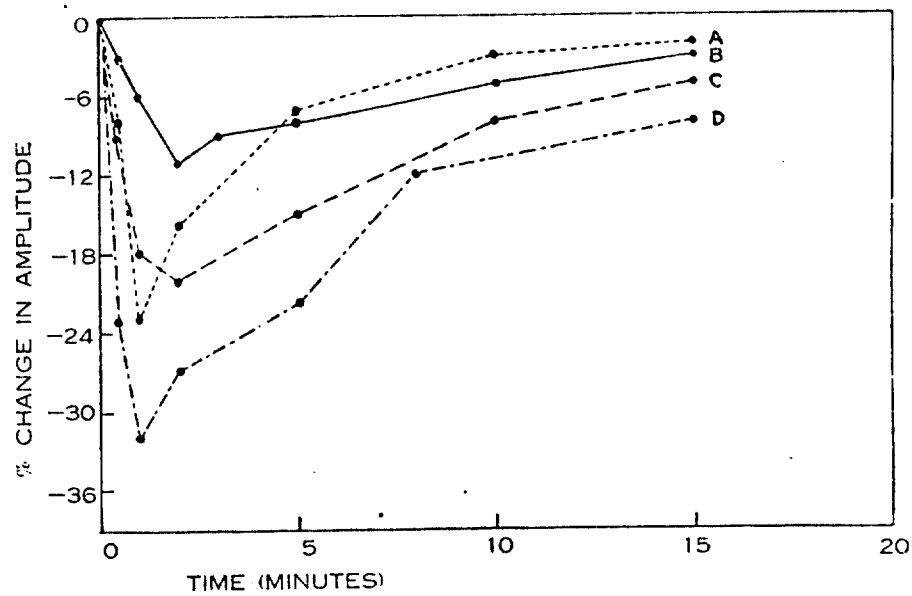


FIG. 2.—Recovery of auricles from substrate depression. (A) acetate 0.01 M, (B) pyruvate 0.005 M, (C) malate 0.01 M, (D) malate 0.02 M.

Consideration of the penetration of these substances into the cardiac cells is of the greatest importance, for, unless the heart cells, like some bacteria, possess enzymatic systems upon their surfaces, it is necessary for the substances to enter the cells in order to produce a direct effect upon the metabolism. Very little work has been done on the permeability of cell membranes to such substances. Furth

mo[illegible] analogies between the penetration of these substances and other weak ac[illegible] be drawn with confidence, inasmuch as cell membranes seem frequently to [illegible]ss selective permeabilities for substances of functional importance. At the [illegible]ly alkaline *p*H of this medium, these substrates exist in solution preponder-anti[illegible] anions; this may interfere with rapid penetration, especially when the sub[illegible]es possess two or three such ionized groups. However, it must be borne in m[illegible] that the permeability to charged molecules may markedly increase in the hea[illegible]ls during the passage of an impulse over the tissue. There is some evidence for [illegible] penetration of such substrates. It will be shown later that in substrate-depl[illegible] auricles there is an almost immediate effect of added pyruvate. Further-mor[illegible]he actions of pyruvate, lactate, β-hydroxybutyrate and other substrates are quit[illegible]pid on exhausted perfused hearts. It will also be shown that iodoacetate and [illegible]roacetate penetrate the auricular cells quite readily. With regard to dicar-boxy[illegible] and tricarboxylic acids, I know of no evidence that is applicable to the pres[illegible]situation, although it seems certain that succinate and malonate are able to pen[illegible]e into heart slices. Fig. 1 shows no correlation between effect and the nu[illegible] of carboxyl groups. I believe that the evidence available points to a de[illegible] penetration by these substances. However, equilibrium may not be reached fo[illegible] three minutes, the times of maximum substrate action thus being expres-siv[illegible]he relatively slow rate of penetration compared to uncharged molecules.

[illegible] possible that the effects observed with the substrates may have been partly or[illegible]ly non-metabolic effects. The relationship between cellular membrane pot[illegible]is and the rate of discharge of the pacemaker cells or the degree of contrac-tion [illegible] not understood, but it is conceivable that alterations brought about in the cell [illegible]mbrane by these substrates might influence the auricular functioning. Such organic acids are known to be capable of producing temporary electrical potential changes in nerve, muscle and plant cells. If such a mechanism of action is involved, it is likely that we must interpret the spontaneous recoveries from substrate depres-sion as originating in the gradual restoration of the normal potential by the establish-ing of a concentration equilibrium between the medium and the interior of the cells. That these substrates depress the auricles by increasing in some manner the effective acetylcholine concentration in the tissue is unlikely, since it was clearly shown that atropine, at a concentration capable of blocking all acetylcholine action (1×10^{-5}), failed to alter the actions of pyruvate, citrate, acetate, malate, and fumarate. It is improbable that the depressant action originates in a simple increase in osmotic pressure of the medium, for glucose may be added up to a concentration of 0.03 M without alteration of the beat. Furthermore, if an osmotic effect were involved, we should expect the various substrates to produce similar degrees of depression at equivalent concentrations; this, however, is not the case. Since the concentra-tion-action curves for the various substrates, as shown in Fig. 1, are not only at different positions along the concentration axis, but also possess markedly different curvatures, it would seem unlikely that the depressant effects were due to any general, non-specific type of action.

Some of the substrates, particularly acetate, malate, and propionate, showed a tendency to induce arrhythmias in the auricles. These arrhythmias were occasion-ally completely irregular, but usually they were of the "pulsus alternans" type, the amplitude of contraction regularly alternating each beat between two different

values. Sometimes such a rhythm was caused by the regular beating of one auricle of a pair while the other auricle beat only every other contraction, but more frequently this was not so. Rapid recoveries from such arrhythmias were observed when the substrate was removed from the bath.

The action of succinate was only depressant in these experiments. Depression of the amplitude was observed by Salant, Livingston, and Connet (1917) on the perfused frog heart (succinate 0.01–0.02 M) and by Forssmann and Lindstén (1946) on the isolated and perfused rabbit heart (succinate 0.01–0.04 M). They did not find in these preparations such marked depression of the rate as was here observed on the rabbit auricles. Forssmann and Lindstén (1946) found a mild stimulation (+15 per cent) of the amplitude by succinate in lower concentrations (0.0005–0.005 M). In the auricles no such stimulation was observed within a similar concentration range; this possibly indicates a difference in behaviour between the auricles and ventricles of the rabbit, inasmuch as they measured the contractions of the left ventricle. No stimulation was found in the isolated and perfused rat heart (Nakamura *et al.*, 1949). Despite the rapid oxidation of succinate by cardiac tissue, it would appear that the energy derived from this is, at least in the normal auricle, of no consequence in the functioning of the auricles. It will be shown later that this energy cannot be utilized to revive substrate-depleted auricles.

The relatively marked depression produced by malate on the auricles is in accord with the results of Salant, Livingston, and Connet (1917) on the frog heart; the frog heart would seem to be more sensitive to the action of this substrate than the rabbit auricle. A definite difference in the behaviour of the two tissues is seen however, in the recovery of the rabbit auricles from the malate depression, whereas the frog heart was reported to remain in a constant state of depression as long as the malate was present (in one case for 62 minutes).

The action of citrate on the rabbit auricle is very interesting, there being a marked stimulation of the rate while the amplitude is simultaneously reduced. This phenomenon occurs only at higher concentrations of citrate (above 0.005 M); below this there is little effect on the rate, although the amplitude is depressed. The acceleration of the rate occurs quite suddenly. For example, in one experiment using 0.01 M-citrate, the rate was unchanged 30 seconds after the addition of the citrate, but it suddenly increased by 22 per cent at 40 seconds, and was up by 39 per cent at 60 seconds, after which it remained relatively constant. The depression of the amplitude observed here is very similar to that reported by Forssmann and Lindstén (1946) on the entire heart, but, unfortunately, they did not include their results on the rate changes. How much of the action of citrate was due to a decrease in the concentration of ionized calcium in solution is difficult to say. Added calcium will counteract the depression produced by citrate, but this does not prove that citrate acts by calcium depletion. When the concentration of citrate is 0.001 M or above, there must be a decrease in the ionized calcium, the original concentration of the calcium ion in the medium being 0.0018 M. If one considers the effect on the rabbit auricle of decreasing the calcium concentration (Webb, 1950), and compares this with the concentration-action curve for citrate depression of the amplitude one must admit that the results coincide with what one might expect if citrate were acting by calcium depletion. On the other hand, the stimulation of the rate cannot be due to a decrease of ionized calcium in the medium, for low calcium concentra-

[illegible] ve very little, if any, effect on the rate of the rabbit auricle. There still [illegible] the possibility that the citrate penetrated the pacemaker cells and within [illegible] oduced a disturbance in the calcium equilibrium, thereby producing changes [illegible] arge rate that would not have occurred as the result of a mere decrease in [illegible] calcium.

The effect of glucose deficiency on rabbit auricles

[illegible] abbit auricles were left for 24–48 hours at 30° C. in a glucose-containing [illegible] a marked reduction in the amplitude was usually observed, especially [illegible] medium had been continually replaced, although the rate often remained [illegible] as it was originally. Under such circumstances the auricles stop in [illegible] ed condition of diastole. These auricles could usually be restarted with [illegible] holine or adrenaline (Vane, 1948; Burn and Vane, 1949) but not by any [illegible] substrates that were tried (pyruvate, acetate, succinate, oxalacetate, and [illegible]. This type of exhaustion occurs in the presence of glucose and is probably [illegible] d in any way to depletion of substrate. It is likely that some alterations [illegible] ellular enzyme systems have taken place, such as the depression of the [illegible] n of acetylcholine (Bülbring and Burn, 1949) or a dissociation of the [illegible] of oxidation and phosphorylation.

[illegible] ucose was removed from the medium in which the auricles were beating, [illegible] s no immediate effect. It is to be expected that the auricles would have [illegible] endogenous substrate—glycogen and fat—on which to operate for a period [illegible] The amount of work performed by beating auricles under these conditions [illegible] great, for they were not working against the usual internal pressure of the [illegible] Seven experiments were performed in which the behaviour of the auricles [illegible] bsence of glucose was observed for five to seven hours. In three of these [illegible] ments there was no change of rate, and in four there was some decrease [illegible] 10 to –30 per cent). In three of these experiments there was no change in amplitude, and in four there was some decrease (–30 to –50 per cent). The auricles thus show variation in their behaviour in the absence of glucose, some apparently having a greater supply of endogenous substrate than others. Since there was essentially no change in rate or amplitude over a period of five to seven hours in the presence of glucose, these decreases in rate and amplitude, when they occurred, must be attributed to the lack of glucose. In longer experiments, when the auricles were left without glucose overnight, the exhaustion produced by substrate depletion is complicated by the simultaneous development of the type of exhaustion seen when glucose is present. Furthermore, it seems evident that lack of glucose in the medium produced some irreversible change or damage in the cardiac cells, for although stimulation was observed when glucose was added to such auricles, there was never anything like complete recovery. This type of damage may be related to that occurring when cardiac tissue is subjected to oxygen deficiency (Webb, Saunders, and Thienes, 1949a).

In order to produce rapid substrate depletion, with as little complication from other changes as possible, the auricles were driven with adrenaline (1×10^{-6}) in several experiments for varying periods of time in the absence of glucose. Such a procedure exhausted the auricles to a low level of activity in 15 to 60 minutes. If adrenaline were allowed to act for one to three hours, the auricles, after the

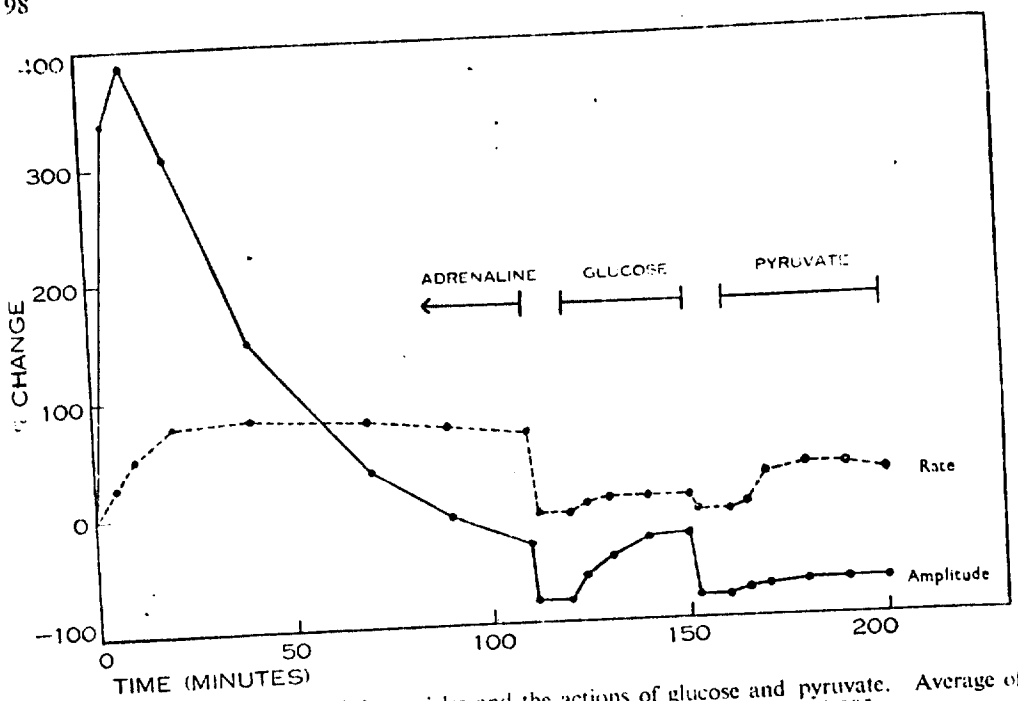


FIG. 3.—Substrate depletion of the auricles and the actions of glucose and pyruvate. Average of four experiments. Adrenaline, 10^{-6}; glucose, 0.0055 M; pyruvate, 0.005 M.

adrenaline had been washed out, beat with a very small amplitude, although the rate was usually relatively unaffected. The averaged results of four experiments are shown in Fig. 3. The rate in the presence of adrenaline remained at a high level, while the amplitude rapidly decreased to below the normal level. After the adrenaline had been removed, the rate returned to nearly its original value and the amplitude was very depressed. Exhaustion with adrenaline in the absence of glucose seems to affect the amplitude much more than the rate, the latter being quite stable. In spontaneous exhaustion in the absence of glucose the amplitude was also more easily depressed. The question whether adrenaline produces cardiac failure by depletion of substrate under such circumstances has never been settled. Some investigators have found that adrenaline-driven hearts lose their glycogen and attribute the failure to this, while others have shown that the heart may fail when there is sufficient glycogen present (Chang, 1936; Fieschi, 1933; Bogue, Evans and Gregory, 1937; and others). This problem might be solved by determining the extent to which the cardiac tissue will recover when glucose is given. If there is complete, or nearly complete, recovery the failure may be attributed to substrate depletion, while if no recovery is seen it is likely that damage and disorganization have occurred to the functional systems. Any intermediate situation would indicate the extent of substrate depletion or cardiac damage. In the present investigation we shall see that the situation is intermediate and we must conclude that changes have taken place in the auricular tissue in addition to substrate depletion. However for convenience we shall speak of such auricles as depleted.

en the auricles were driven with adrenaline there was invariably a gradual dev ment of contracture as the amplitude decreased. This may also be ex d as an increase in tone or a decreased ability of the auricular muscle to . Auricles brought to almost complete exhaustion with adrenaline in the ence of glucose usually beat at a normal rate with a greatly diminished am de and were in a state of contracture. This inability to relax was also see the longer experiments when glucose was absent and no adrenaline was use ut it was never observed in exhaustion occurring when glucose was present. The oblem of relaxation will be discussed after the results obtained with various metabolic inhibitors have been presented.

Effects of metabolic substrates on substrate depleted auricles

Auricles that had been spontaneously depleted or driven with adrenaline in the sence of glucose were invariably stimulated by the addition of 0.0055 M glu , the concentration normally present in Tyrode solution. Furthermore, the de f contracture of the auricles was lessened; that is, the auricles showed an sed ability to relax. Sometimes the degree of relaxation could be alternate ncreased and decreased by additions and removals of glucose. Complete re to the initial amplitude was never seen, but the rate, if it was decreased, w quently restored to its normal value. The rate, however, in the depleted au was never far below its original value, while the amplitude was markedly dep d to only 10–20 per cent of the initial height. Usually there was a fairly rap se in amplitude over the first two minutes after glucose addition, and this wa llowed by a more gradual increase to reach a maximum value after 10–40 min. In terms of percentage, glucose increased the amplitude by 100–300 per cent ver the depressed value. This is illustrated in Fig. 3, where the action of gluc e may be compared with that of pyruvate.

Pyruvate likewise stimulated the substrate depleted auricles but not so well as glucose. However, the effect upon the rate was more marked with pyruvate, the rate being occasionally increased over the normal original value, while a good effect was sometimes obtained on the rate with little or no stimulation of the amplitude. The stimulation of the amplitude by pyruvate was usually between 10–60 per cent, and when compared with glucose on the same auricles this amounted to about one-fifth of the glucose effect. Pyruvate also induced relaxation of the auricles but was not so effective as glucose in this respect. The optimal concentration of pyruvate for such stimulation was found to lie between 0.001–0.005 M.

Only acetate and β-hydroxybutyrate of all other substrates tested were capable of producing any recovery in the substrate depleted auricles, and these effects were small and variable. Neither of these substances was found to induce relaxation. With acetate, the concentration had to be low (0.001 M) for any stimulation to be observed, for if higher concentrations were used only further depression resulted. In six experiments with 0.001 M-acetate, the average increase in amplitude was 18 per cent whereas the rate was not stimulated. Substrates with no ability to revive depleted auricles were fumarate, succinate, citrate, malate, oxalacetate, propionate, butyrate, and glutamate.

It is interesting to note that glucose and pyruvate would occasionally facilitate relaxation of the auricular muscle although the auricles were not beating. Here we may confidently say that depletion of substrate increases the tone of the cardiac muscle and that the presence of glucose or pyruvate decreases the tone. When the auricles were beating, the amplitude of contraction usually decreased while the tone was increasing, and vice versa, but this was not always true, and we must conclude that there is no necessary relationship between the degree of contraction, and the ability of the muscle to relax. Failure of the muscle to relax sufficiently naturally inhibited the development of a full contraction, but it was observed occasionally, when glucose or pyruvate were added while the contracture was proceeding, that the amplitude may increase without simultaneous relaxation.

The results presented here on the rabbit auricle agree well with those obtained in the heart-lung preparation and in the isolated and perfused mammalian heart, not only with respect to the substrates that are utilizable by the cardiac tissue, but also with the comparative abilities of these substrates to revive the depleted heart muscle. However, on no other tissue are the differential actions of glucose and pyruvate on the rate and amplitude so easily observed. On may conclude that auricular and ventricular functional metabolism is qualitatively similar. It is likely, however, that somewhat different systems are involved in the discharge of impulses by the pacemaker cells and in the processes of contraction.

Effects of metabolic inhibitors on normal auricles

Curves showing the relationship between the concentration of inhibitor and the action produced are presented in Figs. 4 and 5. The time course of inhibit.

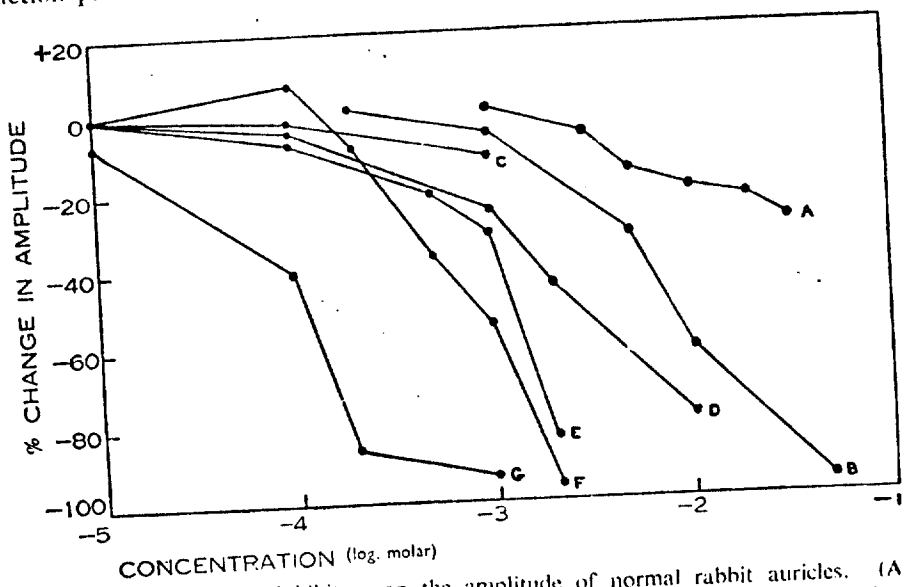
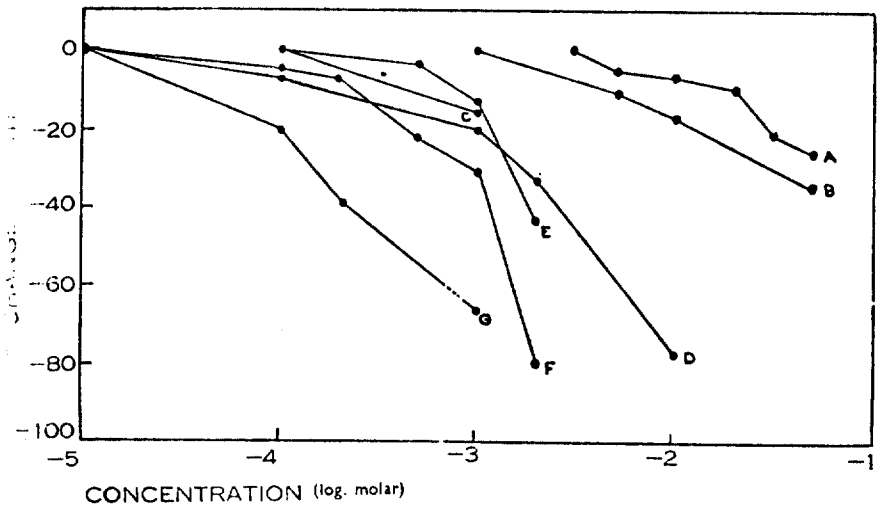


FIG. 4.—The action of inhibitors on the amplitude of normal rabbit auricles. (A) malonate—2 min., (B) fluoride—4 min., (C) phlorizin—5 min., (D) fluoroacetate—25 min., (E) azide—4 min., (F) cyanide—2 min., (G) iodoacetate—25 min. The times refer to the duration of inhibitor action.



. 5.—The action of inhibitors on the rate of normal rabbit auricles. Same designations as in Fig. 4.

is illustrated in Figs. 6 to 12, where the actions upon rate and amplitude e compared. These are all average curves obtained from three to six ments.

le

anide invariably produced a temporary stimulation before it began to depress ricles, this stimulation being more marked in the amplitude than in the rate. egree and duration of stimulation varied with the concentration of cyanide: w concentrations (0.00001–0.0001 M) the stimulation was small but persisted, not being followed by a depression, but as the concentration was increased period of stimulation became shorter and shorter, so that at concentrations 0.001 M the maximum stimulation was reached 20–40 seconds after addition and was followed immediately by a rapidly developing depression. Such temporary stimulation was also seen when the auricles were made anoxic.

The rapid development of the depression would indicate that the available supply of high-energy phosphate bonds in the auricle, at least with respect to some process, is not great, unless cyanide, through some unrecognized action, prevented the direct utilization of this energy. A concentration of cyanide of 0.001 M produces a final depression of about 90 per cent in the amplitude and 50 per cent in the rate; this concentration was reported to inhibit the respiration of minced rabbit heart 45 per cent (Banga, Schneider, and Szent-Györgyi, 1931) and the respiration of rat heart slices 74 per cent (Webb, Saunders, and Thienes, 1949b). The rabbit auricle will usually stop in a concentration of 0.002 M-cyanide. It is probable that, when the auricles stop, there is still a definite fraction of the respiration proceeding; the level of activity to which the auricles are brought by any concentration of cyanide is not a quantitative measure of the cyanide-resistant respiration. Impulse discharge from the pacemaker cells was not inhibited so readily as the amplitude of contraction; this has been observed with other cardiac

preparations (Loewi, 1897; Beresin, 1913; and others). Most workers have reported that hearts poisoned by cyanide stop in diastole (Loewi, 1897, and others) but some have observed systolic standstill (Fujimaki, 1924). In the rabbit auricle both may be observed according to the conditions of the inhibition. When the auricles were stopped rapidly by higher concentrations of cyanide, they stopped in the relaxed condition of diastole; however, if the action of cyanide at concentrations just below those required to stop the auricles was prolonged, the auricles began to go into contracture and eventually stopped in systole. Occasionally both phenomena were observed in the same experiment. In one instance, the auricles were stopped in 80 seconds by 0.002 M-cyanide, but they slowly and partially recovered in the presence of cyanide, so that at 30 minutes the rate was depressed to only 40 per cent of its original value and the amplitude 92 per cent of its original value. At this stage the auricles were in a state of partial contracture, an inability to relax having been developed during this time. Eventually they stopped in systole. However, contracture is less commonly observed with cyanide than with inhibitors like iodoacetate, fluoroacetate, and fluoride.

Spontaneous recovery from depression in the presence of cyanide was very seldom observed, the example cited above being the only definite instance. Normally the auricles would reach a certain level of depression and remain there, or, if the depression were sufficient, they would slowly pass into a state of contracture. The auricular tissue seems to have very little ability to adjust itself to conditions in which the oxygen utilization is impaired; no ability to adjust to anoxic conditions was observed. During very slight cyanide depressions, there was occasionally some recovery, but this may be explained by loss of cyanide from the medium, some being carried off by the vigorous gassing. When the cyanide was removed from the medium, the auricles recovered completely, the rapidity of recovery depending on the concentration of cyanide and the duration of exposure. After marked depression following exposures of three to six minutes to 0.001 M-cyanide, the auricles would recover completely within ten minutes but if the exposure time was only one minute the recovery took place within three to five minutes. No irreversible damage to the cells was observed at these concentrations, unless full contracture was allowed to occur. After the cyanide had been washed out a stimulation of the rate during the recovery phase was frequently observed, amounting to a 20-30 per cent increase over the normal rate before the cyanide had been added. Various arrhythmias commonly occurred during this recovery phase. It is possible that during the depression some energy containing or energy-supplying substance had accumulated in the cells and that when oxygen was again available this resulted in a temporary stimulation of activity; this stimulation, however, was observed only with regard to the rate.

When excess calcium was added to the medium there was a marked stimulation of the amplitude, the rate remaining unchanged (Webb, 1950). Cyanide (0.001 M) antagonized this action, so that lower concentrations of calcium (0.0001–0.0005 M) were almost without effect, while the action of higher concentrations was markedly diminished. Cyanide also diminished the stimulating action of adrenaline on the auricles. In one experiment, adrenaline (10^{-6}) normally increased the rate by 69 per cent and the amplitude by 390 per cent, but in the presence of cyanide (0.001 M) the observed effects of adrenaline were zero and 105 per cent respectively

A:

s interesting to compare the actions of cyanide and azide because of the us accepted similarity of their enzymatic inhibitions. Azide was not as potent an ibitor of the auricles as cyanide. In equal concentrations, azide acted more slo than cyanide and at any particular time produced only 1/2 to 1/3 the de on that would have been produced by cyanide. In Figs. 6 and 7 comparison ma made of their actions at 0.0005 M. Azide did not produce the temporary sti ation observed with cyanide, nor did low concentrations ever stimulate. As h cyanide, azide depressed the amplitude more than the rate. This was also bserved on the isolated hearts of cats and rabbits by Graham (1949).

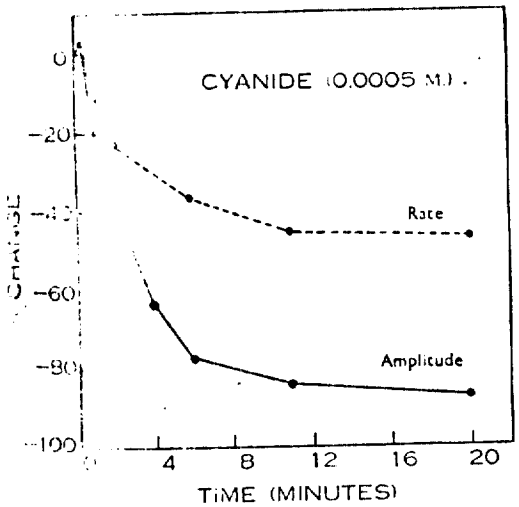


FIG. 6. The action of cyanide on the rabbit auricle.

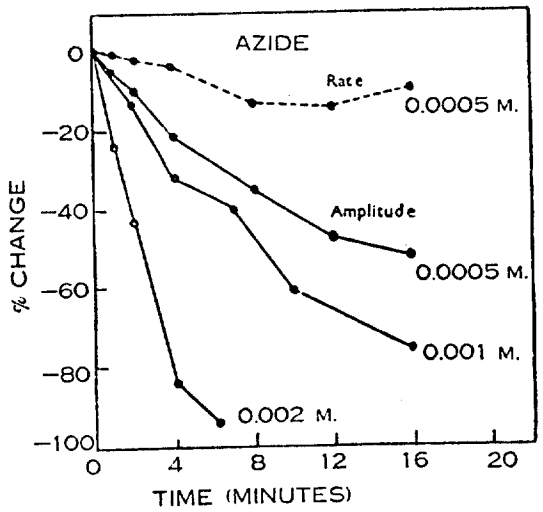


FIG. 7.—The action of azide on the rabbit auricle.

Recovery of the auricles after the azide had been removed was slower than with cyanide; 10 minutes were required after an exposure of 12 minutes to a concentration of 0.0005 M, and 20 minutes after a four-minute exposure to a concentration of 0.002 M, indicating that the azide is more tightly bound than cyanide.

Armstrong and Fisher (1940) concluded that cyanide and azide acted differently on fish hearts because the cyanide-stable respiration was different from the azide-stable respiration. However, this may be merely an expression of the wider range of action of cyanide at higher concentrations, for it is known that cyanide above a concentration of 0.001 M begins to inhibit dehydrogenases in addition to its action on metal-containing enzymes. However, it is clear that even in lower concentrations there was some difference in behaviour between the two inhibitors in their actions on the rabbit auricle.

Fluoride

Fluoride at concentrations between 0.005–0.01 M first depressed both the rate and amplitude of the auricles, but after approximately five minutes the amplitude began to increase and eventually reached values above the normal original level

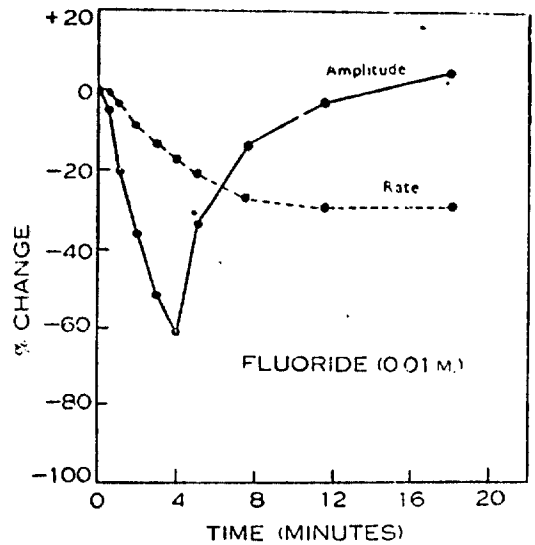


FIG. 8.—The action of fluoride on the rabbit auricle.

(see Fig. 8). Meanwhile, the rate remained at its depressed level or continued to decrease slightly. After 20–40 minutes the auricles were beating slowly but with a large amplitude. However, occasionally contracture began and the amplitude again decreased owing to the increasing inability of the auricles to relax. Previous reports on the action of fluoride on cardiac tissue have been equivocal. Several investigators have found only depression: Vernon (1910a, b) with 0.24–1.2 M-fluoride on the perfused tortoise heart, De Nitto (1928) with 0.00012–0.0012 M-fluoride on isolated mammalian hearts, and Chang (1938) with 0.0078 M-fluoride on rabbit auricles. Gottdenker and Rothberger (1935a, b) found depression of the perfused frog heart and the dog heart-lung by fluoride, but on guinea-pig and rabbit auricular strips they usually observed an increase in the amplitude when the fluoride concentration was 0.003 M. Ferrannini (1936) also found fluoride (0.0024–0.0072 M) to increase the force of contraction of rabbit auricular strips. In most work it has been observed that cardiac tissue is able to function surprisingly well in the presence of fluoride at concentrations which should inhibit glycolysis completely. Chang (1938) stated that fluoride, except for a slight depression, had little effect on the mechanic ability of rabbit auricles over a period of two to three hours as long as oxygen was present. Furthermore, he apparently observed no increases in amplitude. In the present work with the same tissue such a concentration of fluoride produced a marked effect. No explanation can be offered for these discrepancies.

With regard to the mechanism of fluoride action, most workers have concluded that depletion of calcium from the medium is not important. However, at least in the present experiments, it is difficult to see how it could fail to be of some importance. The calcium concentration in Tyrode solution is 0.0018 M; if sodium fluoride is added to the medium in a concentration of 0.005 M, there are approximately equivalent amounts of calcium and fluoride to form CaF_2. Since CaF_2 is soluble to the extent of 0.00022 M and since it is very little ionized (solubility product is 3.4×10^{-11}), it would seem likely that a definite fraction of the calcium would be withdrawn from the medium. When more fluoride is added the calcium should be reduced even more. No visible precipitation of CaF_2 was observed but the walls of the tissue bath slowly became covered with a white layer. Reduction of calcium in the medium leads to a marked depression of the auricle with respect to amplitude, but has no effect on the rate (Webb, 1950). Thus it would seem that the observed depression of rate and the subsequent increase

ampl de, in the presence of fluoride, must have been due to an action of fluoride other han that of altering the calcium concentration in the medium. As to how muc be initial depression of amplitude is due to decreased calcium it is impossible to s.

S fluoride inhibits cholinesterase, the possibility exists that at least part of t depressant action was due to prevention of destruction of endogenous acety oline. However, fluoride was found to give a typical depression of the auric in the presence of physostigmine (10^{-6}), when the cholinesterase was alrea nhibited, and also in the presence of atropine (10^{-6}), when the action of acety oline was completely prevented. It is thus unlikely that the action of fluor is related to the acetylcholine equilibrium in the auricles.

Such concentrations of fluoride as were found to have definite actions on the auricles (0.005–0.05 M) should inhibit glycolysis completely. Muscle enolase is usuall nhibited 50 per cent by 0.001 M-fluoride and completely by 0.005 M-fluoride. A cert decrease in fluoride concentration would result from combination with calcium but in the higher concentrations this could not have been very great. We therefore, conclude that the auricles can function quite well in the absen of the formation of phosphopyruvate from 2-phosphoglycerate. One possib is that the auricles used fat for the necessary energy. Another possibility at the 2-phosphoglycerate was utilized through some other system without the f ion of pyruvate. It is interesting to speculate that the secondary increase in au de is due to this new source of energy, brought into play because of the e block. In rat heart slices the respiration was never depressed more than er cent by fluoride at concentrations as high as 0.1 M (Webb, Saunders, and es, 1949b), so it is evident that in cardiac tissue there is an appreciable fract f the metabolism insensitive to fluoride.

Fl de inhibition is reversible, and after the fluoride had been washed out recov was usually complete within 5–10 minutes. After long exposures there was occasi lly some stimulation of the rate above the normal figure during the post-wash every stage. It was also noticed that the auricles during this period frequently had difficulty in relaxing and the tone varied correspondingly. In passing, it may be remarked that fluoride (0.03 M) quite effectively diminished the action of adrenaline (10^{-7}–10^{-6}), the stimulation of amplitude being almost abolished while the rate effect remained but was made weaker.

Iodoacetate

Iodoacetate in concentrations between 0.0001–0.001 M is a slowly acting depressant of the rabbit auricle, requiring from three to five minutes for appreciable reduction of the amplitude and from 10 to 20 minutes for a slowing of the rate. At certain stages of the depression, the amplitude was markedly reduced while the rate remained unaffected, but eventually both were depressed almost equally before the auricles stopped. During the later stages of this depression the auricles began to go into contracture and they stopped in systole, a phenomenon that has been invariably observed in work with iodoacetate (Weicker, 1934; Chang, 1938; and others). Although slight depressions produced by low concentrations of iodoacetate acting for short periods of time could be partially reversed by removing the iodoacetate, the usual result was that after the iodoacetate had been washed out the inhibition

proceeded, exactly as if the inhibitor were still present in the medium. This is, of course, what one would expect from considerations of the irreversible cellular reactions of iodoacetate, and it confirms previous results on other tissues and preparations.

Iodoacetate is not a specific inhibitor of glycolysis in mammalian cardiac tissue (Webb, Saunders, and Thienes, 1949b), and even in low concentrations it is likely that other enzyme systems are involved in its action. Not only is the synthesis of ATP diminished, by inhibition of the utilization of pyruvate and fatty acid breakdown products as well as by prevention of glycolysis, but it is likely that iodoacetate interferes with the utilization of ATP, for Buchtal, Deutsch, and Knappeis (1944) have reported that iodoacetate blocked the stimulating effect of ATP on skeletal muscle. It would not be surprising if iodoacetate reacted directly with myosin, since this protein complex possesses a number of sulphhydryl groups important to its functioning. This may explain the observation (Chang, 1938) that there is some ATP present in rabbit hearts that have been stopped by iodoacetate.

Adrenaline produced very little stimulation of the iodoacetate depressed auricle, and occasionally a reversal of the rate effect was observed when the depression was well advanced. In one typical experiment the concentration of iodoacetate was 0.001 M, the exposure time was 35 minutes, and the auricles were markedly depressed (rate -42 per cent, amplitude -96 per cent); adrenaline (1[illegible]) stimulated the amplitude slightly (28 [illegible]r cent), but the rate [illegible]s decreased to 75 [illegible]r cent of the orig[illegible]l value. It is not [illegible]prising that [illegible]h auricles would fa[illegible] to be stimulated [illegible]y adrenaline, but [illegible]e reversal of the [illegible]te effect was not [illegible]xpected. It is pos[illegible]ble that iodo-ac[illegible]te blocks some step in the series of reactions through which adrenaline normally produces a stimulation of the rate, leaving the systems by which it may depress the [illegible]te at higher conce[illegible]a-tions relatively [illegible]n-affected.

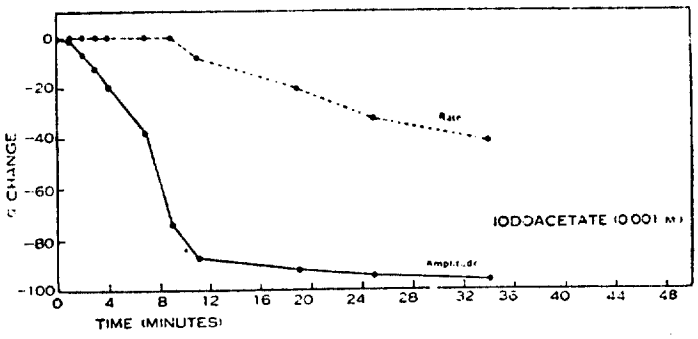


FIG. 9.—The action of iodoacetate on the rabbit auricle.

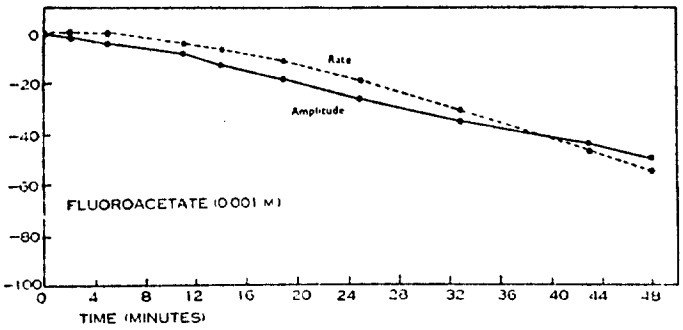


FIG. 10.—The action of fluoroacetate on the rabbit auricle.

Fluoroacetate

Fluoroacetate is the most slowly acting inhibitor of those investigated, depressing the auricles gradually and steadily for a period of two to three hours at a concentration of 0.001 M. It characteristically depressed both rate and amplitude equally, in this respect differing from any other inhibitor used in this study. The metabolic systems involved in impulse discharge and contraction may at some point possess a common path which is attacked by fluoroacetate. At the present time the exact mechanism of action of fluoroacetate is unknown, but it is likely that it inhibits some reaction, or reactions, in the tricarboxylic acid cycle. Accumulation of citrate and acetate during fluoroacetate action has been reported, but the depression of the auricles was not due to these intermediates, as may be seen by comparing the action of fluoroacetate with the actions of these substances. Furthermore, if such were the explanation, we should expect some degree of recovery when fluoroacetate was removed from the bath, but this did not occur. The action of fluoroacetate was quite irreversible. Occasionally the inhibition could be arrested by washing out the inhibitor, but the auricles never showed any tendency to return to their normal rate and amplitude.

Phlorizin

A nearly saturated solution of phlorizin in the medium (0.001M) produced a slight depression of the auricles, the effect on the rate being somewhat greater than on the amplitude, the latter showing some ability to recover spontaneously in the presence of the inhibitor. The mechanism of action of phlorizin at these higher concentrations is not well understood, and therefore it is difficult at the present time to interpret these results or correlate the actions with any particular inhibition of enzymatic systems.

Pyrophosphate

Pyrophosphate at concentrations between 0.002–0.005 M produced a marked depression in the amplitude of the auricles but had little effect on the rate, slight

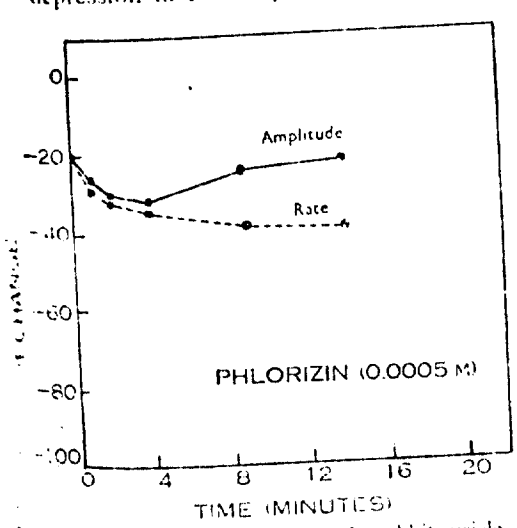


FIG. 11. The action of phlorizin on the rabbit auricle.

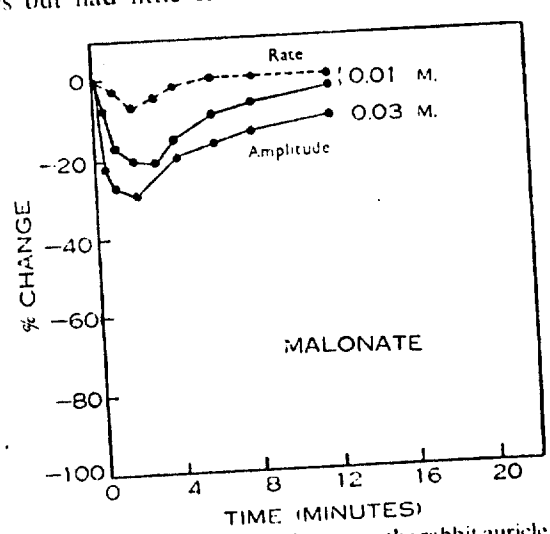


FIG. 12. The action of malonate on the rabbit auricle.

stimulation occasionally being the only effect observed. Since pyrophosphate combines with calcium and the medium became slightly turbid after its addition, the possibility was considered that the action upon the auricle was due to a decrease of ionized calcium in the medium. If calcium was added to the medium during pyrophosphate depression the amplitude returned to normal, but the increased rate was unaffected. Lack of calcium in the medium leads to depression of the amplitude, but the rate is never stimulated and is occasionally slightly reduced. Recovery of the auricles after low calcium was more rapid than after pyrophosphate depression. We may conclude, therefore, that the effects produced by pyrophosphate were partially due to a lack of ionized calcium, but that the pyrophosphate also had a definite action of its own. Since these actions could not be separated conveniently, the action of pyrophosphate was not studied farther and the curves of its action are not included in the Figures.

Malonate

The rabbit auricle was not strongly depressed by malonate: at high concentrations (0.02–0.03 M) a maximum inhibition of only 30 per cent in rate and amplitude was reached. Furthermore, the auricles spontaneously recovered in the presence of malonate at these high concentrations and returned to normal, or nearly normal, rates and amplitudes after 8–10 minutes. When the malonate was washed from the bath during the period of greatest depression, the recovery was very rapid, requiring only two to three minutes. Forssmann and Lindstén (1946) found that malonate in concentrations between 0.01–0.04 M depressed the ventricular contractions of the perfused rabbit heart but had little effect on the rate. It is remarkable that malonate at these concentrations should have so little depressant action on the auricles, for it is generally believed that malonate at a concentration of 0.005 M completely inhibits succinic dehydrogenase and therefore blocks the tricarboxylic acid cycle, a system upon which the cardiac muscle is thought to depend. Recently it has been reported that somewhat higher malonate concentrations, such as those used here, will prevent the oxidation of oxalacetate in homogenates, this action producing an additional derangement of the tricarboxylic acid cycle (Pardee and Potter, 1949). It seems unlikely that failure to penetrate the muscle cells could have been responsible for this lack of action, inasmuch as exposures of 20–30 minutes to malonate did not lead to any marked alteration of auricular function. The strange behaviour of cardiac cells under the influence of malonate was noted previously on rat heart slices (Webb, Saunders, and Thienes, 1949b). In this preparation malonate produced an initial depression of the respiration, but this was followed by a recovery to values near the normal rate. Furthermore, in the rat heart slices it was known that the succinic dehydrogenase was inhibited, for no rapid increase in oxygen uptake was observed when succinate was added as invariably happened when malonate was absent. Rat heart slices can respire adequately in the presence of high concentrations of malonate, and auricles can function quite normally under these conditions. We must conclude that cardiac tissue can respire and function when this particular step in the tricarboxylic acid cycle is blocked. It may well be that when this block is imposed in an organized cellular system, the cycle can be short-circuited; perhaps under such conditions oxalacetate may be formed directly from α-ketoglutarate, or pyruvate or its activated fragment be taken into the

cycl[illegible] combination with something other than oxalacetate. It will be shown late[illegible] that the small amount of depression observed with malonate is probably not due [illegible] the inhibition of succinic dehydrogenase.

Interactions of substrates and inhibitors on the auricles

[illegible]eriments were performed to investigate the abilities of various substrates to c[illegible]ract or antagonize the depressions produced by metabolic inhibitors. The[illegible]ically, if an inhibitor acts specifically on one enzyme in a series of enzymic reac[illegible]s, the addition of the intermediate substrate that is normally formed by the [illegible]on of this enzyme should allow at least partial recovery of the functional activi[illegible] of the tissue. If the inhibitor acts not only on this one enzyme, but upon other[illegible] as well, the amount of recovery produced by the added intermediate will be rough[illegible] proportional to the specificity of the inhibitor effect. The possibility must always be considered that an added substrate, in the presence of an inhibitor, may initi[illegible] energy-forming reactions that are not normally important; thus, a stimulation [illegible] the auricles by a substrate during an inhibitor depression does not necessarily mean [illegible]at there is a direct relationship between the inhibitor and the substrate in the [illegible]lar metabolism.

[illegible]elatively weak effect of malonate on the auricles was puzzling, and attempts were [illegible]efore made to determine whether the depression that was produced was rela[illegible] the commonly accepted action of malonate on succinic dehydrogenase. Ma[illegible] prevents the formation of fumarate from succinate, and recovery from ma[illegible] depression should, therefore, be observed after the addition of fumarate. If m[illegible]e acted simultaneously elsewhere in the tricarboxylic acid cycle this would not [illegible]ssarily be true. Three types of experiment were performed.

[illegible]n two experiments the effects of malonate (0.01–0.03 M) were first determine[illegible] and these were compared with the effects of adding malonate and fumarate (0.00[illegible]0.01 M) together. No modification of the action by fumarate was observed. In a typical experiment the average maximum depression of amplitude produced by malonate alone was 25.7 per cent; when both malonate and fumarate were added together the depression was 27.7 per cent. The changes in rate were likewise comparable.

(2) In the second type of experiment fumarate (0.001–0.01 M) was added to auricles already depressed by malonate (0.02 M). No stimulating effect was observed; in fact, an additional depression was usually seen.

(3) In the third type of experiment the auricles were allowed to remain in contact with fumarate (0.005 M) for five minutes before the addition of the malonate (0.01 M) in order to ensure penetration of the fumarate. Again no change in the response to malonate was observed.

It therefore seems likely that the malonate depression is not due solely to inhibition of succinic dehydrogenase. It is more reasonable, I believe, to assume that malonate at such concentrations exerts a toxic action on the cell surface, a depression such as is seen with any of such substances under similar conditions. Acetate (0.001 M) and pyruvate (0.005 M) were also unable to counteract the depression produced by malonate (0.02 M), only additional depression being observed.

The depression produced by fluoride (0.005–0.02 M) was not counteracted by succinate (0.001 M), acetate (0.001 M), or citrate (0.001 M). However, pyruvate (0.005 M) showed some ability to stimulate the rate of the auricle in the presence of fluoride, but the amplitude was usually unaffected. This is in harmony with most of the results obtained with pyruvate, namely that it acts more particularly upon the mechanisms involved in rate than upon those involved in the amplitude of contraction. Complete antagonism of fluoride with pyruvate was never observed.

Iodoacetate, like fluoride, is generally supposed to act predominantly on the glycolytic stages of metabolism, inhibiting the formation of pyruvate from glycogen or glucose. However, there are reasons for supposing that in cardiac tissue the action is not specific. The heart muscle presumably obtains energy from both glycolytic and post-glycolytic phases of metabolism, although a relatively small amount from the former (approximately 15–20 per cent of the total). If iodoacetate acted only on glycolysis, the addition of pyruvate should restore the activity of the heart muscle to about 80 per cent of its normal value, only the energy from the glycolysis now being lost to the tissues. Three experiments were performed to test this.

(1) In the first experiment the concentration of iodoacetate was quite high (0.001 M), the auricles had been in contact with the inhibitor for 15 minutes, and the resulting depression was severe (rate −40 per cent, amplitude −95 per cent). Addition of pyruvate (0.01 M) gave no stimulation, the rate and amplitude continuing to decrease and contracture to develop. After the auricles were completely stopped at 30 minutes the addition of pyruvate was without effect.

(2) In the second experiment the concentration of iodoacetate was lower (0.0002 M), the time of exposure to the inhibitor was 25 minutes, and the auricles were very depressed (rate −83 per cent, amplitude −87 per cent). The addition of pyruvate (0.005 M) slightly stimulated the rate but had no effect on the amplitude. The normal rate before iodoacetate was added was 72, the iodoacetate had reduced it to 12; pyruvate increased the rate to 18 in two minutes and to 24 in four minutes. Later in the depression pyruvate had no effect at all.

(3) In the third experiment the iodoacetate (0.0002 M) was allowed to act for 15 minutes, the auricles being moderately depressed (rate −19 per cent, amplitude −53 per cent). Pyruvate (0.005 M) slightly stimulated both rate and amplitude, the rate increasing by 15 per cent in two minutes and the amplitude increasing by 32 per cent within 30 seconds; these were the maximum effects and were only temporary, the depression of the auricles proceeding as if the pyruvate had not been present. Later in the depression pyruvate was added at both 0.001 M and 0.01 M without any effect.

One may conclude that pyruvate is only partially able to counteract the depression produced by iodoacetate, and that the higher the concentration of iodoacetate and the longer its action, the less recovery may be observed. These experiments confirm previous conclusions that iodoacetate is not a specific poison of the glycolytic phase of metabolism in mammalian cardiac muscle. Acetate (0.001–0.01 M) showed no ability to counteract the iodoacetate depression.

pression of the auricles produced by fluoroacetate was partially antagonized by pyruvate. This was demonstrated in two experiments.

In the first experiment the concentration of fluoroacetate was 0.001 M, the time of exposure was two hours, and the depression was very marked (rate −79 per cent, amplitude −83 per cent). Pyruvate (0.01 M) increased the rate by 133 per cent and the amplitude by 18 per cent within one minute, the rate returning to half its normal level and the amplitude to only 20 per cent of its normal value. The addition of pyruvate at the same concentration 35 minutes later produced even greater stimulation, the rate increasing by 200 per cent and the amplitude by 126 per cent in two minutes.

In the second experiment the fluoroacetate (0.001 M) was allowed to act for one hour 35 minutes and the auricles were only moderately depressed (rate −24 per cent, amplitude −25 per cent). Pyruvate (0.005 M) gave some stimulation, increasing the rate by 8 per cent and the amplitude by 4 per cent within one minute. After an exposure of 50 minutes, when the auricles were more depressed (rate −41 per cent, amplitude −62 per cent), pyruvate at the same concentration stimulated more successfully, the rate increasing by 40 per cent and the amplitude by 21 per cent. The effect on the amplitude was quite temporary and after three or four minutes the auricles were again being progressively inhibited, but the rate effect lasted for a much longer time.

Discussion

Each metabolic inhibitor used in the present investigation has been found to exert a unique and characteristic action upon the auricle, the effects differing from one another with respect to (*a*) the concentration-action curves, (*b*) the time course of action, (*c*) the relative actions on rate, amplitude of contraction, and muscular relaxation, (*d*) the ability of the cardiac muscle to recover spontaneously in the presence of the inhibitor, and (*e*) the ability to recover after the inhibitor has been removed. Each inhibitor can be distinguished from the rest by its action on the rabbit auricle. The problem, however, is to determine whether any useful information on the mechanisms of cardiac function may be derived from such data in conjunction with the results on substrates, substrate depletion, and anoxia.

The auricles may be stopped in either diastole (state of relaxation) or systole (state of contraction). Substances which arrest the auricles rapidly always stop them in a state of relaxation. The process of contracture, by which the auricles stop in systole, requires time for its development and is not observed early in the action of any substance. There are substances which will rapidly arrest the auricles in diastole and, with continuing action, will induce contracture, the end result being systolic arrest. In the present experiments with rabbit auricles, the following have been found to lead to contracture: iodoacetate, fluoroacetate, fluoride, cyanide, anoxia, and substrate depletion. All of these would be expected to produce a state of reduced metabolic activity with a decrease in the amount of energy available to the muscle. This inhibition of the flow of energy would result in a gradual loss of ATP (and other substances containing high-energy phosphate bonds), such having been observed in the past under similar conditions. Simultaneously with the depletion of ATP the cardiac muscle becomes less and less able to relax, and when the ATP level becomes sufficiently low the muscle goes into complete contracture and

will beat no longer. The ATP concentration need not drop to zero for this to occur. This conception is in harmony with the views of Szent-Györgyi (1947) on skeletal muscle, ATP being required for the relaxation of the muscle fibrils as well as for their contractile activity. The auricular muscle is in a low-energy state when it is contracted and in a high-energy state when it is relaxed. Adding glucose or pyruvate to a muscle in contracture would provide some ATP and the muscle would be able to relax and beat again.

Let us consider auricles that have been stopped in the relaxed state of diastole by some metabolic inhibitor. Why does the muscle not contract? There is certainly present a sufficient concentration of ATP. There is nothing fundamentally wrong with the contractile mechanisms, for the muscle can be made to contract by electrical stimulation, and it will recover from such contractions for a period of time. The initiating reaction for contraction must be depressed. The functioning of cardiac muscle involves not only the mechanisms of contraction and relaxation, but also processes that are rhythmic in nature and serve to stimulate these contractions. Normal contraction is induced by a wave of depolarization (accompanied simultaneously by other changes) which sweeps over the muscle and in some manner causes the actomyosin to be transformed into its low-energy state. This stimulating process can be roughly divided into three phases: the discharge of the impulse from the pacemaker cells, the conduction of this impulse, and the excitatory reaction by which the actomyosin is affected by the impulse. These process are all dependent on metabolic reactions for their energy. Maintenance and restoration of the cellular membrane potentials require energy, and further energy may be required for the synthesis and distribution of substances involved in these processes. It is likely that these excitatory mechanisms are inhibited in auricles that have been arrested in diastole. Occasionally a substance will stop the heart by depressing the discharge of impulses from the pacemaker cells, but usually it would seem to be a matter of depression of conduction or of the excitatory reaction (the process by which the actomyosin is stimulated by the conducted impulse will be here termed the "excitatory reaction"). In many cases the amplitude of the auricles was gradually depressed until arrest occurred; immediately before arrest the rate was depressed relatively little. Observation of such auricles leads one to the conclusion that the pacemaker cells are still discharging after the auricles have ceased to beat, the failure being due to disturbances in conduction or the excitatory reaction. If such is the case, it lends support to the view that partial inhibition of the amplitude is due to the failure of a certain fraction of the contractile units to react, the contraction of each unit being all-or-none in nature. Progressive inhibition of the excitatory reaction would lead to a gradual decrease in the number of units responding to the conducted impulse, and this in turn would lead to a progressive inhibition of the amplitude of contraction. Conduction and the excitatory reaction may be more sensitive than the processes of contraction and relaxation to metabolic inhibitors and anoxia because they do not possess a store of ATP, most of this substance being found in the actomyosin or in the enzymes involved in phosphate transfer.

When the amplitude of contraction of cardiac tissue is reduced to some fraction of its normal value (no change in the degree of relaxation having taken place) we may interpret this in two general ways. Either there is a smaller fraction of

all -none units contracting, or each unit in the muscle is contracting proportionately les The solution to this problem is fundamental to cardiac physiology and ph acology. Does cyanide, for example, when it inhibits the auricular amplitude by per cent, prevent completely the contractions of 20 per cent of the contractile un the remaining units contracting normally, or does it reduce the contraction of h unit an average of 20 per cent? This is not merely the old question of wh er the concentration-action curve is an expression of the action of a sub ance on cellular processes or whether it is derived from the distribution of susceptibilities of the individual cells. A depressant may inhibit the excitatory reaction either by raising the threshold of response of the contractile units or by ducing the excitatory state that is initiated by the passage of the impulse. In the former case we should be measuring a distribution of susceptibilities, but in the latter case it would be a matter of a true action on a cellular process. The is no *a priori* reason why both actions may not be of importance, depending on substance that is producing the depression. It should also be pointed out at, although the contractile units may normally behave in an all-or-none ma r, it is entirely possible that various conditions may modify the degree of traction.

w must we interpret the changes in amplitude brought about by the action of e metabolically important substances on the rabbit auricle? The results of present investigation can be best explained by assuming that these sub ces modify the excitatory reaction and not the contractile mechanisms dir . Only when the cardiac muscle begins to go into contracture is the con tile mechanism itself altered. If this is the correct interpretation, it would ec hat our conceptions of the actions of substances on cardiac muscle must be dified. Such an interpretation would also apply to the actions of drugs on the heart.

Throughout this investigation it was found that the changes in rate and amplitude of the auricles were relatively independent of each other. An increase or decrease in the amplitude had no particular effect on the rate, nor did changes in the rate, within limits, affect the amplitude. Quite frequently one would remain constant during a marked change in the other, and occasionally they would change in the opposite directions. The fundamental energy-forming enzymatic reactions may be quite similar in the processes that control rate and amplitude, but the paths of energy flow and utilization are different. It is true that most substances change the rate and amplitude in the same direction. This is perhaps more readily understandable when we consider changes in amplitude as arising, not from alterations in the contractile mechanisms, but from modifications of some cellular surface phenomenon that is involved in the excitatory reaction, such a surface state being related to the cellular membrane changes occurring in the pacemaker cells. In whatever way we consider the cyclical discharge of impulses, we must assume that there is the development of a depolarized state and that this is propagated over the cardiac muscle, this being responsible, directly or indirectly, for the excitatory reaction and the contraction of the myofibrils that lie in the region over which the impulse is passing. We can thus see a possible connexion between rate and amplitude, a relationship that implies no necessary simultaneous behaviour, but which, in the majority of cases, will lead to parallel

behaviour with respect to direction of change. However, the magnitudes of the changes in rate and amplitude need bear no relationship to one another.

Development of contracture was always a late process, following earlier alterations in the auricular rate and amplitude. We have supposed that this was due to a greater reserve of ATP or other substances containing high-energy bonds. It is indeed possible that the membrane phenomena of impulse discharge, conduction, and the excitatory reaction do not utilize ATP or similar substances, but that the cyclical processes involved are more directly dependent on the oxidative sequence of enzymatic reactions. Most of the metabolic inhibitors investigated, as well as substrate depletion and anoxia, depressed the amplitude more than the rate. This might be explained by assuming that the rate processes require less energy than those involved in the excitatory reaction, but this can hardly be the entire answer since there are instances in which the rate is depressed as much as the amplitude. The metabolic systems involved in the rate and amplitude may also be assumed to be somewhat different, and this is quite likely from a consideration of the characteristic actions of the various inhibitors, but it would explain only the differences in behaviour and not the basic fact that the rate is more resistant to change than the amplitude. Another explanation might be that the rate and amplitude mechanisms alter their functional level to different degrees in response to an equivalent change in some common factor. For example, if we assume that both the rate of discharge and the effectiveness of the impulse on the contractile units depend on changes in the degree of electrical polarization of the cell membrane, it is conceivable that a depression of the resting potential or a depression of the rate of repolarization would inhibit both processes. However, owing to the particular circumstances, the number of units reacting would be depressed more readily than the discharge rate. The excitatory reaction is probably quite delicately adjusted to the changes occurring at or near the cell surface. This delicate balance assures a ready adaptability of the cardiac muscle.

We might expect, for the same reasons, that glucose and pyruvate would stimulate the amplitude more than the rate in substrate depleted auricles. This was found to be true for glucose, but pyruvate acted more particularly on the rate. Quantitative comparisons are complicated by the third action of these substrates, the stimulation of relaxation, an action somewhat more readily produced by glucose than by pyruvate. However, it was clear that pyruvate, both in substrate depleted auricles and in those poisoned by inhibitors, stimulated the rate more effectively than the amplitude and more effectively than it produced relaxation of the muscle. From this it might be concluded that the oxidations in the tricarboxylic acid cycle are the main source of energy for the rate processes. Supplying glucose does not lead to a sufficiently rapid formation of pyruvate, and therefore giving pyruvate directly has a greater stimulating effect. Why, however, is pyruvate less effective than glucose in stimulating the amplitude? The energy derived from the breakdown of glucose to pyruvate must be of greater importance to the processes involved in the contraction and relaxation of the muscle than it is to the rate processes. It is doubtful whether we are measuring the effect of either substrate on the excitatory reaction under such circumstances, the stimulation of ATP formation being the primary action in increasing activity of contraction. According to Szent-Györgyi (1947) the actomyosin requires so much ATP to

b. ive at all, aside from the ATP that is utilized for relaxation. Since ATP c. formed from energy derived from the breakdown of glucose to pyruvate it ot so surprising that glucose is somewhat more effective than pyruvate in th spect. However, in the cellular membrane processes of impulse discharge ar e excitatory reaction the conditions are different; as we have seen, it is p le that these mechanisms do not require ATP and may operate directly fr he oxidative systems, especially the tricarboxylic acid cycle. Since the tricarbe acid cycle can continue to operate in the metabolism of fat, the observation th the rate is more steady during inhibition of glycolysis (iodoacetate) than it is ring inhibition of the tricarboxylic acid cycle (fluoroacetate) is explained. Th remain, however, a number of problems—for example, the anomalous bel viours of malonate and fluoride—and these ideas are presented only as a w ng hypothesis.

e metabolic activities of contracted and relaxed cardiac muscle may be di t. The metabolism may occur in cycles, in phase with contraction and re ion, so that the conception of the metabolism as a steady state would be er us. Deductions based on steady state conditions would not necessarily b licable, nor would arguments based on limiting reactions always be valid. T ust be kept in mind in considerations of the action of metabolic substrates a hibitors on the heart. Determinations of the ability of heart slices to o various substrates may give information on only one phase of the cycle; it ely that such slices are in a state of contracture. The same reasoning w be applicable to the actions of inhibitors. Substrates that are well oxidized by contracted muscle may produce no stimulation of the normally beating au e because their oxidation occurs at phases of the cycle where their energy cannot be utilized; only when the cardiac muscle has become abnormal, when the cyclical processes have become distorted, or when the muscle has gone into contracture will these substrates be oxidized with the production of utilizable energy. Similar metabolic cycles of activity probably occur in the pacemaker cells during rhythmic discharge of impulses and near the surfaces of the muscle cells as the propagated impulses pass.

Furthermore, one is not justified in assuming that there is a general synthesis and supply of ATP upon which all the processes in the cardiac cell must draw. ATP is probably used in only certain functional mechanisms and is synthesized in these particular regions. The concentration of ATP as determined by analysis of the entire muscle can seldom be correlated with the functional activity of the tissue for this reason. When one considers the various processes which control the beat of cardiac tissue, the different metabolic requirements of these processes, the spatial relationships, and the cyclical nature of the metabolism, it is easy to understand why it is so difficult to correlate analyses for substances containing high-energy bonds and for other metabolic intermediates with the level of activity of the cardiac muscle.

Summary

1. The actions of thirteen metabolic substrates and intermediates on the rabbit auricle have been investigated, and it was found that they all depress the normal auricle, with regard to both rate and amplitude, except citrate which at higher concentrations stimulated the rate.

2. The behaviour of the beating auricles during depletion of substrate was observed and the abilities of various substrates to revive the auricles have been determined. It was found that only glucose and pyruvate produced marked effects; glucose produced a more marked effect on muscular relaxation, and pyruvate acted particularly upon the rate.

3. The actions of eight metabolic inhibitors on the rabbit auricle have been investigated and each inhibitor was found to produce a characteristic effect. The actions of these inhibitors on the functioning of the auricles does not always parallel their commonly accepted actions on enzymatic systems, and it is concluded that unknown pathways of metabolism exist in cardiac muscle.

4. The abilities of substrates to revive auricles that have been depressed by inhibitors was studied, and the bearing of these results on the specificity of inhibitor action and the nature of myocardial enzyme systems are discussed.

5. None of the observed actions of metabolic substrates and inhibitors are due to an action on the acetylcholine equilibrium in the auricular muscle, because the presence of atropine did not modify them.

6. The mechanisms by which the auricles can be stopped in diastole or systole are discussed in the light of the information obtained on the actions of substrates and inhibitors.

7. It is questioned whether changes in amplitude are directly related to contractile mechanisms. The results obtained could be best explained by assuming that the actions of the substances investigated were upon the mechanisms by which the propagated impulse stimulates the actomyosin to contract into its low-energy state. This important phase of the cardiac functioning is termed the "excitatory reaction." Depression of this excitatory reaction, either by reducing the excitatory state of the cellular membrane or by increasing the stimulation threshold of the contractile units, leads to a reduced number of units contracting and consequently to a reduced amplitude.

8. Changes in rate, amplitude, and degree of relaxation were shown to be relatively independent of each other. From the actions of substrates and inhibitors as well as substrate depletion and anoxia, on the auricles, an attempt was made to correlate certain phases of metabolism with the mechanisms involved in rate, amplitude, and relaxation. It was concluded that contraction and relaxation are dependent on a certain supply of ATP but that the rhythmic discharge of impulses and the excitatory reaction are more directly dependent on the oxidative reactions of metabolism, particularly the tricarboxylic acid cycle.

9. Some general problems in the correlation of cardiac metabolism with cardiac function are discussed.

References

Armstrong, C. W. J., and Fisher, K. C. (1940). *J. cell. comp. Physiol.*, **16**, 103.
Banga, I., Schneider, L., and Szent-Györgyi, A. (1931). *Biochem. Z.*, **240**, 454.
Beresin, W. J. (1913). *Pflüg. Arch. ges. Physiol.*, **150**, 549.
Bogue, J. Y., Evans, C. L., and Gregory, R. A. (1937). *Quart J. exp. Physiol.*, **27**, 27.
Buchtal, F., Deutsch, A., and Knappeis, G. G. (1944). *Acta physiol. scand.*, **8**, 271.
Bülbring, E., and Burn, J. H. (1949). *J. Physiol.*, **108**, 508.
Burn, J. H., and Vane, J. R. (1949). *J. Physiol.*, **108**, 104.

(g, I. (1936). *Quart. J. exp. Physiol.*, **26**, 285.
(g, I. (1938). *Quart. J. exp. Physiol.*, **28**, 3, 137.
I sitto (1928). *Riv. Pat. sper.*, **3**, 294.
I rnini, A. (1936). *Arch. int. Pharmacodyn.*, **54**, 295.
I i, A. (1933). *Z. ges. exp. Med.*, **86**, 408.
I mann, S., and Lindsten, T. (1946). *Acta pharmacol. toxicol.*, **2**, 329.
: aki, Y. (1924). *Arch. exp. Path. Pharmak.*, **104**, 73.
(denker, F., and Rothberger, C. J. (1935a). *Arch. exp. Path. Pharmak.*, **179**, 24.
(enker, F., and Rothberger, C. J. (1935b). *Arch. exp. Path. Pharmak.*, **179**, 38.
Gi an, J. D. P. (1949). *Brit. J. Pharmacol.*, **4**, 1.
L , O. (1897). *Arch. exp. Path. Pharmak.*, **38**, 127.
Na aura, K., Saunders, P. R., Webb, J. L., Lawson, H. C., and Thienes, C. H. (1949). *Amer. J. Physiol.*, **158**, 269.
L A. B., and Potter, V. R. (1949). *J. biol. Chem.*, **178**, 241.
S , W., Livingston, A. E., and Connet, H. (1917). *J. Pharmacol.*, **10**, 129.
Sz Györgyi, A. (1947). *Chemistry of Muscular Contraction.* New York.
Va I. R. (1948). *Brit. J. Pharmacol.*, **3**, 341.
V , H. M. (1910a). *J. Physiol.*, **40**, 295.
V n, H. M. (1910b). *J. Physiol.*, **41**, 194.
W L., Saunders, P. R., and Thienes, C. H. (1949a). *Arch. Biochem.*, **22**, 444.
W L., Saunders, P. R., and Thienes, C. H. (1949b). *Arch. Biochem.*, **22**, 458.
: L. (1950). (To be published.)
V , B. (1934). *Arch. exp. Path. Pharmak.*, **174**, 383.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 240, No. 3, March 1965
Printed in U.S.A.

243-

Metabolic Characteristics of Isolated Rabbit Heart Mitochondria*

R. W. VON KORFF†

From the Departments of Biochemistry and Pediatrics, Medical School, University of Minnesota, Minneapolis 14, Minnesota

(Received for publication, April 27, 1964)

The quantitative significance of individual oxygen-consuming reactions in mitochondrial respiration studies with rapid platinum electrode polarographic recording techniques usually is defined poorly. While the use of the platinum electrode polarograph with the open type of cell has become commonplace in mitochondrial studies since its application to this purpose by Chance and Williams (3), the simultaneous use of ^{14}C-labeled substrates to measure $^{14}CO_2$ production, and to study changes in the substrate composition, has not been reported.

Jones and Gutfreund (4), using mitochondria from guinea pig liver, mammary gland, and kidney, have reported studies on the oxidation of succinate-^{14}C. Although these investigators used the platinum electrode polarograph for studying oxygen utilization, changes in substrate composition were measured in reaction media incubated in Warburg vessels. Organic acids were separated by paper chromatography, which, in general, does not yield the high degree of resolution characteristic of column chromatography.

Although respiratory activity and changes in substrate composition were not measured, Bellamy (5) analyzed mitochondria from various sources for endogenous substrates; he commented upon possible changes in endogenous substrate composition and noted that such substrates may affect studies of mitochondrial respiration with exogenous substrates.

With the use of the platinum electrode polarograph with several ^{14}C-labeled substrates (pyruvate, α-ketoglutarate, succinate), we have noted rapid and extensive changes in substrate composition. The data indicate that measurement of oxygen consumption with known added substrates is not sufficient to define the quantitative nature of the oxidative reactions.

The effects of the inhibitors malonate, parapyruvate (6), fluoroacetate, and Amytal on the isotopic substrate pattern are reported also. With pyruvate as substrate, malonate causes large increases in β-hydroxybutyrate and acetoacetate, as well as the formation of some acetate. Under the same conditions, parapyruvate results in the formation of acetate as the major product, with little or no formation of acetoacetate.

Marked differences in respiratory stimulation by various substrates and the accumulation of compounds such as alanine, β-hydroxybutyrate, glutamate, succinate, malate, and citrate in variable amounts are believed to be important with respect to the probable nature of the endogenous substrates of mitochondria. Metabolic studies with isolated mitochondria are probably influenced to a major degree by the presence and variable nature of endogenous substrates.

EXPERIMENTAL PROCEDURE

Materials and Methods—Radioactive substrates were obtained from New England Nuclear Corporation, Nuclear-Chicago Corporation, and Calbiochem. All samples were checked for radiopurity by chromatography on Dowex 1-X8 (Cl^-) columns before use. The purity and stability of pyruvate-^{14}C is of particular importance and initially was a cause of great difficulty. The problem was solved by the finding that pure sodium pyruvate-^{14}C is readily stabilized by addition of the theoretical amount of HCl necessary to convert the salt to the free acid (7).

Whenever possible, the radioactive substrates were analyzed by enzymatic methods for the true concentration of substrate. Pyruvate was determined by means of the reaction with NADH and lactate dehydrogenase (8). α-Ketoglutarate was determined by means of the reaction with NADH and glutamate dehydrogenase in the presence of NH_4Cl (9).

The specific activities of the original substrates were determined by persulfate oxidation and plating as $BaCO_3$ (10). Planchets were counted with a Nuclear-Chicago D-47 gas flow counter with a Micromil window. All results were calculated to a thickness of 6.0 mg per cm^2.

Oxygen consumption was measured with the Oxygraph (Gilson Medical Electronics, Middleton, Wisconsin) at 28° with an open cell and lightly coated electrode (11). The medium contained KCl, 0.15 M; $MgCl_2$, 4 mM; and phosphate buffer, pH 7.3, 2 to 3 mM. Substrates and nucleotides were added as indicated for the individual experiments. Just prior to addition of reagents and during the mixing, the servorecorder was turned off to avoid the recording of mixing artifacts. During this period, the pen traced horizontal lines. Although some of the traces show discontinuities resulting from traces of oxygen stirred in during mixing or occasionally due to an electrical disturbance of the event marker, the interpretation of the results is not altered by these irregularities in the trace. $^{14}CO_2$ was determined by withdrawing 0.5 to 1.0 ml of sample and transferring it to a Cavett flask containing 0.05 ml of 70% $HClO_4$ per ml of sample and 0.20 ml of 4 N KOH in the center well. After diffusion for 24 hours at room temperature, the KOH was transferred to a test tube and 1.0 ml of 0.1 M Na_2CO_3 was added as carrier. $BaCO_3$ was precipitated, the solution was neutralized, and the precipitate

* These studies were supported by grants-in-aid from the United States Public Health Service (Grant HE 02080-09), from the Minnesota Heart Association, and from the Graduate School of the University of Minnesota. Preliminary reports of some aspects of this work have appeared (1, 2).

† United States Public Health Service Research Career Development Awardee (GM-K3-15,397-04).

was filtered for counting. All samples were normalized to a standard and corrected to a thickness of 6.0 mg per cm². Samples for organic acid determinations were deproteinized with 0.05 ml of 70% $HClO_4$ per ml of sample, centrifuged, and stored at −20° until analyzed.

Mitochondria were isolated from rabbit heart (2- to 3-pound animals). The rabbits were killed with the use of 100% CO_2, and the trimmed hearts were passed through a disk with holes 1 mm in diameter by means of a stainless steel tissue press. The mince was homogenized in a Dounce-type homogenizer with a loosely fitting pestle (Blessig Glass Specialties, Rochester, New York).

The isolation medium contained KCl, 0.18 M; EDTA, 0.002 M; and Fraction V of bovine serum (Armour Pharmaceutical Company), 1 g/100 ml. A 5% stock solution of Fraction V, dialyzed against water, was used for this purpose. After three to four passes of the pestle, the homogenate was centrifuged at 200 × *g* (average) for 10 minutes. The supernatant suspension of mitochondria was centrifuged at 5000 × *g* (average) for 10 minutes. The pellet was resuspended in 10 ml of the homogenizing medium with the use of a small Dounce homogenizer with loosely fitting pestle. After collection of the mitochondria, they were suspended in 1.5 to 2.0 ml of 0.18 M KCl with the loosely fitting pestle and small homogenizer.

The greatly improved morphology of mitochondria isolated in this manner as compared to those shown previously (12) is evident from Fig. 1.

Chromatographic Separation of Acids—Partial separation of the acids was performed with a Dowex 1-X8 (Cl^-), 200 to 400 mesh (AG grade, Bio-Rad Laboratories), column. This resin frequently is contaminated with traces of HCl, leading to early elution and displacement of the peaks. The resin should be washed with water and neutralized with dilute NaOH until the pH of the supernatant solution is 6.0. A column (1 × 14 cm) was formed by pouring a water suspension of resin into a tube (1 × 30 cm) having a sintered disk, a standard taper (Ŧ) stopcock, and a ball joint. About 2 mm of water were allowed to remain above the resin.

FIG. 1. Electron micrograph of isolated rabbit heart mitochondria (× 18,000).

An aliquot of the perchloric acid supernatant solution from the reaction mixture was neutralized with 4 N KOH to pH 6.5 to 7.0 and cooled to permit precipitation of $KClO_4$. The neutralized solution was added to the resin column and allowed to run into the resin. The $KClO_4$ was rinsed with 0.5 ml of cold water, and this solution was added to the column. When this solution had just entered the resin bed, the column was carefully filled with water to the ball joint and connected to a 250-ml Erlenmeyer flask (Ŧ 24/40 neck and right-angle side arm with ball joint to fit column) filled with water and containing a magnetic stirring bar. A Ŧ 24/40, 150-ml separatory funnel, the stem of which just cleared the stirring bar, was filled with 0.100 N HCl and connected to the water reservoir. A magnetic mixer was mounted about ¼-inch below the mixing flask. The elution was started, and 2-ml fractions were collected. Suitable aliquots of the neutralized eluates were plated and counted as infinitely thin samples. Development of the chromatogram may also be monitored with the use of anthracene scintillation flow cell counting equipment. With some components, neutralization may result in marked decomposition (see "Results").

For further separation of the organic acids and for cochromatography of ^{14}C-labeled acids with known nonradioactive carrier acids, a 1-butanol-chloroform-silicic acid system similar to that described by Bulen, Varner, and Burrell (13) was employed. Silicic acid (Mallinckrodt Chemical Works), 100 mesh (labeled "suitable for chromatographic analyses by the method of Ramsey and Peterson"), was washed to remove fines as described by Bulen *et al.* The washed material must be thoroughly dried at 105°. The stationary phase was prepared as described by Kinnory, Takeda, and Greenberg (14). Silicic acid, 8.8 g, was mixed in a mortar with 5.4 ml of 0.05 N H_2SO_4 until a finely divided powder was obtained. Chloroform was washed in a separatory funnel with 0.05 N H_2SO_4, and the chloroform layer was passed through dry filter paper to remove any water droplets. The silicic acid was slurried with chloroform in a beaker and poured into a column (1.4 × 50 cm) that had a small glass wool plug, a stopcock at the lower end, and a 24/40 Ŧ joint at the top. The neutralized sample, not over 0.6 ml in volume, containing 5 to 10 μmoles of added nonradioactive acids as carrier for acids to be identified, was acidified with 0.05 ml of 3 N H_2SO_4 and mixed well with 1.0 g of dry silicic acid in a 10-ml beaker. A chloroform slurry of the sample was prepared and added to the top of the silicic acid column, which had previously been drained so that only 1 to 2 mm of chloroform remained above the silicic acid bed. After the sample had settled well, a 5-cm layer of chloroform was added to the column, which was connected to a reservoir containing 250 ml of acid-washed chloroform and a magnetic mixing bar. A separatory funnel containing 1-butanol-chloroform, 40:60 (v/v), to which 1 ml of 0.05 N H_2SO_4 had been added per 100 ml of mixed solvent, was connected to the reservoir. Fractions (3 ml) were collected into tubes containing 1.0 ml of a carefully neutralized methyl red indicator solution (a 1:20 dilution with water of a stock solution of 0.04% methyl red in ethanol). The diluted methyl red should be neutralized to an orange color. Carrier acids were readily detected by the red color produced by even traces of acidity in the

[1] Recent work has shown a marked improvement in resolution when 0.050 N HCl is used until 50 fractions have been collected. At this point the 0.050 N HCl is replaced with 0.100 N HCl.

eluates. The peaks were located by titration of the acidic tubes with 2×10^{-3} N NaOH with the use of a Vortex mixer to extract all acid into the aqueous layer during the titration.

For identification of lactate and succinate, the respective tubes from the Dowex column were neutralized and concentrated to dryness. The desired carrier acid (5 μeq) was added, and the sample was taken up in 0.5 ml of water and acidified with 0.05 ml of 3 N H_2SO_4. The solution was taken up in 1 g of dry silicic acid and added as a slurry in benzene to a silicic acid column prepared as above but eluted with about 50 ml of benzene. The acids were eluted with 1-butanol-benzene, 25:75 (v/v) (13). A gradient was not used in this case. Samples (3 ml) were collected into a methyl red solution as described above. Succinate was eluted just prior to lactate in this system.

Amino acids were separated and identified with the earlier chromatographic system described by Moore and Stein (15) with the use of Dowex 50-X8 (minus 400 mesh) ion exchange resin. For the purpose of this work, a 50-cm column was found to be adequate. The elution volumes were observed to be approximately one-half those given by Moore and Stein. The pH 3.42 citrate buffer was used for these experiments.

RESULTS

Pyruvate-^{14}C as Substrate—Pyruvate, at concentrations as low as 10μM in the presence of ADP, stimulated the oxygen consumption of these preparations, while concentrations above 100 μM and the addition of malate did not increase the State 3 rate. Fig. 2 illustrates the polarographic recording for an experiment in

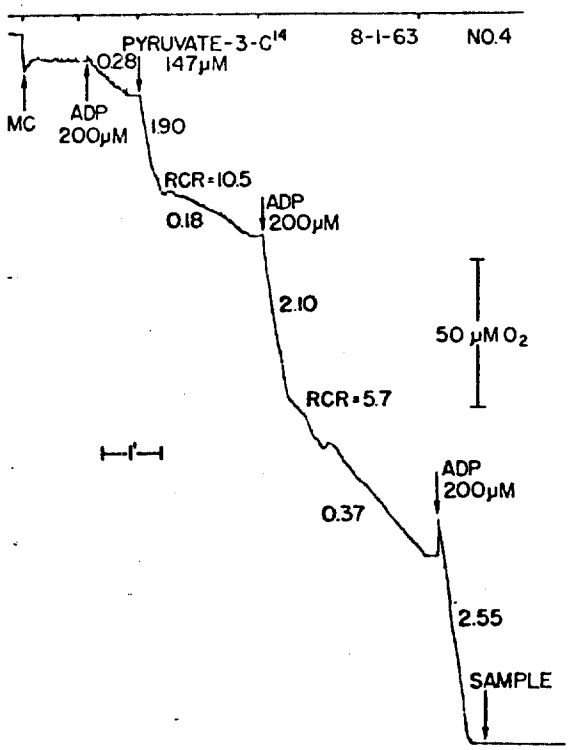


FIG. 2. Platinum electrode polarographic trace of oxygen consumption by heart muscle mitochondria with pyruvate-3-^{14}C (147 μM) as substrate. The medium contained KCl, 0.15 M; $MgCl_2$, 4 mM; and phosphate buffer, pH 7.3, 2 mM; 2.2 mg of mitochondrial protein were used. *Figures beside trace* indicate oxygen consumption in micromolar concentration per second. Time between addition of pyruvate-^{14}C and sampling was 5.0 minutes. *RCR*, respiratory control ratio; *MC*, mitochondria.

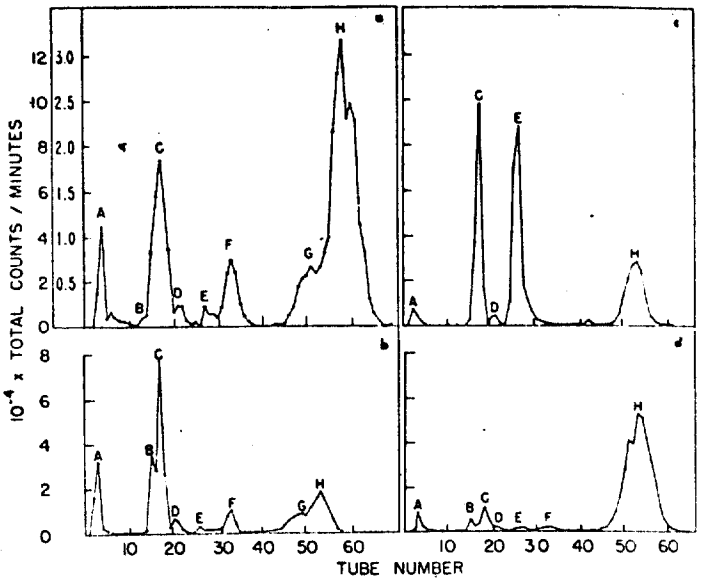


FIG. 3. *a*, organic acid profile of reaction mixtures from the experiment of Fig. 2. *Peaks A* to *D* were obtained from Dowex 1-Cl^- columns and are identified as follows: *A*, alanine, *B*, glutamate + aspartate; *C*, β-hydroxybutyrate + acetate; *D*, lactate; *E*, succinate + acetoacetate; *F*, malate; *G*, citrate; *H*, pyruvate. *b*, organic acid profile of reaction mixture with pyruvate-2-^{14}C (185 μM) as substrate in the absence of added adenine nucleotide. The amount of mitochondrial protein was 4.0 mg. Time between addition of pyruvate and sampling was 5.0 minutes. *c*, organic acid profile of reaction mixture from the malonate-inhibited reaction shown in Fig. 4. *d*, organic acid profile of reaction mixture from the fluoroacetate-inhibited reaction shown in Fig. 6.

which pyruvate-3-^{14}C was added to a concentration of 147 μM. Endogenous substrate was the sole source of dicarboxylic acid providing oxaloacetate for condensation with acetyl-CoA. A marked effect of the prior exhaustion of endogenous substrate on the State 3 (16) rate and the respiratory control ratio was reported earlier (17).[2] The respiratory controls (ratio of State 3 rate to State 4 rate) were 10.5 and 5.7, the decrease being due to an increase in the State 4 rate. For this particular preparation, an endogenous substrate respiration in the absence of ADP was not evident, although an appreciable rate frequently is observed (see Figs. 7 and 8). Substantial phosphorylation of ADP by endogenous substrate was evident from the short duration of State 3 when ADP was added prior to the pyruvate. After approximately 5 minutes, samples were withdrawn for measurement of organic acids and $^{14}CO_2$.

The organic and amino acids formed in the reaction mixture are shown in Fig. 3*a*. With pyruvate as substrate, alanine consistently was formed. Alanine presumably arises by transamination of pyruvate, the amino group being derived either from endogenous sources of amino acids or possibly from glutamate formed via α-ketoglutarate, glutamate dehydrogenase, and traces of ammonia present in the isolated mitochondria. A small peak preceding the β-hydroxybutyrate plus acetate peak contained glutamate with smaller amounts of aspartate. β-Hydroxybutyrate plus variable amounts of acetate were found consistently. Addition of malate (250 μM) in addition to pyruvate reduced but did not eliminate the β-hydroxybutyrate peak.

The small peak following β-hydroxybutyrate was identified as lactate by the addition of nonradioactive carrier succinate and

[2] E. Tarjan and R. W. Von Korff, manuscript in preparation.

TABLE I

Estimated concentrations of ^{14}C-labeled substrates present 5.0 minutes after addition of pyruvate-^{14}C

Results are given as (counts per minute in peak, calculated for a 1-ml reaction volume)/(counts per minute per ml, for 1 μmole of pyruvate-^{14}C per liter when plated and counted as were the column eluates).

Substrate	Experiment of	
	Fig. 3*a*	Fig. 3*b*
	μM	μM
Alanine	4.7	13.5
Glutamate + aspartate	0.5	12.0
β-Hydroxybutyrate + acetate	12.4	29.0
Lactate	1.4	6.7
Succinate	1.4	1.4
Malate	6.3	6.9
Citrate	7.0	9.7
Pyruvate	50.5	20.8
Other small peaks	1.4	0.5
Total	85.6	100.5
$^{14}CO_2$ recovered	32	22
Total "pyruvate" recovered	118	123
Accounted for	80%	66%
Ratio of residual substrates other than pyruvate to total residual substrates	0.41	0.79

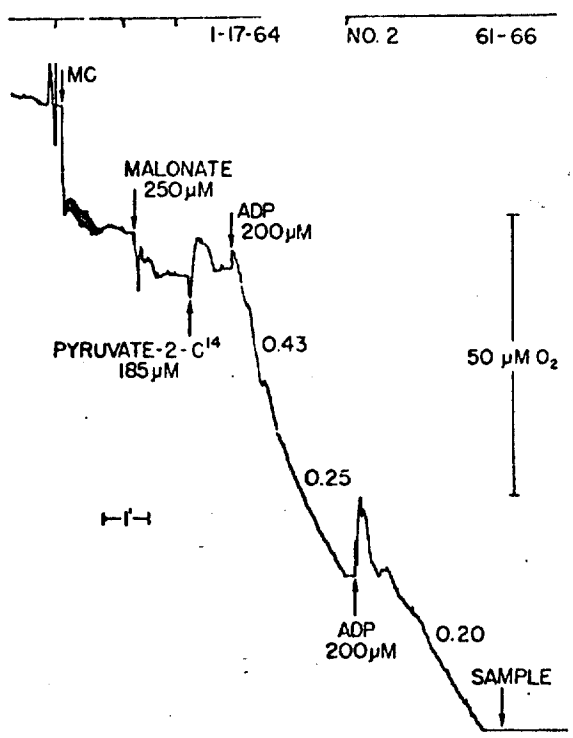


FIG. 4. Polarographic trace of oxygen consumption in a malonate (250 μM)-inhibited system with pyruvate-2-^{14}C (185 μM) as substrate. The medium was the same as in Fig. 2; mitochondrial protein, 1.0 mg. Time between addition of pyruvate and sampling was 6.2 minutes. *MC*, mitochondria.

lactate and chromatography with the silicic acid-1-butanol-benzene system of Bulen *et al.* (13). The small amount of lactate formed is consistent with the fact that lactate dehydrogenase is essentially absent from these mitochondrial preparations (18) and the fact that intramitochondrial NADH is ineffective in the extramitochondrial lactate dehydrogenase reaction (18).

The small peak in the region of tube 27 contained traces of succinate (as evidenced by chromatography with nonradioactive succinate on a silicic acid column with 1-butanol-chloroform) and acetoacetate. Only traces of acetoacetate appeared unless Krebs cycle activity was restricted by malonate.

Malate was found invariably, and may in part represent a substrate pool, although recent preliminary results suggest that malate increases with time. Citrate (tubes 45 to 53) also usually was present. Experiments with unlabeled pyruvate and $H^{14}CO_3^-$ have shown that CO_2 fixation is not a source of dicarboxylic acids in these preparations.

$^{14}CO_2$ recovered in the experiment of Fig. 2 amounted to 59,600 c.p.m. ($BaCO_3$ = 6.0 mg per cm²), equivalent to the complete oxidation of 32 μM of the original 147 μM pyruvate-3-^{14}C which was added; however, the ratio of micromoles of $^{14}CO_2$ liberated (at the specific activity of the pyruvate added) to microatoms of oxygen utilized was 0.08, indicating incomplete oxidation of the pyruvate-3-^{14}C. Complete oxidation would yield a ratio of 0.20. In a series of six experiments with pyruvate-1-^{14}C, the ratio of increase in $^{14}CO_2$ (micromoles) to decrease in O_2 (microatoms) ranged from 0.22 to 0.33 (average, 0.27), while in a comparable number of experiments with pyruvate-2- and -3-^{14}C, the ratio ranged from 0.08 to 0.17 (average, 0.10) (see "Discussion").

Fig. 3*b* illustrates the chromatogram from an experiment in which pyruvate-2-^{14}C (185 μM) was the substrate in the absence of added adenine nucleotide. After addition of substrate, the oxygen consumption rate was constant and equal to 0.37 μM per second. The ratio of micromoles of pyruvate converted to $^{14}CO_2$ to microatoms of oxygen utilized was 0.11. The organic acid pattern was essentially identical with that of Fig. 3*a*, although, on a relative basis, more radioactivity was found in the alanine, glutamate, and β-hydroxybutyrate peaks. The larger amount of amino acids in the experiment of Fig. 3*b* may reflect the greater concentration or amount of mitochondrial protein used, or both. It can be estimated[3] that in the experiments shown in Fig. 3, *a* and *b*, the approximate micromolar concentrations (micromoles per liter) of substrates present at the time of sampling was as shown in Table I. A large fraction of the added ^{14}C not oxidized to $^{14}CO_2$ was recovered in substrates other than pyruvate.

Effect of Malonate on Oxidation of Pyruvate—The effect of malonate on the respiration with pyruvate-2-^{14}C as substrate is shown in Fig. 4. Although dicarboxylic acid was not added and although malonate addition preceded the addition of pyruvate and its concentration exceeded that of pyruvate (250:185 μM), 200 μM ADP to some extent stimulated oxygen consumption

[3] The calculation involves two unlikely assumptions: (*a*) that the specific activities of the residual substrates are equal to that of the added pyruvate and (*b*) that the residual substrates are equally distributed throughout the mitochondrial volume and the medium. Recent experiments in this laboratory by Mr. Merle Olson have shown that the substrate composition within the mitochondria differs from that found in the medium.

for about 1 minute, although at a rate greatly decreased from that observed in the absence of malonate. A second addition of ADP did not stimulate oxygen consumption, although the rate continued at 0.20 μM per second. The organic acid profile from this experiment is shown in Fig. 3c. There was an increase in β-hydroxybutyrate plus acetate and a large increase in acetoacetate. The latter, in addition to acetoacetate, contained a trace of succinate and occasionally small amounts of as yet unidentified products detectable by rechromatography on silicic acid. An acetoacetate-^{14}C peak does not appear when pyruvate-1-^{14}C is the substrate. Marked decomposition of the acetoacetate peak occurred when the eluate was neutralized to an alkaline pH and stored. In this case, some acetate was formed. Elevation of the pH to 10 to 11 for 1 minute or less leads to marked decomposition. Acetoacetate-3-^{14}C purified on a Dowex 1 column did not exhibit a similar instability. Either substances less stable than acetoacetate were present, or substances may have been formed which catalyzed the decomposition of the acetoacetate present. Reduction of the material with borohydride caused most of the ^{14}C to appear in the β-hydroxybutyrate peak, although other unidentified compounds also appeared.

On rechromatography of the pyruvate peak from a malonate-inhibited experiment, some acetate is found. This acetate and variable amounts in the β-hydroxybutyrate peak may arise by decomposition of unstable precursor compounds (compare inhibition by parapyruvate).

Effect of Parapyruvate on Oxidation of Pyruvate—Parapyruvate or γ-methyl-γ-hydroxy-α-ketoglutarate is a potent inhibitor (6) of the Krebs cycle. Fig. 5 (*inset*) illustrates the inhibitory action of parapyruvate on pyruvate oxidation (in the absence of parapyruvate a rate of 1.6 μM O_2 per second was observed.) The organic acid profile yielded the interesting result that, in contrast to a malonate block, only a trace of β-hydroxybutyrate was formed and acetoacetate did not accumulate. However, a large

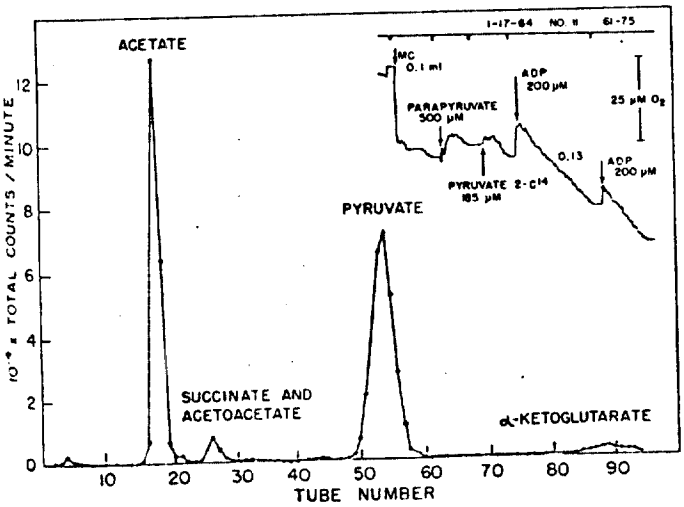


FIG. 5. The *inset* shows a polarographic trace of oxygen consumption in a parapyruvate (500 μM)-inhibited system with pyruvate-2-^{14}C (185 μM) as substrate. The medium was the same as in Fig. 2; mitochondrial protein, 1.0 mg. The *graph* shows the organic acid profile of the reaction mixture from the experiment shown in the *inset*, obtained by chromatography on Dowex 1-Cl⁻. α-Ketoglutarate was identified by rechromatography on silicic acid with carrier α-ketoglutarate. Time between addition of pyruvate and sampling was 6.1 minutes. *MC*, mitochondria.

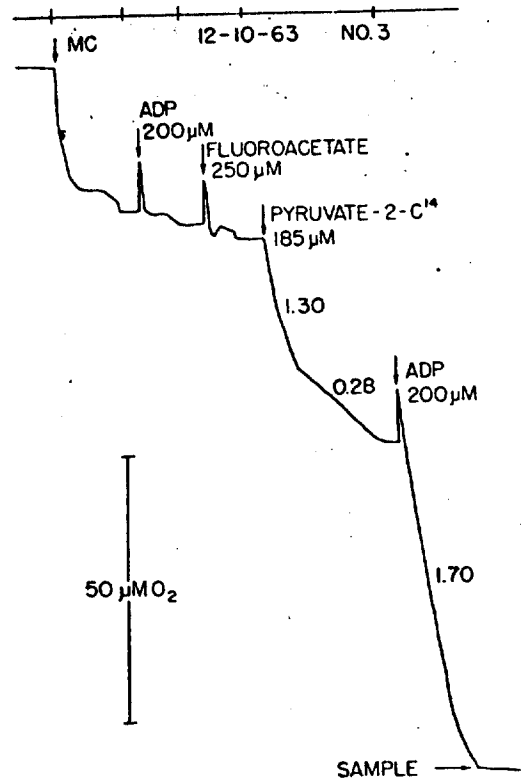


FIG. 6. Polarographic trace of oxygen consumption in a fluoroacetate-inhibited system with pyruvate-2-^{14}C as substrate. The medium was the same as in Fig. 2; mitochondrial protein, 2.0 mg. Interval between addition of pyruvate and sampling was 3.1 minutes. *MC*, mitochondria. (This figure was redrawn to double the width to height ratio.)

amount of acetate was found. The acid of tubes 83 to 94 was identified as α-ketoglutarate. Parapyruvate has been stated to inhibit the α-ketoglutarate oxidase system (19). The large increase in the formation of acetate suggests that parapyruvate may have another effect, previously undescribed.

Effect of Fluoroacetate on Oxidation of Pyruvate—Fluoroacetate at 250 μM in this system had only a small effect on the oxidation of pyruvate-2-^{14}C (185 μM). Fig. 6 illustrates the polarographic trace. The oxygen consumption rate was inhibited about 40% compared to that with pyruvate alone. The recovery of $^{14}CO_2$ was reduced to about the same extent. The organic acid profile, illustrated in Fig. 3d, shows that the principal change was a decrease in β-hydroxybutyrate formation. Acetoacetate did not accumulate. Fluoroacetate leads to the formation of fluorocitrate, which blocks the aconitase reaction (20). The failure of acetoacetate and β-hydroxybutyrate to accumulate in the parapyruvate-inhibited reaction, the failure of acetoacetate to accumulate, and the decrease of β-hydroxybutyrate in the fluoroacetate-inhibited system can be explained if the availability of succinate is decreased by parapyruvate or fluoroacetate and if succinate is necessary for the deacylation of acetoacetyl-coenzyme A to free acetoacetate prior to its reduction to β-hydroxybutyrate.[4] The presence of Krebs cycle inhibitors acting at the substrate level appeared to reduce the accumulation of citrate. In

[4] The author is indebted to Dr. Joseph Stern for suggesting this explanation for the failure of acetoacetate and β-hydroxybutyrate to accumulate in the presence of fluoroacetate or parapyruvate.

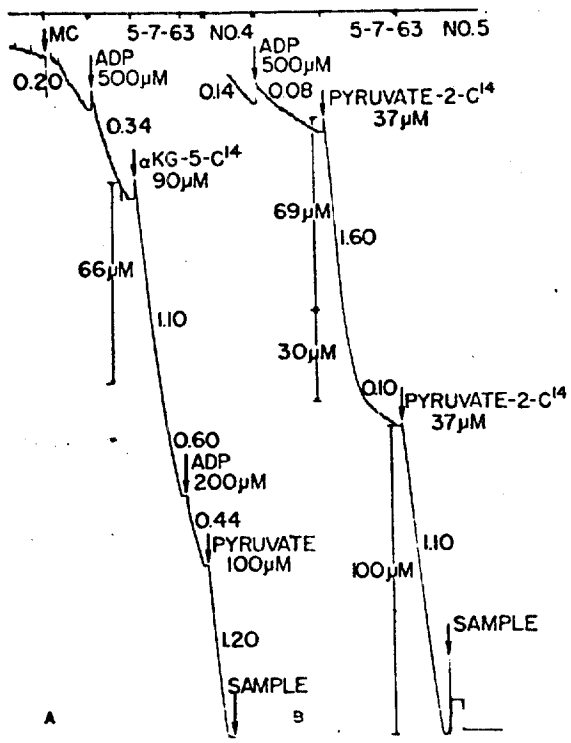


FIG. 7. *Left*, polarographic trace of oxygen consumption with α-ketoglutarate-5-^{14}C (*αKG-5-C*^{14}) (90 μM) as substrate. Excess ADP was present throughout the experiment. The mitochondria for this experiment were isolated in a solution of 0.25 M sucrose, 2 mM EDTA, 1% Fraction V of bovine serum, and 2 mM phosphate buffer. The pellet was washed once with the same solution and the mitochondria were suspended in 0.25 M sucrose. The medium was the same as in Fig. 2; mitochondrial protein, 1.8 mg. *MC*, mitochondria. *Right*, polarographic trace of oxygen consumption with pyruvate-2-^{14}C (37 μM) as substrate.

the fluoroacetate experiment when tubes 41 to 51 were rechromatographed on silicic acid, only traces of citrate were found (compare Fig. 3, *a* to *d*, and Fig. 5).

Effect of Amytal on Oxidation of Pyruvate—Amytal (1 mM) decreased the rate of oxygen consumption with pyruvate-2-^{14}C (185 μM) to about 20% of that obtained with pyruvate alone. A large decrease in the β-hydroxybutyrate peak was the only notable change in the organic acid profile. These observations are in conformity with the known action of Amytal on NAD-linked systems and an inhibition of the initial oxidation of pyruvate to acetyl-CoA.

α-Ketoglutarate and Succinate as Substrates—Neither of these substrates is as effective as pyruvate in stimulating respiration of rabbit heart mitochondria. Although α-ketoglutarate-5-^{14}C at 90 μM was about two-thirds as effective as pyruvate at the same concentration, after approximately 1 μatom of oxygen per μmole of α-ketoglutarate had been utilized, the rate decreased (Fig. 7, *left*) was not increased by further addition of ADP, but was markedly increased by 100 μM pyruvate. Fig. 7 (*right*) illustrates a control with 37 μM pyruvate. In this case, oxygen consumption was equivalent to 5.3 μatoms of oxygen per μmole of pyruvate added. Oxygen consumption was restored by a second addition of 37 μM pyruvate.

The organic acid profiles for these experiments showed that the α-ketoglutarate to a large extent was oxidized to succinate and malate. In the case of pyruvate, even though oxygen consumption exceeded the theoretical amount, alanine and β-hydroxybutyrate were present in the medium. Endogenous substrate pools probably account for the excess oxygen consumption. Although the mitochondria for this experiment were isolated in sucrose, similar results were obtained when they were isolated in KCl.

In agreement with the accumulation of succinate from α-ketoglutarate, succinate consistently was a poor substrate in these systems. The experiment of Fig. 8 shows that although 780 μM succinate did not increase the Q_{O_2} above the endogenous oxidation rate, 25 μM pyruvate yielded a rate of 0.95 μM O_2 per second. The rate decreased sharply after 90 μatoms of oxygen per liter had been utilized. The rate was not changed by ADP but was increased by a second addition of pyruvate. In this case, an even sharper change in rate occurred after 80 μatoms of oxygen per liter had been utilized. Control by pyruvate clearly is evident from this experiment, which also suggests that endogenous substrate is more effective than exogenous dicarboxylic acid in stimulating pyruvate oxidation since successive additions of 25 μM pyruvate consumed decreasing amounts of oxygen. Little increase over the endogenous rate was observed even at 1 to 2 mM succinate. Only when the concentration of succinate reached 3 to 5 mM was the rate of oxygen consumption appreciably stimulated.

DISCUSSION

Three principal aspects of the metabolic behavior of isolated rabbit heart mitochondria were evident from these experiments.

1. When pyruvate was the substrate and the polarographic procedure was used for measuring oxygen consumption, endogenous substrates supplied a source of dicarboxylic acid to maintain a complete Krebs cycle at a maximum rate.

In the usual manometric procedures the endogenous substrates may be oxidized during the temperature equilibration period, producing a requirement for added dicarboxylic acids.

The endogenous substrates contributed to the oxygen consumption and may have been present as pools of substrate reserve available to maintain a necessary minimal oxidative rate and source of energy, possibly for maintenance of structural integrity. Weinbach (21) has suggested that the capacity of liver mitochondria for oxidative phosphorylation by endogenous substrates may be related to their stability. Chance and Hollunger (22) have noted the variability in endogenous substrate content of various mitochondrial preparations and have commented on the difficulty of interpreting observations in experiments in which endogenous substrates contribute to the metabolic reactions. Bellamy (5) reported that glutamate and aspartate constituted the principal endogenous substrates of most mitochondria and that small amounts of fumarate, α-ketoglutarate, and succinate were found in some types of mitochondria.

In preliminary studies (2) we have found that isotopic substrates present during isolation of the mitochondria continue to be metabolized, leading to an accumulation of isotopic compounds in (or on) the mitochondria. The identities of these acid-soluble substrates are currently under investigation.

2. A marked difference in the ability of substrates at low concentrations to stimulate oxygen utilization by rabbit heart mitochondria is apparent. Under the conditions of these experiments, pyruvate has been the most effective substrate in stimulating oxygen utilization of the rabbit heart mitochondria. α-Ketoglutarate was the next most effective substrate, although, to a large extent, it was oxidized to succinate. In addition exogenous succinate was a poor substrate except at relatively high concentrations.

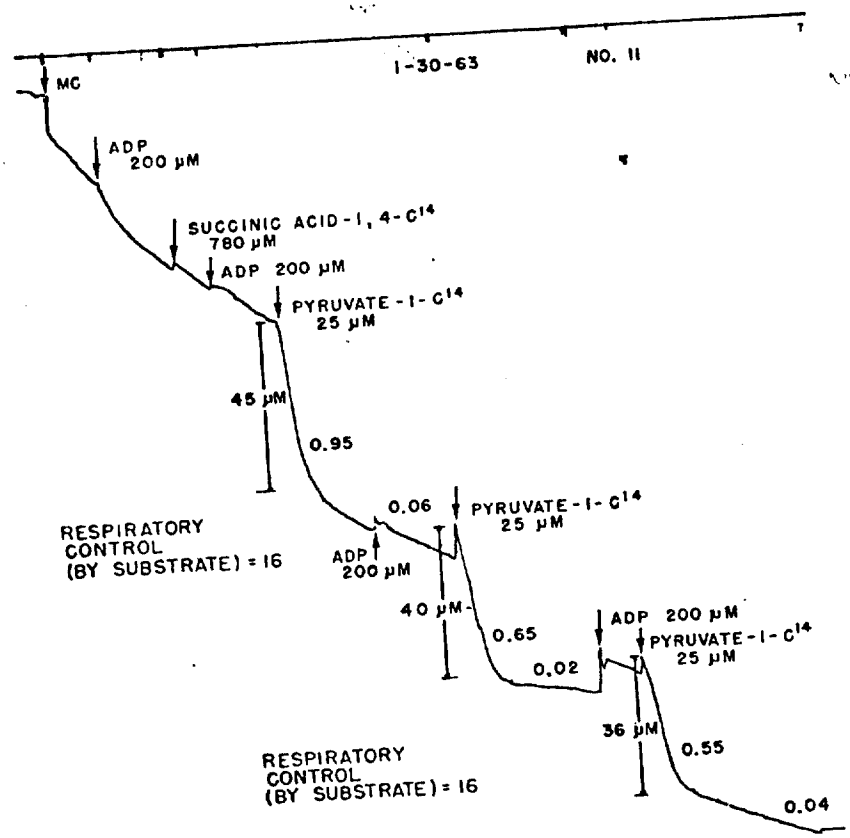


FIG. 8. Comparison of succinate (780 μM) and pyruvate (25 μM) as substrates (platinum electrode trace). The medium contained KCl, 0.13 M; $MgCl_2$, 3 mM; and phosphate buffer, pH 7.3, 3.7 mM; mitochondrial protein, 2.0 mg. *Figures beside trace* indicate micromolar concentration of O_2 per second. Total O_2 consumed, in micromoles per liter, for several additions of pyruvate is also indicated.

Chance (23) previously had noted that the affinity of mitochondria for most substrates is so low that control of electron transfer by small additions of substrate is difficult to demonstrate. α-Ketoglutarate, however, was found to be capable of yielding cyclic control of respiration (3). Pyruvate[5] also belongs in this category and, in our studies with rabbit heart mitochondria, was even more effective than α-ketoglutarate.

The rapidity with which pyruvate was metabolized even at 2° (2) and the low reactivity of succinate may well account for the difference in the apparent permeability of mitochondria for these substrates as reported by Amoore (24).

It is of interest to compare the relative abilities that these substrates have shown in stimulating mitochondrial respiration with the relative concentrations reported in plasma (25): pyruvate, in the rabbit, 210 μM, and in man, 140 μM; α-ketoglutarate, in man, 62 μM; and succinate, in man, 42 μM.

The customary procedure of using substrates in the concentration range of 5 to 10 mM in studying mitochondrial respiration may have little bearing on the situation which obtains within the intact cell.

3. The measurement of oxygen consumption alone in short term oxygen electrode experiments provides insufficient information to define the quantitative nature of the oxidative reactions at the substrate level.

The complete oxidation of pyruvate requires 5 atoms of oxygen per mole of pyruvate, while the formation of acetate or acetoacetate requires only 1 atom of oxygen per mole of pyruvate disappearing. β-Hydroxybutyrate accumulation reduces the net utilization to 0.5 atom of oxygen per mole of pyruvate disappearing. Obviously, oxygen consumption measurements may lead to a large underestimation of the pyruvate utilized if a 5:1 ratio is assumed. In like manner, $^{14}CO_2$ liberation measurements may be grossly misleading without an examination for organic acids in the medium.

The oxidation of pyruvate to acetyl-CoA obviously was not rate-determining since it was observed to exceed the rate of the Krebs cycle as a whole, leading to the formation of acetoacetate, acetate, and β-hydroxybutyrate. The residual oxygen utilization with either malonate or parapyruvate present most likely was due largely to the formation of acetoacetate, β-hydroxybutyrate, and acetate, respectively.

In these experiments β-hydroxybutyrate probably was formed by reduction of the acetoacetate formed as the primary product. Intramitochondrial NADH produced during the oxidative reactions can effect this reduction, a now well known process first demonstrated for isolated mitochondria in 1955 by Chance and Williams (16). Although in recent years most investigators have been concerned with the reduction of acetoacetate by electrons originating from succinate (see for example, Ernster *et al.* (26) and Klingenberg and von Häfen (27)), the reduction by electrons from pyruvate has also been described (28).

The inhibition of β-hydroxybutyrate formation by Amytal in the present study is not comparable to the lack of inhibition with succinate as substrate observed by Azzone, Ernster, and Weinbach (28), since in their experiments acetoacetate was added and an anaerobic oxidation-reduction system was operative. Azzone *et al.* also reported that with the liver mitochondria used, the

[5] Dr. U. Fugmann Rasmussen has observed similar effects with pyruvate and pigeon heart mitochondria (personal communication).

reduction of acetoacetate by pyruvate was enhanced when malate was added. In addition, only 0.1 μmole of acetoacetate was formed from pyruvate in either the presence or absence of Amytal.

The substantial conversion of pyruvate to β-hydroxybutyrate in the experiment of Fig. 3*b* suggests that a cycle converting pyruvate to β-hydroxybutyrate may facilitate oxidation of pyruvate when the availability of adenine nucleotide is limited.

The mechanism of acetate formation is not known. It is possible that the alkali lability of, and acetate formation from, substances present in the acetoacetate and pyruvate peaks eluted from the Dowex columns indicate the presence of substances responsible for acetate formation during the incubation period. Acetate formation by a kidney mitochondrial system has been reported by Bartley (29). Some years ago (30) we observed acetate production in manometric experiments at high pyruvate to malate concentration ratios. In those longer term manometric experiments, formation of acetoacetate and β-hydroxybutyrate was not observed.

The ratios of $^{14}CO_2$ production to oxygen utilization with pyruvate labeled at C-1, C-2, and C-3 are in agreement with the observed accumulation of ^{14}C-labeled substrates in the media and a rapid rate of pyruvate oxidation. With pyruvate-1-^{14}C the ratio of moles of $^{14}CO_2$ formed to atoms of oxygen utilized is greater than 0.20, while with pyruvate-2- and -3-^{14}C the ratios are less than 0.20. A ratio of less than 0.20 can be attributed to the accumulation of ^{14}C-labeled intermediates from pyruvate labeled at C-2 and C-3, while a ratio of greater than 0.20 for pyruvate-1-^{14}C is compatible with a rate of decarboxylation exceeding the rate of oxidation of the carbon atoms 2 and 3 and the observed accumulation of β-hydroxybutyrate and other labeled compounds.

An accumulation of succinate from low concentrations of α-ketoglutarate has also been reported by Chance and Hollunger (31), who used the appearance of the malonate sensitivity of intramitochondrial NAD reduction to detect the succinate.

SUMMARY

Isolated rabbit heart mitochondria were studied at low concentrations of ^{14}C-labeled substrates. Oxygen utilization (measured with the platinum electrode) $^{14}CO_2$ formation, and the accumulation of ^{14}C-labeled substrates (separated by column chromatography) were correlated.

The accumulation of ^{14}C-labeled substrates from pyruvate-^{14}C (notably β-hydroxybutyrate, malate, citrate, and alanine) and the observed ratios for micromoles of $^{14}CO_2$ liberated to microatoms of oxygen utilized indicated that measurements of oxygen utilization are inadequate to define the nature of the metabolic reactions occurring in such systems.

The effects of malonate, parapyruvate, and fluoroacetate on the oxidation of pyruvate (100 to 200 μM) were also tested. Malonate diverted oxidation to acetoacetate and β-hydroxybutyrate, while parapyruvate directed oxidation, by an unknown route, to acetate. Fluoroacetate was only slightly inhibitory at the concentrations tested, and did not significantly alter the pattern of substrates observed in its absence. α-Ketoglutarate was oxidized primarily to succinate and malate. Succinate at 2 mM or less did not increase the oxygen utilization rate over that due to endogenous substrate.

The observations on substrate accumulation suggest that the endogenous substrates of mitochondria are likely to be variable in composition and may alter experiments in which exogenous substrates are used, to a greater extent than would be expected solely from the oxygen utilization rate in the absence of exogenous substrate.

Acknowledgments—The procedure for isolation of the mitochondria is a modification of the procedure developed in this laboratory by Mr. Endre Tarjan. The author is indebted to Mr. Charles Good for the electron micrograph and to Miss Charlotte Rothnem for technical assistance.

REFERENCES

1. Von Korff, R. W., *Abstracts of the 145th Meeting of the American Chemical Society, New York, September 1963*, p. 107C.
2. Von Korff, R. W., *J. Cell Biol.*, **19**, 72A (1963).
3. Chance, B., and Williams, G. R., *J. Biol. Chem.*, **217**, 383 (1955).
4. Jones, E. A., and Gutfreund, H., *Biochem. J.*, **87**, 639 (1963).
5. Bellamy, D., *Biochem. J.*, **82**, 218 (1961).
6. Montgomery, C. M., and Webb, J. L., *J. Biol. Chem.*, **221**, 359 (1956).
7. Von Korff, R. W., *Anal. Biochem.*, **8**, 171 (1964).
8. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193**, 481 (1951).
9. Bernt, E., and Bergmeyer, H. U., in H. U. Bergmeyer (Editor), *Methods of enzymatic analysis*, Academic Press, Inc., New York, 1963, p. 384.
10. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E., *Isotopic carbon*, John Wiley and Sons, Inc., New York, 1949, p. 94.
11. *Gilson Medical Electronics Oxygraph model K instruction manual*, 1960, p. 9.
12. Deshpande, P. D., Hickman, D. D., and Von Korff, R. W., *J. Biophys. Biochem. Cytol.*, **11**, 77 (1961).
13. Bulen, W. A., Varner, J. E., and Burrell, R. C., *Anal. Chem.*, **24**, 187 (1952).
14. Kinnory, D. S., Takeda, Y., and Greenberg, D. M., *J. Biol. Chem.*, **212**, 379 (1955).
15. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951).
16. Chance, B., and Williams, G. R., *J. Biol. Chem.*, **217**, 409 (1955).
17. Tarjan, E., and Von Korff, R. W., *Federation Proc.*, **21**, 236 (1962).
18. Von Korff, R. W., and Twedt, R. M., *Biochim. et Biophys. Acta*, **23**, 143 (1957).
19. Montgomery, C. M., Fairhurst, A. S., and Webb, J. L., *J. Biol. Chem.*, **221**, 369 (1956).
20. Morrison, J. F., and Peters, R. A., *Biochem. J.*, **58**, 473 (1958).
21. Weinbach, E. C., *J. Biol. Chem.*, **236**, 1526 (1961).
22. Chance, B., and Hollunger, G., *J. Biol. Chem.*, **238**, 418 (1963).
23. Chance, B., in G. E. W. Wolstenholme and C. M. O'Connor (Editors), *Ciba Foundation symposium on the regulation of cell metabolism*, Little, Brown and Company, Boston, 1959, p. 91.
24. Amoore, J. E., *Biochem. J.*, **70**, 718 (1958).
25. Albritton, E. C. (Editor), *Standard values in blood*, W. B. Saunders Company, Philadelphia, 1952, p. 91.
26. Ernster, L., Azzone, G. F., Danielson, L., and Weinbach, E. C., *J. Biol. Chem.*, **238**, 1834 (1963).
27. Klingenberg, M., and von Häfen, H., *Biochem. Z.*, **337**, 120 (1963).
28. Azzone, G. F., Ernster, L., and Weinbach, E. C., *J. Biol. Chem.*, **238**, 1825 (1963).
29. Bartley, W., *Biochem. J.*, **53**, 305 (1953).
30. Von Korff, R. W., and Macpherson, E. H., *Federation Proc.*, **13**, 315 (1954).
31. Chance, B., and Hollunger, G., *J. Biol. Chem.*, **236**, 1555 (1961).

other than those of
respect it apparently
e leukemia viruses,
riety of cells under
ental conditions and
ular antigens charac-
2,10,11). A more ac-
lanation is that the
variant virus that is
e strains. This might
mutant MTV with
(b) a mutant MTV
plicating in leukemia
does not, (c) a re-
TV and a leukemia
e leukemia virus re-
maturation. The oc-
eukemias with the G
(6) indicates that in-
Gross virus does occur
ng the opportunity for
ween MTV and leu-
leukemias have cel-
d w wild-type mu-
ot do not, so this
virus determining ML
ML+ leukemias do
kemias of probably all
or may not be G+.
e is a good case for
virus inducing ML
unique, its relation to
ruses is still uncertain.
mmary Leukemia) cel-
by specific cytotoxic
the cells of mammary
d mice and on the cells
ias (ML+ leukemias).
leukemias are rejected
compatible mice not in-
not by MTV-infected
type. Heightened resist-
mammary tumors can
pient mice by immuno-
is from donors that have
anted leukemias.

A., Old, L. J., and Cars-
1033 (1964).
se, E. A., Federation Proc.

d, , Moore, D., Geer-
rolo , 1 (1967).

4. Boyse, E. A., Transplantation Bull. 7, 100 (1960).
5. Old, L. J., Boyse, E. A., Clarke, D. A., and Carswell, E., Ann. N. Y. Acad. Sci., 101, 80 (1962).
6. Old, L. J., Boyse, E. A., and Stockert, E., Cancer Res. 25, 813 (1965).
7. Morton, D. L., Proc. Amer. Assoc. Cancer Res. 5, 46 (1964).
8. Lavrin, D. H., Blair, P. B., and Weiss, D. W., Cancer Res., 26, 293 (1966).
9. Stück, B., Old, L. J., Boyse, E. A., Nature 202, 1016 (1964).
10. Boyse, E. A., Old, L. J., Aoki, T. in "Treatment of Burkitt's Lymphoma," U.I.C.C. Monograph Vol. 8, Burchenal, J. H. and Burkitt, D. P., eds., p. 248. Springer, Berlin, 1967.
11. Hall, W. T., Andresen, W. F., Sanford, K. K., Evans, V. J., and Hartley, J. W., Science 156, 85 (1967).

Received October 12, 1967. P.S.E.B.M., 1968, Vol. 127.

Anaerobic Rat Heart: Effect of Glucose and Krebs Cycle Metabolites on Rate of Beating* (32612)

J. CASCARANO, W. L. CHICK, AND I. SEIDMAN[1]

Department of Zoology, University of California, Los Angeles, Calif. 90024, and Department of Pathology, N.Y.U. Medical Center, New York, N. Y. 10016

Energy production in mammalian hearts exposed to conditions of anaerobiosis has been considered insufficient to maintain beating (1–5). This phenomenon has also been held to be common to other endergonic functions occurring in a variety of mammalian cells (6). However, it has since been shown in an investigation of one such case, active cation transport in anaerobic rat liver slices, that electron accepting metabolites could maintain activity at a level comparable to that attained in oxygenated tissues (7,8). The rationale behind these studies was that electron accepting metabolites should theoretically enhance anaerobic energy production by stimulating (A) mitochondrial phosphorylation and (B) regeneration of cytoplasmic NAD. However, the ability to stimulate anaerobic energy production might be peculiar to liver since many of its cells appear to have a low requirement for oxygen. The intent of the present investigation was to determine if a comparable anaerobic stimulation of cellular function could be achieved in a very active tissue having a high requirement for oxygen. Heart was selected due to its overriding importance in the maintenance of life processes. In addition the automaticity of myocardial contractions provides a physiological parameter which could be easily observed under controlled conditions *in vitro*. Heart perfusions were conducted under an oxygen atmosphere followed by a period of anaerobiosis with media designed to stimulate glycolytic and mitochondrial ATP production. Myocardial contractions under anaerobiosis required the presence of glucose; rate of beating was concentration dependent. Anaerobic myocardial rate was significantly elevated by the further addition of mitochondrial metabolites.

The perfusion procedure was essentially that of Morgan *et al.* (4). Hearts were from male, Sprague-Dawley rats (300–350 gm). A pulsatile pump maintained coronary flow at 7 ml/min while the pressure was allowed to vary. Electrodes were implanted in the walls of the right and left ventricle and connected to an Offner dynograph recorder. Beating was found to be regular in almost all preparations. Arrhythmias occurred infrequently and appeared to bear no relationship to any specific experimental protocol. Rates of ventricular contractions obtained by direct visual observation were identical to those obtained by counting recorded R waves. Heart rates were

* This study was supported by U.S. Public Health Service Grant HE-5166 as well as the Health Research Council of the City of New York (U-1328).

[1] Career Scientist of the Health Research Council of the City of New York (I-275).

[2] Glucagon-free crystalline zinc insulin, lot PJ-4609, obtained through the courtesy of Eli Lilly & Co.

TABLE I. Dependence of Anaerobic Heart Rate on Glucose Concentration.

The first 10.0 ml of perfusate was discarded. Perfusion was then continued with 15.0 ml of recirculated Krebs-Ringer-bicarbonate (KRB) for 21 min under 95% O_2, 5% CO_2. At 21 min, the gas phase was changed to 95% N_2, 5% CO_2, and 20.0 ml of control or experimental KRB was added to the perfusion reservoir. The hearts were then maintained under anoxia for 65 min (21-86 min of perfusion time). At 86 min of perfusion time the gas phase was changed back to 95% O_2, 5% CO_2 and the experiment was continued for an addition 30 min.

Protocol:		O_2, KRB ⟷	Substrate or KRB ⟷	O_2 ⟷
		0-21 min	21-86 min	86-116 min
Recording time[a] (min)		5-21	45-86	100-116
		(Av. beats/min)		
O_2 throughout				
Substrate free	(5)	159 ± 22.3	165 ±23.0	163 ± 30.0
N_2 (21–86 min)				
Glucose (m*M*)				
None	(5)	168 ± 5.7	0	0
1.25	(8)	145 ± 16.0	0	—[b]
2.5	(8)	150 ± 6.3	4 ± 1.3	93 ± 12.8
5.0	(8)	155 ± 11.0	7 ± 1.8	122 ± 8.0
5.0 + Insulin	(8)	169 ± 13.2	17 ± 2.8	117 ± 15.0
10.0	(8)	147 ± 7.3	23 ± 4.2	133 ± 13.5
20.0	(14)	163 ± 6.3	30 ± 2.9	144 ± 7.0
40.0	(7)	180 ± 12.6	27 ± 6.6	132 ± 13.0

[a] Heart rates were determined every 5 min and averaged over the periods indicated to allow for equilibration and stabilization following experimental innovation. Values shown are ± SEM. Number of experiments are in parentheses. Insulin was used at a final concentration of 0.1 unit/ml.

[b] Only one heart showed any recovery—53 beats/min.

determined for every 5-th min throughout the experiment. These values were then averaged for the time periods indicated in the tables. The details of the experimental protocol are indicated in Table I.

The perfusing solution was Krebs–Ringer–bicarbonate (9), and contained substrate under experimental conditions. Glucose was dissolved in the Krebs–Ringer–bicarbonate to give the desired concentration. Acid substrates were first neutralized with NaOH to pH 6.5 and substituted for NaCl in the Krebs–Ringer–bicarbonate. The Na^+ concentration was constant at 142 meq/liter. Substrate concentrations indicated in the tables are final concentration after tip-in. Final pH of the perfusing solutions was 7.4.

The first experiment was designed to determine the influence of anoxia on hearts perfused with a metabolite-free medium. When hearts were oxygenated for the full 116 min beating continued throughout the experiment. However, beating steadily diminished in anoxic hearts and ceased entirely after the 40th min of perfusion time. Reinstitution of oxygen did not restore myocardial contraction (Table I).

Although there appeared to be sufficent endogenous metabolite to maintain aerobic tissue it was thought that failure of automaticity under conditions of anoxia might in part be the result of an insufficient metabolite pool for anaerobic energy producing reactions(5). Hearts were therefore perfused with glucose over a concentration range of 1.25–40 m*M* (Table I). Except for the lowest concentration studied, beating continued throughout the entire 65 min of anaerobiosis. Rate was concentration dependent and achieved a maximum at 20 m*M*. Addition of insulin[2] to the 5 m*M* glucose medium or increasing glucose concentration to 10 m*M* significantly accel-

TABLE II. Combined Effect of Glucose and Various Metabolites on Anaerobic Heart Rate.[a]

Protocol:		O_2, KRB	N_2, Substrate	O_2 Restored
		⟷	⟷	⟷
Recording time (min):		0-21 min 5-21 min	21-86 min 45-86 min	86-116 min 100-116 min
		(Av. beats/min)		
Glucose (5 m*M*)	(8)	155 ± 11.0	7 ± 1.8	122 ± 8.0
+F	(11)	157 ± 13.9	14 ± 2.2[b] $p < .01$	106 ± 9.0
+F+M	(10)	176 ± 12.9	26 ± 3.5 $p < .05$	187 ± 15.0
+F+M+G	(10)	185 ± 13.8	38 ± 3.8 $p < .001$	146 ± 15.1
+M+G	(10)	156 ± 15.8	16 ± 1.8	73 ± 19.1
+O	(12)	184 ± 20.5	24 ± 4.0[b] $p < .025$	136 ± 14.1
+O+K	(8)	166 ± 13.8	46 ± 8.0 $p < .001$	120 ± 15.3
+O+K+DNP	(8)	169 ± 11.3	3 ± 0.9	33 ± 11.5
Glucose (20 m*M*)	(14)	163 ± 6.3	30 ± 2.9 $p < .05$	144 ± 7.0
+F+M+G	(10)	163 ± 13.6	41 ± 4.6	141 ± 9.6
+O+K	(4)	142 ± 14.3	45 ± 5.4	90 ± 24.0

[a] Abbreviations: F, fumarate (15 m*M*); M, L-malate (10 m*M*); G, glutamate (15 m*M*); O, oxalacetate (15 m*M*); K, α-ketoglutarate (15 m*M*); DNP, 2,4-dinitrophenol (10 μ*M*);

[b] Significantly different than 5 m*M* glucose control: *F*, $P < .025$; O, $p < .005$. The *p* values in the table compare experiments immediately above and below. All other conditions identical to Table I.

erated anaerobic heart rate above the 5 m*M* control ($p < .01$). These results are consistent with the glucose uptake and phosphorylation data of Morgan *et al.* (4). It is also evident that restoration of oxygen resulted in an increase in heart rate at 2.5 m*M* glucose and above. At 10 m*M* glucose there was no significant difference between initial and final rates ($0.3 < p < 0.4$).

In addition to the well-recognized contribution of glycolysis to anaerobic energy production, there is experimental evidence implicating the importance of mitochondria in this regard. Sanadi and Fluharty (10) obtained anaerobic ATP synthesis when beef heart submitochondrial particles used fumarate as electron acceptor, and NADH as electron donor. A comparable reaction was demonstrated in sarcosomes of *Ascaris lumbricoides;* formation of ATP was coupled to a dismutation of malate (11). In both studies it was found that 2,4-dinitrophenol uncoupled ATP synthesis. These reactions apparently involve a functional portion of the electron transport chain with a coupled phosphorylation.

Hearts were perfused with glucose-free solutions containing furmarate, malate and glutamate. These metabolites were completely ineffective in the absence of glucose. However, combination with 5 m*M* glucose produced a pronounced stimulation of anaerobic beating (Table II). Each substrate clearly contributed to enhancement of activity; a 5.4-fold increase was obtained when all three were combined with glucose. Combination with 20 m*M* glucose did not elevate the maximal response.

In view of the previously mentioned mitochondrial studies a reasonable explanation of this phenomenon is that the added metabolites stimulated mitochondrial ATP production under anaerobiosis. The importance of fumarate in promoting anaerobic beating is obvious

since both its addition and removal from the perfusing medium caused drastic changes in response. Enhancement by glutamate most likely resulted from its ability to stimulate removal of oxalacetate, the inhibitory product of malate oxidation (12).

The possibility exists that these reactions generate α-ketoglutarate and ATP is then formed by a substrate-level phosphorylation following oxidation of α-ketoglutarate to succinyl-CoA. To test this hypothesis experiments were conducted with NaCN and hydrazine. Since these compounds react with both aldehydes and ketones, it was presumed that their overall effect might be detrimental because they would react with glucose as well as with oxalacetate. For this reason cyanide and hydrazine were not simply substituted for glutamate. It was reasoned that hearts should be first perfused with glucose, fumarate and malate under anaerobiosis allowing oxalacetate to accumulate. If ATP production is coupled to oxidation of malate and reduction of fumarate, then addition of cyanide or hydrazine should remove the inhibitory product, oxalacetate, and accelerate oxidation of malate. This would in turn produce a transient stimulation of heart rate before substantial levels of glucose are depleted. On the other hand, if the ATP producing reaction is a succinyl-CoA substrate-level phosphorylation then addition of these materials should precipitously reduce heart rate, since they will remove oxalacetate, α-ketoglutarate. and succinyl-CoA. Results of this experiment are shown in Fig. 1. Both of these agents resulted in an immediate marked stimulation of heart rate. Cyanide was alkaline (pH 11.1) and changed the pH of the perfusion medium from 7.4 to 9.0. Its effect, though marked, was transient and hearts stopped beating completely within 2 or 3 minutes. Hydrazine sulfate was neutralized to pH 7.4 before introduction. It also produced a rapid stimulation of heart rate, which subsequently diminished but remained substantially higher than the original anaerobic level. Addition of NaCl instead of NaCN or hydrazine produced no change in the anaerobic response. In the light of these experiments, the succinyl-CoA substrate-level phosphorylation hypothesis is not tenable for the stimulation produced by fumarate, malate, and glutamate.

Another ATP producing reaction, has been described by Hunter (13) for liver and kidney mitochondria and involves reduction of oxalacetate and oxidation of α-ketoglutarate. This is apparently a substrate-level phosphorylation since no ATP is produced in a system having β-OH butyrate substituted for α-ketoglutarate (14).

Perfusions conducted with oxalacetate and α-ketoglutarate in a glucose-free medium were also completely ineffective in either maintaining automaticity or restoring beating upon reoxygenation. Combination with 5 m*M* glucose was extremely effective in elevating the anaerobic response, both metabolites clearly augmenting the 6.6-fold elevation (Table II). Incorporation with 20 m*M* glucose did not elevate maximal activity.

The response to α-ketoglutarate and oxalacetate was abolished by 10 μ*M* DNP, a concentration that stimulates respiration of heart (15). Although this response might suggest that uncoupling of phosphorylation has occurred, the influence of DNP must be interpreted with caution. Even though DNP does not inhibit substrate-level phosphorylation, the possibility does exist that it diminishes ATP levels by stimulating mitochondrial adenosine triphosphatase (16). The latter factor might account for the inhibition of heart rate.

It should be pointed out that a cytoplasmic malic dehydrogenase has been demonstrated in mammalian heart (17,18). It is conceivable that oxalacetate serves to stimulate regeneration of extramitochondrial NAD and this in turn increases overall glycolysis. However, according to Morgan *et al.* (4), anaerobic glycolysis is rate limited by glucose at concentrations of 5.56 m*M* or lower since no free intracellular glucose is detectable in either the presence or absence of insulin. On this basis it would appear that at 5 m*M* glucose, glycolysis could not be facilitated by accelerating regeneration of cytoplasmic NAD. The stimulation of anaerobic beating by oxalacetate and α-ketoglutarate can best be explained by a mitochondrial activity (coupled phosphorylation, substrate-level phosphorylation or both).

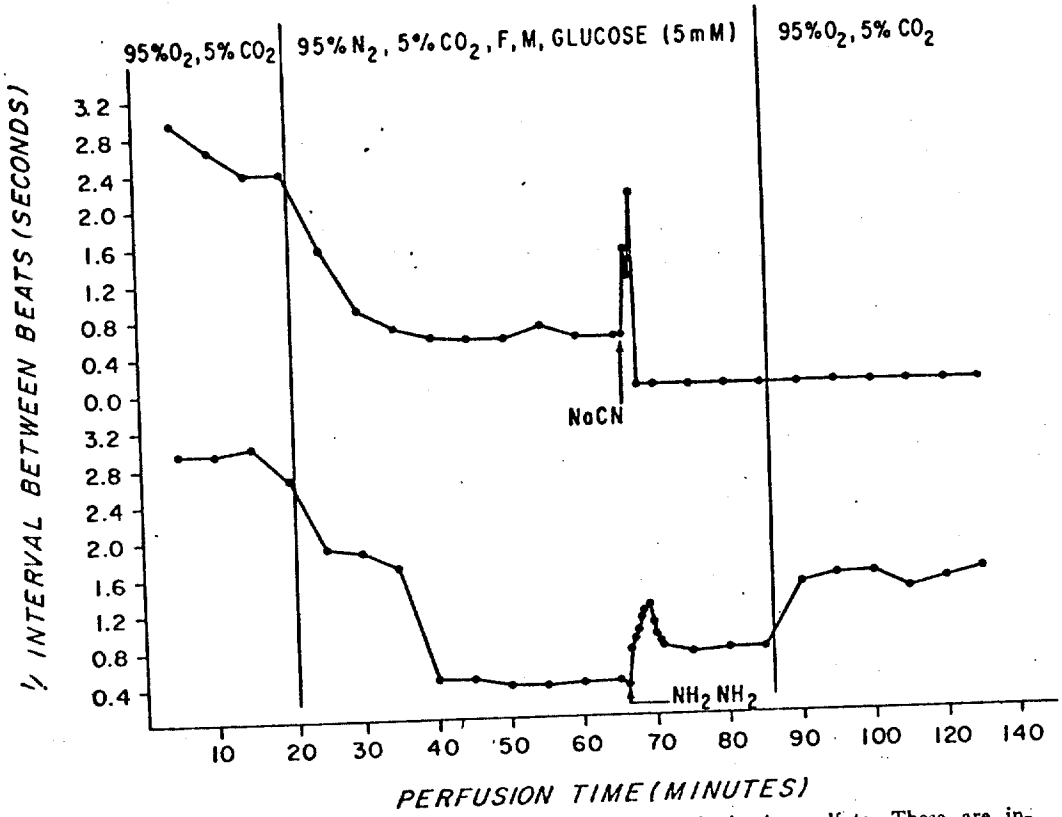


FIG. 1. Stimulation of anaerobic heart rate by NaCN and hydrazine sulfate. These are individual representative experiments. Indentical responses were obtained in all repetitions (4 for each experiment.) The perfusion protocol is the same as that outlined in Table I. At 21 min, glucose (5 m*M*) was introduced in combination with F, fumarate (15 m*M*) and M, malate (10 m*M*). At 66 min, 1.0 ml of either 0.36 *M* NaCN (pH 11.1) or 0.72 *M* hydrazine sulfate (pH7.4) was added to give a final perfusing concentration of 10 m*M* NaCN and 20 m*M* hydrazine sulfate. Final pH of NaCN perfusate was 9.0. The reciprocal of the interval between beats in seconds is plotted as the ordinate; this is comparable to beats per second.

The present study illustrates the overwhelming importance of metabolite environment in maintaining myocardial beating under anaerobiosis. It has been demonstrated that automaticity can be maintained under prolonged anoxia with little loss of responsiveness when hearts are restored to oxygen. Although the importance of glucose to anaerobic energy metabolism has a long established acceptance, no such role has been assigned to mitochondria. The data presented indicate that mitochondrial production of ATP under anaerobiosis might be of considerable physiological importance. This could conceivably have significant implications with regard to certain pathological conditions as well as various forms of physiological adaptation (19).

Summary. Perfused rat hearts subjected to anoxia rapidly stopped beating and did not recover when restored to oxygen 65 min later. However, anoxic beating was promoted by glucose in a concentration dependent fashion; maximal response was achieved at 20 m*M*. Addition of insulin or select Krebs cycle metabolites to the glucose (5 m*M*) perfusing medium further elevated anaerobic beating.

1. Winterstein, L., Z. Physiol. 4, 333 (1904).
2. Rusch, H., Pfluegers Arch. Ges. Physiol. 73, 533 (1898).
3. Bing, R. J., Physiol. Rev. 45, 171 (1965).
4. Morgan, H. E., Henderson, M. J., Regen, D. M., and Park, C. R., J. Biol. Chem. 236, 253 (1961).
5. Fisher, R. B. and Williamson, J. R., J. Physiol. 158, 86 (1961).

6. Whittam, R., in "The Cellular Functions of Membrane Transport," Hoffman, J. F., ed., p. 139. Prentice-Hall, Englewood Cliffs, New Jersey, 1964.

7. Cascarano, J. and Seidman, I., Biochem. Biophys. Acta 100, 301 (1965).

8. Seidman, I. and Cascarano, J., Am. J. Physiol. 211, 1165 (1966).

9. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques," 4th ed., p. 132. Burgess, Minneapolis, Minnesota, 1964.

10. Sanadi, D. R., and Fluharty, A. L., Biochemistry 2, 523, (1963).

11. Seidman, I. and Entner, N., J. Biol. Chem. 236, 915 (1961).

12. Colowick, S. P. and Kaplan, N. O., "Methods in Enzymology," Vol. 2, p. 184. Academic Press, New York, 1955.

13. Hunter, F. E., J. Biol. Chem. 177, 361 (1949).

14. Hunter, F. E., in "Phosphorous Metabolism," McElroy and W. D. Glass, B., ed., Vol. 1, p. 297. Johns Hopkins Press, Baltimore, Maryland, 1951.

15. Fisher, R. B. and Williamson, J. R., J. Physiol. 158, 102 (1961).

16. Lehninger, A. L., "The Mitochondrion," p. 93. Benjamin, New York. 1964.

17. Sacktor, B. and Dick, A. R., Science, 145, 606 (1964).

18. Pette, D. and Luh, W., Biochem. Biophys. Res. Commun., 8, 283 (1962).

19. Rubin, A. D., Cascarano, J., and Zweifach, B. W., J. Exptl. Med. 114, 685 (1961).

Received September 15, 1967. P.S.E.B.M., 1968, Vol. 127.

Role of Monocytes in the Mixed Leukocyte Culture Reaction* (32613)

JULIUS GORDON[1] (Introduced by Lloyd D. MacLean)
Department of Experimental Surgery, McGill University, Montreal, Canada

The mixed leukocyte culture reaction (MLCR) refers to, and is detected by, the transformation and mitosis of some of the lymphocytes, when suspensions of peripheral white blood cells from two genetically different donors are allowed to interact in culture (1). In this reaction the part played by white blood cells other than lymphocytes is not known. Experiments reported in this communication show that lymphocytes isolated from suspensions of peripheral leukocytes from two donors do not transform when cultured together. The reactivity of these purified lymphocytes in mixed cultures can be revealed by the addition of purified monocytes or suspensions of peripheral leukocytes in which the lymphocytes had been rendered unreactive by 2000 R X-irradiation (2) or mitomycin (3). Furthermore, reactions will also occur between purified lymphocytes of one donor and monocyte preparations, devoid of reactive lymphocytes, of another.

* This investigation was supported by grants from The John A. Hartford Foundation, Inc. and the Medical Research Council of Canada.

[1] Research Associate of the Medical Research Council of Canada.

Heparinized human peripheral leukocytes were fractionated on columns of glass powder (4) and cotton wool. Leukocytes, 50–200 $\times 10^6$, in autologous plasma were applied on columns of glass powder. The first fraction eluted with plasma contained predominantly lymphocytes (lymphocytes from glass). A second fraction, eluted with a solution of ethylenediaminetetraacetate (EDTA), contained monocytes and neutrophils and no lymphocytes (monocytes–neutrophils from glass). In two experiments, this second fraction was incubated for 4–7 days at 37°C to allow degeneration of the neutrophils. Cells recovered from these cultures were essentially pure macrophages. The lymphocytes from glass were further purified by passage on a column (1 $\times$ 25 cm) loosely packed with cotton wool. The first fraction, eluted with medium 199 (Microbiological Associates, Bethesda, Md.) contained pure lymphocytes (lymphocytes from cotton wool), whereas a second fraction, eluted with EDTA, contained monocytes and neutrophils and trapped lymphocytes (leukocytes from cotton). In an alternative procedure the lymphocytes were purified directly on cotton wool. Leukocytes

Vascular effects of the Krebs intermediate metabolites[1]

EDWARD D. FROHLICH[2]
Medical Service, VA Research Hospital, and Department of Medicine, Northwestern University Medical School, Chicago, Illinois

FROHLICH, EDWARD D. *Vascular effects of the Krebs intermediate metabolites.* Am. J. Physiol. 208(1): 149–153. 1965.—The vascular effects of seven intermediary products of oxidative metabolism (acetate, citrate, fumarate, malate, α-ketoglutarate, oxalacetate, and succinate) were studied on the constantly perfused kidney or forelimb in 32 dogs. Significant vasodilation was obtained at submaximal dosages, usually 2.47 μmole/min, in both vascular beds for all agents without changing systemic pressure. At maximal infusion rates renal resistance decreased approximately 20%. Saline had no effect. When segmental resistances were calculated for the forelimb by measuring pressures in large and small arteries and veins, active dilation was localized to small vessel segment (25% fall). Arterial and venous segment resistances were unchanged. It is concluded that the Krebs intermediates should be included among those agents termed "vasodilating metabolites," and that these compounds could be exceedingly important in the local regulation of blood flow.

vascular resistance hemodynamics vasoactive metabolites renal circulation forelimb circulation acetate citrate fumarate malate α-ketoglutarate oxalacetate succinate local regulation of blood flow

EVER SINCE 1879, when Roy and Brown suggested that there may be a local mechanism independent of the central nervous system for the control of blood flow to tissue (16), investigators have been searching for substances which dilate vessels during increased metabolic activity. To date these substances remain unknown and have therefore been referred to as "unidentified vasodilator metabolites" (17).

In previous studies bicarbonate and lactate failed to produce vasodilation; but, in high dosages, infusions of the adenosinephosphates (AMP, ADP, and ATP), acetate, citrate, and pyruvate resulted in dilation in the dog forelimb and coronary circulation (7, 13). Unfortunately, in these studies, the concentration of each anion greatly exceeded physiological concentrations. Because of this factor, and the notion that dilation occurred with anions closely related to the tricarboxylic acid cycle, this observation was repeated and extended using more physiological concentrations of seven intermediate metabolites.

The effects of these substances were first studied in the kidney, an organ of low vascular resistance. If dilation were to occur, this effect would be all the more significant. The amount of each metabolite infused at the highest dosage was calculated to approximately double the reported normal plasma concentrations (1). Having demonstrated a vascular effect in the kidney, the dose-response effects were also obtained in the dog forelimb to localize their site of action to specific vascular segments.

METHODS

The local effects of each intermediate metabolite upon vascular resistance were determined in 32 mongrel dogs (avg wt, 17.7 kg) by holding blood flow constant either to the right kidney or left forelimb and measuring the perfusion pressure responses to the local intra-arterial infusion of equimolar, isotonic solutions of each agent. The animals were anesthetized with sodium pentobarbital (35 mg/kg); heparin sodium (5 mg/kg) was administered intravenously.

Renal perfusion. The right kidney of 10 dogs was exposed retroperitoneally through a flank incision and retracted medially to expose the renal artery. The preparation has been described in an earlier communication (8). A Sigmamotor pump was then interposed between the right femoral and renal arteries, the flow of which was held constant at a level which approximated renal arterial perfusion pressure with systemic arterial pressure. At no time was blood flow to the kidney interrupted for more than 1 min, and particular care was taken to avoid trauma to the periarterial nerves. At the termination of each experiment the blood pump was calibrated to re-

Received for publication 27 May 1964.

[1] This work was completed during a tenure of a Veterans Administration Clinical Investigatorship and was supported, in part, by a grant from the Chicago Heart Association.

[2] Veterans Administration Clinical Investigator and Associate in Medicine, Northwestern University School of Medicine. Present address: Research Division, Cleveland Clinic, Cleveland, Ohio.

TABLE 1. *Characteristics of the infused solutions*

Solution*	Osmolality, milliosmols/liter	Metabolite Concn, mg/ml
Chloride	308	
Acetate	298	0.059
Citrate	298	0.190
Fumarate	296	0.114
α-Ketoglutarate	296	0.114
Malate	297	0.132
Oxalacetate	295	0.130
Succinate	296	0.116

* Each solution was prepared to pH 7.4 as the sodium salt and has the molar concentration of 1 μmole/liter.

check blood flow, and each kidney was weighed to determine the blood flow per gram of renal tissue. The average renal blood flow was 75 ml/min (range: 55–110 ml/min), which provided an average flow of 1.3 ml/min per gram of kidney tissue. Systemic and renal arterial perfusion pressures were measured from a cannulated left femoral artery and the perfusion tubing just proximal to the cannula, respectively, using two Statham pressure transducers attached to a direct-writing oscillograph. Even though renal vein pressure was not measured, an index of renal vascular resistance was obtained by dividing the arterial perfusion pressure by the blood flow and expressed as millimeters Hg per milliliter per minute.

Each agent was prepared as the isotonic sodium salt in a concentration of 1 μ mole/ml by dissolving 1½ mmole of the acid of each intermediate in 500 ml normal saline. The pH of each solution was corrected to 7.4 by careful titration with a small quantity of 20% NaOH. Each substance was infused into the renal artery perfusion tubing upstream from the pump, in randomized sequence, at the following rates: 0.49, 1.23, 2.47, 4.94, and 9.89 ml/min, in that order. Pressures in the renal artery and aorta were recorded simultaneously during the 1-min infusion of each metabolite at each dosage rate. Pressures of six consecutive pulsations at 30 sec and 1 min were averaged to calculate the changes in renal vascular resistance.

Forelimb preparation. The left forelimb of 22 mongrel dogs was prepared for constant perfusion using the technique described by Haddy et al. (11). In brief, the blood pump was interposed between the left femoral and brachial arteries, the flow of which was adjusted to approximate forelimb perfusion pressure with systemic arterial pressure, as measured from a catheter inserted through the right femoral artery into the abdominal aorta. A small artery and vein in the footpad were cannulated, and the large cephalic vein and arterial perfusion tubing were needled. The small artery cannula was attached to one pressure transducer for continuous measurement of pressure; a second pressure transducer was attached to a multiple stopcock arrangement so that large or small vein pressures could be measured continuously by switching from one venous pressure to the other at varying intervals; a third transducer was similarly attached to the large artery and aorta. By obtaining the pressure gradients across the arterial, venous, small vessel, and

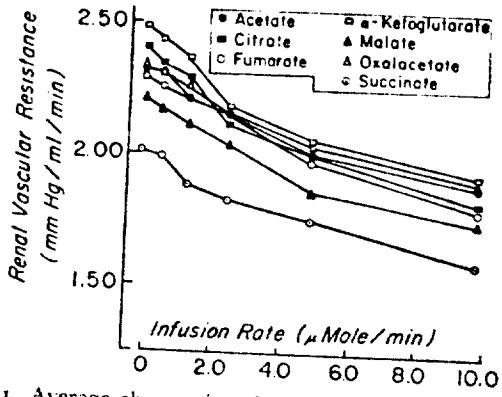


FIG. 1. Average changes in calculated renal vascular resistance after 30 sec infusion at each rate. Each curve represents the average change in calculated resistance obtained from seven animals when each metabolite was infused locally over the range 0.49–9.89 μmole/min. Average renal blood flow, 75 ml/min.

total circulations, and dividing them by blood flow, segmental vascular resistances were calculated and expressed as millimeters Hg per milliliter per minute. Average forelimb blood flow was 106 ml/min (range: 51–157 ml/min). Each metabolite was infused into the brachial artery perfusion tubing upstream from the pump, in randomized sequence, at the same rates used for the kidney, except that the infusion was maintained for only 30 sec at each rate, at which time pressures were measured and segmental resistances calculated. To provide appropriate control for the volume infused and for the sodium ion, isotonic sodium chloride was also infused. Table 1 details the pH, osmolality, and concentration of each solution infused.

RESULTS

Renal vascular bed. The dose-response effects of the seven intermediate metabolites, after 30 sec and 1-min infusion at each rate, are shown in Fig. 1. The fall in renal vascular resistance over the entire infusion range was approximately the same for each metabolite. In no instance was a fall in renal vascular resistance not observed. At the highest infusion rate, calculated renal vascular resistance declined approximately 20% at 30 sec; prolonged infusion for 1 min did not significantly induce further renal vasodilation.

Forelimb vascular bed. The forelimb vascular effects of the metabolites and the saline control obtained over the same infusion range as in the kidney is presented in Figs. 2 and 3. The infusion rate was maintained for only 30 sec at each level, at which time the dosage was increased to the next higher rate. There was no significant effect on forelimb pressures or calculated vascular resistance over the entire infusion range as a result of the infusion of sodium chloride. These responses with saline can be compared with the observed effects of the metabolites. In every instance, infusion of the metabolite resulted in a significant fall in large and small artery pressures. There was no effect on aortic or forelimb large and small vein pressures. The reduction in total vascular

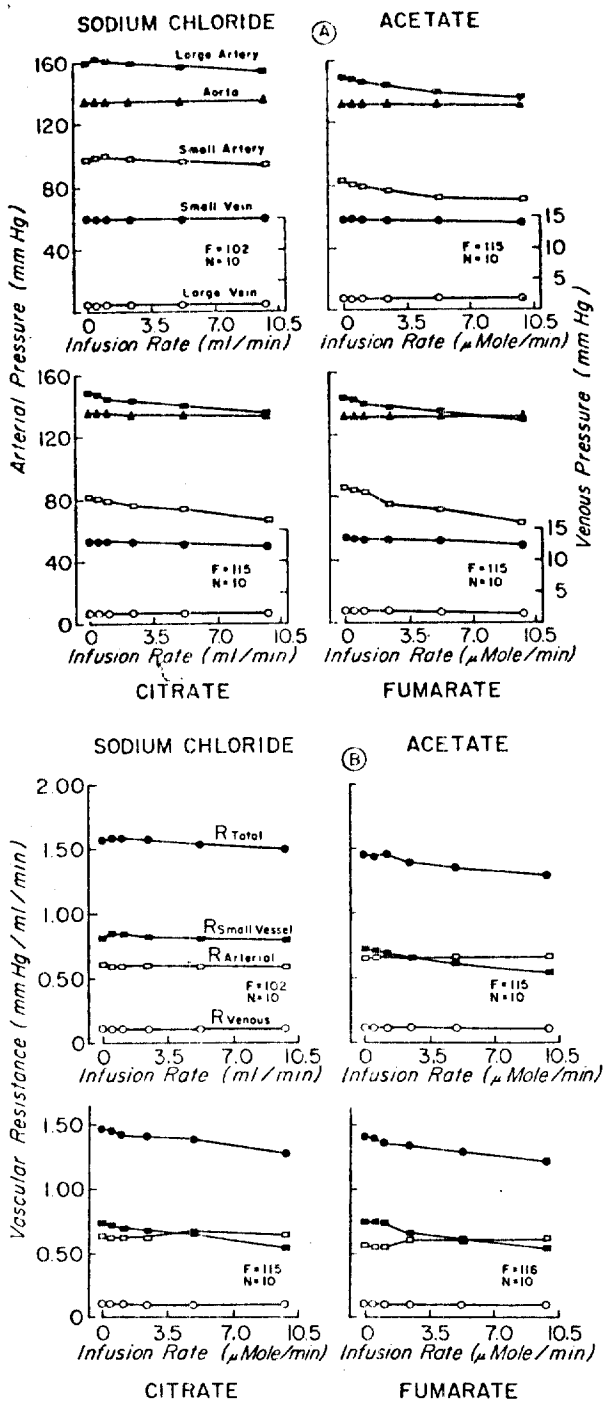


FIG. 2. Average changes in forelimb vascular pressure and resistance values, respectively, obtained during the local infusion of sodium chloride, acetate, citrate, and fumarate over the range 0.49–9.89 μmole/min. Each infusion rate maintained for 30 sec, at which time pressures were steady. N refers to number of animals in each average and F to average forelimb blood flow, in ml/min.

resistance observed with each agent represents a fall of approximately 25% in small vessel resistance. Table 2 details the infusion levels at which a significant decrease in small vessel resistance occurred. Dilation was usually obtained when local plasma concentration was less than doubled and before the maximal infusion rates were

TABLE 2. *Percentage decrease in small vessel resistance*

Metabolite	Infusion Rate, μmole/min. for 30 sec each					
	0	0.49	1.23	2.47	4.94	9.89
Na-chloride*	0	+2	+2	0	1	4
Na-acetate	0	1	4	8†	14	26
Na-citrate	0	1	4	8†	11	26
Na-fumarate	0	0	1	11	16†	28
Na-α-ketoglutarate	0	0	1	3	7†	21
Na-malate	0	0	4†	6	12	25
Na-oxalacetate	0	0	3	6†	10	22
Na-succinate	0	1	4	6†	16	32

* Infusion rate in ml/min. † Statistical significance at least at $P < .05$ level beginning with this infusion level.

begun. For example, dilation was observed with citrate and malate when the concentration was increased by 16 and 18%, respectively (Table 3). These increases in local plasma-metabolite concentration are most likely within the physiological range (1). Plasma concentrations were not measured and were therefore calculated from reported normal concentrations (1), the averaged group forelimb blood flow, and the volume and duration of infusion, ignoring gradual buildup of concentration.

DISCUSSION

The data presented demonstrate that the seven products of oxidative metabolism studied herein significantly decrease the resistance to blood flow in the renal and forelimb vasculatures. This decrease is probably produced by active arteriolar dilation. In both circulations dilation was observed with every infusion. Following each infusion, usually within 30 sec, pressures and calculated resistances returned to preinfusion levels.

Those factors affecting vascular resistance such as temperature, dilution, tonicity, and pH could not have been responsible for the changes, since blood temperature remained constant, volume controls demonstrated insignificant effects, and the solutions were isotonic and of the same pH as plasma. Since small vessel resistance decreased with decreasing intraluminal pressure, the reductions in small vessel resistance caused by these metabolites must have been produced by active small vessel dilation, and in particular, of the arterioles. Since the sodium salt of each agent was used, and isotonic sodium chloride in these and other studies (4, 8, 14, 18) has been shown to be without vascular effect, the dilation observed must have been due to the anionic part of each metabolite.

The mechanism for this dramatic dilation cannot be established from these studies, but the degree of dilation is much greater than observed with higher dosages of the major cations at the same infusion rates (4, 8, 14, 18). One possibility suggests an increased catabolism of these infused metabolites associated with a secondarily induced increase in the concentration of high-energy

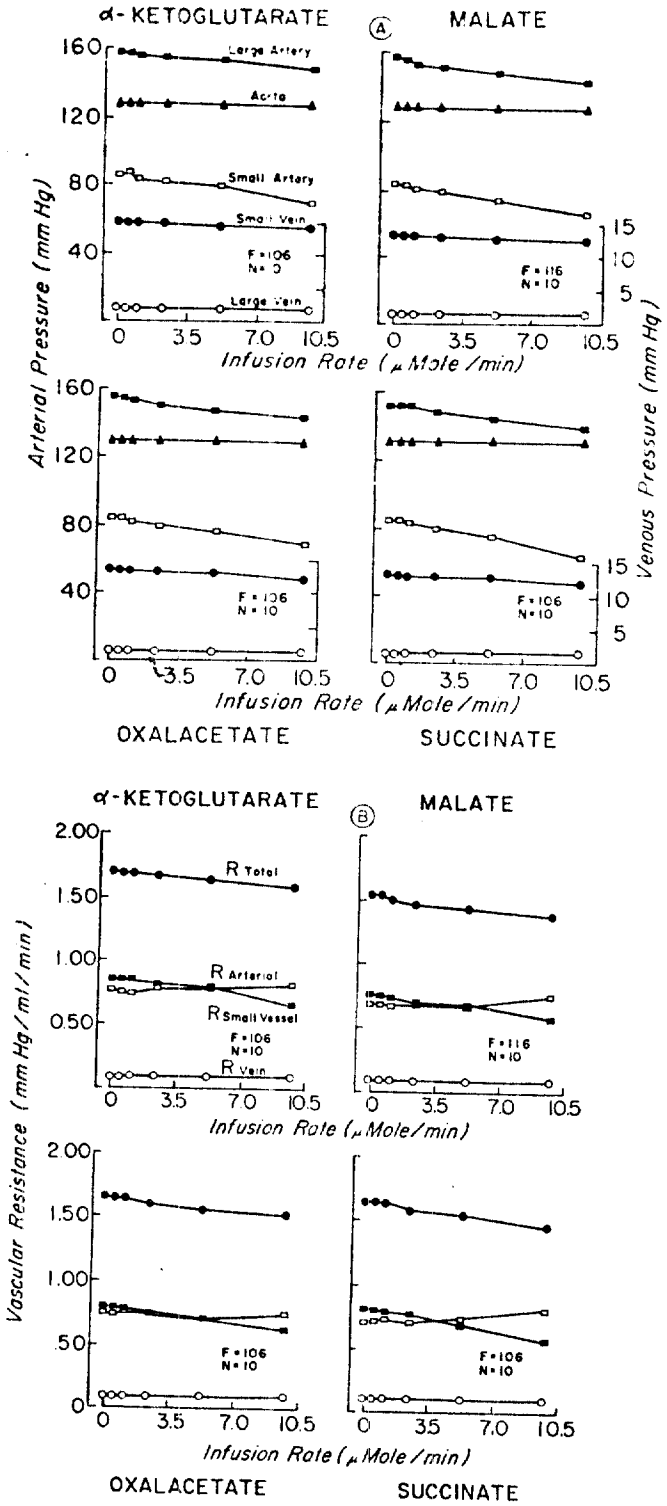


FIG. 3. Average changes in forelimb vascular pressure and resistance values, respectively, obtained during the local infusion of sodium α-ketoglutarate, malate, oxalacetate, and succinate over the range 0.49–9.89 μmole/min. Each infusion rate maintained for 30 sec, at which time pressures were steady. N refers to number of animals in each average and F to average forelimb blood flow, in ml/min.

phosphates necessary for the oxidation of these agents. A previous study confirmed the highly potent vasodilator action of AMP, ADP, and ATP, and localized their site of action to the same vessel segments in the forelimb (7). However, because the response observed is so prompt, it is unlikely that this metabolic mechanism prevails. It seems more likely that these substances have direct local effects on the vascular smooth muscle. Preliminary observations of studies in progress seem to indicate that these agents dilate by binding with local calcium ions. Excess calcium ion, per se, has been shown to constrict both the renal and forelimb vascular beds (8, 14), and when calcium salts of these metabolites are infused intra-arterially into the forelimb, dilation does not occur.

A review of the literature failed to reveal any previous observation demonstrating vasodilation with these agents. Serum lactate and pyruvate levels have been reported to be elevated in various hypotensive states (12). A recent study by Rowe and his associates (15) failed to demonstrate any significant change in calculated total peripheral, coronary, or pulmonary vascular resistance following an intravenous infusion of molar sodium succinate. On the other hand, cases have been reported of sudden death, sensations of warmth, and observed profuse vasodilation following the administration of succinate for the determination of circulation time (2, 9, 19). Another report demonstrated that sodium succinate relieved intermittent claudication while it concurrently precipitated attacks of angina pectoris (6). At that time it was difficult to explain the coincidence of these two phenomena, but in the light of more recent physiological studies it is conceivable that the succinate induced a redistribution of blood flow which precipitated coronary insufficiency (5).

Recent work has suggested that certain phenomena which are believed to be on the basis of local regulation of blood flow (i.e., autoregulation, reactive hyperemia) may well be attributed to the local release of vasoactive metabolites (3, 10). Berne has suggested that adenosine and AMP may be the metabolic mediators in cardiac and skeletal muscle (3). Haddy has suggested that adenosine, AMP, ADP, ATP, magnesium, potassium, increased hydrogen ion concentration, and possibly other unknown metabolites may all participate in local regulation of

TABLE 3. *Amount of metabolite necessary to produce significant vasodilation*

Metabolite	Normal Serum Concn, mg/100 ml†	Infusion Rate, ml/min	Amount Infused, mg/50 sec
Na-citrate	3.00	2.47*	3.24*
Na-fumarate	0.30	4.94	1.19
Na-α-ketoglutarate	0.90	4.94	1.26
Na-malate	0.90	1.23	0.98
Na-succinate	0.50	2.47	0.65

* Values for expected concentration assuming complete mixing of the infusate in the known volume of blood perfusing the forelimb per unit time. † Ref. 1.

blood flow (10). The present study offers some evidence to suggest that the Krebs intermediate metabolites should be included among those substances termed vasodilating metabolites. These compounds could be important in the regulation of local blood flow under a variety of conditions.

The author expresses his appreciation to Leon Kosterka for his excellent technical assistance.

REFERENCES

1. Altman, P. L., and D. S. Dittmer (editors). *Blood and Other Body Fluids*. Washington, D.C.: Fed. Am. Soc. Exptl. Biol., 1961, p. 87.
2. Barrett, R. H. Analeptic effect of sodium succinate on barbiturate depression in man. *Anesthesia Analgesia, Current Res.* 26: 74–81; 105–113, 1947.
3. Berne, R. M. Regulation of coronary blood flow. *Physiol. Rev.* 44: 1–29, 1964.
4. Chou, C. C., E. D. Frohlich, and E. C. Texter, Jr. Effects of cations on the segmental resistances in the mesenteric vascular bed. *Circulation* 28: 703, 1963.
5. Corday, E., J. H. Williams, L. B. De Vera, and H. Gold. Effect of systemic blood pressure and vasopressor drugs on coronary blood flow and the electrocardiogram. *Am. J. Cardiol.* 3: 626–637, 1959.
6. Dwyer, C. B., S. Kronenberg, and M. Saklad. Anginal syndrome during sodium succinate therapy. *Ann. Internal Med.* 31: 148–153, 1949.
7. Frohlich, E. D. Local effect of adenosine mono-, di-, and triphosphate vessel resistance. *Am. J. Physiol.* 204: 28–30, 1963.
8. Frohlich, E. D., J. B. Scott, and F. J. Haddy. Effect of cations on resistance and responsiveness of renal and forelimb vascular beds. *Am. J. Physiol.* 203: 583–587, 1962.
9. Greenfield, J. Sodium succinate as a test of circulatory efficiency. *Ann. Internal Med.* 32: 524–527, 1950.
10. Haddy, F. J. Local regulation of the peripheral vascular system. In: *Shock and Hypotension: Pathogenesis and Treatment*. New York, Philadelphia: Grune & Stratton. In press.
11. Haddy, F. J., A. G. Richards, J. L. Alden, and M. B. Visscher. Small vein and artery pressures in normal and edematous extremities of dogs under local and general anesthesia. *Am. J. Physiol.* 176: 355–360, 1954.
12. Migone, I. Metabolic aspects of shock. In: *Shock, Pathogenesis and Therapy*, edited by K. D. Bock. Berlin: Springer, 1962, p. 76–94.
13. Molnar, J. I., J. B. Scott, E. D. Frohlich, and F. J. Haddy. Local effects of various anions and H^+ on dog limb and coronary vascular resistances. *Am. J. Physiol.* 203: 125–132, 1962.
14. Overbeck, H. W., J. I. Molnar, and F. J. Haddy. Resistance to blood flow through the vascular bed of the dog forelimb. Local effects of sodium, potassium, calcium, magnesium, acetate, hypertonicity, and hypotonicity. *Am. J. Cardiol.* 8: 533–541, 1961.
15. Rowe, G. G., C. A. Castillo, S. Afonso, J. E. Lopez, W. C. Lowe, and C. W. Crumpton. The systemic and coronary hemodynamic effect of molar sodium succinate. *Am. J. Med. Sci.* 246: 180–183, 1963.
16. Roy, C. S., and J. G. Brown. The blood pressure and its variations in the arterioles, capillaries and small veins. *J. Physiol., London*, 2: 323–359, 1879.
17. Rushmer, R. F. Peripheral vascular control. In: *Cardiovascular Dynamics* (2nd ed.). Philadelphia: Saunders, 1961, p. 98–130.
18. Scott, J. B., E. D. Frohlich, R. A. Hardin, and F. J. Haddy. Na^+, K^+, Ca^{++}, and Mg^{++} action on coronary vascular resistance in the dog heart. *Am. J. Physiol.* 201: 1095–1100, 1961.
19. Watson, R. M., C. D. Allen, and M. J. Schwartz. Sudden death after the administration of sodium succinate. *New Engl. J. Med.* 266: 1162–1163, 1962.

more, Kuschke and Gruner's(1) suggestion that reserpine functions as a thyroxine antagonist would lead one to expect that rather than potentiating the toxic effects of thyroid administration reserpine would counteract such effects. In this regard it may be of importance that the dosage of reserpine employed in the present experiment was larger than that employed by others. It is possible, however, that the growth retardation and death obtained when thyroid and reserpine were fed concurrently were due not to a potentiation of thyrotoxicity as a result of reserpine administration but rather a potentiation of reserpine toxicity as a consequence of thyroid feeding.

Summary. Doses of reserpine and desiccated thyroid which were non-lethal to immature rats when administered separately resulted in a 100% mortality within a 2 week period when administered concurrently.

1. Kuschke, H. J., Gruner, H., *Klin. Wchnschr.*, 1954, v32, 563.
2. De Felice, E. A., Smith, T. C., Dearborn, E. H, PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 171.
3. Mayer, S. W., Kelly, F. H., Morton, M. E., *J. Pharmacol. and Exp. Therap.*, 1956, v117, 197.
4. Moncke, C. L., *Medizinische*, (Dec.) 1955, No. 50, 1742.
5. Goodman, J. R., Florsheim, W. H., Tempereau, C. E., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 196.
6. Ford, R. V., Livesay, W. R., Miller, S. I., Moyer, J. H., *Med. Rec.* (Houston), 1953, v47, 608.
7. Ershoff, B. H., Hershberg, D., *Am. J. Physiol.*, 1945, v145, 16.
8. Ershoff, B. H., *Arch. Biochem.*, 1947, v15, 365.

Received May 13, 1958. P.S.E.B.M., 1958, v99.

Effect of Polycarboxylic Acids on Blood Clotting.* (24292)

EDWIN E. GORDON (Introduced by H. L. Blumgart)
The Department of Medicine, Harvard Medical School, the Medical Research Department, Yamins Research Laboratories, and the Medical Service, Beth Israel Hospital, Boston, Mass.

The anticoagulant action of citrate has been known for more than 60 years(1). The similarity in molecular structure of isocitrate and aconitate to citrate suggested that these tricarboxylic acids might also interfere with the clotting mechanism. To test this hypothesis, isocitrate, aconitate and the sodium salts of other polycarboxylic acids were incubated with human blood. Of the acids tested, those with 3 free carboxyl groups were effective in prolonging the clotting time.

Methods. Two ml of venous blood from normal volunteers or from patients without known clotting defects was discharged into tubes containing a solution of 0.5 ml of the appropriate test substance and into control tubes charged with 0.5 ml of 0.85% saline. The tubes were stoppered, inverted several times and placed upright in a rack at room temperature. Clotting time was defined as the interval between termination of mixing and formation of a firm clot which remained at the bottom when the tube was completely inverted. The polycarboxylic acids, obtained from commercial sources,† were put into solution, neutralized to pH 7 (pH Hydrion paper) and made up to volume with distilled water or saline just prior to use. The citric acid content of the commercial *cis*-aconitic acid, *trans*-aconitic acid, dl-Na_3 isocitrate, and tricarballylic acid was found to be less than 0.05%

* Supported by a grant from the Massachusetts Heart Assn.

† Citric acid was purchased from Baker Chemical Co.; *cis*-aconitic acid, dl-malic acid, fumaric acid, tricarballylic acid, malonic acid, dl-isocitric acid lactone and Na_3 isocitrate were purchased from the California Foundation for Biochemical Research, Los Angeles. The source of the other compounds tested was the Nutritional Biochemical Corp., Cleveland, Ohio. *Trans*-aconitic acid, recrystallized from the commercial preparation, was found to be 86% pure by chromatography(2).

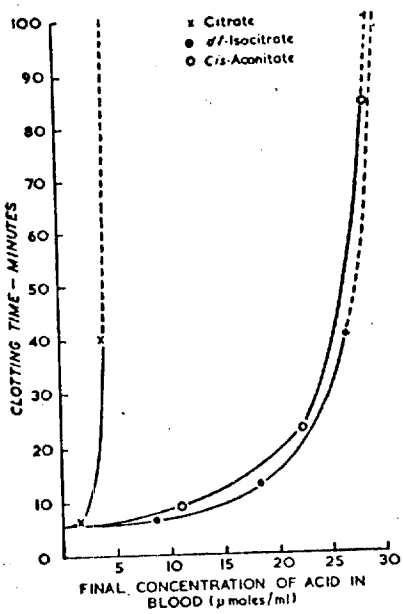


FIG. 1. Effect of varying concentrations of citrate, dl-isocitrate and *cis*-aconitate on clotting time of whole blood. Two ml of blood was added to 0.5 ml of a solution containing the test substance.

as determined by the method of Natelson, Pincus & Lugovoy (3). This amount of citric acid present as a contaminant would not appreciably influence blood clotting time.

Results. Both isocitrate and *cis*-aconitate were found to increase clotting time of whole blood (Fig. 1). The effect was dependent upon the concentration of polycarboxylic acid in the blood. Prolongation of clotting time was identical in the presence of equimolar concentrations of isocitrate and *cis*-aconitate. At a final concentration of 46 μmoles/ml both isocitrate and *cis*-aconitate increased clotting time to over 24 hours (Table I). The unnatural isomer, *trans*-aconitate, prolonged the clotting time, but was less effective than the *cis* isomer. Tricarballylate was also found to influence clot formation. The lactone of isocitric acid was relatively ineffective in increasing the clotting time. Only a slight prolongation of clotting time was noted when each of the dicarboxylic acids, α-ketoglutarate, succinate, fumarate, malate, glutarate, citraconate, glutamate and malonate was added to blood.

In other experiments, 2.0 ml of blood was mixed with 0.5 ml of 0.23 M *cis*-aconitate and permitted to stand for 4 hours. Control tubes contained 0.5 ml of saline and a drop of heparin (1000 U/ml) instead of *cis*-aconitate. At the end of the incubation period the cellular elements were removed by centrifugation, plasma proteins were precipitated with 3 volumes of 20% trichloracetic acid and the citric acid of the supernatant was determined. Plasma that had been incubated with *cis*-aconitate contained 5-7 μg citric acid/ml more than the control. This experiment indicated that a quantity of *cis*-aconitate, insufficient to affect clotting time, had been converted to citrate. Incubation of isocitrate in a similar manner did not reveal conversion of this acid to citrate.

Recalcification times were carried out on plasma from whole blood that had been incubated for 2 hours at room temperature with 0.046 M isocitrate and with *cis*-aconitate. Plasma clots formed between 5 and 10 min-

TABLE I. Effect of Polycarboxylic Acids on Clotting Time of Human Blood. 2 ml of blood mixed with 0.5 ml of 0.23 M acid (neutralized to pH 7). Control tubes contained 2 ml of blood plus 0.5 ml of 0.85% saline. Values are the means of a number (in parentheses) of experiments. Range of values is given beneath mean value in each case.

Acid	Clotting time Control	Clotting time Sample
Tricarboxylic	Min.	
Citric* (4)	5	>24 hr
Cis-aconitic (4)	5	>24 "
Trans-aconitic (4)	7	5–18 "
Isocitric (4)	6	>24 "
Isocitric lactone (4)	5	30 min. 13–49 "
Tricarballylic (4)	5	>24 hr
Dicarboxylic	Min.	Min.
α-ketoglutaric (4)	5	10.5 9–12
Succinic (4)	6	28 18–41
Fumaric (4)	6	8 7–9
Malic (7)	5	21 17–42
Glutaric (4)	6	10.5 4–15
Citraconic (3)	7	27 26–29
Glutamic (2)	5.5	10 8–12
Malonic (5)	5	12 8–16

* Concentration .115 M.

utes after addition of 0.2 ml of 0.02 M $CaCl_2$ to 0.2 ml of plasma. Clot formation was not apparent 4 hours after $CaCl_2$ had been added to plasma from the tricarballylate incubated blood samples.

Discussion. These experiments indicate that blood clotting is inhibited *in vitro* in the presence of isocitrate and *cis*-aconitate as well as in the presence of citrate. None of the dicarboxylic acids tested was effective in prolonging clotting time; the lactone of isocitric acid was similarly ineffective. The mechanism of the inhibition has not been established. Although other interpretations are possible, it is likely that the tricarboxylic acids chelate with ionic calcium through the 2 terminal carboxyl groups as postulated by Heinz(4) to form relatively undissociable calcium complexes in the blood. Tricarballylate has been shown to complex with ionic calcium in pure solutions(5), but it appears from the recalcification experiments that its ability to prevent clotting stems from interference with the coagulation mechanism other than its Ca^{++} binding ability.

In the normal state it is unlikely that *cis*-aconitate or isocitrate play a significant role in prevention of blood clotting *in vivo* because of the small concentration of these substances in blood(6). It is possible, however, that these intermediates of the Krebs cycle might influence the intracellular concentration of ionized calcium. This concept would be especially important in those tissues, such as nerve and muscle, where small changes in concentration of ionized calcium are known to exert profound effects(7). Conversely, increased concentrations of ionized calcium in tissues might influence citric acid cycle metabolism by complexing with citrate, isocitrate or *cis*-aconitate thereby interfering with enzyme substrate formation.

Summary. Human blood was incubated *in vitro* with the sodium salts of a number of di- and tricarboxylic acids and their effect on clotting time was noted. *Cis*-aconitate, *trans*-aconitate, isocitrate and tricarballylate markedly increased clotting time of whole blood. Prolongation of clotting in the presence of isocitrate and *cis*-aconitate was dependent upon their concentration in blood. The possible mode of action of these metabolically active compounds and the implications of the findings were discussed.

1. Pekelharing, G. A., *Untersuchungen über das Fibrinferment,* Amsterdam, 1892.

2. Bulen, W. A., Varner, J. E., Burrell, R. C., *Anal. Chem.,* 1952, v24, 187.

3. Natelson, S., Pincus, J. B., Lugovoy, J. K., *J. Biol. Chem.,* 1948, v175, 745.

4. Heinz, K., *Angew. Chem.,* 1949, v61, 256.

5. Schubert, J., Lindenbaum, A., *Nature,* 1950, v166, 913.

6. Frohman, C. E., Orten, J. M., Smith, A. H., *J. Biol. Chem.,* 1951, v193, 277.

7. Heilbrunn, L. V., *The Dynamics of Living Protoplasm,* Academic Press, Inc., New York, 1956.

Received May 19, 1958. P.S.E.B.M., 1958, v99.

Role of Relaxin in Stimulating Mammary Gland Growth in Mice.* (24293)

HIROSHI WADA† AND CHARLES W. TURNER
(With technical assistance of Mary E. Powell)
Department of Dairy Husbandry, University of Missouri, Columbia

It has been shown that estrogens (E) will stimulate mammary gland duct growth while E and progesterone (P) in suitable amounts and ratios will stimulate lobule-alveolar growth in normal and gonadectomized male and female animals(1-6). It has been suggested that relaxin synergizes with E and P in stimulating lobule-alveolar growth in rats

* Contribution from Mo. Agr. Exp. Sta., Journal Series, No. 1879. Approved by Director.

† Research scholar, Ministry of Education of Japanese Government. This investigation supported in part by grant from Am. Cancer Soc.

Journal of the Fukuoka Medical College, January 1959, pp. 136-151.

The Effect of Organic Acids belonging to the TCA Cycle on the Liver.

By Keiichiro Tsukamoto, Medical Doctor, Third Department of Internal Medicine (Director: Professor Toichi Sawada), College of Medicine, Kyushu University, Fukuoka, Japan.

(For a brief English synopsis, refer to the original text on p. 136.)

Table of Contents

I. Effect of Organic Acids upon Liver Functions.

1. Experimental Method

2. Experimental Result

1) Pigment Exhaust Function (BSP test)

2) Detoxication Test (Snatonin Test)

3) Sugar Metabolism Function (fructose load test)

4) Bile Production Function (determination of urobilinogen in ureine)

3. Summary

II. Effect of Organic Acids upon Liver Glycogen

1. Experimental Method

2. Experimental Result

3. Summary

III. General Summary and Observations

IV. Conclusion

Introduction

In the year 1937, H. A. Krebs (1) proposed a theory regarding the Tricarboxylic Acid Cycle (which will hereafter be abbreviated as the TCA cycle) and a large number of researchers have studied the detailed mechanisms of the cycle since that time. As a result, it has become clear that, not only the hydrocarbons but also the three major nutritional elements including fat and protein enter the TCA cycle from some routes to be completely decomposed, thereby freeing energy.

At our Sawada Internal Medical Institute, the organic acids of this cycle were clinically taken up. Professor Sawada, with an eye upon the fuming dextrose (grape sugar) which corresponds to the entrance to this cycle, hit upon the Sawada urine reaction which is a scientific diagnostic method for the lack of vitamin B_1. In addition, Miyaji et al. carried out a basic and clinical study of each of the organic acids of the TCA cycle.

As this cycle and as organic acids closely related to this cycle, citric acid, (alpha)-keto glutaric acid, succinic acid, fumaric acid, malic acid, oxalacetic acid, acetic acid and lactic acid, etc. can be mentioned. Of these, succinic acid is used for the internal administration of 0.6 grams each as a liver-assisting agent (2). According to the report of Mr. Miyaji et al. (3, 4), further, it also has the quality of being effective against irregular pulsation. In this manner, a new path has been paved for the clinical study of organic acids.

The liver is a major location for metabolism. It has been confirmed that the TCA cycle exists here. If a proper amount of an organic acid belonging to this cycle is given, it appears effective since there will be its metabolism as a substrate. However, they may each have advantages and disadvantages in their use and, moreover, some special functions may be observed if the amount given is increased.

Relative to the above reasoning, Yoshida (5) carried out an examination of citric acid and reported that the injection of 50 milligrams per kilograms into a domesticated rabbit promotes the liver function and markedly increases the detoxication effect. However, no report has been published regarding the other organic acids.

In the present study, therefore, citric acid, succinic acid, malic acid, acetic acid and lactic acid were selected as organic acids belonging to the TCA cycle and as organic acids which are closely related thereto. Regarding these, the effect of the same upon the liver functions of domesticated rabbits,

both healthy and having experimental defects, and upon the increase or decrease in the amount of the liver glycogen of the mice was examined. As a result, some interesting facts were obtained.

I. Effect of Organic Acids upon Liver Functions

In the selection for a liver function test, four kinds which were capable of concisely and accurately showing were selected and, as for the experiments, both covering the normal ones and the defective ones were carried out simultaneously. Since there were variations every day - these variations were sometimes so marked at times - in the test results of experimental animals, efforts were made to clearly attach proper significance in terms of the difference in values both before and after the test in order to make the result accurate.

Even though an examination of the protein metabolism function and the fat metabolism function is highly significant in this case, the experimental values obtained were so very small and extremely variable in the animal experiment that it was difficult to determine the effect of the medicines. Therefore, they were not employed.

I. Experimental Method

1) Experimental Animals

Domesticated male rabbits, each weighing approximately two lilograms, were used after they were domesticated for more than a week.

2) Chemicals Used and Method of Feeding

Four organic acids including acetic acid, succinic acid, l-malic acid and l-lactic acid were selected and, as for the feeding method, the one adopted by Oishi (6) was employed. A one-mol soda solution, 0.5 cubic centimeters per kilogram each was administered into the vein. It should be mentioned in this connection that the same amount of citric acid was not administered because the rabbits would die in many cases.

2. Experimental Results

1) Pigment Discharge Function

Bromsulfurein Test (BSP Test)

(1) Effect of Organic Acids upon the BSP Discharge Function of Regular Domesticated Rabbits.

According to Kurihara (7), there are cases where, when five milligrams per kilograms was injected into rabbits in the BST test, it disappears quickly from the blood, thereby making it difficult to carry out a measurement. Therefore, 10 milligrams per kilogram was injected, the BSP values in the blood serum were measured 10 and 15 minutes later and the remaining rates were obtained to be used as the 1-minute values and the 15-minute values to be used as controls.

After the passage of three hours, an organic acid and BSP were injected at the same time and the 10-minute and 15-minute values were measured and compared with the controls. In order to clarify the effect of the organic acids, further, an organic acid and BSP were inversely injected at the same time, the 10-minute and 15-minute values were measured and, after the passage of three hours, BSP alone was injected and the 10-minute and 15-minute values were measured, and these were compared with the controls.

As is shown in Table 1 and Figure 1, the experimental result shows that the BSP 10-minute and 15-minute values were better in each case in the case of an acetic acid load. In the case of succinic acid, the change was quite small in each example. In average, the 10-minute value was slightly better and the 15-minute value was slightly inferior. The change in each example was small in the case of malic acid, too, and the 10-minute and 15-minute values were slightly better on an average. There are many cases of lactic acid where there was observed a deterioration in each case, albeit to a slight degree. In average value, therefore, the 10-minute and 15-minute values were slightly inferior.

(2) Effect of Acetic Acid upon the BSP Discharge Function of Domesticated Rabbits having Liver Troubles.

Carbon tetrachloride was dissolved at the rate of 20 percent in olive oil and, by using the weight of the domesticated rabbits when they were hungry early in the morning, one cubic centimeter per kilogram (0.2 grams of ccl_4 was contained in one cubic centimeter) was injected into the spinal muscles of domesticated rabbits to cause liver trouble. Day by day, the effect of the acetic acid load upon BSP was examined as in the case of regular domesticated rabbits. It is mentioned in this connection that as the measured values the 10-minute values obtained were used.

(Table 1 The BSP values prior to and after the load of organic acids (regular domesticated rabbits). (The amount of an organic acid was 0.5 cubic centimeters per kilogram of each one-mol soda, quietly injected). a. Domesticated rabbit No. b. Order of Load. c. After 10'. d. After 15'. e. Order of load. f. After 10'. g. After 15'. h. Acetic acid. i. Average. j. Average for the whole group. k. Succinic acid. m. Average. n. Average for the whole group. o. Malic acid. p. Average. q. Average for the whole group. r. Lactic acid. s. Average. t. Average for the whole group. u. Order of load. v. After 10'. w. After 15'. x. Order of load. y. After 10'. z. After 15'. Legend: B: BSP, A: acetic acid. S: succinic acid. M: Malic acid. L. Lactic acid. . Indicates being effective.)

(Figure 1 Comparison of the effects of organic acids by BSP discharge function, regular domexticated rabbits. a. Acetic acid. b. Succinic acid. c. Malic acid. d. Lactic acid.)

As shown in Table 2, in the case of four domesticated rabbits, the BSP was measured at an interval of several days after the 24th hour of the injection of carbon tetrachloride. Twenty-four (24) hours or 48 hours after the injection of carbon tetrachloride, the trouble started to accumulate rapidly and reached the maximum. At first, the rate of recovery was high, becoming milder thereafter in a gradual manner and reaching approximately the regular state on around the 19th day.

Among the 21 measurements, 12 showed improvement, five showed no change and four showed deterioration. Excepting those where no change was registered, 75 per cent showed a better result and 25 per cent showed a deteriorated result. As compared with regular domesticated rabbits, the results were found to be slightly less favorable. Nevertheless, the pigment discharge function by the acetic acid load in the case of domesticated rabbits having troubled livers, as a whole, showed a turn for the better.

(Table 2 The BSP values before and after the acetic acid load (domesticated rabbits having troubled livers). a. Method of the load. Legend: B: BSP. A: 0.5 cubic centimeters per kilogram of one mol soda of acetic acid was quietly injected. The arrow mark indicates the order of enforcement.)

2) Detoxication Function

Santonin Test

Experimental Method

Along with the hippuric acid synthesis test and the BSP test, this test is the most sensitive and significant in knowing the pathological change in the liver. It is a detoxication function test with the oxidation of sodium santonate or the production of (Beta)-oxy-santonin as the target. According to Takasugi (8), the "Santoni" test should sufficiently test the amount of the discharge within a period of approximately six hours since the discharge of pigment in the urine reaches the maximum within a period of from one to two hours, with a gradual decrease thereafter and the complete discharge being achieved in a period of four hours at most and seven hours at the latest.

Even though it is stated that the total amount of pigment discharged in the same domesticated rabbits is generally constant, my experiment showed, as in the case of the BSP test, that the total amounts of the discharge of pigment were different from day to day even in the case of same domesticated rabbits. Thus, the following procedure was adopted in order to make the experi-

mental result as accurate as possible:

As shown in Figure 2, after the discharge of urine by the introduction of urine, 0.1 cubic centimeter per kilogram of Santosol (a product of Nippon Shinyaku, containing 80 milligrams of Santonin per cubic centimeter) was injected quietly through the ear vein and urine was collected thereafter for a period of three hours. Immediately thereafter,the same amount of Santosol was quietly injected and urine was collected thereafter for a period of three hours.

The amounts of (beta)-oxy-santonin which was discharged by being oxidized by the urine obtained the first time and the urine obtained the second time were obtained as the index and those were set as Vo and No respectively. In this manner, No/Vo x 100 was obtained. (In the normal case, Vo is greater than No and No/Vo x 100 is generally constant). A similar test was carried out on the following day; however, an organic acid is quietly injected simultaneously with the second quiet injection of the Santosol. In this manner, (Beta)-oxy-santonin which was obtained the first and second times was set as Vx and Nx, and Nx/Vx x 100 was obtained. In this manner, when Nx/Vx x 100 is greater than No/Vo x 100, it is possible to determine that the substance termed x is certainly effective in promoting the oxidation of Santonin or detoxication.

(Figure 2 Procedure of the Enforcement of the Santonin Test. 1. First day. 2. Santosol. 3. Santosol. 4. Urine discharge. 5. Urine Collection. 6. Urine collection. 7. Second day. 8. Santosol. 9. Santosol plus x. 10. Urine discharge. 11. Urine collection. 12. Urine collection.)

Regarding the measurement of (Beta)-oxy-santonin contained in urine, the pink color which has been produced after the addition of alkali soon fades and, accordingly, it is impossible to carry out a measurement by the conventional method. Therefore, a measurement method using a photoelectric colorimeter as enunciated by Anami (9) was employed. In other words, a color was generated by using 10 per cent caustic soda and, by using the Azorbin-S of the 100,-

000 times as the standard, a measurement was carried out in 10 seconds by using a filter-510 m(mu) and it was obtained as an index. It is mentioned in this connection that the amount of pigment discharged was calculated in the following manner for the sake of convenience.

Amount of pigment discharged equals the amount of urine times the concentration divided by 10.

(1) Effect of Organic Acids on the Santonin Detoxication of Regular Domesticated Rabbits.

As shown in Table 3 and Figure 3, the experimental result shows that the amount of Santonin pigment discharged after the injection of succinic acid was markedly increased as compared with the control, that there was a slight increase in that case of acetic acid and that there was no change in the case of malic acid and lactic acid.

In the case of domesticated rabbits, therefore, it was found that succinic acid promotes the detoxication of Santonin most markedly and that acetic acid promotes the detoxication to a slight extent.

(Table 3 Santonin Detoxication Capacity before and after the Load of Organic Acids. (Regular domesticated rabbits). As for the amount of organic acids administered, 0.5 cubic centimeters per kilograms of each one-mol soda was quietly injected. a. Control. b. Acetic acid. c. Succinic acid d. Malic acid. e. Lactic acid. f. Three hours before Vo oxy-santonin. g. After three hours No. oxy-Santonin. h. Average. i. Average. j. Average. k. Average. m. Average.)

(Figure 3 Comparison of the effects of organic acids in Santonin detoxication capacity (regular domesticated rabbits). a. Control. b. Acetic acid. c. Succinic acid. d. Malic acid. e. Lactic acid.)

(2) Effect of Organic Acids upon the Santonin Detoxication Capacity of Domesticated Rabbits having Liver Trouble.

Regarding seven rabbits, the amount of the santonin pigment discharge before

the liver trouble was obtained. One point zero (1.0) per cent per kilogram of 20 per cent carbon tetrachloride olive oil was injected (only in the case of No. 21, 0.5 cubic centimeter per kilogram was used) and, by using a method which was the same as in the case of regular domesticated rabbits, the development was examined and the amount of the Santonin pigment discharge was measured.

As shown in Tables 4 and 5 and in Figure 4, the experimental result shows that, as compared with the control, succinic acid and malic acid showed a marked increase in the amount of Santonin pigment discharge and that both lactic acid and acetic acid showed a slight increase. In the case of domesticated rabbits having liver trouble, therefore, it can be stated that succinic acid and malic acid most markedly promote the liver detoxication and that lactic acid and acetic acid promote the same to a slight extent.

3) Sugar Metabolism Function

Fructose Load Test

The liver is closely related to sugar metabolism and since the organic acids belonging to the TCA cycle are the end products in the process for the decomposition of sugar, it is believed highly significant to examine the effect of these substrates upon sugar metabolism. Nevertheless, there are few function tests of this kind which are accurate as the BSP test or the detoxication test, withthe galactose test and fructose test being frequently used in actuality.

The former is especially excellent; however, since the clinical significance of the fructose load test is held to be generally similar to the galactose test, I have carried out the determination of fructose contained in urine by using the method of Li (10), who improved the method of Straus. It is mentioned that, in this case, a variation was used wherein the amount of the fructose load was set at 1.0 cubic centimeter of the 20 per cent solution and a photoelectric calorimeter was used for the determination of fructose in urine. Since the amounts of fructose contained in urine were various even in the case of the same domesticated rabbits after the fructose load, the follow-

ing procedure was used in order to examine the effect of organic acids accurately.

In other words, as in the case of the Santonin detoxication test, the amount of fructose contained in urine one hour after the injection of fructose was expressed by Vo, the amount of fructose contained in urine one hour after the quiet injection of the same amount of fructose immediately thereafter was expressed by No and No/Vo x 100 was obtained and used as the control value.

In order to examine the effect of organic acids, the control value was obtained and fructose was quietly injected in a similar manner on the following day and, in this case, 0.5 cubic centimeters per kilogram of a one-mol organic acid sodoum solution was mixed with 0.5 cubic centimeters per kilogram at the time of the quiet injection at the second time. By obtaining the amounts of fructose in the respective urine, Vx and Nx were obtained and Nx/Vx x 100 was compared with the control.

(Figure 4 Comparison of the effect of organic acids by the Santonin detoxication capacity. (A Graphic Presentation of Table 5). a. Control. b. Acetic acid. c. Succinic acid. d. Malic acid. e. Lactic acid.)

(Table 4 Santonin detoxication capacity before and after organic acid loads. (In the case of domesticated rabbits having liver trouble). 1. Three hours before. 2. Three hours after. 3. Three hours before. 4. Three hours after. 5. Three hours before. 6. Three hours after. 7. Three hours before. 8. Three hours after. 9. Three hours before. 10. Three hours after. 11. Three hours before. 12. Three hours after. 13. Three hours before. 14. Three hours after. Legend: 0.5 cubic centimeters per kilogram of each one-mol soda of A acetic acid, S succinic acid, M malic acid and L lactic acid was quietly injected.)

(Table 5 Santonin Detoxication Capacity before and after the Organic Acid Loads. (Domesticated rabbits having liver trouble.) (An orderly rearrangement of Table 4.) a. Control. b. Acetic acid. c. Succinic acid. d. Malic acid. e. Lactic acid. 1. Three hours before Vo oxy Santonin.

2. Three hours after No. oxy Santonin. 3. Average. 4. Three hours before. 5. Three hours after. 6. Average. 7.Three hours before, Vx oxy-Santonin. 8. Three hours after Nx oxy-Santonin. 9. Average. 10. Average, 11. Average.)

(1) In the case where an organic acid is injected in a regular domesticated rabbit.

a. When Organic Acid is injected simultaneously with Fructose.

(Table 6 In the case where an organic acid was quietly injected simultaneously with fructose. (The amount of an organic acid administered was 0.5 cubic centimeter per kilogram of each one-mol soda.) 1. First day. 2. Fructose. 3. Fructose. 4. Urine discharge. 5. Urin collection. 6. Urine collection. 7. One hour. 8. One hour. 9. Second ay. 10. Fuctose. 11. Fructose. 12. Organic acid. 13. Urine discharge. 13. Urine collection. 14. Urine collection. 16. one hour. 17. one hour. a. Control. b. Succinic acid. b. Acetic acid. d. Malic acid. e. Lactic acid. f. Average. g. Average. h. Average. i. Average. j. Average.)

As is shown in Table 6, the experimental result shows that the extent of an increase in urine the case of fructose was the largest when succinic acid was administered as compared with the control, that there was no great difference in the case of acetic acid and that there was a decrease in the case of malic acid and lactic acid. As was pointed out by Li, the amount of the increase in fructose is generally indicative of a reduction in the liver sugar metabolism capacity and, as such, this result seems strange.

However, Yoshida carried out a similar experiment in the case of citric acid and reported that, when citric acid is injected, the amount of fructose further increases as compared with the control. It was believed that this was due to the fact that the treatment of fructose received an interference by the treatment of citric acid, with a result that the amount of the discharge of fructose contained in urine increased, and that a similar increase

was observed in the amountof fructose in the case of dextrose (grape sugar) which was considered to be a liver-function promoting agent. By carrying out a similar experiment, I, too, ascertained the correctness of this. The result obtained is shown in Table 7. It is mentioned that 0.5 cubic centimeters per kilogram of 20 per cent was injected into the vein in the case of dextrose.

(Table 7 When Dextrose was Quietly Injected Along with Fructose. (The amount of dextrose administered was 0.5 cubic centimeters per kilogram of 20 per cent. a. Average.)

As a supplementary experiment, Yoshida further carried out a fructose load test one hour after the injection of citric acid. As compared with the control, there was clearly a decrease in the amount of fructose, thereby showing the result of fructose treatment capacity promotion. Therefore, Yoshida stated that, even though it is admitted that the fructose treatment capacity does indeed decrease immediately after the injection of citric acid, it is not indicative of a decline in the sugar metabolism capacity but that it would rather promote it after the passage of a certain period of time. Therefore, I carried out an experiment in the following manner:

b. In the Case where Fructose was quietly injected One Hour after the quiet injection of organic acids.

As for the experimental method, fructose was injected quietly twice as in the case of a, but an organic acid was individually injected quietly one hour after the first quiet injection of fructose, and the second quiet injection of fructose was carried out one hour after that. As regards the collection of urine, it was carried out one hour after the quiet injection of fructose.

As is shown in Table 8, the experimental result shows that the amount of fructose was slightly reduced in the case where succinic acid was administered but that there was observed an increase in the case of acetic acid, malic acid and lactic acid. There was a decrease in the case of dextrose.

It is mentioned in this case that the above result shows a considerably reduced rate of an increase in the amount of fructose as compared with the

afore-mentioned case where an organic acid was charged simultaneously with fructose.

As pointed out by Yoshida, this result makes one assume the situation in which, if an organic acid is once administered, the subsequent sugar metabolism is promoted. Therefore, an experiment was further carried out by extending the time after the load of organic acids by one hour.

(Table 8 . In the case where fructose was quietly injected one hour after the quiet injection of an organic acid. (The amount of an organic acid administered was 0.5 cubic centimters per kilogram of each one-mol soda in quiet injection.) 1. First day. 2. Fructose. 3. Fructose. 4. Urine discharge. 5. Urine collection. 6. Urine collection. 7. One hour. 8. One hour. 9. One hour. 10. Second day. 11. Fructose. 12. Organic acid. 13. Fructose. 14. Urine collection. 15. Urine collection. 16. one hour. 17. one hour. 18. one hour. a. Control. b. Succinic acid. c. Acetic acid. d. Malic acid. e. Lactic acid. f. Dextrose (grape sugar). g. Average. h. Average. i. Average. j. Average. k. Average. m. Average.)

c. In the case where fructose was quietly injected two hours after the quiet injection of organic acids.

As shown in Table 9 and Figure 5, the experimental result shows that the amount of fructose (no) in urine by the second quiet injection of fructose increased as compared with the value obtained at the first time (Vo) in the case of the control. In the case where dextrose was injected, however, if a quiet injection is carried out two hours after the second quiet injection of fructose, the later amount of fructose was found to be markedly reduced as compared with the previous amount of fructose. This is indicative of the promotion of the sugar-treatment capacity. Therefore, it was decided to quietly inject an organic acid two hours before the second quiet injection of fructose.

By examining the fructose treatment capacity of organic acids in this amnner, the following result was obtained. In the case of regular domesticated

rabbits, succinic acid and acetic acid showed a promotion of the fructose treatment capacity and malic acid and lactic acid rather showed a decline in the fructose treating capacity.

(Table 9 In the case where fructose was quietly injected two hours after the quiet injection of an organic acid. (The amount of organic acids administered was 0.5 cubic centimeters per kilogram of each one-mol soda in quiet injection.) 1. First day. 2. Fructose. 3. Fructose. 4. Urine discharge. 5. Urine collection. 6. Urine collection. 7. One hour. 8. Two hours. 9. One hour. 10. Second day. 11. Fructose. 12. Fructose. 14. Urine discharge. 15. Urine collection. 16. One hour. 17. Two hours. 18. One hour. a. Control. b. Succinic acid. c. Acetic acid. d. Malic acid. e. Lactic acid. f. Dextrose. g. Average. h. Average. i. Average. j. Average. k. Average. m. Average.)

(Figure 5 Comparison of the effects of organic acids by the fructose load test (regular domesticated rabbits). a. Control. b. Acetic acid. c. Succinic acid. d. Malic acid. e. Lactic acid.)

(2) in the case where organic acids were injected into domesticated rabbits having liver trouble.

In order to examine the effects of organic acids in the fructose load test, it was considered that, as in the examination of domesticated rabbits in (1), the method whereb an organic acid was individually injected quietly one hour after the first quiet injection of fructose, the second quiet injection of fructose was carried out two hours thereafter,and the amounts of fructose contained in urine one hour after the quiet injection of fructose are compared was the most suitable. Therefore, this method alone was used in the case of domesticated rabbits having liver trouble.

Regarding five domesticated rabbits, the amounts Vo and No representing the amounts of fructose discharged in urine before the liver trouble were obtained. By injecting 0.5 cubic centimeter per kilogram of 20 per cent carbon tetrachlor-

ide olive oil was injected and, by using the same method as in the case of (1), the amount of fructose was measured. As regards the injection of carbon tetrachloride, 0.25 cubic centimter per kilogram was injected on the third day, 0.25 cubic centimeters per kilogram was injected on the fourth day and 0.2 cubic centimeters per kilogram was injected on the fifth day.

(Table 10 Fructose load test on deomsticated rabbits having liver trouble. Legend: S. succinic acid. A. Acetic acid. M. Malic acid. L. Lactic acid. 0.5 cubic centimeters per kilogram of each one-mol soda salt was quietly injected.)

(Table 11 In the case where fructose was quietly injected two hours after the quiet injection of an organic acid. (An orderly rearrangement of Table 10.) a. Succinic acid. b. Acetic acid. c. Malic acid. d. Lactic acid. e. Control. f. Average. g. Average. h. Average. i. Average. j. Average.)

As shown in Tables 10 and 11 and in Figure 6, the experimental result shows that, as compared with the control, acetic acid and succinic acid show a fructose treatment capacity and that malic acid and lactic acid rather showed a drop in the fructose treatment capacity.

4) Bile Production Function

Determination of Urobilinogen in Urine)

As the method for examining the bile production function, there are the jaundice index measurement method of Mr. Meulengracht, the Serum bilirubin determination method of Mr. Hijmans van den Bergh, and the urobilinogen determination method, etc. Amongst these methods, we used the urobilinogen measurement method (which may hereafter be reffered to as "u-ge"). As for the determination of urobilinogen in urine, the two-hour urine method of Watson (11) was used.

(Figure 6 Comparison of the effects of organic acids by the fructose load test. (Domesticated rabbits having liver trouble). (This

represents a graphic presentation of N/V x 100 in Table 11.) a. Control. b. Acetic acid. c. Succinic acid. d. Malic acid. e. Lactic acid.)

(1) Urobilinogen Value in the Urine of Regular Domesticated Rabbits.

(Table 12 Urobilinogen values in the urine of regular domesticated rabbits (two-hour values). a. At the time of urine collection. c. Amount of U in 12-14 hour urine. c. Amount of U in 14-16 hour urine. d. Average.)

As shown in Table 12, the urobilinogen values in the two-hour urine of healthy domesticated rabbits exists in the range between 0.021 and 0.075 milligrams and the mean value in six examples was 0.052 milligrams.

(2) In the case where an organic acid was loaded in a regular domesticated rabbit.

The urobilinogen values in two-hour urine before the load of an organic acid (pre-urine value) and after the load (post-urine value) were measured and they were compared. The urolbilinogen values of regular domesticated rabbits are extremely small. Therefore, a difference of more than 0.02 milligrams was considered significant.

As shown in Table 13, the experimental result shows that succinic acid alone reduced the urobilinogen value in urine.

(Table 13 Change in urobilinogen values in urine in the case where regular domesticated rabbits were given the load of an organic acid. a. Succinic acid. b. Acetic acid. c. Malic acid. d. Lactic acid. e. Pre-urine value. f. Post-urine value. g. Pre-urine value. h. Post-urine value. Legend: The pre-urine value was between 12 and 14 hours, the post-urine value was between 14 and 16 hours and the amount of an organic acid administered was 0.5 cubic centimeters per kilogram of each one-mol soda in quiet injection.)

(3) In the case where an organic acid was loaded (charged) on domesticated rabbits having liver trouble.

Regarding five domesticated rabbits, the urobilinogen value in urine before

the liver trouble was measured, 0.5 cubic centimeters per kilogram of 20 per cent carbon tetrachloride olive oil was injected and the change in the urobilinogen value in urine due to the effect of the organic acids was examined every day by a method which was similar to theone used in connection with the regular domesticated rabbits. The injection of carbon tetrachloride was carried out at the rate of 0.5 cubic centimeters per kilogram on the first day, 0.25 cubic centimeters per kilogram on the third ay, 0.25 cubic centimeters per kilogram on the fourth day and 0.2 cubic centimeters per kilogram on the fifth day. (In order to facilitate the coloration of urobilinogen, carbon tetrachloride olive oil was injected several times in this fashion.)

(Table 14 Urobilinogen value in the urine of domesticated rabbits having liver trouble. Legend: V indicates the value in two-hour urine and N indicates the value in two-hour urine. A acetic acid, S succinic acid, M malic acid and L lactic acid, 0.5 cubic centimeters per kilogram of each one-mol soda salt was quietly injected.)

(Table 15 Change in the urobilinogen value in the urine in the case where an organic acid was loaded in a domesticated rabbit having liver trouble. (This is an orderly rearrangement of Table 14.) a. Control. b. Succinic acid. c. Acetic acid. d. Malic acid. e. Lactic acid. f. Average. g. Average. h. Average. i. Average. j. Average.)

3. Summary

By the quiet injection of 0.5 cubic centimeters per kilogram of each one-mol soda salt of acetic acid, succinic acid, malic acid and lactic acid, the effect upon the liver functions of the domesticated rabbits having liver trouble by carbon tetrachloride was examined by the BSP test, the Santonin detoxication test, the fructose load test and the determination of urobilinogen in urine. It was observed that the effect of succinic acid was marked in all cases, that acetic acid was similar and that, in the case of malic acid and lactic acid, the effect was not marked or rather there was observed a tendency for deterioration.

II. Effect of Organic Acids upon Liver Glycogen.

Since the TCA cycle is the end system which is produced by the decomposition of glycogen, it can be stated that glycogen can be produced if the process is traced backward.

When 0.15 grams of sodium succinate was given to a mouse, there was observed a marked increase in (the amount of) glycogen in the liver within a period of four hours (12). When C_2 carbon dicaroxylic acid was given to a laboratory rabbit, there was similarlyobserved an increase in (the amount of) glycogen in the liver, according to a certain report (13). When two grams of four different kinds of organic acids was given to rats for a period of three days through the mouth, succinic acid, fumaric acid and malic acid markedly increased (the amount of) glycogen in the liver and acetic acid did not give any effect, according to another report (14). When citric acid is regularly given to a rat, furthermore, there was observed a marked increase in (the amount of) glycogen in the liver (15) and it is known that there is a similar trend in the case of lactic acid.

I administered the soda salt of succinic acid, acetic acid, malic acid, lactic acid and citric acid each to regular mice and the effects of these acids upon the glycogen in the liver were comparately observed.

1. Experimental Method

1) Preliminary Experiment

The results obtained relative to the amount of glycogen in the mouse liver (which will hereafter be abbreviated as the 'g' amount) are variegated for different researchers. In other words, Tanaka (16) reported the value of 2.5 per cent, Makino (17) reported the value of 0.77 per cent (after 36-hour hunger), Kobayashi (18) reported the value of 2.7 per cent (after 20-hour hunger), Kodama (19) reported the value of 0.2 per cent (after 24-hour hunger) and so forth.

Under the circumstances, I measured the amount of glycogen in the liver of a

mouse after 24-hour hunger by the measuring method which will be described later.

(Table 16 Amounts of glycogen in the livers of regular mice after 24-hour hunger. a. Body weight. b. Amount of glycogen. c. Body weight. d. Amount of glycogen. e. Average of 10 examples.)

As the result of the above measurement, an average value of 0.19 per cent for 10 examples was obtained as shown in Table 16. This is roughly in agreement with the report of Kodama. Even though there may be some differences depending upon the measuring methods used, etc., it seems, nevertheless, that the values reported by Tanaka and Kobayashi are excessively high.

The same can be stated about the rabbits or rats. For example, Kurokawa reported the amount of glycogen after 24-hour hunger as 1.5 per cent (in the range between 0.17 and 3.18 per cent) and Fujii reported the same value at 4.26 per cent in the case of domesticated rabbits. In the case of rats, Tanaka reported the value after 24-hour hunger at 0.25 per cent, Okawa at 0.19 per cent, Dittmar at 0.47 per cent, Wetzel at 0.78 per cent and Hasegawa at 0.19 per cent, etc.

In charging a mouse with an organic acid, its quantitative relationship and the method of its feeding become a problem. At first, a mouse was made hungry for a period of 18 hours, 0.35 cubic centimeters of 1M sodium succinate was hypodermically injected and death was brought about in three hours later. As shown in Table 17, the result in the group where sodium succinate was injected was inferior. This is believed to be due to the fact that the amount of succinic acid administered was too excessive.

Next, administration through the mouth was tried. According to Ponsford and Maclean, who administered two grams of the ammonium salt of an organic acid to rats per day for a period of three days, the amount of glycogen in the organic acid group was markedly increased as compared with the control.

Therefore, I impregnated four grams of bread with three cubic centimeters

of 10 per cent sodium succinate (containing 0.3 grams of succinic acid) and fed the same through the mouth for a period of three days. As shown in Table 18, the administration of succinic acid clearly increased the amount of glycogen.

(Table 17 Amount of glycogen in the liver of a mouse after 18-hour hunger and three hours after the hypodermic injection of 0.35 cubic centimeters of 1M sodium succinate. a. Control group. b. Groups tested. c. Body weight. d. Body weight. e. Average. f. Average.)

(Table 18 Amount of glycogen in the liver three days after the administration of 0.3 grams of succinic acid through the mouth. a. Control group, b. Groups tested. c. Body weight. d. Amount of glycogen. e. Body weight. f. Amount of glycogen. g. Average. h. Average.)

By taking into consideration the amount of an organic acid fed on the basis of the above preliminary experiment, our experiment was carried out in the manner to be described below:

2) Experimental Animals and Feeding Method

Four grams of wheat and five grams of cabbage were fed every day and one gram of dried sardines was fed every other day at a certain prescribed time to male regular mice whose weights were betweeen 17 and 25 grams.

3) Method for Feeding Organic Acids

The sodium of M/10 succinic acid, acetic acid, malic acid and lactic acid were injected to the members of the group to be tested at the rate of one cubic centimeter per 20 grams of weight. (When citric acid was made M/10, mice developed tetanic convulsion and diedin many cases). In the control groups, there were created a group of those which received the injection of one cubic centimeter of table salt water and the other group of those which received the injection one cubic centimter of M/10 dextrose. In all cases, the injection was carried out at the rate of once a day for a period of one week on a continuous basis into the spinal muscle of the mice and, 24 hours after the last injection and feeding, the head was cut off to cause death.

It is mentioned in this connection that both the injection and feeding were carried out at five o'clock p.m.

4) Measurement of Glycogen in the Liver

According to the method of Verzar and Wenner (20), liver glycogen was hydrolyzed to make dextrose and a measurement was carried out by using the Hagedorn-Jensen method. The amount of dextrose which was measured was multiplied by 0.925 to express the glycogen content in the liver, in milligram per cent.

2. Experimental Result

The experimental result is shown in Table 19 and Figure 7.

(Table 19 a. Control. b. Succinic acid. c. Acetic acids. d. Malic acid. e. Lactic acid. f. Citric acid. g. Dextrose. h. Body weight. i. Amount of glycogen. j. Body weight. k. Amount of glycogen. m. Body weight. n. Amount of glycogen. o. Body weight. p. Amount of glycogen. q. Body weight. r. Amount of glycogen. s. Body weight. t. Amount of glycogen. u. Body weight. v. Amount of glycogen, w. Average. Legend: One cubic centimeter per 20 grams per the M/10 solution of each organic acid soda was injected, except that citric acid was injected at the rate of one cubic centimeter per 20 grams of the M/20 solution and that dextrose was injected at the rate of one cubic centimeter per 20 grams of the M/10 solution. As for the control, one cubic centimter per 20 grams of table salt water was injected for a period of one week in all cases and a measurement was carried out 24 hours after the final injection.)

(Figure 7 Comparison of the amounts of mouse liver glycogen by the injection of organic acids. a. Control. b. Succinic acid. c. Acetic acid. d. Malic acid. e. Lactic acid. f. Citric acid. g. Dextrose.)

1) Control

The mean value of 10 examples was 170.2 milligrams per cent (in the range between 123.1 and 215.6 milligram per cent).

2) Succinic Acid

The average of the 10 examples was 304.4 milligram per cent (in the range between 95.3 and 254.2 milligram per cent) and, as compared with the control, this represented an increase of only five per cent, thereby showing almost no difference.

4) Malic Acid

The average of 10 examples was 113.0 milligram per cent (in the range between 93.4 and 168.4 milligrams per cent). This showed a decline of 34 per cent as compared with the control. Amongst all the organic acids, this showed the least favorable result.

5) Lactic Acid

The average of 10 examples was 228.2 milligram per cent (in the range between 71.2 and 405.4 milligram per cent). It showed an increase of 34 per as compared with the control. Among the organic acids, its result was the second best (the best being succinic acid).

6) Citric Acid

The average of 10 examples was 205.3 milligram per cent (in the range between 77.0 and 310.8 milligram per cent). As compared with the control, it showed an increase of 21 per cent.

7) Dextrose

The average of 10 examples was 252.1 milligram per cent (in the range between 162.8 and 468.1 milligram per cent). As compared with the control, it showed an increase of 48 per cent.

The above-described result is more or less similar to the result obtained by Ponsford. However,the results pertaining to dextrose and malic acid were not necessarily in conformity with those he had obtained.

According to Ponsford, dextrose showed an increase of 220 per cent as compared with the control and malic acid showed an increase of 110 per cent as compared with the control. According to my own examples, there was no such marked increase in the case of dextrose and malic acid rather showed a

drop.

3. Summary

Acetic acid, succinic acid, malic acid, lactic acid and dextrose, in an amount of 1.0 cubic centimeter per 20 grams of the 1/10 mol solution, and sodium citrate, in an amount of 1.0 cubic centimeter of the 1/20 mol solution, were injected into the mice of 10 examples and the amount of their liver glycogen was measured.

As a result, it was found that succinic acid markedly increased the amount of liver glycogen. This was found to be followed by lactic acid and citric acid, which increased the amount slightly. In the case of acetic acid, there was found almost no difference from the control. It was further learned that, in the case of malic acid, there was rather a drop in the amount.

Even though dextrose showed a marked increase in the amount as compared with the control, its result was not as satisfactory as that of succinic acid. It is well known that the amount of glycogen in the liver increases by dextrose. Nevertheless, our result shows that the increase is greater in the case of succinic acid. I believe that this is a significant result.

III. General Summary and Observations

Zero point five (0.5) cubic centimeters per kilogram of one mol soda each of acetic acid, succinic acid, malic acid and lactic acid was injected to domesticated rabbits and their effect upon the liver function was examined. According to the BSP test, in the case of domesticated rabbits, acetic acid was found to be the best, followed by succinic acid. Both malic acid and lactic acid showed almost no effect at all. In the BSP test, death was brought about in the concha blood vein, thereb making it impossible to carry out a charge for a long period of time. In the case of those rabbtis with liver trouble caused by carbon tetrachloride, therefore, only the effect of acetic acid was examined. As in the case of regular domesticated rabbits, however, BSP was favorably turned.

In the Santonin detoxication test, succinic acid detoxicated them most mark-

kedly in the case of regular domesticated rabbits. As compared with the control, the amount of pigment discharge was approximately three times as great. Even in the case of liver trouble, the amount of the discharge increased to approximately twice. In addition, the effect of malic acid showed no change as compared with the control in the case of domesticated rabbits. It showed an effect as in the case of succinic acid in the case of liver trouble, with the amount of discharge being approximately twice.

In the fructose loading test, succinic acid and acetic acid showed a promotion of the fructose treatment capacity in the case of regular domesticated rabbits as compared with the control and a similar trend was shown in the case of liver trouble.

In the determination of urobilinogen in urine, it is believed impossible to attach significance inasmuch as the amount of urobilinogen in urine is extremely small in the case of regular domesticated rabbits. However, succinic acid showed a decline in the value of urobilinogen. In the case of liver trouble, it was acetic acid where the drop in the urobilinogen value in urine was the greatest. Succinic acid showed no great difference from the control.

According to Matsuo, the pigment discharge function is being mainly carried out in the neighborhood of the small "leaves" of the liver. Also, the metabolism of fructose and other carbohydrates is mainly carried out at the center of the liver's small "leaves", according to a certain report (21). From such a point of view, judging from the fact that succinic acid and acetic acid had a satisfactory effect upon the metabolism of fructose in addition to the pigment, discharge amount, it is believed that they act as a promotor upon the function of the liver as a whole.

In short, the result of the liver function in the case where an organic acid is charged was the best in the case of succinic acid, followed by acetic acid.

It showed that succinic acid had a marked effect, especially, on the

Santonin detoxication capacity. Regarding this detoxication effect, R. G. Mezzich reported that succinic acid has a barbitalic detoxication effect and that, when sodium succinate was administered to a person who had taken a large amount of barbital, there was observed early recovery(22).

When sleep was induced in a domesticated rabbit by injecting barbital and succinic acid was later injected into the same, it was observed that the sleep time was shortened as compared with the control. On the other hand, Ogawa stated that the action of succinic acid upon barbital cannot be termed something peculiar (23).

Nevertheless, it became clear from my experiment that succinic acid shows an effect of promoting detoxication capacity. It can also be proved by the increase in the amount of glycogen as will be stated later.

Generally speaking, there is a parallel relationship between the liver function and the amount of liver glycogen. When the amount of liver glycogen is large, it is believed that the liver function is better. This also became clear from my own experiment. In other words, when one cubic centimeter per 20 grams of the 1/10 mol sodium salt of each of the organic acids was injected into mice, succinic acid was found to have increased the amount of liver glycogen the most among the organic acids.

According to the result obtained by Ponsford, succinic acid and malic acid gave values which were twice as large and dextrose (grape sugar) gave a value which was three times as large as that of the control. According to my own experiment, the succinic acid value was approximately twice and was the highest and this was followed by dextrose (grape sugar), lactic acid and citric acid in that order. Malic acid was rather found to be dissatisfactory.

The above result is approximately in agreement with the result of the effect upon the liver function of domesticated rabbits. My experiment did not show that lactic acid gives a favorable effect upon the liver function. However, it gave a satisfactory result in the case of liver glycogen.

In addition, citric acid and succinic acid are known to have an anti-ketonic function (24) and there are reports stating that the ketonic bodies will be reduced when citric acid and succinic acid are administered (6, 25, 26). This is believed to be due to the fact that both of these increase the (amount of) liver glycogen.

It has been reported that, at the time of a liver trouble, the amounts of fuming dextrose (27), (alpha)-ketoglutaric acid (28,29) and citric acid (30) increase. If sodium succinate is injected at this time, a certain report states that the Sawada urine reaction turns negative.

According to Miyaji et al (31), on the other hand, the effect of a continuous injection of organic acids upon the liver structure was such that citric acid was found to be the worst, with acetic acid and malic acid bringing about a slight change and succinic acid bringing about no change whatsoever. Miyaji et al. also stated (32) that no obstruction at all was observed at the time when 0.1 gram of succinic acid per kilogram was administered to a domesticated rabbit for a period of two months.

Based on the above, succinic acid was considered to have a favorable effect upon the liver function as compared with the other organic acids. The result I obtained clearly confirmed the above belief. Even though succinic acid, citric acid and malic acid are all organic acids belonging to the TCA cycle, it is an interesting fact that succinic acid alone shows a markedly favorable effect upon the liver. It seems that the above is due to the fact that succinic acid is often used as it is harmless as compared with the other organic acids.

IV. Conclusion

By selecting succinic acid, acetic acid, malic acid, lactic acid and citric acid from among those belonging to the TCA cycle and their effect upon the liver function was examined relative to the liver function and liver glycogen. As the methods for examining the liver function, the BSP test, the Santonin

detoxication test, the fructose load test and the urine urobilinogen determination method were used relative to domesticated rabbits. Liver glycogen was examined relative to mice. The following results were obtained in this study.

1. In the case of liver function, succinic acid was found to have the most promoting effect. This was followed by acetic acid. Both malic acid and lactic acid showed various results, registering apparently unfavorable effects in some cases. Particularly, succinic acid showed a markedly promoting effect on Santonin detoxication capacity both in the regular case and in the case involving troubled livers.

2. As for the amounts of liver glycogen after the administration of organic acids, succinic acid showed the most marked increase, followed by lactic acid, and citric acid in this order. Acetic acid showed almost no effect whatsoever. Malic acid rather showed a reducing effect. The result showed that succinic acid increased the amount of liver glycogen to a greater extent than dextrose.

3. To sum up the above, succinic acid gives the best effect upon the liver among all the organic acids mainly belonging to the TCA cycle.

(The essence of this study was published before the 42nd and 43rd Assemblies of the Japanese Society of Digestive Organ Diseases.)

(At the end, the author would like to offer his sincere gratitude to his teacher, Professor Sawada, who was kind enough to offer guidance and read the manuscript. He also offers his deep thanks to Mr. Miyaji, Lecturer of this Department, who offered direct guidance and encouragement in many details.)

References:

1) H. A. Krebs, W. A. Johnson: Enzymologia, 4, 148, 1937.

2) Practical Therapeutics by M. E. Rehfuss and A. H. Price: Second Edition, Baltimore, The Williams and Wilkins Company 1951.

3) Miyaji and Saeki: Clinics and Study (Rinsho to Kenkyu), 32; 1066, 1955.

4) Miyaji: Journal of the Fukuoka Medical Society, 47; 916, 1956.

5) Yoshida: Journal of the Society of Internal Medicine of Japan, 44; 979,

1956.

6) Oishi: Journal of the Okayama Medical Society, 67; 913, 1955.

7) Kurihara: Journal of the Fukuoka Medical Society, 44; 8, 1953.

8) Takasugi: Journal of the Society of Internal Medicine of Japan, 25; 765, 1937.

9) Anami: Nippon Medical Report, No. 1630, 1955.

10) Li: Journal of the Fukuoka Medical Society, 41; 697, 1950.

11) Saito: Clinical Chemical Examinations using Photoelectric Colorimeter, p. 89, 1954.

12) R. Stohr: Hoppe-Seyler Z, 217; 153, 1933.

13) W. Krutscher and M. Schippers: Z. Phisiol. chem. 286; 59, 1950.

14) A. P. Ponsford and I.S. Maclean: From the Biochem. Depart. Lister Institute, London, 1932.

15) Kay, M.: J. B. C. 133; 59, 1940.

16) Tanaka: Journal of the Osaka Medical Society: 31; 3811, 1932.

17) Makino: Vitamins, 4; 316, 1951.

18) Kobayashi: Annual Report of the Scientific Researches of the Tokyo Municipal Hospitals, 2; 57, 1935.

19) Kodama: Clinical Biochemistry, 122, 1952.

20) Verzar and Wenner: Biochem. J. 42; 42, 1948.

21) Matsuo: Nippon Clinical (Nippon Rinsho), 9; 153, 1951.

22) R. G. Mezzich: Rev. facultad farm. y. bioquim, 12, 40, 1950.

23) Ogawa: Modern Medicine, 4; 717, 1949.

24) A. V. Koranyi: ibid., 101, 669, 1937.

25) C. A. Vesce: Arch. Studio fisiopatol. Clin. ricanbio 7, 331, 1937; C.A. 34, 24596, 1940.

26) Kan, Nakajima, Kawai and Matsuoka: Journal of the Society of Internal Medicine of Japan: 42, 214, 1953.

27) E. Bueding, M. H. Stein and H. Wortis: J. Biol. Chem. 140; 697, 1941.

28) L. H. Smith, Jr., R. H. Ettinger and D. Seligson: J. Clin. Invest. 32; 273, 1953.

29) Sawada, Miyaji and Shikagami: Vitamins, 10, 134, 1956.

30) H. Buch: Nord. Med. 5; 125, 1940.

31) Miyaji and Kikuda: Comprehensive Clinical Medicine, 6; 61, 1957.

32) Miyaji, Saeki and Matsuoka: Journal of the Fukuoka Medical Society, 47; 635, 1956.

(The manuscript was received on November 11, 1958, special printing.)

Translated by Carl Demrick Associates, Inc./RO/db

Table 1

a 家兎 No.		b 負荷順序	c 10′後	d 15′後	e 負荷順序	f 10′後	g 15′後
h 酷	1	B	14.0%	5.0%	A	6.4%	3.6%
		A	3.3	2.5	B	10.1	7.5
	2	B	14.1	9.7	A	4.8	4.8
		A	9.7	7.0	B	9.5	4.7
	6	B	29.0	12.8	A	9.0	3.4
		A	13.6	3.1	B	10.2	3.4
	8	B			A	3.6	2.6
		A			B	7.0	5.0
i 酸	平均	B	19.0	9.2	A	7.9	3.6
		A	8.9	4.2	B	9.2	5.2
j	全体平均	B	13.6	7.2			
		A	8.4	3.9			
K コハク酸	1	B	6.4%	3.6%	S	9.5%	4.8%
		S	3.4	1.2	B	6.9	3.4
	4	B	9.3	5.5	S	6.2	5.6
		S	6.9	6.2	B	9.2	5.0
	6	B	12.8	4.0	S	2.5	2.0
		S	12.8	5.9	B	5.0	4.2
	7	B	7.5	6.3	S	12.6	5.8
		S	8.0	7.2	B	14.0	3.8
m	平均	B	9.0	4.9	S	7.7	4.6
		S	7.8	5.1	B	8.8	4.1
h	全体平均	B	8.9	4.5			
		S	7.8	4.9			

No.		u 負荷順序	v 10′後	w 15′後	x 負荷順序	y 10′後	z 15′後
o リンゴ酸	3	B	18.0%	3.6%	M	10.6%	3.9%
		M	11.8	8.4	B	8.9	4.4
	6	B	7.2	2.5	M	10.3	7.3
		M	9.5	2.8	B	12.8	11.2
	8	B	11.4	8.7	M	5.6	4.1
		M	10.6	6.6	B	7.2	5.1
	10	B	6.3	5.1	M	9.5	5.9
		M	10.0	4.4	B	9.5	7.5
p	平均	B	10.7	5.0	M	9.0	5.3
		M	10.5	5.6	B	9.6	7.1
q	全体平均	B	10.2	6.1			
		M	9.8	5.5			
r 乳酸	4	B	5.6%	5.1%	L	12.6%	6.7%
		L	9.3	4.0	B	12.5	5.3
	6	B	6.2	3.9	L	6.6	9.3
		L	6.7	2.2	B	7.5	4.8
	8	B	7.5	5.0	L	7.8	4.7
		L	10.3	8.9	B	15.3	12.1
	9	B	10.9	10.3	L	12.8	8.9
		L	13.6	9.3	B	8.7	6.7
s	平均	B	7.6	6.1	L	10.0	7.5
		L	10.0	8.2	B	11.0	7.2
t	全体平均	B	9.3	6.7			
		L	10.0	7.9			

Table 2

No. (kg)	u 負荷法	前	1	2	3	5	8	9	10	12	15	17	19
4	B	8	87		98				28				
			↑		↓				↑				
(1.9)	A		98		93				27				
7	B	10	92	131		44	27	30	25	22	20	19	12
				↑		↓	↑	↓	↓	↑	↓	↓	↑
(2.4)	A			111		59	26	20	16	23	16	11	12
8	B	7	119		97								
			↓		↑								
(2.1)	A		89		90								
9	B	12	78			46			31	38	34	36	24
			↓			↓			↑	↓	↓	↑	↑
(2.0)	A		49			26			38	22	40	36	16

Table 3

	No. (kg)	前3時間 Vo オキシサントニン (f)	後3時間 No. オキシサントニン (g)	No/Vo×100
a 対照	10	215.6	171.9	79
	(2.1)	131.3	102.3	78
	11	190.0	132.0	69
	(2.0)	57.0	33.8	60
	12	139.5	117.2	84
	(1.9)	126.0	98.2	78
	13	80.5	64.0	80
	(1.8)	110.0	86.0	78
	平均 (h)	131.2	100.7	77

	No. (kg)	前3時間 Vx オキシサントニン	後3時間 Nx オキシサントニン	Nx/Vx×100
b 酢酸	10	164.0	213.7	130
	11	147.9	69.3	47
	12	170.0	284.4	167
	13	173.0	171.0	99
	平均 (l)	204.8	238.1	111
c コハク酸	10	25.3	110.5	437
	11	93.8	339.2	362
	12	116.2	169.4	146
	13	115.5	127.5	110
	平均 (j)	87.7	186.7	222
d リンゴ酸	10	113.4	100.5	88
	11	267.5	170.8	64
	12	166.0	223.3	135
	平均 (k)	182.3	164.9	90
e 乳酸	10	94.0	93.1	99
	11	120.7	95.0	79
	12	177.9	169.9	96
	13	281.4	168.8	60
	平均 (m)	168.5	131.7	78

Table 4

No. (kg)		0	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10	1	119.6		0				44.3	51.1	62.1						120.4
				A				A	A							M
(2.1)	2	81.3		46.9				25.4	55.3	37.8						150.5
12	3	132.0		0												
				S												
(2.0)	4	97.0		16.3												
15	5	131.3		1.0				28.3	37.4	51.8	39.4		60.1		76.1	56.0
				S				S		A					S	M
(2.0)	6	107.6		0				72.5	26.3	72.5	27.6		107.5		45.9	118.8
17	7	197.0		16.2			80.6	1.5					136.1			97.1
								S					A			A
(1.9)	8	106.2		12.5			42.0	12.3					43.0			93.7
18	9	255.0		5.6			7.9	39.0	37.5				123.8		163.8	
				M			M	L	A						S	
(2.0)	10	135.0		9.2			23.5	57.6	35.8				97.5		222.8	
19	11	213.1		11.7			32.1	0								
				L			L	M								
(2.1)	12	184.0		0			56.0	18.4								
21	13	128.2	29.8	69.1	69.7	134.8		122.0								
				L		M		L								
(2.1)	14	103.1	21.4	97.8	48.7	160.6		91.0								

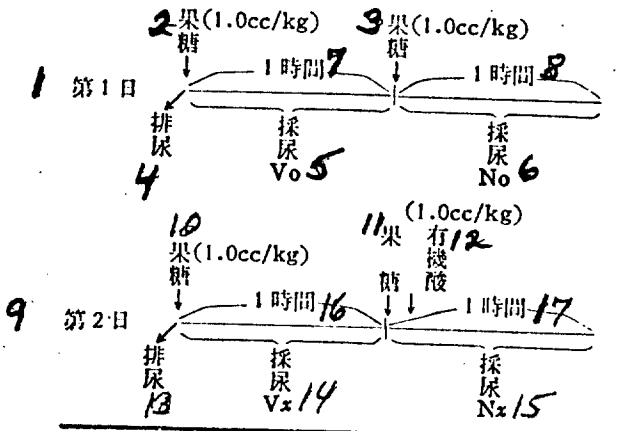
Table 5

	No.	1 前3時間 Vo オキシサントニン	2 後3時間 No オキシサントニン	No/Vo×100
a 対照	10	62.1	37.8	
	15	37.4	26.3	
		39.4	27.6	
	17	16.2	12.5	
		80.6	42.0	
	18	123.8	97.5	
	21	29.8	21.4	
		69.7	48.7	
	3 平均	57.4	39.2	68

	No.	4 前3時間 Vx オキシサントニン	5 後3時間 Nx オキシサントニン	Nx/Vx×100
b 酢酸	10	0	46.9	
		44.3	25.4	
		51.1	55.3	
	15	51.8	72.5	
	17	136.1	43.0	
		97.1	93.7	
	18	37.5	35.8	
	6 平均	59.7	53.2	89

	No.	7 前3時間 Vx オキシサントニン	8 後3時間 Nx オキシサントニン	Nx/Vx×100
c コハク酸	12	0	16.3	
	15	1.0	0	
		28.3	72.5	
		76.1	45.9	
	17	1.5	12.3	
	18	163.8	222.8	
	9 平均	45.1	66.6	148
d リンゴ酸	10	120.4	150.5	
	15	56.0	118.8	
	18	5.6	9.2	
		7.9	23.5	
	19	0	18.4	
	21	134.8	160.6	
	10 平均	54.1	80.1	148
e 乳酸	15	60.1	107.5	
	18	39.0	57.6	
	19	11.7	0	
		32.1	56.0	
	21	69.1	97.8	
		122.0	91.0	
	11 平均	55.7	54.0	97

Table 6



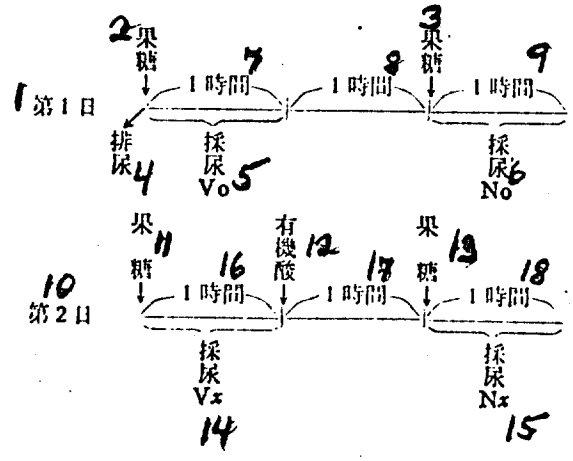
	No.	Vo	No	No/Vo×100
a 対照	10	67.83mg	74.89mg	110%
	17	76.21	101.66	133
	28	52.04	87.34	168
	29	100.29	105.89	106
	30	102.47	121.00	118
	平均 f	79.77	98.16	127

	No.	Vx	Nx	Nx/Vx×100
b コハク酸	10	96.07mg	184.68mg	192%
	15	47.31	78.60	166
	28	74.44	110.68	149
	30	64.99	94.25	145
	平均 g	70.70	117.05	167
c 酢酸	10	112.80mg	104.08mg	97%
	15	134.99	149.82	156
	28	55.29	98.25	178
	29	78.60	136.82	174
	30	62.22	54.35	87
	平均 h	88.58	108.66	123
d リンゴ酸	10	80.42mg	118.65mg	148%
	15	110.62	81.88	74
	28	133.67	99.01	70
	29	125.78	76.00	61
	30	58.94	78.85	134
	平均 i	101.89	90.68	89
e 乳酸	10	129.47mg	39.18mg	31%
	15	92.47	124.82	133
	28	132.99	146.77	111
	29	118.08	78.12	71
	30	66.23	85.09	128
	平均 j	107.85	94.80	87

Table 7

No.	Vx	Nx	Nx/Vx×100
10	78.57mg	255.92mg	325%
15	94.61	132.09	137
29	102.86	176.61	172
30	72.78	120.09	165
a 平 均	62.21	171.18	275

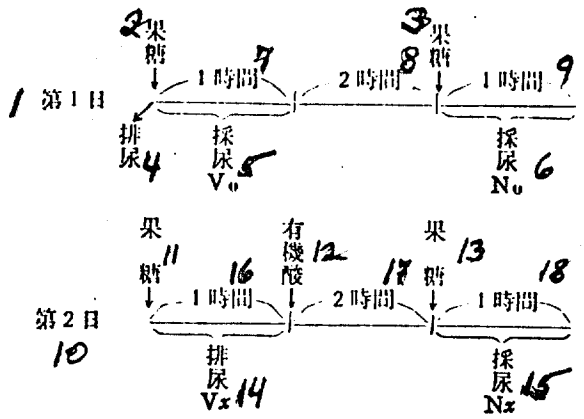
Table 8



	No.	Vo	No	No/Vo×100
a 対照	10	227.09mg	206.70mg	91%
	29	136.09	92.25	68
	30	207.54	147.44	71
	32	91.47	91.02	98
	33	100.18	163.99	164
	平 均 g	152.47	140.28	98

	No.	Vx	Nx	Nx/Vx×100
b コハク酸	10	109.05mg	160.06mg	147%
	28	99.46	140.74	148
	29	142.29	164.56	116
	30	207.24	103.56	50
	平 均 h	139.51	129.73	93
c 酢酸	10	179.40mg	225.40mg	126%
	29	98.26	130.17	132
	30	114.39	127.73	111
	平 均 i	130.68	161.10	123
d リンゴ酸	29	122.58mg	136.47mg	111%
	30	92.00	130.22	142
	平 均 j	107.29	133.34	124
e 乳酸	10	146.48mg	182.49mg	125%
	30	108.12	135.30	125
	平 均 k	129.30	158.90	125
f ブドウ糖	10	85.52mg	89.50mg	105%
	15	202.79	154.84	76
	29	101.52	56.40	56
	平 均 m	129.98	100.25	77

Table 9



	No.	Vo	No	No/Vo×100
a 対照	10	99.42mg	102.48mg	103%
	15	112.86	130.24	115
	29	106.99	118.16	110
	30	98.20	122.83	125
	33	130.76	139.43	107
	平均 g	109.65	122.63	111

	No.	Vx	Nx	Nx/Vx×100
b コハク酸	10	84.03mg	79.45mg	94%
	15	104.08	82.49	79
	29	101.89	102.84	100
	30	131.00	65.11	50
	33	70.17	73.71	104
	平均 h	98.23	80.72	82
c 酢酸	10	99.47mg	55.80mg	51%
	15	102.02	84.15	82
	29	72.88	78.46	106
	30	54.64	66.96	121
	33	66.57	57.32	86
	平均 i	79.12	68.54	87
d リンゴ酸	15	33.96mg	67.25mg	198%
	29	71.98	142.50	198
	30	113.78	165.33	145
	33	31.06	37.37	120
	平均 j	62.69	103.11	164
e 乳酸	10	70.72mg	82.79mg	117%
	15	108.06	153.71	142
	30	32.61	52.16	160
	33	97.26	145.33	149
	平均 k	77.16	108.50	141
f ブドウ糖	15	82.48mg	49.13mg	60%
	30	73.69	49.10	67
	33	97.04	78.25	81
	平均 m	84.40	58.83	70

Table 10

No. (kg)	日	0	2	3	4	5	8
10	V	126.77mg	122.81	350.54	192.81		
			S	A			
	N	144.10mg	185.30	165.45	234.84		
(2.5)	N/V×100	(114%)	(151%)	(47%)	(122%)		
15	V	131.00	72.78	88.59	110.05	149.26	150.31
					M	L	S
	N	117.38	101.45	120.08	235.09	208.36	105.53
(2.0)	N/V×100	(90%)	(139%)	(135%)	(213%)	(139%)	(70%)
29	V	89.28	115.14	117.83	180.35	169.82	111.60
			A	M	L	S	
	N	110.08	124.45	196.41	296.00	131.00	159.72
(2.2)	N/V×100	(123%)	(108%)	(167%)	(164%)	(77%)	(143%)
30	V	53.48	122.82	139.08	166.91	170.79	105.52
			M	L	S	A	M
	N	65.57	151.39	111.16	203.43	161.63	159.22
(2.0)	N/V×100	(123%)	(123%)	(86%)	(122%)	(95%)	(151%)
33	V	63.25	109.17	141.92	142.65	140.70	77.23
			L	S	A	M	L
	N	73.58	145.30	124.45	152.84	189.28	109.27
(2.0)	N/V×100	(116%)	(133%)	(88%)	(107%)	(135%)	(141%)

Table 11

	No.	V	N	$\frac{N}{V}\times100$
a コハク酸	10	122.81mg	185.30mg	151%
	15	150.31	105.53	70
	29	169.82	131.00	77
	30	166.91	203.43	122
	33	141.92	124.45	85
	平均 f	150.35	149.94	100
b 酢酸	10	350.54mg	165.45mg	47%
	29	115.14	124.45	108
	30	170.79	161.63	93
	33	142.65	152.84	107
	平均 g	194.78	152.34	78
c リンゴ酸	15	110.05mg	235.09mg	213%
	29	117.83	196.41	167
	30	122.82	151.39	123
	30	105.52	159.22	151
	33	140.70	189.28	135
	平均 h	119.38	186.28	156

	No.	V	N	$\frac{N}{V}\times100$
d 乳酸	15	110.05mg	235.09mg	213%
	29	180.35	296.00	164
	30	139.08	111.16	86
	33	109.17	145.30	133
	33	77.23	109.27	141
	平均 i	123.18	179.36	146
e 琥珀酸	10	192.81mg	234.84mg	122%
	15	72.78	101.45	139
	15	88.59	120.08	135
	29	111.60	159.72	143
	平均 j	116.44	154.02	132

Table 12

No. \ 採尿時 a	b 12～14時尿 U 量	c 14～16時尿 U 量
10	0.075mg	0.067mg
15	0.063	0.049
29	0.066	0.066
30	0.031	0.025
32	0.052	0.047
33	0.039	0.021
d 平　均	0.054	0.049

Table 13

	No.	e 前尿値	f 後尿値
a コハク酸	10	0.071mg	0.055mg
	15	0.039	0.010
	29	0.059	0.016
	33	0.034	0.014
	平均	0.051	0.024
b 酢酸	10	0.037mg	0.061mg
	29	0.053	0.020
	30	0.020	0.030
	33	0.038	0.029
	平均	0.037	0.035

	No.	g 前尿値	h 後尿値
c リンゴ酸	10	0.076mg	0.033mg
	29	0.075	0.037
	30	0.044	0.104
	33	0.024	0.024
	平均	0.055	0.050
d 乳酸	10	0.051mg	0.050mg
	15	0.041	0.043
	30	0.048	0.022
	33	0.033	0.020
	平均	0.043	0.034

Table 14

No.	日	0	2	3	4	5	6
10	V	mg 0.127	0.210 S	0.107 A	0.190		
	N	mg 0.102	0.269	0.053	0.183		
15	V	0.050	0.033	0.092	0.093 M	0.109 L	0.092 S
	N	0.041	0.022	0.098	0.124	0.062	0.063
29	V	0.063	0.137 A	0.150 M		0.307 S	
	N	0.059	0.025	0.112		0.123	
30	V	0.043	0.049 M	0.068 L	0.031 S	0.065 A	0.047 S
	N	0.046	0.081	0.042	0.036	0.074	0.040
33	V	0.046	0.096 L	0.116 S	0.218 A	0.056 M	0.111 L
	N	0.054	0.043	0.042	0.082	0.117	0.141

Table 15

	No.	前尿値	後尿値
a 対照	10	0.190mg	0.183mg
	15	0.033	0.027
	15	0.092	0.098
	平均 f	0.105	0.103
b コハク酸	10	0.210mg	0.269mg
	15	0.092	0.063
	29	0.307	0.123
	30	0.031	0.036
	33	0.047	0.040
	平均 g	0.117	0.106
c 酢酸	10	0.107mg	0.053mg
	29	0.137	0.025
	30	0.065	0.074
	33	0.218	0.082
	平均 h	0.138	0.059

	No.	Vx	Nx	Nx/Vx×100
d リンゴ酸	15	0.093mg	0.124mg	
	29	0.150	0.112	
	30	0.049	0.081	
	33	0.056	0.117	
	平均 i	0.087	0.109	
e 乳酸	15	0.109mg	0.062mg	
	30	0.068	0.042	
	33	0.096	0.043	
	33	0.111	0.141	
	平均 j	0.096	0.072	

Table 16

No.	a 体重	b「グ」量	No.	c 体重	d「グ」量
	g	mg%		g	mg%
1	18	195.5	6	22	252.5
2	20	83.3	7	18	143.3
3	23	206.6	8	21	291.4
4	20	181.6	9	20	154.2
5	17	132.2	10	19	245.0
	e 10例平均				188.8

Table 17

	a 対照群			b 被検群	
No.	c 体重	「グ」量	No.	d 体重	「グ」量
	g	mg%		g	mg%
11	20	349.6	14	18	59.2
12	19	116.6	15	17	108.2
13	19	188.7	16	20	47.2
e 平均		218.3	f 平均		71.6

Table 18

a 対照群			b 被検群		
No.	c 体重	d「グ」量	No.	e 体量	f「グ」量
	g	mg%		g	mg%
17	20	185.2	21	19	263.7
18	21	251.6	22	19	396.7
19	19	189.2	23	20	100.8
20	20	241.2	24	21	260.9
g 平均		216.8	h 平均		305.5

Table 19

No.	a 対照		b ハク酸		c 酢酸		d リンゴ酸		e 乳酸		f クエン酸		g 葡萄糖	
	h	i	j	k	m	n	o	p	q	r	s	t	u	v
	g	mg%	g	mg%	g	mg%	g	mg%	g	mg%	g	mg%	g	mg%
1	24	161.0	25	452.3	31	199.8	23	156.3	25	188.7	18	186.9	23	468.1
2	25	167.4	19	186.9	24	95.3	21	111.9	22	105.4	16	77.0	23	199.8
3	24	140.4	22	373.8	18	131.4	19	107.1	22	71.2	20	220.2	22	196.1
4	21	215.6	24	386.7	18	176.7	21	96.2	21	203.5	17	224.6	25	334.9
5	22	175.8	25	275.7	21	254.2	25	121.1	19	206.3	18	173.9	25	178.5
6	21	164.0	20	146.2	19	179.5	25	99.9	21	356.1	20	310.8	20	278.0
7	21	123.1	20	87.9	19	97.2	20	51.3	19	320.1	16	181.3	18	235.5
8	18	191.5	21	457.0	21	230.3	18	93.4	18	159.1	18	172.5	25	223.9
9	20	188.6	17	281.1	25	247.0	17	168.4	17	266.4	21	210.6	21	162.8
10	21	174.2	21	396.0	18	183.2	18	124.0	21	405.4	20	195.8	21	243.7
平均	w	170.2		304.4		179.5		113.0		228.2		205.3		252.1

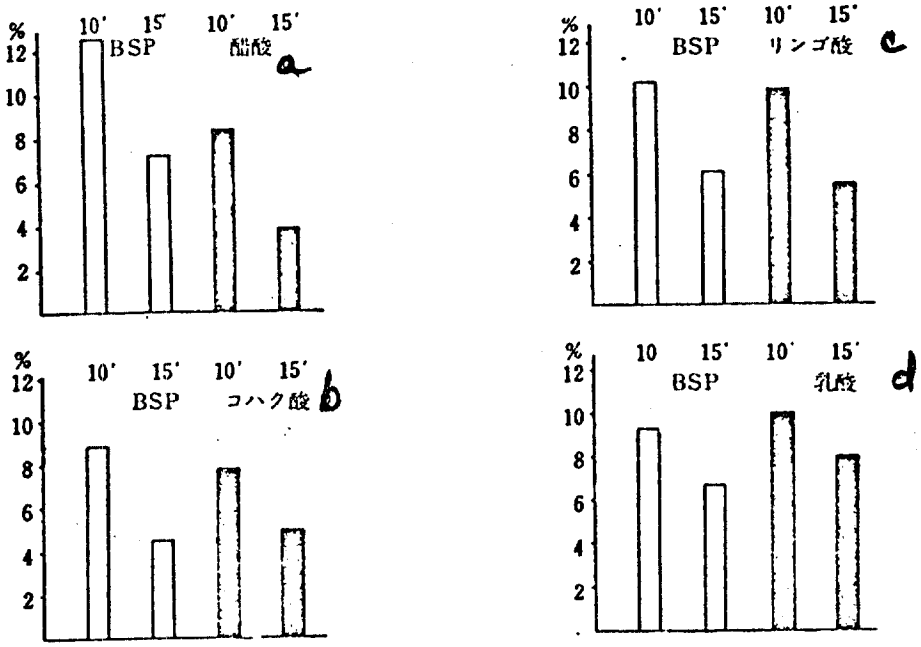


Figure 1

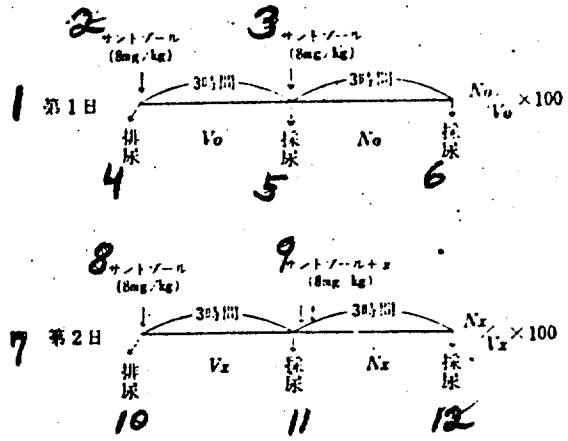


Figure 2

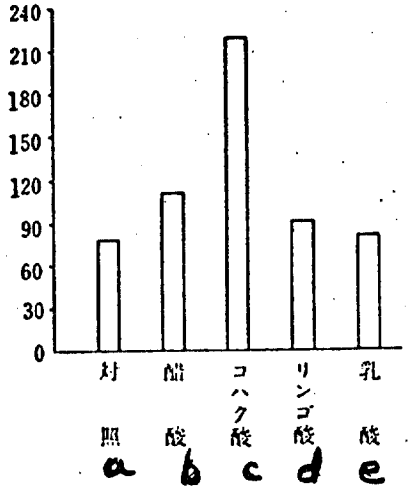


Figure 3

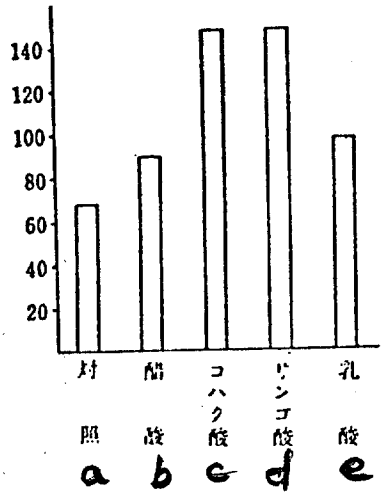


Figure 4

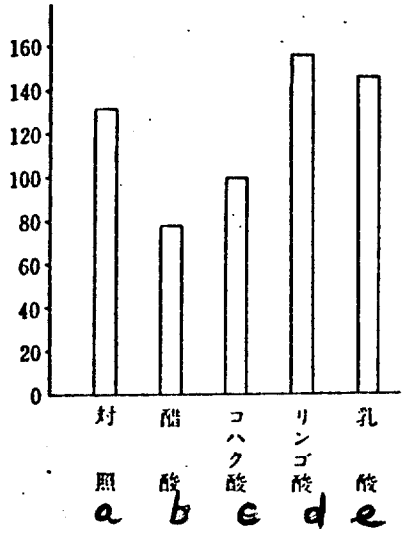


Figure 5

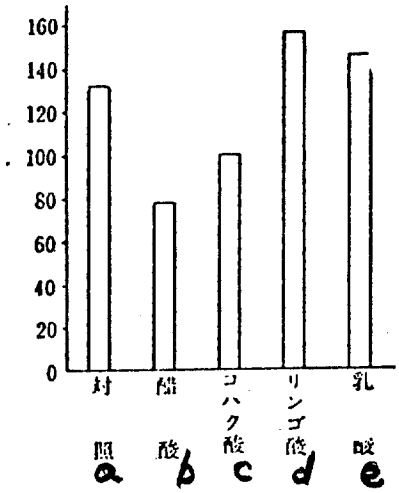


Figure 6

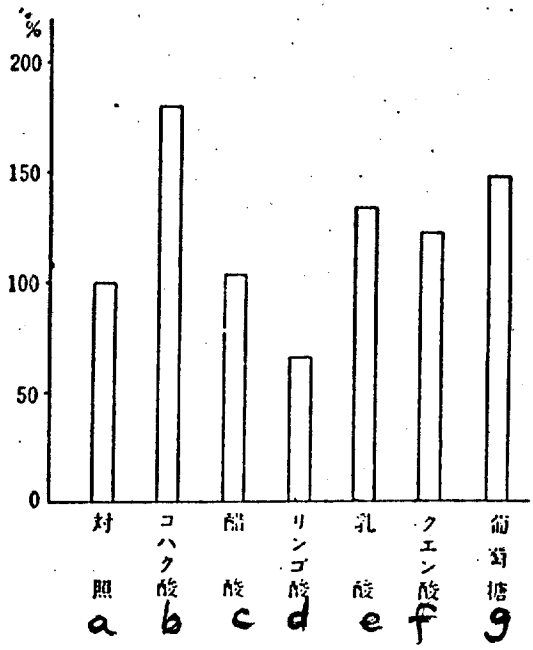


Figure 7

Malic Acid
478

主として TCAサイクルに属する有機酸の肝臓に及ぼす影響

医学士　塚　本　敬　一　郎

九州大学医学部第三内科教室（指導　沢田藤一郎教授）

The Influence of Organic Acids of T C A Cycle on the Liver

Keiichiro TSUKAMOTO

Third Department of Internal Medicine (Director: Prof. Dr. T. SAWADA)
Faculty of Medicine, Kyushu University, Fukuoka, Japan.

Among organic acids, citric acid is frequently used and have been published afew studies upon the relation of citric acid to liver function. And it is found that succinic acid has an effect to arrythmia.

Then I observed and compared the effects of organic acids (succinic acid, acetic acid, malic acid, lactic acid, citric acid) with one another on the function of liver in healthy rabbits and liver damaged ones and on the deposition of liver glycogen in healthy mice.

The following liver function test were performed: B S P test, santonin detoxication test, fructose test and urobilinogen test,

The following results were obtaind.

1) After intravenous injection of 0.5cc/kg. of 1 M sodium salts in rabbits, succinic acid greatly improved several liver functions, especially the ability of detoxication. Acetic acid was pretty good. Malic acid and lactic acid were rather injurious.

2) On the deposition of liver glycogen, after intracutaneus injection of 1cc/20gr of M/10 sodium salts in mice, succinic acid was greatly increased more than the control group. Lactic acid and citric acid accelerated it, too. Acetic acid was analogous to control group and malic acid decreased it.

3) In a word, among organic acids of T C A cycle succinic acid has the best effect to the liver.

目　次

緒　言
I．肝機能に及ぼす有機酸の影響
1．実験方法
2．実験成績
1）色素排泄機能（B S P試験）
2）解毒機能（サントニン試験）
3）糖代謝機能（果糖負荷試験）
4）胆汁生成機能(尿中ウロビリノーゲン定量)
3．小括
II．肝グリコーゲンに及ぼす有機酸の影響
1．実験方法

2．実験成績

3．小括

Ⅲ．総括及び考按

Ⅳ．結　論

緒　言

1937年H．A．Krebs[1]によつて，"Tricarboxylic acid cycle"（以下TCAサイクルと略す）学説が提唱され，其の後多くの人々によつて，この細部の機構が研究され，炭水化物はもちろんのこと，脂肪及び蛋白質の3大栄養素も，幾つかの経路からTCAサイクルに入り，完全に分解されてエネルギーを遊離することが解明されてきた．

沢田内科においてはこのcycleの有機酸を臨床的にとりあげ，沢田教授はこのcycleの入口にあたる焦性葡萄酸に注目して，ビタミンB₁欠乏の化学的診断法であるところの沢田氏尿反応を考案された．また宮地等は更にTCAサイクルの各有機酸の基礎的並びに臨床的研究を行つた．

このサイクル及びこのサイクルに関係深い有機酸としては，クエン酸，α-ケトグルタール酸，コハク酸，フマール酸，リンゴ酸，オキザロ酢酸，酢酸，乳酸等があるが，このうちコハク酸に関しては利胆剤として1回0.6gの内服が用いられているとか[2]，また宮地等[3][4]の報告にみる如く，不整脈に有効である等の性質が最近になつて解明され，有機酸の臨床的研究の新しい方向が開けてきた．

ところで肝臓は代謝の主な場所であり，TCAサイクルの存在が確認されているので，このサイクルに属する有機酸を適量与えれば，基質として代謝されるので有効であると思われる．然し夫々には利用に優劣があろうし，また与える量を多くすれば特有な作用がみられるかもわからない．

之に関しては吉田[5]がクエン酸について行い，家兎に50mg/kg注射すれば肝機能は亢進し特に解毒能は著しく亢進すると報告しているが，他の有機酸については未だ報告がない．

そこで本研究ではTCAサイクに属する有機酸及びこれと関係深い有機酸として，クエン酸，コハク酸，リンゴ酸，酢酸，乳酸をえらび，これについて健康及び実験的肝障害家兎の肝機能に及ぼす影響並びにマウスの肝グリコーゲンの増減に及ぼす影響をしらべ興味ある成績を得た．

Ⅰ．肝機能に及ぼす有機酸の影響

肝機能検査の選択には出来るだけ簡単正確に示し得るもの4種を選び，且つ実験は正常の場合と肝障害の場合とを平行的に行つた．また実験動物は日によつて諸種の検査成績に変動があり，然も時に甚だしいことがあるので，成績を正確ならしめるため出きるだけ前後の値の差ではつきりと意味づけがなされるように試みた．なお蛋白代謝機能，脂肪代謝機能をしらべることは，この場合非常に有意義なことではあるが，動物実験では成績値が極めて小さく，また変動の甚だしきものがあつて薬剤の効果を判定し難いので用いなかつた．

1．実験方法

1）実験動物

体重2kg前後の雄性家兎を何れも1週間以上飼育して馴らした後に使用した．

2）使用薬剤及び投与法

有機酸は酢酸，コハク酸，ℓ-リンゴ酸，d,ℓ-乳酸の4種を選び，投与法は大石の方法[6]に従い，各1モルソーダ溶液を0.5cc/kg宛静脈内投与した．なおクエン酸ソーダーの同量は死亡することが多いので使用しなかつた．

2．実験成績

1）色素排泄機能

ブロムサルフアレイン試験（BSP試験）

(1) 正常家兎のBSP排泄能に及ぼす有機酸の影響

栗原[7]によると，家兎におけるBSP試験は5mg/kg注射法では血中より速かに消失して測定し難いことがあるので，10mg/kg注射し10分及び15分後の血清中のBSP値を測定してその残存率を求め，夫々10分値，15分値とし之を対照とした．次に3時間後に有機酸とBSPを同時に注射して10分値，15分値を測定して対照と比較した．なお更に有機酸の効果の判定を明確にするため，逆に先づ有機酸とBSPを同時に注射して10分値，15分値を測定し，3時間後にBSPのみを注射し10分値，15分値を測定して対照と比較した．

実験結果は第1表，第1図に示す如く，酢酸負荷では各例ともBSP10分値，15分値いづれも好転した．コハク酸では各例とも変化が僅かで，その平均では10分値は僅かに好転，15分値は僅かに悪くなつた．リンゴ酸でも各例の変化は僅かで，平均では10分値，15分値とも僅かに好転した．乳酸は各例とも僅かではあるが悪化する場合が多く，従つて平均では10分値，15分値ともに僅かに悪くなつた．

(2) 肝障害家兎のBSP排泄能に及ぼす酢酸の影響

四塩化炭素を20%の割合にオリーブ油に溶解し，家兎早朝空腹時の体重を標準とし，1cc/kg（1cc中CCl_4 0.2g含有）を家兎脊筋内に注射して肝障害を起さしめ，逐日的に正常家兎の場合と同様にして，酢酸負荷のBSPに及ぼす効果をしらべた．なお測定値は

Table 1

第 1 表　　有機酸負荷前後のＢＳＰ値（正常家兎）

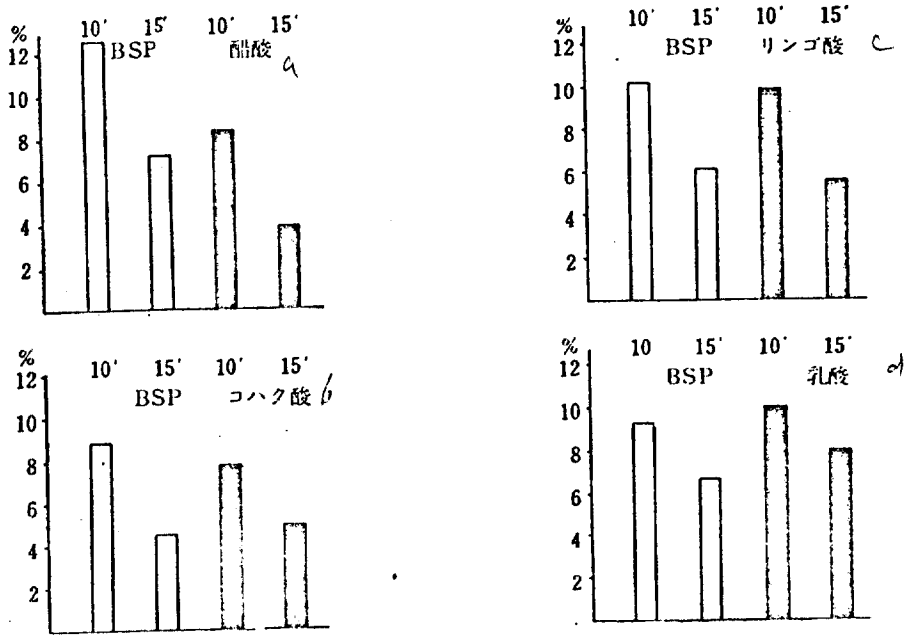
（有機酸の投与量は各１モルソーダ0.5cc/kg静注）

家兎 No.		負荷順序	10′後	15′後	負荷順序	10′後	15′後
酢酸	1	B	14.0%	5.0%	A	・6.4%	・3.6%
		A	・3.3	・2.5	B	10.1	7.5
	2	B	14.1	9.7	A	・4.8	4.8
		A	・9.7	・7.0	B	9.5	4.7
	6	B	29.0	12.8	A	9.0	3.4
		A	・13.6	・3.1	B	10.2	3.4
	8	B			A	・3.6	・2.6
		A			B	7.0	5.0
	平均	B	19.0	9.2	A	・7.9	・3.6
		A	・8.9	・4.2	B	9.2	5.2
	全体平均	B	13.6	7.2			
		A	8.4	3.9			
コハク酸	1	B	6.4%	3.6%	S	9.5%	4.8%
		S	・3.4	・1.2	B	6.9	3.4
	4	B	9.3	5.5	S	・6.2	5.6
		S	・6.9	6.2	B	9.2	5.0
	6	B	12.8	4.0	S	・2.5	・2.0
		S	12.8	5.9	B	5.0	4.2
	7	B	7.5	6.3	S	12.6	5.8
		S	8.0	7.2	B	14.0	3.8
	平均	B	9.0	4.9	S	7.7	4.6
		S	7.8	5.1	B	8.8	4.1
	全体平均	B	8.9	4.5			
		S	7.8	4.9			

No.		負荷順序	10′後	15′後	負荷順序	10′後	15′後
リンゴ酸	3	B	18.0%	3.6%	M	10.6%	3.9%
		M	・11.8	8.4	B	8.9	4.4
	6	B	7.2	2.5	M	10.3	7.3
		M	9.5	2.8	B	12.8	11.2
	8	B	11.4	8.7	M	・5.6	4.1
		M	10.6	6.6	B	7.2	5.1
	10	B	6.3	5.1	M	9.5	5.9
		M	10.0	4.4	B	9.5	7.5
	平均	B	10.7	5.0	M	9.0	5.3
		M	10.5	5.6	B	9.6	7.1
	全体平均	B	10.2	6.1			
		M	9.8	5.5			
乳酸	4	B	5.6%	5.1%	L	12.6%	6.7%
		L	9.3	4.0	B	12.5	5.3
	6	B	6.2	3.9	L	6.6	9.3
		L	6.7	2.2	B	7.5	4.8
	8	B	7.5	5.0	L	・7.8	・4.7
		L	10.3	8.9	B	15.3	12.1
	9	B	10.9	10.3	L	12.8	8.9
		L	13.6	9.3	B	8.7	6.7
	平均	B	7.6	6.1	L	10.0	7.5
		L	10.0	8.2	B	11.0	7.2
	全体平均	B	9.3	6.7			
		L	10.0	7.9			

B：BSP　A：酢酸　S：コハク酸　M：リンゴ酸　L：乳酸　・有効を示す

Fig. 1 第 1 図　　ＢＳＰ排泄能による有機酸の効果比較（正常家兎）



第2表　醋酸負荷前後のBSP値（肝障害家兎）

No.（kg）	負荷法	前	1	2	3	5	8	9	10	12	15	17	19
4	B	8	87		98				28				
			↑		↓				↑				
(1.9)	A		98		93				27				
7	B	10	92	131		44	27	30	25	22	20	19	12
				↑		↓	↑	↓	↓	↑	↓	↓	↑
(2.4)	A			111		59	26	20	16	23	16	11	12
8	B	7	119		97								
			↓		↑								
(2.1)	A		89		90								
9	B	12	78			46			31	38	34	36	24
			↓			↓			↑	↓	↓	↑	↑
(2.0)	A		49			26			38	22	40	36	16

B：BSP　A：醋酸の1モルソーダ0.5cc/kg静注
→：実施の順序を示す

10分値を採用した．

第2表に示す如く4匹の家兎につき，四塩化炭素注射後24時間目から数日おきにＢＳＰを測定した．四塩化炭素注射後24時間または48時間で障害は急速に進み最高に達した．恢復は始めはその傾向が大で，その後次第に緩慢な経過をたどり，19日目頃にはほゞ正常域に近くなつた．

測定回数21回のうち，好転12，不変5，悪化4で，不変例を除けば好転75%，悪化25%であり，正常家兎の場合に比較すればやゝ成績は劣るも，肝障害家兎における醋酸負荷による色素排泄機能は大体好転を示した．

2）解毒機能

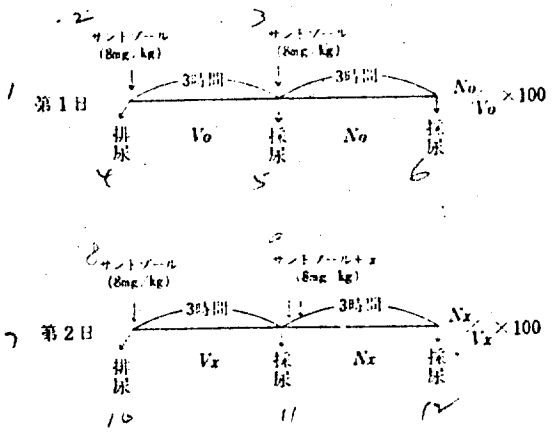
サントニン試験

実験方法

この試験は馬尿酸合成試験と同様，ＢＳＰ試験とともに肝の病変を知る上に最も鋭敏且つ有意義なものであり，肝におけるサントニン酸ソーダの酸化，即ちβ-oxy-santonin の生成を目標とした解毒機能検査である．サントニ試験は高杉[8]によれば，色素の尿中排出は1時間から2時間位で最高に達し，後漸次減少して最も早きは4時間，遅くとも7時間で排泄はほとんど止むので，大体6時間迄の排泄量を検すれば足るという．然して同一の家兎における色素排泄総量は大体一定しているといつているが，私の実験ではＢＳＰ試験の場合と同様に，同一家兎においても日によつてその色素排出量は異つているので，私は実験成績をできるだけ正確ならしめるため次のように行つた．

すなわち第2図に示す如く，先ず導尿により排尿さ

第2図　サントニン試験実施の要領



せてからサントゾール（日本新薬発売の1cc中純サントニン80mg含有）を0.1cc/kg宛耳静脈より静注し，その後3時間の尿を集める．次で直ちに再び同量のサントゾールを静注し更にその後3時間の尿を集める．第1回の尿及び第2回目の尿に酸化されて排泄されるβ-oxy-santonin の量を指数として求め，それぞれVo，Noとする．かくしてNo/Vo×100を求めておく，（正常の場合Vo>Noで，同一の家兎ではNo/Vo×100は大体一定している）．翌日同様の実験を行うが，第2回目のサントゾール静注時に同時に有機酸を静注する．かくして第1回目と第2回目の尿中に排泄されるβ-oxysantonin をそれぞれ Vx，Nx とし $Nx/Vx\times100$ を求める．このようにすれば $Nx/Vx\times100>No/Vo\times100$ の場合は，x という物質は確かにサントニンの酸化，すなわち解毒を促進すると判断することができる．

尿中β-oxy-santonin の測定であるが，アルカリ添加後生じた紅色はまもなく褪色するために従来の方法では測定が不充分なので，阿南の光電比色計による測定法[9]を用いた．すなわち10%苛性ソーダで発色させ，10万倍の Azorbin-S を標準として，フイルター510mμを用いて10秒内に測定し，指数として求めた．なお色素排泄量を便宜上次の様に算出した．

$$色素排泄量=\frac{尿量\times濃度}{10}$$

(1)　正常家兎のサントニン解毒能に及ぼす有機の影響

実験結果は第3表，第3図に示す様に，コハク酸注射後のサントニン色素排泄量は対照に比し著明に増加し，醋酸は軽度に増加，リンゴ酸及び乳酸は不変であつた．従つて正常家兎においてはコハク酸は最も著明にサントニンの解毒を亢進せしめ，醋酸は軽度に亢進せしめることがわかつた．

Table 3

第3表　有機酸負荷前後のサントニン解毒能
（正常家兎）
有機酸の投与量は各1モルソーダ
0.5cc/kg静注

	No. (kg)	前3時間 Vo オキシサントニン	後3時間 No. オキシサントニン	No/Vo×100
対照	10	215.6	171.9	79
	(2.1)	131.3	102.3	78
	11	190.0	132.0	69
	(2.0)	57.0	33.8	60
	12	139.5	117.2	84
	(1.9)	126.0	98.2	78
	13	80.5	64.0	80
	(1.8)	110.0	86.0	78
	平均	131.2	100.7	77

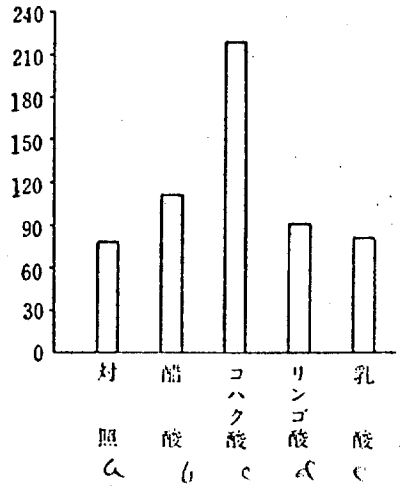
	No. (kg)	前3時間 Vx オキシサントニン	後3時間 Nx オキシサントニン	Nx/Vx×100
醋酸	10	164.0	213.7	130
	11	147.9	69.3	47
	12	170.0	284.4	167
	13	173.0	171.0	99
	平均	204.8	238.1	111
コハク酸	10	25.3	110.5	437
	11	93.8	339.2	362
	12	116.2	169.4	146
	13	115.5	127.5	110
	平均	87.7	186.7	222
リンゴ酸	10	113.4	100.5	88
	11	267.5	170.8	64
	12	166.0	223.3	135
	平均	182.3	164.9	90
乳酸	10	94.0	93.1	99
	11	120.7	95.0	79
	12	177.9	169.9	96
	13	281.4	168.8	60
	平均	168.5	131.7	78

(2)　肝障害家兎のサントニン解毒能に及ぼす有機酸の影響

7匹の家兎につき先づ肝障害前のサントニン色素排泄量を求めた後，20%四塩化炭素オリーブ油を1.0cc/kg（No. 21のみ0.5cc/kg）注射し，正常家兎の場合と同様の方法で経過を追つてサントニン色素排泄量を測定した．

Fig. 3

第3図　サントニン解毒能による有機酸の効果比較（正常家兎）



実験結果は第4表，第5表，第4図に示すように，対照に比レコハク酸及びリンゴ酸はサントニン色素排泄量の著明な増加を示し，乳酸及び醋酸は軽度に増加を示した．従つて肝障害家兎においては，コハク酸及びリンゴ酸は最も著明に肝解毒能を亢進せしめ，乳酸及び醋酸は軽度に亢進せしめる．

3）糖代謝機能

果糖負荷試験

肝臓は糖代謝に密接な関係を有し，TCAサイクルに属する有機酸は糖の分解過程における終末産物なの

第4図　サントニン解毒能による有機酸の効果比較（肝障害家兎）
（第5表を図示したもの）

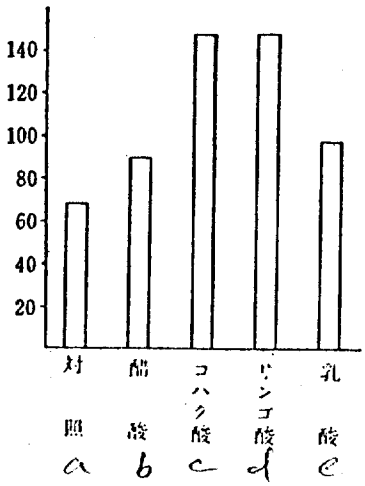


Table 4

第 4 表　有機酸負荷前後のサントニン解毒能（肝障害家兎）

No. (kg)		0	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10	前3時間	119.6		0				44.3	51.1	62.1						120.4
				A				A	A							M
(2.1)	後3時間	81.3		46.9				25.4	55.3	37.8						150.5
12	前3時間	132.0		0												
				S												
(2.0)	後3時間	97.0		16.3												
15	前3時間	131.3		1.0				28.3	37.4	51.8	39.4		60.1		76.1	56.0
				S				S		A					S	M
(2.0)	後3時間	107.6		0				72.5	26.3	72.5	27.6		107.5		45.9	118.8
17	前3時間	197.0		16.2			80.6	1.5					136.1			97.1
								S					A			A
(1.9)	後3時間	106.2		12.5			42.0	12.3					43.0			93.7
18	前3時間	255.0		5.6			7.9	39.0	37.5				123.8		163.8	
				M			M	L	A						S	
(2.0)	後3時間	135.0		9.2			23.5	57.6	35.8				97.5		222.8	
19	前3時間	213.1		11.7			32.1	0								
				L			L	M								
(2.1)	後3時間	184.0		0			56.0	18.4								
21	前3時間	128.2	29.8	69.1	69.7	134.8		122.0								
				L		M		L								
(2.1)	後3時間	103.1	21.4	97.8	48.7	160.6		91.0								

A醋酸，Sコハク酸，Mリンゴ酸，L乳酸の各1モルソーダを0.5cc/kg静注

Table 5

第5表　有機酸負荷前後のサントニン解毒能（肝障害家兎）（第4表を整理したもの）

	No.	前3時間 Vo オキシサントニン	後3時間 No オキシサントニン	No/Vo×100
a 対照	10	62.1	37.8	
	15	37.4	26.3	
		39.4	27.6	
	17	16.2	12.5	
		80.6	42.0	
	18	123.8	97.5	
	21	29.8	21.4	
		69.7	48.7	
	平均	57.4	39.2	68

	No.	前3時間 Vx オキシサントニン	後3時間 Nx オキシサントニン	Nx/Vx×100
b 醋酸	10	0	46.9	
		44.3	25.4	
		51.1	55.3	
	15	51.8	72.5	
	17	136.1	43.0	
		97.1	93.7	
	18	37.5	35.8	
	平均	59.7	53.2	89

	No.	前3時間 Vx オキシサントニン	後3時間 Nx オキシサントニン	Nx/Vx×100
c コハク酸	12	0	16.3	
	15	1.0	0	
		28.3	72.5	
		76.1	45.9	
	17	1.5	12.3	
	18	163.8	222.8	
	平均	45.1	66.6	148
d リンゴ酸	10	120.4	150.5	
	15	56.0	118.8	
	18	5.6	9.2	
		7.9	23.5	
	19	0	18.4	
	21	134.8	160.6	
	平均	54.1	80.1	148
e 乳酸	15	60.1	107.5	
	18	39.0	57.6	
	19	11.7	0	
		32.1	56.0	
	21	69.1	97.8	
		122.0	91.0	
	平均	55.7	54.0	97

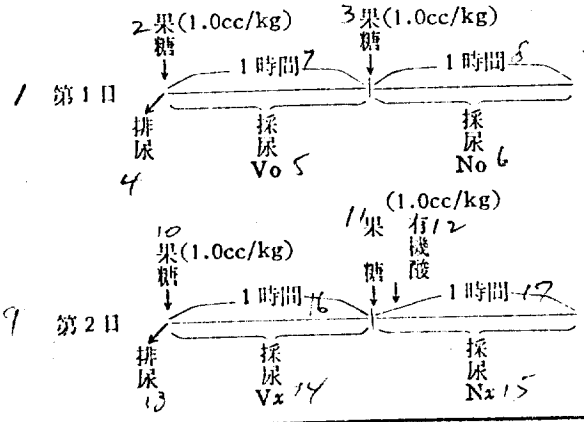
で，之らの基質をあたえることによつて糖代謝に如何なる影響を及ぼすかをみることは極めて有意義であると思われる．然るにこの種の機能検査はＢＳＰ試験や解毒試験のようには適確なものが少なく，ガラクトーゼ試験と果糖試験が実際上よく用いられる．ことに前者は優れているが，果糖負荷試験の臨床的意義は大体ガラクトーゼ試験と同様とされるところから，私はStrausの方法を改良した李の方法[10]に従い尿中果糖定量を行つた．たゞ果糖負荷量を20%液1.0cc/kgとし，尿中果糖定量に光電比色計を応用せる変法を用いた．なお果糖負荷後の尿中果糖量は同一の家兎でも日によつて一定しなかつたので，有機酸の影響を正確にみるために次のようにした．

すなわちサントニン解毒試験の場合の如く果糖静注後1時間尿中の果糖量をVoとし，直後に再度同量の果糖を静注して1時間尿中の果糖量をNoとし，No/Vo×100を求めて対照値とした．有機酸の効果をしらべるには，対照値を求めた後，翌日再度同様にして果糖を静注するが，この場合は2回目の静注時に1モル有機酸ソーダ液を0.5cc/kg果糖に混じて静注し，夫々の尿中果糖量を求めてVx，NxとしNx/Vx×100を対照と比較した．

(I)　正常家兎に有機酸を注射した場合

a．有機酸を果糖と同時に静注した場合

第6表　有機酸を果糖と同時に静注した場合
（有機酸の投与量は各1モルソーダ0.5cc/kg静注）



	No.	Vo	No	No/Vo×100
対照	10	67.83mg	74.89mg	110%
	17	76.21	101.66	133
	28	52.04	87.34	168
	29	100.29	105.89	106
	30	102.47	121.00	118
	平均	79.77	98.16	127

	No.	Vx	Nx	Nx/Vx×100
コハク酸	10	96.07mg	184.68mg	192%
	15	47.31	78.60	166
	28	74.44	110.68	149
	30	64.99	94.25	145
	平均	70.70	117.05	167
醋酸	10	112.80mg	104.08mg	97%
	15	134.99	149.82	156
	28	55.29	98.25	178
	29	78.60	136.82	174
	30	62.22	54.35	87
	平均	88.58	108.66	123
リンゴ酸	10	80.42mg	118.65mg	148%
	15	110.62	81.88	74
	28	133.67	99.01	70
	29	125.78	76.00	61
	30	58.94	78.85	134
	平均	101.89	90.68	89
乳酸	10	129.47mg	39.18mg	31%
	15	92.47	124.82	133
	28	132.99	146.77	111
	29	118.08	78.12	71
	30	66.23	85.09	128
	平均	107.85	94.80	87

実験結果は第6表に示すように，コハク酸投与の場合が対照に比して尿中果糖増加の程度最も高く，醋酸では大差なく，リンゴ酸と乳酸では寧ろ減少した．一般的には李の云う如く果糖量の増量は肝糖質代謝機能の減退を意味するのでこの成績は奇異に見える．然し吉田はクエン酸について同様の実験を行い，クエン酸を注射した場合には対照に比し果糖量は更に増加すると報告し，これは果糖の処理がクエン酸の処理により干渉をうけて尿中果糖の排泄量がますのであろうと考察し，肝機能亢進剤と考えられる葡萄糖でも同様に果糖量の増加を示した．私も同様の実験でその真なることを確めた．すなわち第7表に示す通りである．葡萄糖は20%を0.5cc/kg静脈内注射した．

更に吉田は追加実験として，クエン酸注射後1時間目に果糖負荷試験を行つてみると，対照に比し果糖量は明らかに減量してをり，果糖処理能亢進という成績をみた．よつて吉田はクエン酸注射直後の果糖処理能

Tab. 7

第7表　葡萄糖を果糖と同時に静注した場合（ブドウ糖の投与量は20%を0.5cc/kg静注）

No.	Vx	Nx	Nx/Vx×100
10	78.57mg	255.92mg	325%
15	94.61	132.09	137
29	102.86	176.61	172
30	72.78	120.09	165
平均	62.21	171.18	275

は確かに減退するが，これは直ちに糖代謝機能の減退を意味するものではなく，寧ろ或る時間経過後は亢進せしめるものであろうといつている．そこで私は次のように実験してみた．

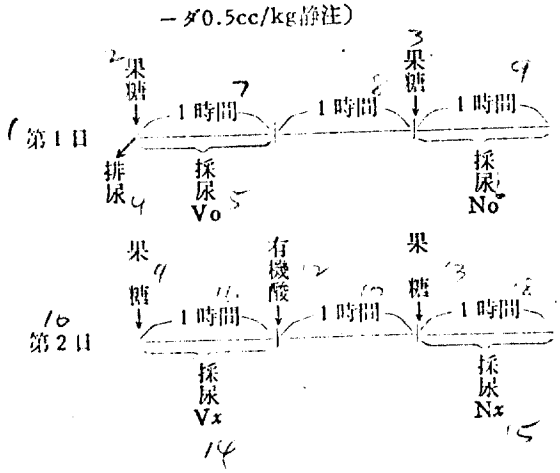
b．有機酸静注後1時間して果糖を静注した場合

実験方法はa．の場合と同様に果糖を2回静注するが，有機酸は第1回目の果糖静注1時間後に単独で静注し，第2回目の果糖静注は更にその後1時間経つて静注した．採尿は果糖静注後夫々1時間の尿とした．

実験結果は第8表に表すように，コハク酸投与の場合は果糖排泄量はやゝ減少したが，醋酸，リンゴ酸，乳酸では増加した．ブドウ糖では減少した．なおこの成績は有機酸を果糖と同時に負荷した前記の場合に比すれば可成り果糖量の増加の程度が少なくなつた．この成績は吉田のいう如く，あらかじめ有機酸を投与しておけば，その後の糖代謝は亢進するようになることを推測せしめる．そこで今度は有機酸負荷後の時間を更に1時間延長せしめて実験を行つてみた．

Tab. 8

第8表　有機酸静注後1時間して果糖を静注した場合（有機酸の投与量は各1モルソーダ0.5cc/kg静注）



No.	Vo	No	No/Vo×100
対照 10	227.09mg	206.70mg	91%
29	136.09	92.25	68
30	207.54	147.44	71
32	91.47	91.02	98
33	100.18	163.99	164
平均	152.47	140.28	98

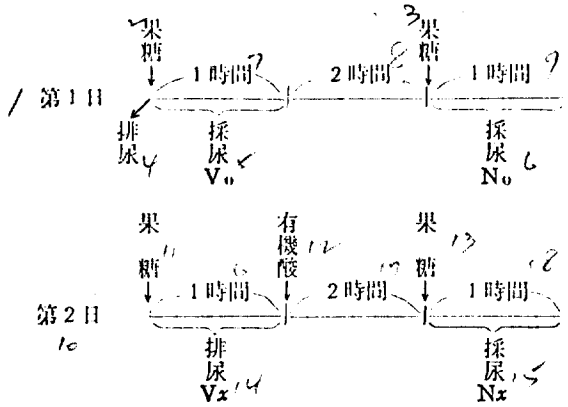
No.	Vx	Nx	Nx/Vx×100
コハク酸 10	109.05mg	160.06mg	147%
28	99.46	140.74	148
29	142.29	164.56	116
30	207.24	103.56	50
平均	139.51	129.73	93
醋酸 10	179.40mg	225.40mg	126%
29	98.26	130.17	132
30	114.39	127.73	111
平均	130.68	161.10	123
リンゴ酸 29	122.58mg	136.47mg	111%
30	92.00	130.22	142
平均	107.29	133.34	124
乳酸 10	146.48mg	182.49mg	125%
30	108.12	135.30	125
平均	129.30	158.90	125
ブドウ糖 10	85.52mg	89.50mg	105%
15	202.79	154.84	76
29	101.52	56.40	56
平均	129.98	100.25	77

c．有機酸静注後2時間して果糖を静注した場合

実験結果は等9表，第5図に示すように，対照では2回目の果糖静注による尿中果糖量（No）は1回目の量（Vo）よりも増加した．しかし葡萄糖を注射した例では，逆に2回目の果糖静注2時間前に静注すれば，後の果糖量は前の果糖量よりも著明に減少した．これは果糖処理能の亢進を意味するものである．それで2回目の果糖静注の2時間前に有機酸を静注することにした．このようにして有機酸の果糖処理能を検討して次の結果を得た．すなわち正常家兎においてはコハク酸，醋酸は果糖処理能亢進を示し，リンゴ酸，乳

Table 9

第9表　有機酸静注後2時間して果糖を静注した場合
（有機酸の投与量は各1モルソーダ0.5cc/kg静注）



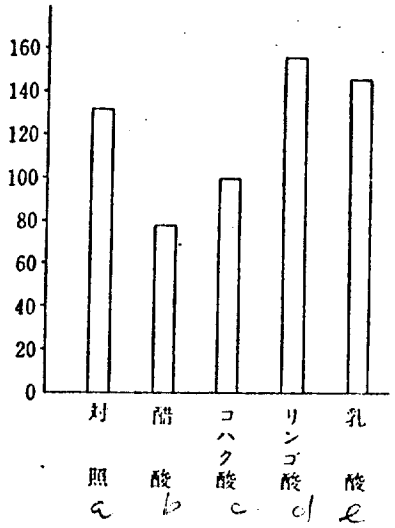
	No.	Vo	No	No/Vo×100
対照	10	99.42mg	102.48mg	103%
	15	112.86	130.24	115
	29	106.99	118.16	110
	30	98.20	122.83	125
	33	130.76	139.43	107
	平均	109.65	122.63	111

	No.	Vx	Nx	Nx/Vx×100
コハク酸	10	84.03mg	79.45mg	94%
	15	104.08	82.49	79
	29	101.89	102.84	100
	30	131.00	65.11	50
	33	70.17	73.71	104
	平均	98.23	80.72	82
醋酸	10	99.47mg	55.80mg	51%
	15	102.02	84.15	82
	29	72.88	78.46	106
	30	54.64	66.96	121
	33	66.57	57.32	86
	平均	79.12	68.54	87
リンゴ酸	15	33.96mg	67.25mg	198%
	29	71.98	142.50	198
	30	113.78	165.33	145
	33	31.06	37.37	120
	平均	62.69	103.11	164

	No.	Vx	Nx	Nx/Vx×100
乳酸	10	70.72mg	82.79mg	117%
	15	108.06	153.71	142
	30	32.61	52.16	160
	33	97.26	145.33	149
	平均	77.16	108.50	141
ブドウ糖	15	82.48mg	49.13mg	60%
	30	73.69	49.10	67
	33	97.04	78.25	81
	平均	84.40	58.83	70

Fig 5

第5図　果糖負荷試験による有機酸の効果比較（正常家兎）



酸はむしろ果糖処理能減退を示した．

(2)　肝障害家兎に有機酸を注射した場合

果糖負荷試験による有機酸の効果をみるには(1)の正常家兎でしらべた如く，1回目の果糖静注後1時間して有機酸を単独静注し，しかる後2時間たつてから2回目の果糖静注を行い，夫々果糖静注後の1時間尿中果糖量を比較する方法が適切であると考えられるので，肝障害家兎ではこの方法のみを用いることにした．

5匹の家兎につき先づ肝障害前の尿中果糖排泄量Vo，Noをもとめた後，20%四塩化炭素オリーブ油を0.5cc/kg注射し，(1)の場合と同様の方法で日を追つて果糖量を測定してみた．四塩化炭素注射は3日目0.25cc/kg，4日目0.25cc/kg，5日目0.2cc/kgを行つた．

Table 10

第10表　肝障害家兎の果糖負荷試験

No. (kg)		0	2	3	4	5	8
10	V	126.77mg	122.81	350.54	192.81		
			S	A			
	N	144.10mg	185.30	165.45	234.84		
(2.5)	N/V×100	(114%)	(151%)	(47%)	(122%)		
15	V	131.00	72.78	88.59	110.05	149.26	150.31
					M	L	S
	N	117.38	101.45	120.08	235.09	208.36	105.53
(2.0)	N/V×100	(90%)	(139%)	(135%)	(213%)	(139%)	(70%)
29	V	89.28	115.14	117.83	180.35	169.82	111.60
			A	M	L	S	
	N	110.08	124.45	196.41	296.00	131.00	159.72
(2.2)	N/V×100	(123%)	(108%)	(167%)	(164%)	(77%)	(143%)
30	V	53.48	122.82	139.08	166.91	170.79	105.52
			M	L	S	A	M
	N	65.57	151.39	111.16	203.43	161.63	159.22
(2.0)	N/V×100	(123%)	(123%)	(86%)	(122%)	(95%)	(151%)
33	V	63.25	109.17	141.92	142.65	140.70	77.23
			L	S	A	M	L
	N	73.58	145.30	124.45	152.84	189.28	109.27
(2.0)	N/V×100	(116%)	(133%)	(88%)	(107%)	(135%)	(141%)

S．コハク酸　A．醋酸　M．リンゴ酸　L．乳酸の各1モルソーダ塩を0.5cc/kg静注

第11表　有機酸静注後2時間して果糖を静注した場合（肝障害家兎）（第10表を整理したもの）

	No.	V	N	$\frac{N}{V}\times100$
a コハク酸	10	122.81mg	185.30mg	151%
	15	150.31	105.53	70
	29	169.82	131.00	77
	30	166.91	203.43	122
	33	141.92	124.45	85
	平均	150.35	149.94	100
b 醋酸	10	350.54mg	165.45mg	47%
	29	115.14	124.45	108
	30	170.79	161.63	93
	33	142.65	152.84	107
	平均	194.78	152.34	78
c リンゴ酸	15	110.05mg	235.09mg	213%
	29	117.83	196.41	167
	30	122.82	151.39	123
	30	105.52	159.22	151
	33	140.70	189.28	135
	平均	119.38	186.28	156
d 乳酸	15	110.05mg	235.09mg	213%
	29	180.35	296.00	164
	30	139.08	111.16	86
	33	109.17	145.30	133
	33	77.23	109.27	141
	平均	123.18	179.36	146
e 対照	10	192.81mg	234.84mg	122%
	15	72.78	101.45	139
	15	88.59	120.08	135
	29	111.60	159.72	143
	平均	116.44	154.02	132

実験結果は第10表，第11表及び第6図に示すように，対照に比し醋酸，コハク酸は果糖処理能亢進を示し，リンゴ酸，乳酸はむしろ果糖処理能減退を示した．

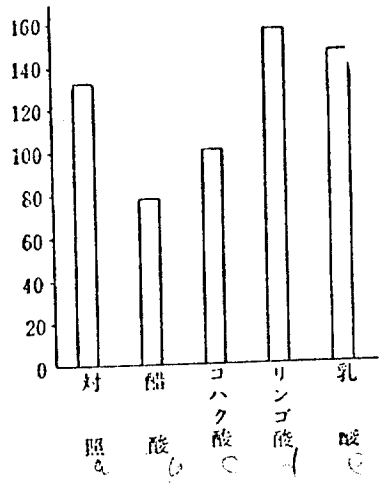
4）胆汁生成機能

尿中ウロビリノーゲン定量

胆汁生成機能を調べる方法としては，Meulengracht 氏黄疸指数測定法，Hijmans van den Bergh氏

Fig 6

第6図　果糖負荷試験による有機酸の効果比較（肝障害家兎）
（第11表のN/V×100を図示したもの）



血清ビリルビン定量法及びウロビリノーゲン定量等があるが，このうちウロビリノーゲンの測定（以下ウ・ゲと略記）を行つた．尿中ウ・ゲ定量はWatson 2時間尿法[11]を用いた．

(1)　正常家兎の尿中ウ・ゲ値

Table 12

第12表　正常家兎の尿中ウ・ゲ値（2時間値）

No. ＼ 採尿時	12～14時尿 U量	14～16時尿 U量
10	0.075mg	0.067mg
15	0.063	0.049
29	0.066	0.066
30	0.031	0.025
32	0.052	0.047
33	0.039	0.021
平　均	0.054	0.049

第12表に示す如く健康家兎の2時間尿中ウ・ゲ値は0.021～0.075mgの間にあり，6例の平均は0.052mgであつた．

(2)　正常家兎に有機酸を負荷した場合

有機酸の負荷前（前尿値）及び負荷後（後尿値）の各2時間尿中ウ・ゲ値を測定して夫々を比較した．正常家兎のウ・ゲ値は極めて小さいので0.02mg以上の差を有意とした．

実験結果は第13表に示すように，コハク酸のみが尿中ウ・ゲ値を減少せしめた．

第13表　Table 13　正常家兎に有機酸を負荷した場合の尿中ウ・ゲ値の変化

	No.	前尿値	後尿値
コハク酸	10	0.071mg	0.055mg
	15	0.039	0.010
	29	0.059	0.016
	33	0.034	0.014
	平均	0.051	0.024
酢酸	10	0.037mg	0.061mg
	29	0.053	0.020
	30	0.020	0.030
	33	0.038	0.029
	平均	0.037	0.035
リンゴ酸	10	0.076mg	0.033mg
	29	0.075	0.037
	30	0.044	0.104
	33	0.024	0.024
	平均	0.055	0.050
乳酸	10	0.051mg	0.050mg
	15	0.041	0.043
	30	0.048	0.022
	33	0.033	0.020
	平均	0.043	0.034

前尿値は12時～14時迄の間，後尿値は14時～16時迄の間　有機酸の投与量は各1モルソーダ0.5cc/kg静注

(3)　肝障害家兎の有機酸を負荷した場合

5匹の家兎につき先づ肝障害前の尿中ウ・ゲ値を測定した後，20%四塩化炭素オリーブ油を0.5cc/kg注射し，正常家兎におけると同様の方法で日を追つて有機酸の影響による尿中ウ・ゲ値の変化を追求した．四塩化炭素の注射は1日目0.5cc/kg，3日目0.25cc/kg，4日目0.25cc/kg，5日目0.2cc/kg行つた．（ウロビリノーゲンの発色を容易にするためにこのように数回四塩化炭素オリーブ油を注射した）

実験結果は第14表，第15表に示すように，酢酸が最もよく尿中ウ・ゲ値を減少せしめ，次いで乳酸であり，次いでコハク酸であつた．リンゴ酸ではかえつてウ・ゲが僅かながら増加した．

Table 14

第14表　肝障害家兎の尿中ウ・ゲ値

No.	日	0	2	3	4	5	6
10	V	mg 0.127	0.210 S	0.107 A	0.190		
	N	mg 0.102	0.269	0.053	0.183		
15	V	0.050	0.033	0.092	0.093 M	0.109 L	0.092 S
	N	0.041	0.022	0.098	0.124	0.062	0.063
29	V	0.063	0.137 A	0.150 M		0.307 S	
	N	0.059	0.025	0.112		0.123	
30	V	0.043	0.049 M	0.068 L	0.031 S	0.065 A	0.047 S
	N	0.046	0.081	0.042	0.036	0.074	0.040
33	V	0.046	0.096 L	0.116 S	0.218 A	0.056 M	0.111 L
	N	0.054	0.043	0.042	0.082	0.117	0.141

Vは前2時間尿，Nは後2時間尿中の値
A醋酸，Sコハク酸，Mリンゴ酸，L乳酸の各1モルソーダ塩0.5cc/kg静注

第15表　肝障害家兎に有機酸を負荷した場合の尿中ウ・ゲ値の変化（第14表を整理したもの）

Table 15

	No.	前尿値	後尿値
対照	10	0.190mg	0.183mg
	15	0.033	0.027
	15	0.092	0.098
	平均	0.105	0.103
コハク酸	10	0.210mg	0.269mg
	15	0.092	0.063
	29	0.307	0.123
	30	0.031	0.036
	33	0.047	0.040
	平均	0.117	0.106
醋酸	10	0.107mg	0.053mg
	29	0.137	0.025
	30	0.065	0.074
	33	0.218	0.082
	平均	0.138	0.059
リンゴ酸	15	0.093mg	0.124mg
	29	0.150	0.112
	30	0.049	0.081
	33	0.056	0.117
	平均	0.087	0.109
乳酸	15	0.109mg	0.062mg
	30	0.068	0.042
	33	0.096	0.043
	33	0.111	0.141
	平均	0.096	0.072

3．小　括

醋酸，コハク酸，リンゴ酸，乳酸の各1モルソーダ塩を0.5cc/kg静注し，正常家兎及び四塩化炭素による肝障害家兎の肝機能に及ぼす影響をBSP試験，サントエン解毒試験，果糖負荷試験，尿中ウロビリノーゲン定量によつてしらべ，コハク酸はいづれの場合にも効果が顕著で，醋酸もほゞ同程度であり，リンゴ酸，乳酸では効果があまりはつきりせず，かえつて悪化するようにみえる場合もあつた．

II．肝グリコーゲンに及ぼす有機酸の影響

TCAサイクルはグリコーゲンの分解によつて生じた終末系なので，これを逆にたどればグリコーゲンが生成される．

ネヅミにコハク酸ソーダ0.15gを与えたところ，4時間以内に肝内グリコーゲンは著明に増加し[12]，モルモットにC_4炭素ヂカルボン酸を与えたところ，同様に肝内グリコーゲンが増加したという報告があり[13]，またラッテに四種の有機酸アンモニウム塩を2g 3日間経口投与したところ，コハク酸，フマール酸，リンゴ酸は著明に肝内グリコーゲンを増加せしめ，醋酸はほとんど影響を与えなかつたという報告がある[14]．またクエン酸を正常ラッテに投与すると肝グリコーゲンの著明な増加がみられ[15]，乳酸にもこの傾向のあることが知られている．

私はコハク酸，醋酸，リンゴ酸，乳酸，クエン酸の各ソーダ塩を正常マウスに投与して肝グリコーゲンに及ぼす影響を比較観察した．

1．実験方法

1）予備実験

マウス肝グリコーゲン量（以下「グ」量と略記）については発表者によつて成績がまちまちである．すな

わち田中[16]は2.5%，牧野[17]は0.77%（36時間飢餓後），小林[18]は2.7%（20時間飢餓後），児玉[19]は0.2%（24時間飢餓後）等の報告がある．

そこで私は後述の測定方法により，24時間飢餓のマウス肝内「グ」量を測定した．

第16表　正常マウス24時間飢餓後の肝内「グ」量

No.	体重	「グ」量	No.	体重	「グ」量
	g	mg%		g	mg%
1	18	195.5	6	22	252.5
2	20	83.3	7	18	143.3
3	23	206.6	8	21	291.4
4	20	181.6	9	20	154.2
5	17	132.2	10	19	245.0
		10例平均			188.8

その結果第16表に示す如く10例平均0.19%の成績を得た．之は大体児玉の報告と一致する．測定法等により幾分の差異はあるかも知れぬが，それにしても田中，小林の値は高すぎるように思われる．然し同様なことは実兎やラッテにおいてもいえることであり，例えば家兎では24時間飢餓後の肝「グ」量は黒川では，1.5%（0.17～3.18%），藤井では4.26%と報告されており，ラッテでは24時間飢餓後の値は田中は0.25%，大河は0.19%，Dittmar は0.47%，Wetzel は0.78%，長谷川は0.19%等と報告している．

さてマウスに有機酸を負荷するにあたり，その量的関係，投与方法が問題になるが，先づマウスを18時間飢餓後1Mコハク酸ソーダ0.35cc皮下注射して3時間後致死せしめると，第17表に示す如くコハク酸ソーダ注射群の方が成績が悪い．之れはコハク酸の投与量が過量のためと思われる．

次に径口投与についてやつてみた．Ponsford & Maclean によると，ラッテに3日間有機酸のアンモニウム塩を毎日2g投与して「グ」量を測定すると，対照に比し有機酸群が著明に増加せしめたという．そこで私は10%コハク酸ソーダ3cc（コハク酸0.3g含有）をパン4gに浸して3日間経口投与したところ，第18表に示す如くコハク酸投与群が明らかにグリコーゲンを増加せしめた．

第17表　マウス18時間飢餓後1Mコハク酸ソーダ0.35cc皮注して3時間後の肝「グ」量

a対照群			b被検群		
No.	体重	「グ」量	No.	体重	「グ」量
	g	mg%		g	mg%
11	20	349.6	14	18	59.2
12	19	116.6	15	17	108.2
13	19	188.7	16	20	47.2
平均		218.3	平均		71.6

第18表　コハク酸0.3g径口投与3日間後の肝「グ」量

a対照群			b被検群		
No.	体重	「グ」量	No.	体重	「グ」量
	g	mg%		g	mg%
17	20	185.2	21	19	263.7
18	21	251.6	22	19	396.7
19	19	189.2	23	20	100.8
20	20	241.2	24	21	260.9
平均		216.8	平均		305.5

以上の予備実験から有機酸投与量を考慮して，次にのべる如き方法によつて実験を行つた．

2）実験動物及び食餌投与法

17～25gの雄性正常マウスを用い，食餌は小麦4g，白菜5gを毎日，煮干1gを隔日に一定時間に与えた．

3）有機酸投与法

被検群にM/10コハク酸，醋酸，リンゴ酸，乳酸各ソーダ，M/20クエン酸ソーダ（クエン酸をM/10とするとマウスはテタニー様の発作を起して死亡する場合が多い）を体重20gにつき1cc注射した．対照群には生理的食塩水1cc注射群とM/10ブドウ糖1cc注射群をもうけた．いづれも連続1週間毎日1回マウス脊筋内に注射し，最後の注射並びに食餌投与後24時間目に断頭致死せしめた．なお注射及び食餌投与は午後5時に行つた．

4）肝グリコーゲンの測定

Verzár & Wenner の方法[20]に従い肝グリコーゲンを加水分解してブドウ糖になした後，Hagedorn-Jensen 法で測定し，測定したブドウ糖量に0.925を乗じて肝グリコーゲン含有mg%として表わした．

2．実験成績

実験結果は第19表，第7図に示すように，

1）対照

10例の平均170.2mg%（123.1～215.6mg%）．

2）コハク酸

10例の平均304.4mg%（87.9～457.0mg%）で対照に比し79%の増加を示し，且つ有機酸のうちでは最も

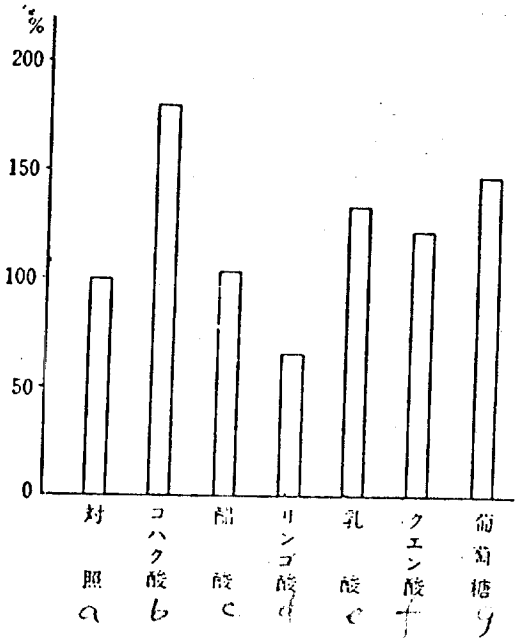
Table 19

第19表　有機酸注射によるマウス肝「グ」量の消長

No.	対照		コハク酸		醋酸		リンゴ酸		乳酸		クエン酸		葡萄糖	
	体重	「グ」量	体重	「グ」量	体重	「グ」量	体重	「グ」量	体重	「グ」量	体重	「グ」量	体重	「グ」量
	g	mg%	g	mg%	g	mg%	g	mg%	g	mg%	g	mg%	g	mg%
1	24	161.0	25	452.3	31	199.8	23	156.3	25	188.7	18	186.9	23	468.1
2	25	167.4	19	186.9	24	95.3	21	111.9	22	105.4	16	77.0	23	199.8
3	24	140.4	22	373.8	18	131.4	19	107.1	22	71.2	20	220.2	22	196.1
4	21	215.6	24	386.7	18	176.7	21	96.2	21	203.5	17	224.6	25	334.9
5	22	175.8	25	275.7	21	254.2	25	121.1	19	206.3	18	173.9	25	178.5
6	21	164.0	20	146.2	19	179.5	25	99.9	21	356.1	20	310.8	20	278.0
7	21	123.1	20	87.9	19	97.2	20	51.3	19	320.1	16	181.3	18	235.5
8	18	191.5	21	457.0	21	230.3	18	93.4	18	159.1	18	172.5	25	223.9
9	20	188.6	17	281.1	25	247.0	17	168.4	17	266.4	21	210.6	21	162.8
10	21	174.2	21	396.0	18	183.2	18	124.0	21	405.4	20	195.8	21	243.7
平均		170.2		304.4		179.5		113.0		228.2		205.3		252.1

各有機酸ソーダM/10液1cc/20g注，但しクエン酸はM/20液1cc/20g注，ブドウ糖はM/10液1cc/20g注
対照は生理的食塩水1cc/20g注いづれも1週間注射し，最後の注射後24時間して測定

第7図　有機酸注射によるマウス肝「グ」量の比較



著明に肝「グ」量を増加せしめた．

3）醋酸

10例の平均179.5mg%（95.3～254.2mg%）で対照に比し僅かに5%の増加でほとんど大差なかつた．

4）リンゴ酸

10例の平均113.0mg%（93.4～168.4mg%）で対照に比し34%の減少を示し，有機酸のうちでは最も成績が劣つていた．

5）乳酸

10例の平均228.2mg%（71.2～405.4mg%）で対照に比し34%の増加を示し，有機酸のうちではコハク酸に次いで成績がよかつた．

6）クエン酸

10例の平均205.3mg%（77.0～310.8mg%）で対照に比し21%の増加を示した．

7）ブドウ糖

10例の平均252.1mg%（162.8～468.1mg%）で対照に比し48%の増加を示した．

以上の成績は大体 Ponsford の成績と似ているが，ブドウ糖とリンゴ酸の成績が必ずしも一致しなかつた．

すなわち Ponsford によればブドウ糖は対照に比し220%の増加を示し，リンゴ酸は110%の増加を示したといつているが，私のやつた例ではブドウ糖の場合はそのように著明な増加がなかつたし，リンゴ酸は寧ろ減少を示した．

3．小　括

醋酸，コハク酸，リンゴ酸，乳酸，ブドウ糖の各1/10モル液を1.0cc/20g，クエン酸ソーダは1/20モル液1.0cc/20gを各10例のマウスに7日間毎日1回注射し，肝「グ」量を測定した結果，コハク酸は著明に肝グリコーゲンを増加せしめ，次いで，乳酸，クエン酸の順に軽度に増加せしめ，醋酸は対照と大差なく，リンゴ酸は却つて減少せしめることを知つた．なおブドウ糖は対照に比し著しく増加を示したが，コハク酸より劣つた成績を示した．ブドウ糖によつて肝の

グリコーゲンが著明に増加することは衆知のことである．然るに之よりもコハク酸が多く増加せしめたという成績になつたが，之には意義深いものがあると思う．

III. 総括及び考按

醋酸，コハク酸，リンゴ酸，乳酸の各1モルソーダ塩を 0.5cc/kg 家兎に注射して肝機能に及ぼす影響をしらべたところ，BSP試験では正常家兎の場合には醋酸が最もよく次いでコハク酸が好影響を示し，リンゴ酸と乳酸はほとんど影響なかつた．BSP 試験は耳介血管に壊死をきたし長期にわたつての負荷は困難であつた．それで四塩化炭素による肝障害家兎では醋酸の影響だけしかしらべなかつたが，正常の場合と同様に BSP を好転させた．

サントニン解毒試験では，正常家兎の場合にはコハク酸が最も著明に之を解毒し，色素排泄量は対照に比して約3倍となつた．肝障害の場合にもコハク酸によつてその排泄量は約2倍となつた．またリンゴ酸の影響は正常家兎の場合には対照に比較すると不変であるが，肝障害の場合にはコハク酸と同様に効果を示し約2倍の排泄量を示したことは興味がある．

果糖負荷試験では，正常家兎の場合にはコハク酸と醋酸は対照に比較して果糖処理能亢進を示し，肝障害の場合にも同様の傾向を示した．

尿中ウロビリノーゲンの定量では，正常家兎の場合には尿中・ゲ含量が極めて少ないので意味づけは困難と思われるが，コハク酸はウ・ゲ値の減少を示した．肝障害の場合には尿中ウ・ゲ値の減少の最も著しいものは醋酸であつた．コハク酸は対照と大差なかつた．

松尾によれば色素排泄機能は主として肝小葉周辺部において営まれるといゝ，また果糖その他の炭水化物の代謝は主として肝小葉中心部において営まれるとの報告がある[21]．かかる点から考えればコハク酸及び醋酸が色素排泄量のみならず果糖代謝にも好影響を及ぼしたことにより，肝全体の機能に対して亢進的に作用するものと思われる．

要するに有機酸を負荷した場合の肝機能成績は，コハク酸が最もよく次いで醋酸であつた．

そしてコハク酸は特にサントニン解毒能に対しては著明な効果を示したが，この解毒能に関しては R. G. Mezzich はコハク酸にはバルビタール解毒作用があり，バルビタールを大量服用した人にコハク酸ソーダを投与したところ早期に快復せしめたと報告している[22]．また家兎にバルビタールを注射して睡眠に陥らしめ，コハク酸を注射すると，対照に比し睡眠時間の短縮することが認められている．之に対し小川はバルビタールに対するコハク酸の作用は特有のものとはいゝ難いと云つている[23]．然しながらコハク酸が解毒能亢進を示すことは私の実験でも明らかであり，次に述べる肝グリコーゲン量の増加からも裏付けられる．

一般に肝機能と肝グリコーゲン量とは平行的な関係にあり，肝グリコーゲン量の多い時は肝機能がよいといわれている．このことは私の実験においても明らかである．すなわちマウスに各有機酸の $1/10$ モルのソーダ塩を 1cc/20g 7日間注射したところ，コハク酸は他の有機酸に比して肝グリコーゲン量を最も多く増加させた．

Ponsford の成績によると，対照に比しコハク酸，リンゴ酸は大体2倍，葡萄糖は3倍という値を得たと云つているが，私の実験ではコハク酸は約2倍で最も良く，次いで葡萄糖，乳酸，クエン酸の順であり，リンゴ酸は寧ろ悪かつた．この成績はさきの家兎における肝機能に及ぼす効果の成績と大体一致している．乳酸は肝機能には好影響を及ぼすという成績は私の実験では得られなかつたが，肝グリコーゲンについては良い結果が得られた．

またクエン酸，コハク酸には抗ケトン体作用のあることが知られてをり[24]，クエン酸，コハク酸を投与すればケトン体が減少するという報告[6][25][26]がある．これは両者が何れも肝グリコーゲンを増加させることによるためであろう．

肝疾患時には血中及び尿中の焦性葡萄酸[27]，α-ケトグルタール酸[28][29]，クエン酸[30]は増加すると報告されている．この時コハク酸ソーダを注射しておくと，沢田氏尿反応は陰性になるものがかなりあるとの報告もある．

一方宮地ら[31]によると有機酸を連続注射した場合の肝組織に及ぼす影響は，クエン酸が最も悪く，醋酸，リンゴ酸等は軽度の変化をきたし，コハク酸は全く変化をきたさない．また宮地ら[32]は家兎にコハク酸を 0.1g/kg 2カ月間投与した場合にも何らの障害が認められなかつたといつている．

以上のことからもコハク酸は他の有機酸に比して肝臓に良い影響をあたえるだろうと考えられていたが，私の成績はこのことを明らかにしたものである．かようにコハク酸，クエン酸，リンゴ酸は同じTCAサイクル上の有機酸でありながら，コハク酸のみが肝に対して顕著な好影響を示すことは興味深いことである．このことはコハク酸が他の有機酸に比して無害なため

によく利用されるからであろうと思われる．

IV. 結　論

主としてTCAサイクルに属する有機酸のうち，コハク酸，醋酸，リンゴ酸，乳酸，クエン酸の5種を選び，これらの肝臓に及ぼす影響を肝機能と肝グリコーゲンについてしらべた．肝機能検査法としてBSP試験，サントニン解毒試験，果糖負荷試験，尿ウロビリノーゲン定量を用い家兎についてしらべた．肝グリコーゲンはマウスについてしらべた．そして次の結果を得た．

1．肝機能においてはコハク酸が最も亢進的に作用し，次いで醋酸であり，リンゴ酸と乳酸は効果が区々で，みかけ上悪い成績を示す場合もあつた．ことにコハク酸は，サントニン解毒能を正常の場合にも肝障害の場合にも著明に亢進した．

2．有機酸投与後の肝グリコーゲン量は，コハク酸が最も著明に増加せしめ，次いで乳酸，クエン酸の順に増加せしめ，醋酸はほとんど影響なく，リンゴ酸は寧ろ減少せしめた．然してコハク酸はブドウ糖よりも肝グリコーゲン量を増加せしめる成績を得た．

3．之を要するに主としてTCAサイクルに属する有機酸の中では，コハク酸が肝臓に最も良い影響を与えることを知つた．

（本研究の要旨は第42回及び第43回日本消化機病学会において発表した．）
（擱筆するにあたり，終始御懇篤なる御指導，御校閲を賜りたる恩師沢田教授に深甚の謝意を捧げると共に，教室の宮地講師には詳細に亙り直接の御指導，御鞭撻を賜りあわせて謝意を表します．）

文　献

1) H. A. Krebs, W. A. Johnson : Enzymologia, 4, 148, 1937.　2) Practical Therapeutis by M. E. Rehfuss and A. H. Price : Second Edition. Baltimore, The Williams & Wilkins Company 1951.　3) 宮地・佐伯：臨床と研究．32; 1066, 昭30.　4) 宮地：福岡医学雑誌．47; 916, 昭31.　5) 吉田：日本内科学会雑誌．44; 979, 昭31.　6) 大石：岡山医学雑誌．67; 913, 昭30.　7) 栗原：福岡医学雑誌．44; 8, 昭28.　8) 高杉：日本内科学会雑誌．25; 765, 昭12.　9) 阿南：日本医事新報．No. 1630. 昭30.　10) 李：福岡医学会雑誌．41; 697, 昭25.　11) 斉藤：光電比色計による臨床化学検査．P89. 昭29.　12) R. Stöhr : Hoppe-Seyler Z, 217; 153, 1933.　13) W. Kutscher and M. Schippers : Z. Phisiol. chem. 286; 59, 1950.　14) A. P. Ponsford and I. S. Maclean : From the Biochem. Depart. Lister Institute, London, 1932.　15) Kay. M. : J. B. C. 133; 59, 1940.　16) 田中：大阪医学雑誌．31; 3811, 1932.　17) 牧野：ビタミン．4; 316, 1951.　18) 小林：東京市立病院学術業績年報．2; 57, 1935.　19) 児玉：臨床生化学．122, 1952.　20) Verzár & Wenner : Biochem. J. 42; 42, 1948.　21) 松尾：日本臨床．9; 153, 1951.　22) R. G. Mezzich : Rev. facultad farm. y. bioquim, 12, 40, 1950. C. A., 45, 2099i, 1951.　23) 小川：最新医学．4; 717, 昭24.　24) A. V. Korányi : ibid., 101, 669, 1937.　25) C. A. Vesce : Arch. Studio fisiopatol. Clin. ricanbio 7, 331, 1937. : C. A. 34, 24596, 1940.　26) 乾・中島・河合・松岡：日本内科学会雑誌．42, 214, 1953.　27) E. Bueding, M. H. Stein and H. Wortis : J. Biol. Chem. 140; 697, 1941.　28) L. H. Smith, Jr, R. H. Ettinger and D. Seligson : J' Clin. Invest. 32; 273, 1953.　29) 沢田・宮地・鹿上：ビタミン．10, 134, 昭31.　30) H. Buch : Nord. Med. 5; 125, 1940.　31) 宮地・菊田：綜合臨床．6; 61, 昭32.　32) 宮地・佐伯・松岡：福岡医学雑誌．47; 635, 昭31.

（昭和33年11月11日受付，特掲）

Oxidative Metabolic Pattern of Normal and Ketotic Cow Liver Incubated With 1-C^{14}-Labeled Aliphatic Acids.* (24666)

A. C. CHUNG, F. G. HUETER AND J. C. SHAW
Dept. of Dairy Husbandry, University of Maryland, College Park

It is well accepted that the ruminant derives the major portion of its energy from volatile fatty acids (VFA) produced in the rumen as a result of fermentation of ingested feed by rumen microflora. By and large, the VFA produced in the rumen are absorbed as such and reach the liver directly through the portal system. The major VFA concerned are acetic, propionic, butyric and valeric acids, listed in their normal descending order of production and absorption. In addition, formic acid is also an important metabolite for ruminants since we showed it was produced in rather marked amounts by perfused goat livers(4). If one assumes that the ruminant liver is very similar in its behavior, in a given set of conditions, to the liver of the monogastric animal, then the major metabolic dissimilarities between these animals must arise from differences in substrates available to livers through the portal blood (glucose for monagastrics and VFA for ruminants) and in any enzymatic and endocrine adaptation to these differences. In addition it is commonly accepted that liver function is disturbed(5,6) when ruminants, particularly dairy cattle, exhibit clinical ketosis, a primary metabolic disorder or syndrome believed associated with stress(7). For above reasons, studies were initiated concerning oxidative metabolic pathways of various VFA by normal and ketotic ruminant liver slices.

Methods. The methods used including chromatographic - autoradiographic technic were described by Katz and Chaikoff(3) and Brown *et al.*(1). Independent incubations of liver slices (0.5 g) were made in duplicate with the following 1-C^{14} labeled acids: formic, acetic, propionic, butyric and valeric. For each incubation, 25 μM of substrate containing 25 μc of C^{14} was employed. The flasks were incubated 2 hours at 39 and 37°C for cow and rat liver respectively. The relative position of non-volatile compounds separated by 2-dimensional chromatography is shown in Fig. 1.

Results. In all incubations the volatile fraction contained the largest percentage (83-98%) of recovered C^{14}. Since this fraction represents non-utilized substrate, it can be assumed that large excesses of substrate were present during all incubations. In addition, no significant lipogenesis was observed during incubations.

Formate-C^{14}. Formate carbon was incorporated to greatest extent into glucose, followed by what appear to be phosphate esters (Table I). A marked decrease occurred in $C^{14}O_2$ production and in incorporation of formate into non-volatile compounds in ketotic cow liver slices. Compared to the nor-

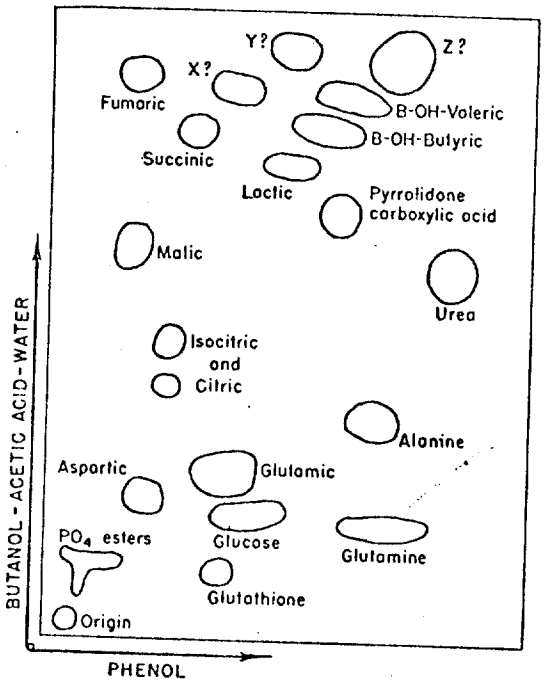


FIG. 1. Diagrammatic representation of location and identity of radioactive spots observed on various autoradiograms.

* Scientific Article No. A724, Contrib. No. 2960, Md. Agric. Exp. Station.

Supported by U. S. Atomic Energy Comm. and by funds of Northeast Regional Project.

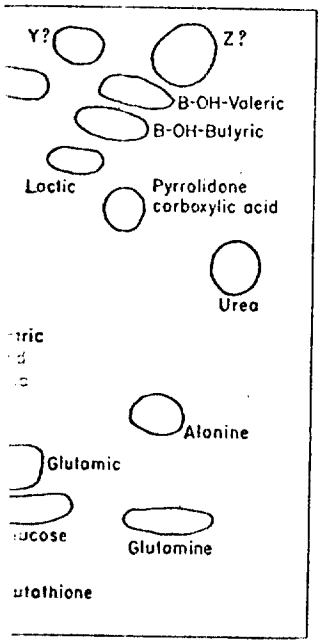
Cow Liver Incubated
(24666)

HAW
College Park

hours at 39 and 37°C for
respectively. The relative
olatile compounds separated
chromatography is shown

ll incubations the volatile
the largest percentage (83-
d C^{14}. Since this fraction
ilized substrate, it can be
excesses of substrate were
incubations. In addition,
enesis was observed during

Formate carbon was incor-
st extent into glucose, fol-
ear to be phosphate esters
rked decrease occurred in
and in incorporation of
vo' compounds in ke-
es. mpared to the nor-



representation of location
tive spots observed on var-
ora grams.

TABLE I. Percentage Incorporation of Isotope from Formate-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

	Liver sources: "Normal" cow*		Ketotic cow A		Ketotic cow B		Rat	
	Flask I	Flask II	Flask I	Flask II	Flask I	Flask II	Flask I	Flask II
Glucose	55.3	55.2	82.4	83.3	31.8	35.2	59.6	62.9
β-Hydroxybutyrate	3.7	2.2	.0	.0	5.8	7.0	Tr	.0
β-Hydroxyvalerate	.0	.0	.0	.0	8.3	4.9	.0	.0
Malate	4.6	2.7	.0	.0	Tr	Tr	3.8	3.2
Aspartate	.0	.0	.0	.0	21.7	11.1	.0	4.9
Glutamate + glutamine	7.2	7.1	.0	.0	9.4	7.7	3.0	2.9
Isocitrate + citrate	1.3	2.5	.0	.0	Tr	Tr	.0	.0
Lactate	3.5	3.8	.0	.0	4.5	4.9	4.2	3.6
Alanine	.0	.0	.0	.0	.0	.0	2.9	.0
Succinate	2.2	1.8	.0	.0	.0	.0	Tr	.0
Fumarate	Tr	Tr	.0	.0	.0	.0	.0	.0
PO_4 esters	8.1	10.4	Tr	Tr	6.4	16.1	13.0	11.3
Urea	4.9	3.6	.0	.0	Tr	5.9	5.5	7.0
Glutathione	.0	.0	.0	.0	.0	.0	3.4	.0
Unidentified	2.3	3.1	.0	.0	.0	.0	.0	.0
Origin	5.5	6.9	Tr	Tr	Tr	Tr	Tr	4.2
Total chromatogram radioactivity (cpm)	2,308	1,699	158	140	668	603	1,405	1,263
Major fractions	% of recovered C^{14}							
Respired CO_2	1.9	2.0	.5	.4	2.2	1.4	1.0	1.2
Nonvolatile fraction	2.4	3.5	3.3	1.3	2.4	5.2	1.1	1.2
Volatile "	95.7	94.5	96.2	98.3	95.4	93.4	97.9	97.6
Total recovered C^{14} (cpm × 10^6)	14.0	14.0	4.0	3.8	3.5	3.6	15.0	16.0

* Liver sample obtained by biopsy. Tr = Trace.

TABLE II. Percentage Incorporation of Isotope from Acetate-1-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

	Liver sources: "Normal" cow-S*		Ketotic cow A		Ketotic cow B		Rat	
Chromatographic compounds	Flask I	Flask II	Flask I	Flask II	Flask I	Flask II	Flask I	Flask II
Glucose	4.8	4.2	18.7	16.9	11.0	11.3	6.0	5.0
β-hydroxybutyrate	48.0	48.5	12.5	6.1	31.6	32.9	50.1	53.1
β-hydroxyvalerate	.0	.0	5.6	12.4	.0	Tr	.0	.0
Malate	7.5	6.3	7.7	7.5	6.8	7.0	5.3	6.2
Aspartate	7.5	9.5	9.7	7.4	11.2	12.8	3.5	3.7
Glutamate + glutamine	15.2	16.9	12.0	21.8	11.8	14.0	13.9	15.6
Isocitrate + citrate	10.5	11.0	19.7	16.9	13.8	9.9	8.3	6.2
Lactate	4.2	Tr	Tr	Tr	2.5	Tr	Tr	Tr
Alanine	.0	.0	.0	.0	.0	.0	"	"
Succinate	.0	Tr	Tr	Tr	3.6	Tr	2.0	1.6
Fumarate	.0	.0	"	"	Tr	"	Tr	Tr
PO_4 esters	.0	.0	5.0	"	.0	"	"	"
Urea	.0	Tr	.0	.0	.0	.0	2.9	2.7
Glutathione	.0	.0	.0	.0	.0	.0	Tr	Tr
Pyrrolidone carboxylic acid	.0	.0	Tr	Tr	2.5	2.9	.0	.0
Origin	Tr	.0	"	"	3.1	2.1	Tr	Tr
Total chromatogram radioactivity (cpm)	720	874	875	1,106	1,452	1,594	2,198	2,780
Major fractions	% of recovered C^{14}							
Respired CO_2	.4	.3	.1	.1	.1	.1	.1	.2
Nonvolatile fraction	1.7	2.1	1.4	1.5	2.1	2.7	2.5	3.2
Volatile "	97.9	97.6	98.5	98.4	97.8	97.2	97.4	96.6
Total recovered C^{14} (cpm × 10^6)	4.8	4.9	5.8	5.7	5.4	5.1	5.9	6.1

* Liver sample obtained from abbatoir.

TABLE III. Percentage Incorporation of Isotope from Butyrate-1-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

Chromatographic compounds	Liver sources: "Normal" cow-S* Flask I	"Normal" cow-S* Flask II	"Normal" cow† Flask I	"Normal" cow† Flask II	Ketotic cow A Flask I	Ketotic cow A Flask II	Ketotic cow B Flask I	Ketotic cow B Flask II	Rat I
Glucose	10.1	9.8	.0	Tr	.0	Tr	Tr	Tr	.0
β-hydroxybutyrate	74.3	74.8	77.0	76.5	57.1	36.9	47.0	48.6	77.4
β-hydroxyvalerate	.0	.0	1.9	2.6	5.2	9.4	4.3	4.7	1.6
Malate	2.8	2.8	2.2	1.6	2.2	2.3	3.4	4.0	2.0
Aspartate	3.3	4.7	2.9	3.3	8.5	11.7	13.0	12.4	1.0
Glutamate + glutamine	4.9	5.5	11.7	12.4	21.9	29.7	21.7	21.7	10.1
Isocitrate + citrate	.0	.0	Tr	1.1	Tr	Tr	Tr	Tr	Tr
Lactate	Tr	1.4	"	Tr	"	"	3.3	3.5	"
Alanine	"	.0	.0	.0	.0	.0	Tr	.0	"
Succinate	"	Tr	Tr	Tr	Tr	Tr	"	Tr	"
Fumarate	.0	.0	"	"	"	.0	"	"	"
X ‡	.0	.0	"	"	"	Tr	1.3	"	"
Y ‡	.0	.0	1.2	1.1	.0	"	.0	.0	.0
Urea	.0	.0	.0	.0	.0	"	Tr	Tr	2.2
Glutathione	.0	.0	.0	.0	.0	.0	.0	.0	Tr
Pyrrolidone carboxylic acid	.0	.0	.0	.0	.0	Tr	Tr	Tr	"
Origin	Tr	.0	Tr	Tr	Tr	"	"	"	"
Total chromatogram radioactivity (cpm)	2,049	3,324	6,193	5,905	1,717	1,281	3,347	2,604	4,639
Major fractions	% of recovered C^{14}								
Respired CO_2	.4	.6	.5	.5	.3	.4	.7	.6	.4
Nonvolatile fraction	3.9	4.9	13.6	13.4	3.9	3.6	6.3	6.0	8.8
Volatile "	95.7	94.5	85.9	86.0	95.8	96.3	93.0	93.4	90.7
Total recovered C^{14} (cpm × 10^6)	3.7	3.8	3.1	3.0	3.5	3.2	3.1	3.3	3.5

* Liver sample obtained from abbatoir. † Liver sample obtained by biopsy.

mal cow, the ketotic cow B liver slives incorporated a smaller % of formate into glucose and a larger % into aspartate, glutamine, β-hydroxybutyric acid (BHBA) and β-hydroxyvaleric acid (BHVA). Of the 2 ketotic cows, cow B exhibited the most marked hypoglycemia and ketonuria. The different distribution pattern observed in the case of ketotic cow A may have been due to extremely low incorporation of C^{14} (159 cpm) into the non-volatile components.

Acetate-C^{14}. Total incorporation of acetate label into the non-volatile components was low (Table II). This agrees with results by McCarthy *et al.*(4) which showed that labeled acetate passes through the perfused goat liver with only slight utilization. The data are similar to those observed for rat liver by Katz and Chaikoff(3) and mouse liver by Brown *et al.*(1), except for a lesser amount of label in glucose. The principal difference between normal and ketotic cows was in the higher incorporation into glucose and the lower incorporation into BHBA of labeled acetate by ketotic cow liver slices. The increased incorporation of acetate label into glucose by the ketotic liver slices is of interest in connection with the observation that hypoglycemia in ketotic cows can be ameliorated by oral administration of acetate (7).

Butyrate-1-C^{14}. Results from rat liver slice incubations with butyrate are in close agreement with those of normal cow liver slices (Table III). No marked differences in labeled CO_2 production were noted for the various liver slices. On the average, normal cow liver incorporated about twice as much butyrate into non-volatile compounds as ketotic cow liver. BHBA and the glutamate-glutamine group accounted for the bulk of the label from butyrate with lesser amounts occurring in aspartate, malate and BHVA. Very slight incorporation into C_3 intermediates was observed. The major differences between normal and ketotic cow liver were in incorporation of isotope into non-volatile compounds. Expressed as percentage incor-

TABLE IV. Percentage Incorporation of Isotope from Propionate-1-C^{14} into Chromatographic Non-volatile Compounds and Various Major Fractions by Liver Slices.

Chromatographic compounds	Liver sources: "Normal" cow-S* Flask I	Ketotic cow A Flask I	Ketotic cow A Flask II	Ketotic cow B Flask I	Ketotic cow B Flask II
Glucose	1.0	.0	Tr	Tr	Tr
β-hydroxybutyrate	Tr	3.4	3.1	2.6	3.3
β-hydroxyvalerate	1.6	4.3	2.4	5.8	2.1
Malate	34.0	9.7	6.4	16.8	17.5
Aspartate	25.9	24.0	18.8	35.6	35.3
Glutamate + glutamine	2.0	1.6	1.6	2.1	2.0
Isocitrate + citrate	.0	Tr	1.3	4.8	2.5
Lactate	6.1	9.4	5.1	8.7	8.1
Alanine	1.1	Tr	Tr	3.1	1.9
Succinate	3.0	9.2	11.2	3.6	4.3
Fumarate	5.1	2.0	Tr	2.8	2.7
X ?	7.6	17.6	23.3	5.5	6.1
Y ?	10.4	16.4	21.5	6.1	6.5
Z ?	.0	.0	Tr	.0	5.0
PO_4 esters	.0	.0	.0	Tr	Tr
Urea	Tr	Tr	Tr	1.3	1.3
Glutathione	"	.0	"	.0	Tr
Origin	"	Tr	"	Tr	"
Total chromatogram radioactivity (cpm)	3,317	2,937	2,241	8,454	7,308
Major fractions		% of recovered C^{14}			
Respired CO_2	.3	.1	.1	.2	.2
Nonvolatile fraction	3.3	1.7	1.4	4.0	3.2
Volatile fraction	96.4	98.2	98.5	95.8	96.6
Total recovered C^{14} (cpm × 10^6)	4.6	13.0	13.0	13.0	13.0

* Liver sample obtained from abbatoir.

poration, ketotic liver, compared to normal, produced (a) one third less BHBA, (b) twice the relative amount of glutamate-glutamine and BHVA, and (c) 3 times the relative amount of aspartate. Only in the case of the abbatoir cow were significant amounts of label from butyrate found in glucose. This may have been a physiological response to fasting since this animal had been fasted more than 24 hours. Comparing the results obtained with butyrate with those of acetate would suggest that degradation of butyrate into 2 acetate units is far from the complete story concerning metabolism of butyrate by bovine liver slices.

Propionate. Malate, aspartate and lactate contained most of the label from propionate with lesser amounts in BHVA, BHBA and succinate (Table IV). The unidentified compounds designated as X? and Y? (Fig. 1) also contained an appreciable portion of the label. Lower incorporation of label into malate was obtained with ketotic than with normal liver slices. A high incorporation of label was observed in the succinate and X and Y compounds of ketotic cow A.

Valerate-1-C^{14}. The most striking observation with this substrate was its wide distribution into non-volatile intermediates and its marked utilization by cow liver slices (Table V). BHVA and BHBA contained most of the label from valerate except for the ketotic cows in which the label in BHBA was low. It is of considerable interest to note (Table II) that incorporation of acetate label into BHBA by ketotic cow liver tissue was also low. The incorporation of valerate label into malate and glutamate (plus glutamine) was also lower for the ketotic cows. Perhaps the major difference between normal and ketotic liver with this substrate was its large incorporation into an unidentified compound, designated Z (Fig. 1), with negligible incorporation into this compound by normal cow liver slices. The spotting of this substance on the radiogram encompassed the greatest total area, suggesting a large turnover. A slight incorporation of labeled propionate into this substance by ketotic liver slices will be noted in Table IV. Identification of this compound may provide an important clue to the etiology of bovine ketosis. As previously stated, ketotic liver slice incubations resulted in exceptionally marked decreases in oxidation and incorporation of formate. This altered formate metabolism must be an important part of any metabolic explanation concerning boving ketosis.

It should be noted that the ketotic liver slices resulted in increased incorporation of formate, acetate and butyrate into aspartate. An increased production of glutamate was also observed for ketotic liver slices incubated with labeled butyrate. This increased production of aspartate and glutamate may be related to faulty gluconeogenesis in ketotic cow liver slices. This is of interest in relation to the reports of Shaw(7) concerning the

TABLE V. Percentage Incorporation of Isotope from Valerate-1-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

Chromatographic compounds	Liver sources: "Normal" cow* Flask I	"Normal" cow* Flask II	Ketotic cow A Flask I	Ketotic cow A Flask II	Ketotic cow B Flask I	Ketotic cow B Flask II
Glucose	Tr	Tr	Tr	Tr	.5	.7
β-hydroxybutyrate	27.5	23.9	4.1	2.7	5.5	7.0
β-hydroxyvalerate	43.8	46.9	57.8	52.8	48.7	42.7
Malate	3.6	3.1	.8	1.1	1.4	1.6
Aspartate	4.3	3.9	2.1	4.4	4.0	4.7
Glutamate + glutamine	9.8	10.0	4.0	5.9	6.0	5.5
Isocitrate + citrate	1.8	2.7	2.3	3.4	2.6	2.2
Lactate	1.1	1.1	.8	Tr	1.9	1.7
Alanine	.0	Tr	Tr	.0	Tr	Tr
Succinate	1.0	1.0	1.6	5.0	1.2	"
Fumarate	.7	.8	.0	.0	Tr	"
X ?	1.1	1.1	3.7	10.2	2.7	1.2
Y ?	3.0	2.7	.0	3.4	2.5	3.2
Z ?	Tr	Tr	18.7	6.7	19.5	25.7
PO_4 esters	"	.6	Tr	Tr	Tr	Tr
Urea	.7	.5	2.1	1.8	1.3	1.1
Pyrrolidone carboxylic acid	.6	.7	1.1	1.4	1.2	.9
Origin	Tr	.5	Tr	Tr	Tr	Tr
Total chromatogram radioactivity (cpm)	12,179	10,718	9,195	5,474	9,545	10,178
Major fractions			% of recovered C^{14}			
Respired CO_2	.5	.5	.4	.4	.4	.4
Nonvolatile fraction	5.6	5.0	3.7	3.4	3.8	4.0
Volatile "	93.9	94.5	95.9	96.2	95.8	95.6
Total recovered C^{14} (cpm × 10^6)	13.0	14.0	13.0	13.0	13.0	13.0

* Liver sample obtained by biopsy.

effective treatment of ketosis by administration of ACTH and glucocorticoids.

The overall decreased metabolic activity *in vivo* studies by Robertson *et al.*(6) and Peeters *et al.*(5).

Conclusions. 1) The oxidative metabolic patterns of normal and ketotic cow liver studies with 1-C^{14}-labeled C_1 through C_5 aliphatic acids, were determined using a chromatographic-autoradiographic technic. 2) The label from each metabolite had its own distinctive distribution pattern as evidenced by autoradiograms. 3) Normal cow liver slices incorporated carboxyl-labeled carbons of the various aliphatic acids, to the greatest extent, into non-volatile compounds as follows: formate, into glucose and what appear to be phosphate esters; acetate, into β-hydroxybutyrate, glutamate, citrate and small amounts of glucose and lactate; butyrate, into β-hydroxybutyrate and glutamate-glutamine with little incorporation into C_3 intermediates; propionate, into malate, aspartate and lactate; valerate, into β-hydroxybutyrate and β-hydroxyvalerate with a very wide distribution into the other intermediates chromatographically obtainable. 4) Ketotic cow liver slices exhibited a marked reduction in utilization of formate and an altered distribution in labeling of intermediates when incubated with various labeled aliphatic acids.

1. Brown, G. W., Jr., Katz, J., Chaikoff, I. L., *Cancer Res.*, 1956, v16, 509.

2. Entenman, C., Lerner, S. R., Chaikoff, I. L., Dauben, W. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 364.

3. Katz, J., Chaikoff, I. L., *J. Biol. Chem.*, 1954, v206, 887.

4. McCarthy, R. D., Shaw, J. C., Lakshmanan, S., PROC. SOC. EXP. BIOL. AND MED., in press.

5. Peeters, G., Oyaert, W., Bouckaert, J., Haeck, H., *Zentr. fur Veterinarmed.*, 1958, v5, 43.

6. Robertson, W. G., Mixner, J. P., Bailey, W. W., Lennon, H. D., Jr., *J. Dairy Sci.*, 1957, v40, 977.

7. Shaw, J. C., *ibid.*, 1956, v39, 402.

Received October 17, 1958. P.S.E.B.M., 1959, v100.

512

FURTHER STUDIES ON THE ADRENAL CORTEX AND CARBOHYDRATE METABOLISM

W. W. WINTERNITZ, R. DINTZIS AND C. N. LONG

Departments of Physiology and Internal Medicine, Yale University, New Haven, Connecticut

WE HAVE already reported in a preliminary communication (39) that the subcutaneous injection of epinephrine into fasting adrenalectomized rats is followed by a greater fall in muscle glycogen than in intact rats and by practically no accumulation of liver glycogen in the four hour period following the injection of the hormone. Since the blood glucose and blood lactate changes are similar to those in intact rats, it follows that in the adrenalectomized animal a much larger proportion of the carbohydrate lost from the muscles has been disposed of over pathways that do not lead to liver glycogen accumulation.

Since the subcutaneous injection of epinephrine may be regarded as a test of the capacity of the organism to dispose of lactate, in this case derived from its own muscle glycogen, this paper reports not only more extended observations on the different response of intact and adrenalectomized rats to this hormone but also differences in the capacity of intact and adrenalectomized rats to form liver glycogen from other precursors.

METHODS

Male rats of the Wistar strain weighing 180–220 gm. were used throughout. They were all fasted for 24 hours prior to the experiment. Adrenalectomy was performed in one stage and the animals maintained for 7–14 days either by giving 1% NaCl as drinking fluid or by the injection of desoxycorticosterone acetate (DCA) (0.25 mg./day).

Alloxan diabetes was produced by the intravenous injection of 4–5 mg. of alloxan per 100 gm. of body weight. The diabetic animals were kept in separate metabolism cages and the severity of the diabetes was determined from the urinary glucose excretion. Only those with high blood glucose levels at the end of a 24-hour fast were used.

All epinephrine injections, except where otherwise stated, were made into unanesthetized animals, the quantity given being 0.02 mg. of a freshly diluted solution of l-epinephrine hydrochloride (Adrin, Sharp and Dohme) which was injected subcutaneously.

At the end of the experimental periods all animals were deeply anesthetized by intraperitoneal injections of Nembutal. After a blood sample had been obtained from the vena cava or tail veins, one gastrocnemius muscle was rapidly removed, placed in a previously tared tube containing 2 ml. of 30% KOH. The tube was placed immediately into a boiling water bath until dissolution of the tissue was complete. Approximately 1 gm. of the right lobe of the liver was also removed and treated in the same manner as

Received June 5, 1957.

the muscle sample. The remainder of the liver was excised and weighed so that the total glycogen content of this organ could be determined.

The determination of muscle and liver glycogen was carried out by a modified Pfluger method and the glucose resulting from its hydrolysis, as well as blood glucose, was estimated by the Nelson method. Glycogen values are expressed as glucose. Blood lactic acid was determined by the method of Barker and Summerson.

In the experiments where various substances were infused, the animals were anesthetized with Nembutal, and a polyethylene cannula was inserted into a femoral vein. The substance was infused by means of a pump geared to deliver 2 ml. of fluid per hour. A total of 3 ml. was infused into each animal. The total time of infusion in all cases was 90 minutes.

Urine was collected during the infusion from a polyethylene catheter that was tied into the bladder. Urine samples were collected in small vials and frozen until analyzed.

The following substances were infused. (a) D-Glucose, A.R. anhydrous, (b) D-Fructose, twice recrystallized from acetone and water, (c) L-Lactic acid (Nutritional Biochemicals) partially neutralized with NaOH; the final pH of the solution administered was 4.0, (d) Glycerol, A.R., (e) L-Malic acid, which was also neutralized to pH 4.0 before injection. 1.67 mEq. of glucose and fructose and 3.34 mEq. of glycerol, lactic and malic acid dissolved in 3 ml. of water were given to each rat during the 90 minute infusion.

When hydrocortisone was administered it was given subcutaneously as a finely divided suspension in 0.9% NaCl, 5 hours prior to the sacrifice of the animals.

Calculation of total carbohydrate changes. The method introduced by Cori and Cori (7) for the calculation of the total muscle glycogen, free glucose and lactate in the body fluids has been used, with some modifications, for the calculation of the absolute changes in these constituents as a result of epinephrine injection.

These calculations depend on three assumptions: (a) that the muscles constitute 50% of the body weight of fasted animals; (b) that the free glucose and lactate is distributed in a volume equivalent to 25% of the body weight; (c) that the glycogen content of the gastrocnemius muscle may be used as the mean figure for the glycogen content of the whole skeletal musculature.

The experimental evidence justifying these assumptions is as follows: (a) Donaldson (9) found by actual measurement that in male rats weighing 220 gm. that the skeletal musculature constituted some 43% of the body weight. Since these were fed rats a figure of 50% is a reasonable estimate of the muscle mass in rats of this weight at the end of a 24-hour fast. (b) It has been shown in dogs and rabbits by means of radio-active glucose infusion, that the space occupied by diffusible glucose is about 25–30% of the body weight (35, 44). This agrees well with the values obtained by Sveinsson (36) by another method. In the fasted male rat Gey (13) has determined the true glucose content of a number of organs including the skeletal muscles and liver and related them to the true glucose content of the plasma. From his figures it is possible to calculate that the total free glucose content of the whole animal must be between 20–30% of the body weight. The use of the figure of 25% of the body weight, instead of 50%, as used by Cori and Cori would appear to give a more correct estimate of the total free glucose (and lactate) in the body. (c) The use of the glycogen content of a single gastrocnemius muscle as a measure of the average glycogen content of the whole of the skeletal musculature is the factor that would seem most open to question. There is, however, good reason to believe that such an estimate in the rat is not subject to such large and variable errors as in the case of the cat (11). Undoubtedly this is due to the greater uniformity in body size, nutritive state, etc. of the inbred laboratory reared animal. For example, Cori (8) observed in the rat that there was good agreement between an estimate of total muscle glycogen calculated from the content of the gastrocnemius and the total amount determined in the whole carcass. A similar relationship was also obtained by Evans (12),

while Long and Grant (25) found that the sum of the glycogen and lactic acid of the carcasses of both resting and exercised fasting rats is of the order of 230–240 mg. per 100 gm. of initial body weight. This would correspond to an initial gastrocnemius glycogen content of 460–480 mg.%, a value which is generally accepted as in the normal range for this muscle in the fasting animal.

RESULTS

THE EFFECT OF EPINEPHRINE ON THE CARBOHYDRATE METABOLISM OF FASTED NORMAL AND ADRENALECTOMIZED RATS

In Table 1 are shown the glycogen content in per cent of the wet weight of the liver and gastrocnemius muscles of intact and adrenalectomized

TABLE 1. THE EFFECT OF EPINEPHRINE ON THE LIVER AND MUSCLE GLYCOGEN, BLOOD GLUCOSE AND LACTIC ACID OF NORMAL AND ADRENALECTOMIZED FASTING RATS

	Number of rats	Liver glycogen	Muscle glycogen	Blood glucose	Blood lactic acid
Normal rats					
Controls	22	136 ± 16*	567 ± 11	71 ± 2	22 ± 1
1 hr. after Epinephrine	12	877 ± 209	374 ± 18	177 ± 10	55 ± 4
4 hrs. after Epinephrine	13	1694 ± 120	385 ± 22	115 ± 5	26 ± 3
8 hrs. after Epinephrine	12	570 ± 23	424 ± 32	76 ± 4	—
Adrenalectomized rats					
Controls—1% NaCl	18	70 ± 8	528 ± 12	49 ± 4	18 ± 1
1 hr. after Epinephrine—1% NaCl	12	36 ± 9	202 ± 16	123 ± 10	81 ± 5
4 hrs. after Epinephrine—1% NaCl	12	156 ± 51	225 ± 18	100 ± 4	19 ± 1
8 hrs. after Epinephrine—1% NaCl	14	20 ± 5	237 ± 16	43 ± 2	—
Controls—DCA	6	6 ± 3	518 ± 12	62 ± 2	18 ± 1
4 hrs. after Epinephrine—1% NaCl	6	201 ± 78	190 ± 16	96 ± 10	—

All values mg. per 100 gm. tissue.
* Standard error of the mean.

rats 1, 4 and 8 hours after the injection of the hormone, together with the blood glucose and lactic acid levels at these times.

It will be seen that 1 hour after the injection into intact rats there has occurred a 34% fall in muscle glycogen and a six fold increase in liver glycogen. This is accompanied by marked hyperglycemia and a large increase in blood lactic acid. Four hours after the injection, the loss of muscle glycogen is the same as at 1 hour but the liver glycogen is now increased some twelve fold over the initial level. The blood glucose is still elevated but the blood lactic acid has returned to the initial level. Eight hours later the liver glycogen has declined to one-third of its level at the fourth hour. The muscle glycogen still has not increased over the level reached the first hour after the injection.

In adrenalectomized rats the situation is very different. While the initial levels of liver and muscle glycogen and blood glucose are lower in

adrenalectomized animals, the fall in muscle glycogen, 1 hour after epinephrine, is twice as great (62%) as in intact animals. At this time there has been no significant increase, in fact a small decrease, in the concentration of glycogen in the liver. The levels of muscle and liver glycogen are essentially unchanged at the 4th and 8th hour after the injection, although there is some indication of a slight increase in liver glycogen at the 4th hour. Maintenance of the adrenalectomized rats by the daily injection of desoxycorticosterone acetate (DCA), instead of by the use of 1% NaCl as drinking fluid does not alter this atypical response to epinephrine (Table 1). Similar differences in the effect of epinephrine on the muscle

TABLE 2. CARBOHYDRATE BALANCES IN NORMAL AND ADRENALECTOMIZED RATS AT VARIOUS TIMES AFTER THE INJECTION OF EPINEPHRINE

	Liver glycogen	Muscle glycogen	Glucose in body fluids	Lactate in body fluids	Total	Net change
Normal rats						
Controls	5 ± 0.6*	284 ± 5	18 ± 0.6	6 ± 1	313 ± 5	—
1 hr. after Epinephrine	31 ± 7	187 ± 9	44 ± 2.4	14 ± 1	276 ± 10	− 37
4 hrs. after Epinephrine	56 ± 6	193 ± 11	29 ± 1.4	7 ± 0.3	285 ± 14	− 28
8 hrs. after Epinephrine	20 ± 3	212 ± 16	19 ± 1	(6)†	257 ± 15	− 56
Adrenalectomized rats						
Controls—1% NaCl	2 ± 0.3	265 ± 7	12 ± 0.5	5 ± 0.2	284 ± 7	—
1 hr. after Epinephrine—1% NaCl	1 ± 0.3	101 ± 8	31 ± 2.5	21 ± 1.2	154 ± 7	−130
4 hrs. after Epinephrine—1% NaCl	5 ± 1	113 ± 9	25 ± 1.6	5 ± 0.2	148 ± 8	−136
8 hrs. after Epinephrine—1% NaCl	1 ± 0.2	119 ± 8	11 ± 0.2	(5)†	136 ± 7	−148
Controls—DCA	0.2 ± 0.2	259 ± 6	15 ± 0.2	5 ± 0.3	279 ± 6	—
4 hrs. after Epinephrine—1% NaCl	7 ± 0.3	95 ± 8	24 ± 2.6	(5)†	131 ± 10	−148

All values mg. per 100 gm. body weight.
* Standard error of the mean.
† Assumed to be the same as the initial value.

glycogen of intact and adrenalectomized rats have previously been reported by Collip, Thomson and Toby (5).

By use of the assumptions as to muscle mass, average muscle glycogen content and distribution volume of glucose and lactic acid in the body, it is possible to calculate the total changes in muscle and liver glycogen as well as in free glucose and lactic acid in the bodies of intact and adrenalectomized rats injected with epinephrine. These calculations are to be found in Table 2.

In the case of intact rats such calculations show the well known effect of epinephrine on the preformed carbohydrate stores of the body. Thus at the end of the first hour the loss of muscle glycogen is almost all accounted for by the increase in liver glycogen, blood glucose and lactic acid. There is, however, a small but significant amount of carbohydrate (37 mg. per 100 gm. of body weight) that is unaccounted for in these terms. At the fourth hour the difference in the total carbodyrate content of the controls and injected animals is no longer significant. Eight hours after the injection there is a significant loss of carbohydrate amounting to some 56 mg. per 100 gm. of rat.

In the adrenalectomized animals, by reason of the greater loss of muscle glycogen and its failure to appear as liver glycogen, there is at all periods examined a large proportion of the carbohydrate lost from the muscles that is unaccounted for as liver glycogen, glucose or lactate. This is about 100 mg. per 100 gm. of rat greater than is found in the intact rats at comparable periods. In fact at the 1- and 4-hour periods the carbohydrate unaccounted for in the adrenalectomized rat represents 78% and 90% respectively of the glycogen lost from the muscles under the action of epinephrine. It is also evident from the figures in Tables 1 and 2 that the disappearance of muscle carbohydrate occurs quite rapidly after the administration of epinephrine, in fact the major loss has occurred 1 hour after the injection. Presumably, in the absence of the adrenal cortical hormones a large portion of the carbohydrate, derived from the muscles, is utilized over pathways of metabolism that do not allow any significant portion to be accumulated as liver glycogen.

TABLE 3. EFFECT OF EPINEPHRINE ON THE MUSCLE AND LIVER GLYCOGEN LEVELS OF ANESTHETIZED INTACT AND ADRENALECTOMIZED RATS. MUSCLES REMOVED 1 HOUR APART

	No.	Muscle glycogen, mg.%			Liver glycogen, mg.%
		Initial	Final	Diffce.	
Normal	6	534 ± 18	287 ± 25	−247 ± 8 (−46%)	1105 ± 145
Adrex.	5	484 ± 15	107 ± 12	−377 ± 8 (−78%)	62 ± 28

In another series of experiments the procedure was varied. Rats were anesthetized and one gastrocnemius muscle was removed along with a blood sample. Epinephrine was then injected and the animals kept under anesthesia for 1 hour at which time the second muscle along with a portion of the liver was removed for analysis. The results are in Table 3 and again show that in the adrenalectomized animals there is a significantly greater loss of muscle glycogen and a failure to deposit liver glycogen.

The failure of adrenalectomized animals to respond in a normal manner to epinephrine is not due to an inability of the hormone to release the muscle glycogen as lactic acid nor to a failure of the liver to increase the blood glucose from lactic acid. This is evident from the data on blood glucose and lactic acid given in Table 1 and is confirmed by a closer analysis of the time relationships of the response of these blood constituents after the injection (Fig. 1).

Except for a greater increase in the blood lactic acid 1 hour after injection into the adrenalectomized animals, and perhaps a somewhat sluggish response of the blood glucose, the curves are identical. In each case the peak of the blood lactic acid is reached at 1 hour and the initial levels are restored in 4 hours. The peak of the blood glucose does not occur until the

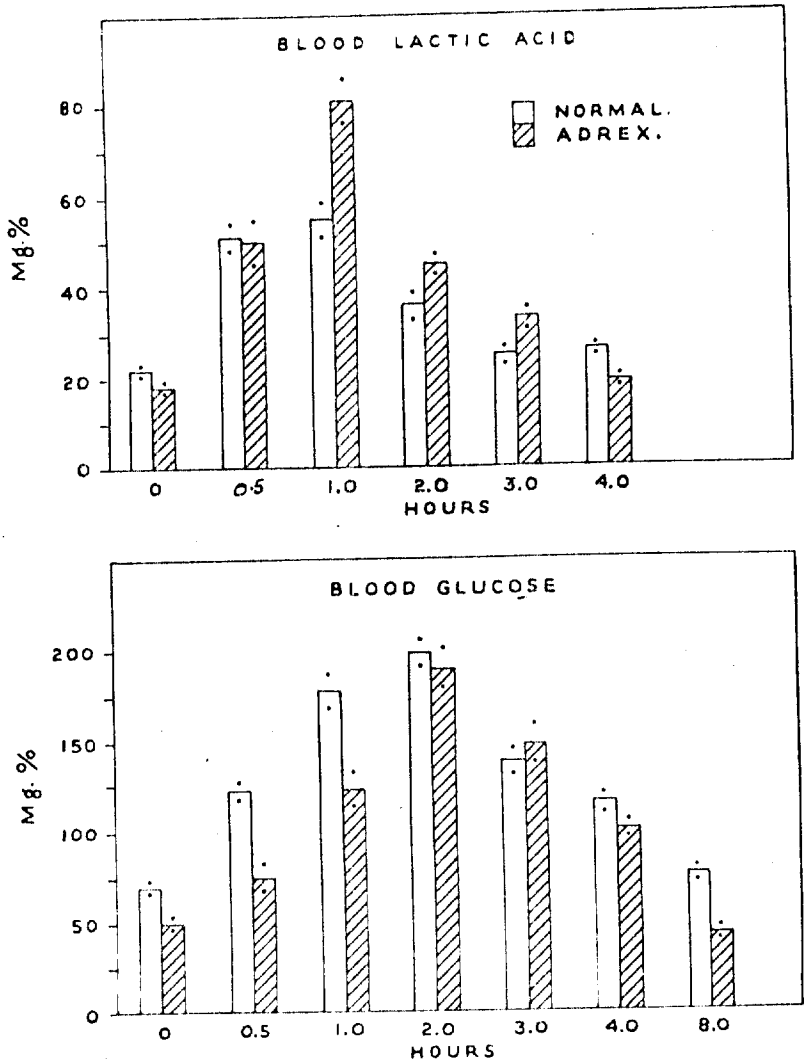


FIG. 1. The response of the blood glucose and lactic acid of fasted intact and adrenelectomized rats to the subcutaneous injection of epinephrine.

second hour and the initial levels are not reached again until somewhere between the fourth and eighth hour. There is no apparent difference, once the peak has been reached, in the rate of removal from the blood of lactic acid or glucose.

Effect of replacement therapy. It was noted above that the daily injection of DCA from the time of adrenalectomy did not restore the normal pattern of response of the carbohydrate metabolism to the injection of epinephrine. The effect of cortisone and adrenal cortical extract is shown in Table 4.

In all the experiments contained in Table 4 fasted adrenalectomized rats were used. They were either: (a) implanted at the time of operation

with cortisone pellets of about 9 mg. Some days later they were injected with epinephrine and sacrificed 4 hours later; (b) injected with 1 ml. an hour of aqueous cortical extract (ACE) during the 4-hour period of epinephrine action; (c) injected every 2 hours for 12 hours with 1 ml. of adrenal cortical extract prior to the injection of epinephrine, the injection of ACE being continued during the ensuing 4-hour period. These three groups, therefore, represent (a) the effect of a small, continuous but supportive amount of cortisone, (b) the effect of suddenly increased levels of adrenal steroids during the period of epinephrine action and (c) the effects

TABLE 4. EFFECT OF ADRENAL CORTICAL HORMONES ON THE RESPONSE OF THE ADRENALECTOMIZED RAT TO EPINEPHRINE

Group	Treatment	No.	Liver glycogen	Muscle glycogen	Glucose	Net
A	Cortisone pellet	2	2	303	15	
	Cortisone pellet + EPI.	4	32 ± 5*	111 ± 11	24 ± 1	−153
B	ACE/ml./hr./4 hrs.	7	17 ± 1	256 ± 16	19 ± 1	
	ACE/ml./hr./4 hrs. + EPI.	7	17 ± 1	98 ± 8	35 ± 2	−111
C	ACE 1 ml./2 hrs./16 hrs.	5	43 ± 6	286 ± 16	19 ± 1	
	ACE 1 ml./2 hrs./16 hrs. + EPI.	7	92 ± 4	187 ± 9	19 ± 1	− 39

All values mg. per 100 gm. B.W.
* Standard error of the mean.

of excess of the cortical hormones both before and during the epinephrine period.

The effect of a small pellet of cortisone (Table 4A) is to partially restore the capacity of the liver to retain part of the muscle lactate as glycogen. It does not, however, prevent the excessive depletion of the muscle glycogen. In consequence there is still a large net loss of carbohydrate during the period.

The administration of cortical extract over a 4 hour period of epinephrine action increases the liver glycogen of the control group (Table 4B) and allows significant deposition of liver glycogen in the group given epinephrine. It does not, however, prevent the excessive loss of muscle glycogen and again there occurs a large net loss of carbohydrate.

On the other hand, rats pretreated with ACE for 12 hours prior to the injection of epinephrine, with continuance of the A.C.E. during the epinephrine period, show a pattern of carbohydrate response similar to that of intact rats (Table 4C). That is to say that in addition to a normal increment in liver glycogen there was also a considerably reduced loss of muscle glycogen (35% as compared to over 60% in Groups A and B). It should also be noted that in both Groups A and B that the increase in liver glycogen occurs in spite of the fact that in the control groups the level is already elevated by treatment with ACE alone.

The effect of epinephrine on eviscerated rats. In order to determine whether the greater loss of muscle glycogen in adrenalectomized rats given epinephrine was due to a greater sensitivity of the glycogenolytic mechanism of the muscles in the absence of the adrenal cortical hormones, the following experiment was performed.

One group of rats were adrenodemedullated, 3–4 weeks before use, in order to allow time for adequate regeneration of the cortical portion of the gland. This also ensures that the subsequent evisceration will not release large amounts of epinephrine and initiate the glycogenolytic process before the standard dose of epinephrine could be given. Another group of rats were totally adrenalectomized 1 week before use.

At the time of the experiment both groups were anesthetized and eviscerated. A subcutaneous injection of glucose, amounting to 20 mg. per 100 gm. of rat in the case of the adrenodemedullated animals and 40 mg. per 100 gm. in the case of the adrenalectomized animals was given. One gastrocnemius muscle and a blood sample was removed and the epinephrine injected subcutaneously. One hour later the second muscle and blood sample were taken.

It is apparent (Table 5) that the presence or absence of adrenal cortical tissue does not alter the rate of muscle glycogenolysis under the action of epinephrine. Consequently, the greater loss of carbohydrate observed in non-eviscerated adrenalectomized animals must be associated with the presence of one of the visceral organs.

TABLE 5. EFFECT OF EVISCERATION ON THE RESPONSE OF THE EVISCERATED RAT TO EPINEPHRINE

	No. of animals	Muscle glycogen, mg.%			Blood lactic acid, mg.%			Final blood glucose, mg.%
		Initial	Final	Decrease	Initial	Final	Increase	
Adrenodemed.	9	577±24	364±33	213±16	22±2	124± 7	102	62±10
Adrex.	8	568±21	329±26	239±16	18±2	130±10	112	68±12

The effect of epinephrine on alloxan diabetic rats

Since it is well known that adrenalectomized animals are unduly sensitive to insulin, there exists the possibility that the differences between them and intact animals is due to the unopposed action of insulin, secreted in response to the hyperglycemia produced by epinephrine. While there is, in fact, no evidence in these experiments of excessive insulin responses in the adrenalectomized animals, nevertheless, it was felt that this possibility should be definitely excluded.

Rats were rendered diabetic by the injection of alloxan. Animals were

selected that had exhibited severe glycosuria and had a high blood glucose at the end of a 24-hour fast. These were divided into four groups. (A) Control diabetic animals injected with saline and killed 4 hours later. (B) Diabetic animals who received epinephrine and were sacrificed 4 hours later. (C) A group of diabetic animals that were adrenalectomized 1 week before the period of fasting, injected with saline and killed 4 hours later. (D) A similar group of adrenalectomized-diabetic animals that were injected with epinephrine and sacrificed 4 hours later.

TABLE 6. EFFECT OF EPINEPHRINE ON THE CARBOHYDRATE METABOLISM OF ALLOXAN DIABETIC AND ADRENALECTOMIZED ALLOXAN DIABETIC RATS

Group	No.	Liver Glycogen	Muscle Glycogen	Glucose in body fluids	Urine glucose mg/4 hrs.	Total	Net change
A) Allox-diab.	11	108 + 16*	250 ± 16	66 ± 7	8 ± 2	432 ± 19	--
B) Allox-diab. +EPI. (4 hrs.)	7	45 ± 8	89 ± 15	114 ± 11	113 ± 21	361 ± 19	− 71
C) Adrex allox.-diab.	5	1 ± 0.6	226 ± 11	14 ± 0.5	0	241 ± 12	—
D) Adrex allox.-diab. +EPI. (4 hrs.)	8	0.7 ± 0.4	39 ± 20	39 ± 8	0	79 ± 14	−162

All values mg. per 100 gm. body weight.
* Standard error of the mean.

The bladders of all animals were emptied by pressure prior to the injection of saline or epinephrine. Urine voided during the experimental period was collected and any urine remaining in the bladder at the time of sacrifice was also obtained.

The results in Table 6 are expressed as mg. of carbohydrate per 100 gm. of body weight. In the first place it will be noted that alloxan diabetic rats have, at the end of a 24-hour fast, much higher levels of liver glycogen than normal animals. This has been observed by several investigators (cf. 40). The average blood glucose of Group A at the end of the fast was 264 ±27 mg. %, while the urinary glucose excretion during the 4 hour period averaged about 2–3 mg. per 100 gm. body weight an hour, values comparable to those observed by Scow (32) in fasting totally depancreatized rats. The total carbohydrate in the body of these fasting diabetic rats was therefore significantly greater than that of fasting normal rats. It should also be noted that the muscle glycogen content was slightly decreased.

The injection of epinephrine into such diabetic animals (Group B; Table 6) is followed by marked hyperglycemia, so that 4 hours later the blood glucose averaged 457 mg. % (Range 348–648). This was associated with heavy glycosuria indicating that a large proportion of the pre-existing stores of liver and muscle glycogen had been lost to the body. This is borne out by the observation that the liver glycogen falls in these animals under the action of epinephrine, in contrast to the increase that occurs in normal animals (Tables 1 and 2). There is also an exaggerated loss of muscle glycogen as compared to normal animals. This indicates, what is well known, that in normal animals part of the muscle glycogen lost under the

immediate action of epinephrine is restored to them by the action of the increased insulin secretion evoked by the hyperglycemia. In severely diabetic animals this part of the Cori cycle is largely inoperative, and a large proportion of the glucose, formed from lactate, that would normally replenish the muscle glycogen is now found in the body fluids and urine.

The important point, however, is that the net balance of carbohydrate is not unduly different from that of intact rats. The net loss of total carbohydrate (Table 6) as the result of epinephrine action in diabetic rats was

TABLE 7. EFFECT OF THE INFUSION OF VARIOUS SUBSTANCES ON CARBOHYDRATE METABOLISM OF INTACT, ADRENALECTOMIZED, AND ADRENALECTOMIZED RATS TREATED WITH HYDROCORTISONE

Substance infused	Amount Mg./100 gm./B.W.	No.	Liver glycogen Mg.%	Liver glycogen Mg./100 gm./B.W.	Muscle glycogen, Mg.%	Blood sugar, mg.% Initial	Blood sugar, mg.% Final	Urinary excretion, mg./100 gm./B.W.
Normal								
None	—	4	117	4	478 ± 43	—	—	—
Glucose	122	6	1130	38	502 ± 11	73 ± 4	137 ± 7	0
Fructose	155	7	1900	71	530 ± 17	80 ± 6	153 ± 11	10 ± 3
Glycerol	119	6	1180	40	471 ± 14	69 ± 4	95 ± 4	—
Lactate	136	5	1420	44	528 ± 25	70 ± 2	87 ± 1	5 ± 2
Malate	142	5	570	18	479 ± 33	78 ± 3	98 ± 2	—
Adrex.								
None	—	11	27	1	457 ± 8	—	—	—
Glucose	130	6	500	16	489 ± 9	68 ± 6	198 ± 14	Trace
Fructose	124	6	810	25	477 ± 7	63 ± 4	151 ± 18	4 ± 0.8
Glycerol	130	7	280	9	406 ± 8	55 ± 3	85 ± 5	—
Lactate	119	8	128	3	444 ± 12	57 ± 3	84 ± 5	2 ± 0.5
Malate	149	6	31	1	539 ± 15	61 ± 3	101 ± 10	—
Adrex. + hydrocortisone								
None	—	4	1000	31	451 ± 10	—	—	—
Glucose	146	6	1980	66	454 ± 8	94 ± 5	259 ± 24	4 ± 4
Fructose	148	6	2970	108	467 ± 15	93 ± 2	179 ± 20	13 ± 1
Glycerol	145	5	1800	60	436 ± 18	85 ± 5	116 ± 8	—
Lactate	129	6	1730	54	484 ± 11	83 ± 1	114 ± 7	6 ± 2
Malate	151	6	1450	46	490 ± 19	96 ± 2	109 ± 12	—

71 mg. per 100 gm. as compared to a net loss of 28 mg. per 100 gm. in non-diabetic rats (Table 2). This difference is probably due to the difficulties of obtaining accurate measurements of urinary excretion of glucose during the period of action of epinephrine.

Groups C and D (Table 6) were adrenalectomized-alloxan diabetic rats given either saline or epinephrine. The control group exhibited the characteristic low blood glucose and liver glycogen and the absence of any glycosuria in the fasting state. A comparable group given epinephrine (Group D) showed, 4 hours later, a moderate degree of hyperglycemia, no accumulation of liver glycogen, a profound fall in muscle glycogen, and only traces of glucose in the urine. Consequently, there is a net loss of body carbohydrate of 162 mg. per 100 gm., an amount quite comparable to that unaccounted for in adrenalectomized rats treated in the same manner.

It is, therefore, apparent that loss of the adrenal cortical hormones affects the disposition of the carbohydrate mobilized by epinephrine in a

different manner than does the withdrawal of insulin secretion. In the case of the former, a large proportion of the carbohydrate is evidently disposed of over pathways that do not result in its appearance in the components determined in these experiments. Insulin lack, on the other hand, merely prevents the utilization of the glucose, formed from lactate, for muscle and perhaps liver glycogen synthesis, and in consequence large amounts of glucose appear in the body fluids and urine. Finally, these experiments emphasize that the effect of withdrawal of cortical hormones on carbohydrate metabolism in not dependent on the presence of insulin; a conclusion that is in keeping with the observation of Miller (26) that the administration of excess of adrenal cortical extract to adrenalectomized alloxan diabetic rats still results in a marked accumulation of liver glycogen.

Liver glycogen formation from various precursors in adrenalectomized rats

There have been several reports that after adrenalectomy both the intact animal and the isolated liver slice exhibit a diminished capacity to form liver glycogen from glucose, lactic, pyruvic acid and alanine (2, 3, 12, 16, 19, 24, 43). Conversely, the injection of adrenal cortical extracts into adrenalectomized animals or their addition to the medium containing liver slices, or even to the fluid perfusing isolated livers, has been shown to increase the liver glycogen content when lactate, glucose or alanine are used as glycogen precursors (6, 20, 28, 29, 33).

In view of the marked difference shown by adrenalectomized rats in the disposition of lactic acid released into the organism under the action of epinephrine, the ability of normal and adrenalectomized rats, and adrenalectomized rats treated with hydrocortisone, to dispose of several well known precursors of liver glycogen and blood glucose was reinvestigated. In these experiments, the details of which are described under Methods, the various meterials were infused at a constant rate into the animals over a relatively short period of time ($1\frac{1}{2}$ hours). The substances chosen for study in this manner were glucose, fructose, glycerol, lactate and malate. Table 7 shows the measured changes in liver and muscle glycogen as well as in the blood glucose as a consequence of the infusion of these substances into the three classes of animals employed. In Table 8 the amount and percentage of the infused material recovered as liver glycogen or sugar in the body fluids is calculated. It should be noted that with the amounts of the various substances infused and the relatively short period of infusion employed the changes in muscle glycogen have no significance. Consequently, the experiments test the relative capacity of the various groups of animals to form or retain a portion of the infused material as liver glycogen or, in the case of glycerol, lactate and/or malate, to also form glucose from them.

Glucose. At the amount and rate of glucose infused, normal rats retained some 28% as liver glycogen and added some 13% to the glucose in the body

fluids. After adrenalectomy only 12% of the material was accounted for as liver glycogen, but since the blood glucose rose to a greater degree some 25% was recovered as glucose in the body fluids. This apparent reduction in glucose tolerance after adrenalectomy in rats, as measured by blood glucose changes, has also been reported by Minibeck and Verzar (27) and Evans (12). The latter also noted the marked decrease in liver glycogen formation under these conditions. The administration of hydrocortisone (5 mg.) $3\frac{1}{2}$ hours before the infusion elevated to normal the deposition of

TABLE 8. DISPOSITION OF VARIOUS SUBSTANCES BY INTACT AND ADRENALECTOMIZED RATS

Substance infused	Amount infused*	Deposited as liver glycogen*	in body fluids*	% of amount infused as: Liver glycogen	Sugar in body fluids	% Accounted for
			Normal			
Glucose	122	34	16±2.5	27.9±2.1	13.1	41.0
Fructose	155	67	18±2.2	43.2±3.7	11.6	54.8
Glycerol	119	36	7±2.0	30.3±4.4	5.9	36.2
Lactate	136	40	4±0.8	29.4±2.1	2.9	32.3
Malate	142	14	5±1.0	9.9±2.4	3.5	12.4
			Adrex.			
Glucose	130	15	33±3.3	11.5±1.5	25.3	38.8
Fructose	124	24	22±4.8	19.4±1.9	17.7	37.3
Glycerol	130	8	8±1.5	6.2±1.7	6.2	12.4
Lactate	119	2	7±1.5	1.7±0.9	5.9	7.6
Malate	149	0	10±2.0	0	6.7	6.7
			Adrex.+Hydrocortisone			
Glucose	146	35	41±6.3	24.0±5.2	28.1	52.1
Fructose	148	77	22±2.2	52.0±3.8	14.9	66.9
Glycerol	145	29	8±1.0	20.0±7.0	5.5	25.5
Lactate	129	23	8±1.8	17.8±4.1	6.2	24.0
Malate	151	14	3±2.2	9.9±3.7	2.0	11.9

* Mg. per 100 gm. of body wt.

liver glycogen without significant effect on the quantity of glucose in the body fluids.

Fructose. Fructose is known to be a much better precursor of liver glycogen than glucose in intact rats. This is again illustrated in Tables 7 and 8 where the infusion of this sugar into intact animals resulted in the deposition of 43% of the amount given as liver glycogen in the period studied. Some 12% of the sugar was accounted for as reducing sugars in the body fluids (glucose+fructose). In the adrenalectomized animals the amount of reducing sugar rose slightly during the infusion but the proportion recovered as liver glycogen was reduced to less than half of that found in intact rats. Prior administration of hydrocortisone to the adrenalectomized

rats returned the liver glycogen deposition to normal amounts without any change in the amount of free sugar in the body.

Glycerol. In intact rats, this substance is as good a precursor of liver glycogen as glucose. It also brings about a moderate increase in the blood glucose level. After adrenalectomy, the same proportion of the amount infused is present as blood glucose, or in other words, the capacity of the liver to form blood glucose from glycerol is not impaired by adrenalectomy. However, there is a marked fall in amount of liver glycogen deposited. The prior administration of hydrocortisone, although not influencing the amount of glucose formation, returns the proportion of glycogen formed to amounts comparable to those observed in intact rats.

Lactic acid. The disposition of infused lactic in normal and adrenalectomized rats is entirely similar to that observed when an excess of lactate is liberated into the circulation by the administration of epinephrine (cf. above). That is to say, in intact animals a high proportion is recovered as liver glycogen while a smaller portion of the amount infused is accounted for by the increase in free glucose. After adrenalectomy, the quantity of infused lactate converted to free glucose is slightly increased while the proportion accounted for as liver glycogen undergoes a most striking decline. The prior administration of hydrocortisone to adrenalectomized animals, while it did not entirely restore the liver glycogen deposition to that found in intact rats, nonetheless brought about a substantial and unmistakable increase.

Malic acid. Even in intact animals malate is not a very effective precursor of liver glycogen, since only 10% of the infused material can be accounted for as the polysaccaride. It does, however, under the conditions used in these experiments, cause a moderate increase in the blood glucose. After adrenalectomy, liver glycogen deposition is not detectable at all, while glucose formation is unimpaired. Administration of hydrocortisone restored the proportion of liver glycogen deposition to normal levels.

The reduction from normal in liver glycogen formation in adrenalectomized rats per unit of precursor infused is 59% for glucose; 55% for fructose; 80% for glycerol; 94% for lactate and 100% for malate. The capacity to form free glucose in the body fluids from glycerol, lactate or malate is unchanged while in the case of glucose a relative inability to dispose of this sugar appears to be present after adrenalectomy.

The urinary excretion of glucose, fructose and lactate was measured in all three groups of animals studied. As Table 7 shows the differences between intact and adrenalectomized animals could not be accounted for by a more rapid and extensive excretion of these compounds. In the case of lactate, the blood levels, before and at the end of the infusion, were measured. In intact animals the increase in blood lactate was 50 ± 8 mg. %; in adrenalectomized animals it was 48 ± 4 mg. %; and in adrenalectomized animals treated with hydrocortisone it was 57 ± 4 mg. %. Evidently there

is no difference in the ability of these various groups of animals to remove infused lactate from the blood. This observation is in agreement with that found in the case of the blood lactate clearance after epinephrine injection (cf. Figure 1.).

DISCUSSION

These studies indicate that the adrenal cortical hormones have a marked effect on the disposition of carbohydrate. The change in the pattern of metabolism of glucose, fructose, glycerol, lactate, and malate following adrenalectomy suggests that there is a particular pathway or pathways of metabolism that operate to greater than normal degree in the absence of the cortical hormones, and conversely are diminished when these hormones are present in excess.

The experiments reported here while they do not permit any precise localization of the site of action of these hormones do allow certain deductions of the general nature of their effect. In the first place the differences in the response to epinephrine injection are not due to any change in the glycogenolytic activity of this hormone on muscles; nor are they due to any gross failure of the liver to form glucose from the lactate reaching it from the muscles. Rather, the atypical "Cori cycle" in adrenalectomized rats is characterized by a failure to deposit any significant amount of the glucose formed from lactate either as muscle or liver glycogen, a failure that is restored by the administration of the 11-17 hydroxy adrenal steroids. Consequently, since neither lactate or glucose accumulate in the body fluids to an excess degree, it must follow that they have been utilized to a far greater extent than normal over other pathways of metabolism. Furthermore, the presence or absence of insulin does not alter the consequences of either adrenal cortical insufficiency or excess. It may, therefore, be concluded that although both insulin and adrenal cortical insufficiency have certain overall affects on carbohydrate metabolism, such as hypoglycemia, in common, the mechanisms operating to produce them are not interdependent nor necessarily related.

This atypical disposition of the lactate produced by the action of epinephrine is also seen when other materials known for their capacity to increase liver glycogen in normal rats are infused into animals deprived of their adrenals. In the case of the two sugars, glucose and fructose, the proportion recovered as liver glycogen undergoes a large decrease while the decline in liver glycogen formation from glycerol, lactate, and malate is even greater. In the case of all these compounds there is again no evidence that this difference is due to a failure to clear them from the blood at an adequate rate or to excessive urinary loss. Nor in the case of the last three is it due to a failure to form glucose, at least as judged by the normal increase in blood glucose after their infusion. Since a normal quantity of liver glycogen deposition occurs if the adrenalectomized rats are pretreated with hydro-

cortisone it must be concluded that adrenalectomy results in an excessive utilization of either carbohydrates or their precursors over some other pathway.

There is also evidence from these experiments to suggest that the liver is the organ upon which these effects of the adrenal cortical hormones are first exerted. This is indicated by the absence of any notable differences in the lactate metabolism of eviscerated and adrenalectomized eviscerated animals. While it might be argued that this would be anticipated if it was the glucose utilization of the muscles alone that was affected by adrenalectomy, it should be noted that Russell (31) was unable to observe any notable differences in the glucose tolerance of adrenalectomized eviscerated rats maintained on DCA when compared to normal eviscerated animals, although she found that adrenal cortical extract reduced the glucose tolerance of saline-maintained adrenalectomized and of hypophysectomized animals.

Wick and Drury (45) and Cohn *et al.* (4) reported no effect of adrenalectomy or of adrenal steroids on the glucose metabolism of eviscerated rabbits and dogs. Ingle *et al.* (18) found that adrenal cortical extract had a small, delayed effect on the glucose tolerance of eviscerated rats. Selye and Dosne (34) have reported that adrenal cortical extract does not alter the rate of fall of blood glucose in such animals. Furthermore Levine and his colleagues (22) found that the extreme insulin sensitivity of adrenalectomized rats was abolished after evisceration. There is also no indication that adrenalectomy alters the glucose uptake of the isolated rat diaphragm (4, 19, 21, 23, 38). It should be remembered that the surgical procedure is drastic and that important metabolic activity of the carcass may be suppressed to a minimum by evisceration. The lack of a consistent immediate or striking effect of adrenalectomy on the carbohydrate metabolism of the muscles is in sharp contrast to the prompt effect of these hormones on the carbohydrate metabolism of intact animals and on that of the perfused liver or liver slice.

The alternate pathways of metabolism, the extent of whose operation is subject to control by the adrenal cortical hormones is not known. It has been suggested by some and denied by others that lipogenesis in the liver is such a pathway. In the experiments reported in this paper, the change in the fatty acid content of the liver 4 hours after giving epinephrine to adrenalectomized rats was determined. No significant increase was found over that of untreated animals, yet in this period about 100 mg. of carbohydrate was unaccounted as glycogen or glucose.

The most obvious pathway by which the temporary excess of carbohydrate or its precursors might be utilized is by increased oxidation to CO_2. Winternitz and Kline (41) have reported that adrenalectomized rats do have a higher respiratory quotient than normal animals after epinephrine

injection. Russell (30) did not observe any higher RQ in glucose fed adrenalectomized animals although Thorn *et al.* (37) found such an increase in individuals with Addison's disease.

One other possibility is that these hormones control the proportion of glucose that is metabolized through the phospho-gluconate shunt. There is some indirect evidence that they have such an effect. Glock and McLean (14) found that the content of glucose 6-phosphate and 6-phospho-gluconate dehydrogenases of the liver was decreased in alloxan diabetic rats, a condition in which there is a relative or even an absolute increase in adrenal cortical activity. A hormonal effect exerted on this pathway of carbohydrate metabolism would have far reaching consequences on the overall metabolism of both carbohydrate and protein since not only would carbohydrate utilization be affected but what is more important the production of pentose phosphates. Since the latter are essential components of nucleotides and nucleic acids, widespread alterations in protein metabolism could be expected to follow variations in the level of cortical hormones in the body. Furthermore, such an effect might explain the well known relationship between the carbohydrate and protein metabolism that is such a characteristic feature of either a lack or excess of these hormones. There is already some evidence that the nucleotide and nucleic acid metabolism is influenced by cortical hormones. Glock and McLean (15) observed that the total diphosphopyridine nucleotide content of the liver was reduced in alloxan diabetic rats, while several papers have reported that excess of cortisone reduced the ribose nucleic acid content of liver and other tissues (1, 10, 17, 42).

It may well be that the association of a depression of carbohydrate utilization and negative nitrogen balance which is such a characteristic feature of excess of cortical hormones, as well as a consequence of injury of various kinds, has its origin in these alterations in nucleic acid and nucleotide metabolism; alterations that are in part at least determined by the changing levels of these hormones in the body.

SUMMARY

The subcutaneous injection of epinephrine into fasted adrenalectomized rats is followed by a decline in muscle glycogen almost twice as large as in intact animals. Furthermore, in contrast to intact animals there is practically no accumulation of liver glycogen. Injection of adrenal cortical hormones restores the normal pattern of response.

This difference in response is not due (a) to a failure to remove lactate from the blood or (b) to an inability to form glucose from lactate.

In consequence, in adrenalectomized rats there is a large portion of the muscle glycogen, mobilized by epinephrine, that is not accounted for as liver glycogen or increased glucose in the body fluids.

Eviscerated adrenalectomized rats have a normal response to epinephrine. Evidently, a changed pattern of metabolism in the liver is responsible for the effects observed.

The increased, and unaccounted for, disappearance of muscle glycogen is also found in adrenalectomized alloxan-diabetic rats.

The infusion of glucose, fructose, glycerol, lactate and malate into fasted adrenalectomized rats is followed by much less glycogen deposition than in intact animals. This is restored by prior treatment with hydrocortisone. The increase in blood glucose after glycerol, lactate or malate infusion is not changed by adrenalectomy.

It is suggested (a) that the liver is the site of the immediate effect of the adrenal cortical hormones on carbohydrate metabolism; (b) that these hormones may regulate both in the liver and other organs the extent to which glucose is utilized over alternate pathways of metabolism. Possible pathways that may be affected are discussed.

Acknowledgments

We are indebted to the Research Grants Committee of the Lilly Research Laboratories for their generous support of this research.

REFERENCES

1. Baker, B. L., D. J. Ingle, C. H. Li and H. M. Evans: *Amer. J. Anat.* **82**: 75. 1948.
2. Bendall, J. R. and H. Lehmann: *Biochem. J.* **36**: XIV. 1942.
3. Buell, M. V., I. A. Anderson and M. B. Strauss: *Amer. J. Physiol.* **116**: 274. 1936.
4. Cohn, C. and M. Kolinsky: *Metabolism* **2**: 146. 1953.
5. Collip, J. B., D. L. Thomson and G. Toby: *J. Physiol.* **88**: 191. 1936.
6. Corey, E. L. and S. W. Britton: *Amer. J. Physiol.* **131**: 783. 1940–41.
7. Cori, C. F. and G. T. Cori: *J. Biol. Chem.* **79**: 343. 1928.
8. Cori, G. T.: *Amer. J. Physiol.* **94**: 557. 1930.
9. Donaldson, H. H.: "The Rat." Memoirs of the Wistar Institute, Philadelphia, No. 6.
10. Dunn, C. E., A. D. Bass and H. McArdle: *Fed. Proc.* **15**: 418. 1956.
11. Evans, C. L., C. Tsai and F. G. Young: *J. Physiol.* **73**: 67. 1931.
12. Evans, G. T.: *Endocrinology* **29**: 731. 1941.
13. Gey, K. F.: *Biochem. J.* **64**: 145. 1956.
14. Glock, G. E. and P. McLean: *Biochem. J.* **61**: 390. 1955.
15. Glock, G. E. and P. McLean: *Biochem. J.* **61**: 397. 1955.
16. Holmes, E. G. and H. Lehmann: *Brit. J. Exper. Path.* **21**: 196. 1940.
17. Hull, N. and A. White: *Endocrinology* **51**: 210. 1952.
18. Ingle, D. J., M. C. Prestrud, J. E. Nezamis and M. H. Kuizenga: *Amer. J. Physiol.* **150**: 423. 1947.
19. Koepf, G. F., H. W. Horn, C. L. Gemmill and G. W. Thorn: *Amer. J. Physiol.* **133**: 353. 1941.
20. Koepf, G. F., H. W. Horn, C. L. Gemmill and G. W. Thorn: *Amer. J. Physiol.* **135**: 175. 1941.
21. Krahl, M. E. and C. F. Cori: *J. Biol. Chem.* **170**: 607. 1947.
22. Levine, R., B. Simkin and N. Cunningham: *Amer. J. Physiol.* **159**: 111. 1949.

23. Li, G. H., C. Kalman and H. M. Evans: *Arch. Biochem.* **22**: 357. 1949.
24. Lipsett, M. N. and F. J. Moore: *J. Biol. Chem.* **192**: 743. 1951.
25. Long, C. N. H. and R. Grant: *J. Biol. Chem.* **89**: 553. 1930.
26. Miller, A. M.: *Proc. Soc. Exper. Biol. & Med.* **72**: 635. 1949.
27. Minibeck, H. and F. Verzar: *Helv. Med. Acta* **5**: Suppl. 7. 1940.
28. Needham, D. M. and C. Y. Chiu; *Biochem. J.* **46**: 114. 1950.
29. Okada, M.: *Jikeiki Med. J.* **1**: 158. 1955.
30. Russell, J. A.: *Amer. J. Physiol.* **128**: 552. 1940.
31. Russell, J. A.: *Amer. J. Physiol.* **140**: 98. 1943.
32. Scow, R. O.: *Endocrinology* **60**: 359. 1957.
33. Seckel, H. P. G.: *Endocrinology* **26**: 97. 1940.
34. Selye, H. and C. Dosne: *Amer. J. Physiol.* **128**: 729. 1940.
35. Steele, R., J. S. Wall, R. C. DeBodo and N. Altszuler: *Amer. J. Physiol.* **187**: 15. 1956.
36. Sveinsson, S. L.: *Skand. Arch. Physiol.* **83**: 188. 1940.
37. Thorn, G. W., G. F. Koepf, R. A. Lewis and E. Olsen: *J. Clin. Invest.* **19**: 813. 1940.
38. Villee, C. A., V. K. White and A. B. Hastings: *J. Biol. Chem.* **195**: 287. 1952.
39. Winternitz, W. W. and C. N. H. Long: *Proc. Soc. Exper. Biol. & Med.* **81**: 683. 1952.
40. Winternitz, W. W. and W. E. Lattanzi: *Endocrinology* **58**: 232. 1956.
41. Winternitz, W. W. and D. L. Kline: *Fed. Proc.* **15**: 202. 1956.
42. Williams, W. L., C. U. Lowe and L. Thomas: *Anat. Rec.* **115**: 247. 1953.
43. Wang, F. C. *Nature*, London **165**: 277. 1950.
44. Wick, A. N., D. R. Drury and E. M. Mackay: *Amer. J. Physiol.* **163**: 224. 1950.
45. Wick, A. N. and D. R. Drury: *Ann. New York Acad. Sc.* **54**: 684. 1951.

166

which are probably inactivated by different types of enzymes, the theory of substrate competition will not explain the mechanism, unless the potentiating compound were capable of reacting with the various enzymes. This does not seem probable in view of the degree of specificity exhibited by enzyme systems. The effector cell may be sensitized by these potentiators. This raises a fundamental question with regard to the mechanism of action of the various sympathomimetic agents. A completely satisfactory answer has not yet been found. However, it does not seem unlikely that compounds containing long aliphatic chains might alter permeability by surface action and thereby increase the sensitivity of the cell to other compounds.

Summary. 1. A series of 19 N-substituted hexylamines have been studied for their effect on blood pressure and on potentiation of the pressor response of some sympathomimetic compounds. 2. N-methyl-hexylamine was found to have the greatest pressor action with di-hexylamine next most active in the aliphatic series. 3. N-(gamma-cyclohexylpropyl)-hexylamine has the greatest pressor action of those compounds containing a ring. In general, compounds containing a cyclohexyl ring were more active than those containing a phenyl ring or with only aliphatic groups. 4. Many of the compounds in this series have been shown to potentiate the pressor response of epinephrine, beta-phenylethylamine, N-methyl-beta-cyclohexylethylamine, ephedrine and some other sympathomimetic agents. The potentiation of epinephrine was more variable than that of the other sympathomimetic compounds. 5. The possible mechanisms of the potentiation phenomenon are discussed. The amine-oxidase theory of potentiation does not appear to apply to the compounds in this series. 6. Acute toxicity tests indicate that those compounds containing a cyclohexyl ring are more toxic than those with a phenyl ring. Increasing the length of the chain between the ring and the nitrogen increases the toxicity. Di-n-hexylamine is no more toxic than n-hexylamine.

The author is indebted to Mrs. Kathryn Z. Hooper for assistance in carrying out some of the experiments and to Miss V. Loraine Nash for the determination of acute toxicity.

15317

Protective Action of Certain Compounds Against Dietary Hepatic Damage in the Rat.*

JOHN GREEN AND A. BRUNSCHWIG.

From the Department of Surgery, University of Chicago.

In a previous communication[1] experimental studies were reported to show that the protective action of protein (casein) and methionine against chloroform hepatic damage in the dog could be accounted for by the presence of —SH since thioglycollic acid conferred similar protection to the above and glycollic acid, aspartic acid, glutamic acid and sodium thiosulphate did not afford protection. This conclusion was further strengthened by hitherto unpublished experiments in which thiomalic and thiolactic acids conferred protection but malic and lactic acids failed to afford protection. In a second report[2] it was shown that thioglycollic acid conferred protection in the rat against acute hepatic damage by carbontetrachloride but glycollic acid conferred almost an equal degree of protection.

* These studies were conducted under the Charles and Mary F. S. Worcester Fund. The University of Chicago.

[1] Brunschwig, A., *et al.*, *Arch. Path.*, 1945, **40**, 81.

[2] Brunschwig, A., Johnston, C., and Nichols, S., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 388.

TABLE I.
Summary of Experiments Upon Protective Action of Various Agents Against Parenchymal Necrosis in Liver and of Lipotropic Action.

Supplement to basic diet avg per animal		No. rats	Avg % wt lost	Hepatic cell necrosis series		Fatty infiltration of liver: Moderate* series		Fatty infiltration of liver: Severe† series	
				No.	%	No.	%	No.	%
Basal diet only		48	17.7	6	12.5	13	27	15	31.2
Choline	41.8 mg	20	8.6	0		0		0	
Choline / Cystine	20 / 32.3	20	3.2	0		5	25	0	
Methionine	40	18	2.3	0		5	27.8	1	5.5
Thioglycollic acid	24.6	25	30	1	4.0	3	12	10	40.
Glycollic acid	23.3	20	14	1	5.0	7	35	10	50.
Thiomalic acid	31.75	20	18.6	2	10.0	0		19	95.
Malic acid	35.8	19	13.0	2	10.5	5	26.3	9	47.5
Thiolactic acid	28.45	19	15.0	3	15.8	4	21.0	11	58.0
Total No. rats		209							

* ++ fatty infiltration (see text).
† +++ or ++++ fatty infiltration (see text).

Other —SH compounds were also tested but a close correlation between the presence of —SH and protection against hepatic damage was not here observed. Methionine in large doses did not afford protection. The following studies were conducted to determine if certain —SH and other compounds might afford protection against hepatic necrosis of dietary deficiency in the rat and also to observe if such compounds might possess lipotropic action.

Methods. White rats of inbred strain weighing at least 150 g were maintained upon the following diet for 70 days:

	%
Casein (vitamin free)	8
Crisco	38
Glucose	48
Cod liver oil	2
Salt mixture[3]	4

Vitamins added to afford each animal approximately the following daily intake: Thiamine hydrochloride, 75 μg; pantothenic acid, 100 μg; riboflavin, 40 μg; pyridoxine, 65 μg; niacinamid .5 mg. (These were supplied in vitamin B complex-oral, Lederle Lab. Inc.)

This diet has been shown[4] to result in varying degrees of fatty infiltration of the liver, cell necrosis and if maintained for sufficient periods, cirrhosis.

The animals were kept in groups of 8 to 10 in a cage. The daily individual intake of food was not measured but the animals were weighed at weekly intervals and the average of each cage recorded. This served as the indicator of food ingested.

Supplements consisting of the various agents to be tested for protective action on the liver were added to the several portions of the basic diet as indicated in Table I. The quantities of sulphur in the —SH compounds added were equivalent to the S in 40 mg of methionine.

In a few instances the animals died before the expiration of the experiment, the remainder were killed at the termination of the period stated and the livers were fixed in 5% formaldehyde embedded in paraffin and sections stained with hematoxylin and eosin, and in some instances by Mallory's connective tissue stain. Frozen sections for staining with scarlet red were also made in a number of instances.

The features noted were: degree of necrosis (+ to ++++), degree of fatty infiltration (a few scattered vacuolated cells, +; larger groups of vacuolated cells, ++; wide zones of vacuolated cells, +++; practically every cell vacuolated, ++++. The fact that

[3] Hubbell, R. B., *et al.*, *Am. J. Nutrition*, 1937, **14**, 273.

[4] Gyorgy, P., and Goldblatt, H., *J. Exp. Med.*, 1942, **75**, 355.

these vacuoles represented fat was controlled by appropriate stains since in marked protein depletion the cytoplasm of hepatic cells often appears vacuolated but does not contain fat). The presence of cirrhosis was also sought for but this was not frequently encountered due in all probability to the duration of the experiments. It was observed only in 3 instances, one in each of the groups receiving glycollic, thiolactic and thiomalic acids respectively.

The results are summarized in Table I.

Discussion. From the table it is noted that cellular necrosis occurred in 12.5% of the control series and moderate to severe fatty infiltration was present in 58%. Choline afforded absolute protection against necrosis and fatty infiltration. Choline in reduced quantity plus cystine protected against necrosis and also exhibited lipotropic action but not as well as larger doses of choline alone. Methionine exhibited approximately the same effects as choline plus cystine. Thioglycollic and glycollic acids afforded appreciable but not absolute protection against necrosis and *did not exhibit lipotropic action.* Indeed glycollic acid afforded a higher incidence of fatty infiltration than in the control series. Thiomalic, malic and thiolactic acids afforded no protection against necrosis and resulted in a higher incidence of fatty infiltration than in the control series.

The results with thioglycollic and glycollic acids are of interest since by their use it would appear that the lipotropic factor and necrosis protecting factor are shown not to be necessarily identical. For further support of this is the fact that methionine, which has been repeatedly shown to be lipotropic, does not protect against the acute hepatic cell damage caused by carbontetrachloride in rats.

The explanation of the protective action of thioglycollic and glycollic acids against acute hepatic cell damage by carbontetrachloride and by dietary deficiency is not apparent.

Summary. Both thioglycollic and glycollic acids afford a degree of protection against hepatic cell necrosis due to dietary deficiency in rats. These compounds are not lipotropic. The above studies indicate that in certain dietary deficiencies in rats leading to hepatic fatty infiltration and parenchymal necrosis, the factors responsible for both these changes are not necessarily identical.

15318

Experiments with Protoporphyrin.

I. ZELIGMAN. (Introduced by J. G. Hopkins.)

From the Departments of Dermatology and Biochemistry, Columbia University, College of Physicians and Surgeons, and from the Skin and Cancer Unit, New York Post-Graduate Medical School and Hospital, Columbia University.

It has recently been shown[1] that the chromogen of hemoglobin is synthesized *in vivo* from acetate and glycine, but little is known with certainty of the metabolic origin of the porphyrins of the excreta. It has been variously suggested that these are formed (a) during the breakdown of endogenous hemoglobin to bile pigments,[2] (b) by conversion of ingested chlorophyll and hemoglobin[3] and (c) by processes involved in the synthetic production of hemoglobin.[4]

[1] a. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, 1945, **159**, 45; b. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, 1945, **159**, 567.

[2] a. Schreus, H. T., and Carrié, C., *Klin. Wchnschr.*, 1933, **12**, 745; b. Herold, L., *Arch. f. Gynäk.*, 1934, **158**, 213.

[3] a. Snapper, J., *Arch. f. Verdauungskr.*, 1919, **25**, 230; b. Schumm, O., *Z. f. physiol. Chem.*, 1926, **153**, 225; c. Kämmerer, H., *Dtsches. Arch. f. klin. Med.*, 1924, **145**, 257; d. Vannotti, A., *Porphyrine und Porphyrinkrankheiten*, Berlin, J. Springer, 1937.

ay have been a specific effect of the pres-
ly well accounted for as a consequence of
more acidic than the pH of the tissues.
4 hours of culture at pH 5, the concentra-
ues rose to approximately 0.14 M, and the
me interference with the metabolic reac-
oduct may therefore occur after the con-
s sufficiently high.
uggests that malonic acid is to some ex-
ent in tobacco leaves, but the exact role

LIOGRAPHY

Ges., 14, 1183 (1881).
., J. Am. Chem. Soc., 47, 2044 (1925).
., J. Am. Chem. Soc., 53, 1040 (1931).
952).
R., Biochem. J., 22, 689 (1928).
(1936).
J. Biol. Chem., 133, 183 (1940).
., 2 937).
1937).
Burris, R. H., J. Biol. Chem., 178, 977 (1949).
rch. Biochem., 10, 497 (1946).
ophys., 47, 314 (1953).
(1952).
(1948).
(1949).
urner, J. S., Australian J. Scient. Res., Series

S., and Bliss, C. I., Plant Physiol., 24, 335

onnecticut Agr. Exp. Sta., Bull. 569 (1953).
. Sta., Bull. 589 (1955).
nal. Chem., 26, 208 (1954).
m. Soc., 2153 (1931).
Biol. Chem., 221, 1019 (1956).
5).
. Biol. Chem., 221, 79 (1956).
. Biol. Chem., 218, 225 (1956).
323 (1955).
A., II, J. Biol. Chem., 197, 121 (1952).
83 (1955).
4 (1949).

254

THE ORGANIC ACIDS OF BONE

By HELEN LEES* and ADRIAN C. KUYPER

(From the Department of Physiological Chemistry, Wayne State University College of Medicine, Detroit, Michigan)

(Received for publication, October 4, 1956)

Dickens (1), Thunberg (2), and others have reported that bone contains a large amount of citrate, usually about 0.7 per cent. This high concentration may be due to coprecipitation of citrate with bone salt during the process of calcification, a process which occurs *in vitro* (3). It may also be due to increased formation of citrate, the result of a greater activity of citrate-forming than of citrate-removing enzymes in bone (4, 5). It seemed of interest to determine whether the acids associated with citric acid in other tissues are also present in high concentration in bone, the extent to which they may be coprecipitated with bone salt, and what part they play in the oxidative metabolism of bone.

EXPERIMENTAL

Determination of Organic Acids in Bone

Preparation of Bone Samples—Dogs and rabbits were killed with Nembutal, and either the femora or humeri were removed as quickly as possible, sawed into slices, and scraped free from marrow. The cancellous bone was separated from the compact bone, and approximately 15 gm. of each type were covered with ether and allowed to stand for 2 days to remove most of the fat. The ether was poured off, the bone was dissolved in 5 ml. of 4 N HCl per gm. of bone at room temperature, and the solution was allowed to stand for at least a week to permit *cis*-aconitic acid to change to *trans*-aconitic acid. This conversion, previously studied by Krebs and Eggleston (6), was confirmed by experiments in which the amounts of the two acids in a solution were determined by analysis of the pH-titration curve. At equilibrium about 90 per cent of the acid is present as the trans isomer. Aconitic acid was found to be heat-labile when refluxed in ether and is thus decomposed during continuous extraction. The cis form has an unfavorable distribution coefficient and is lost during the necessary continuous extraction, whereas the trans isomer has a favorable distribution coefficient and may be quantitatively recovered by pouring ether through

* Taken from a dissertation presented to the Graduate School of Wayne State University by Helen Lees in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Supported in part by Research Grant A-613 from the National Institutes of Health.

the solution (see below). Conversion of *cis*-aconitic acid to the trans isomer makes the quantitative determination of this acid possible.

Extraction of Acids—The solution of bone was filtered, and a 25 ml. aliquot was transferred to a Kutscher-Steudel liquid-liquid extractor in which the outside diameter of the inner tube was about 7 mm., the inside diameter of the outer tube about 10 mm., and the total height of the extractor about 7 feet. Two extracts were obtained in the following manner.

Extract A—Ether distilled from anhydrous potassium carbonate was allowed to drip into the inside tube of the extractor from a separatory funnel and then to pass upward through the aqueous phase and into a flask connected to the side arm until 200 ml. of ether were collected. This extract contained almost all of the fumaric and aconitic acids, which are decomposed on continuous extraction, and portions of the other acids (Table I).

TABLE I

Recoveries of Organic Acids by Extraction and Chromatography

Acid	Recovery in Extract A	Recovery by chromatography alone		Recovery by extraction (Extracts A and B) and chromatography	
		Fraction Nos.	Mean	Mean	Range*
	per cent		*per cent*	*per cent*	*per cent*
Acetic		14–18	99	93†	79–107
Fumaric	99	24–29	98	99	97–102
Lactic	34	41–46	98	89†	85–95
Succinic	65	44–49	99	98	95–102
α-Ketoglutaric	56	49–54	94	83	81–85
trans-Aconitic	93	59–65	97	92	89–95
cis-Aconitic	39	80–87	100	92‡	
L-Malic	14	91–102	96	98	98–99
Citric	5	111–128	96	98	95–100

* Range of from three to six determinations.

† Includes recovery of "volatile" fractions. See the text.

‡ The *cis*-aconitic acid was allowed to stand for a week in 3 N HCl before extraction to allow its conversion to *trans*-aconitic acid. 91 per cent was recovered as *trans*-aconitic acid and 1 per cent as *cis*-aconitic acid.

Extract B—A 100 ml. flask containing about 50 ml. of ether was connected to the side arm, a condenser was connected, and continuous extraction was carried out for 40 hours. This completed the extraction of the acids.

Column Chromatography—The procedure used was similar to that of Marvel and Rands (7). Because of the presence in Extract B of small amounts of substances which interfered with the determination of fumaric

nversion of *cis*-aconitic acid to the trans iso-
etermination of this acid possible.
ution of bone was filtered, and a 25 ml. ali-
cher-Steudel liquid-liquid extractor in which
er tube was about 7 mm., the inside diame-
mm., and the total height of the extractor
are obtained in the following manner.
from anhydrous potassium carbonate was
tube of the extractor from a separatory fun-

TABLE I

ids by Extraction and Chromatography

	Recovery by chromatography alone		Recovery by extraction (Extracts A and B) and chromatography	
	Fraction Nos.	Mean	Mean	Range*
		per cent	*per cent*	*per cent*
	14–18	99	93†	79–107
	24–29	98	99	97–102
	[illegible]–46	98	89†	85–95
	[illegible]–49	99	98	95–102
	49–54	94	83	81–85
	59–65	97	92	89–95
	80–87	100	92‡	
	91–102	96	98	98–99
	111–128	96	98	95–100

erminations.
fractions. See the text.
ed to stand for a week in 3 N HCl before extrac-
s-aconitic acid. 91 per cent was recovered as
cis-aconitic acid.

rough the aqueous phase and into a flask
l 200 ml. of ether were collected. This
the fumaric and aconitic acids, which are
raction, and portions of the other acids

ontaining about 50 ml. of ether was con-
enser was connected, and continuous ex-
hours. This completed the extraction of

procedure used was similar to that of
se of the presence in Extract B of small
erfered with the determination of fumaric

and aconitic acids, Extracts A and B were chromatographed separately. Each column was prepared by mixing 20 gm. of dry silicic acid (Mallinckrodt analytical reagent, 100 mesh) with 10 ml. of 0.05 N H_2SO_4. The mixture was suspended in 100 ml. of chloroform, poured into an 18 × 485 mm. chromatography tube and packed to a final height of about 160 mm. The ether was evaporated from the extracts of bone by a stream of dry, carbon dioxide-free air, and the residue was transferred to the column in 1 ml. of 40 per cent butanol in chloroform, the flask being washed successively with 1 ml. of 10 per cent butanol-chloroform and four 1 ml. portions of chloroform. The columns were developed by forcing through them, under 12 pounds pressure of nitrogen gas, successive 100 ml. portions of 0, 5, 7, 10, 12, 15, 20, 25, and 30 per cent *n*-butanol in chloroform and finally 500 ml. of 35 per cent butanol in chloroform. 10 ml. fractions were collected, and to each were added 1 ml. of water and a drop of phenol red. These were titrated with 0.005 N NaOH, the values being corrected for a blank which varied directly with the amount of butanol in the solutions from about 0.01 to 0.03 ml.

Because the lactate and succinate fractions are not completely separated from each other, they were rechromatographed. After being titrated, the aqueous layers of these fractions were pooled and evaporated to dryness under a fan. The salts were dissolved in 0.5 ml. of 0.1 N H_2SO_4 and mixed with 1 gm. of silicic acid, suspended in chloroform, and poured on a silica gel column. The acids were eluted with 700 ml. of 7 per cent *tert*-amyl alcohol in chloroform, followed by 100 ml. of 15 per cent *n*-butanol in chloroform. 10 ml. fractions were collected. Lactic acid was eluted into Fractions 41 to 54 and succinic acid into Fractions 56 to 70.

Recoveries of Mixtures of Known Acids—All of the acids were satisfactorily recovered by simple column chromatography (Table I). In the procedure of extraction plus chromatography, fumaric, succinic, malic, and citric acids were quantitatively recovered. The low recovery of aconitic acid (92 per cent) is probably due to the formation of a small amount of the cis isomer and its decomposition during extraction. About half of the α-ketoglutaric acid is subjected to continuous extraction with ether. Decomposition during this extraction may be related to the double bond in the enol form of the acid. The variable recoveries of acetic and lactic acids include portions of these acids volatilized on evaporation of the ether and trapped in dilute alkali. Analyses for these acids in bone are not recorded because the amounts found were highly variable, probably owing to their origin in uncontrolled reactions.

Paper Chromatography—Identification of the acids of bone collected by column chromatography was confirmed by paper chromatography with two different solvents, *n*-butanol-formic acid-water in a ratio of 10:2:15 (8)

and absolute ethanol-NH_4OH (concentrated)-water in a ratio of 80:5:15 (9). Known acids were run simultaneously with the acids from bone. The keto acids of bone were identified by paper chromatography of their 2,4-dinitrophenylhydrazones prepared from a hydrochloric acid solution of bone (10), with *n*-butanol-ethanol-0.5 N NH_4OH in a ratio of 70:10:20 (11). A disadvantage of this method is the appearance of two spots due to the syn- and antiisomers of the hydrazones (12).

Coprecipitation of Organic Acids with Bone Salts in Vitro

Sufficient KH_2PO_4, $CaCl_2$, $MgCl_2$, $NaHCO_3$, and NaCl were dissolved in volumes of 2 liters to give solutions containing 25 mg. per cent of P, 54 mg. per cent of Ca, 4 mg. per cent of Mg, 360 mg. per cent of $NaHCO_3$, and 0.1 M NaCl. In order to avoid immediate precipitation during the preparation of these solutions, they were saturated with carbon dioxide before the addition of the bicarbonate. Various concentrations of organic acid (Table III) were added, and the solutions were warmed to 38° and aerated with 6 per cent carbon dioxide-air mixture. Precipitation soon began and was allowed to continue at 38° with continuous aeration and occasional manual shaking for about 7 hours. The supernatant solutions then had an ionic strength of 0.16 and contained about 1.5 mg. of P, 9 mg. of Ca, and 210 mg. of $NaHCO_3$ per 100 ml., approximately the composition of normal blood serum. The precipitates were removed by filtration through a Büchner funnel, weighed, and dissolved in 20 ml. of 4 N HCl. Solutions containing *cis*-aconitic acid were allowed to stand for at least a week before analysis to allow conversion to *trans*-aconitic acid. The organic acids were determined as already described. Corrections were made for the organic acids present in the supernatant solutions held by the wet precipitates.

Incorporation of Radioacetate

Dogs were killed with Nembutal, and their femora were removed as quickly as possible and chilled. The compact bone of the shaft was freed from all other tissue and sawed with a four-bladed hacksaw to a coarse powder. 5 gm. of the powdered bone were suspended in 18 ml. of Krebs-Ringer phosphate buffer, pH 7.4, containing 0.5 mg. of diphosphopyridine nucleotide, 18.3 mg. of nicotinamide, 0.41 mg. of adenosine triphosphate, 0.1 mg. of coenzyme A, and 0.5 mg. of sodium acetate-1-C^{14} equivalent to 25 μc. of radioactivity. When used as controls, bone samples were autoclaved for 20 minutes at 15 pounds pressure before the addition of the coenzymes and radioactive acetate. Bone suspensions were incubated for 5 hours at 37° with gentle shaking, and then 6 ml. of concentrated HCl were added to the flasks. After a week, the organic acids were determined

H (concentrated)-water in a ratio of 80:5:15 multaneously with the acids from bone. The ified by paper chromatography of their 2,4- pared from a hydrochloric acid solution of anol-0.5 N NH_4OH in a ratio of 70:10:20 method is the appearance of two spots due the hydrazones (12).

anic Acids with Bone Salts in Vitro

$MgCl_2$, $NaHCO_3$, and NaCl were dissolved lutions containing 25 mg. per cent of P, 54 cent of Mg, 360 mg. per cent of $NaHCO_3$, avoid immediate precipitation during the they were saturated with carbon dioxide bonate. Various concentrations of organic nd the solutions were warmed to 38° and dioxide-air mixture. Precipitation soon inue at 38° with continuous aeration and bout 7 hours. The supernatant solutions 6 and contained about 1.5 mg. of P, 9 mg. per 100 ml., approximately the composi- pitates were removed by filtration ned, and dissolved in 20 ml. of 4 N HCl. acid were allowed to stand for at least a nversion to *trans*-aconitic acid. The or- ready described. Corrections were made he supernatant solutions held by the wet

ion of Radioacetate

tal, and their femora were removed as The compact bone of the shaft was freed with a four-bladed hacksaw to a coarse one were suspended in 18 ml. of Krebs- containing 0.5 mg. of diphosphopyridine de, 0.41 mg. of adenosine triphosphate, g. of sodium acetate-1-C^{14} equivalent to ed as controls, bone samples were auto- ds pressure before the addition of the e. Bone suspensions were incubated ng, and then 6 ml. of concentrated HCl week, the organic acids were determined

as already described. Radioactivity of the various fractions obtained on column chromatography was determined by drying the aqueous layers from each tube on planchets and then counting in a gas flow counter. Radioactivity not identified with the organic acids was found in many of the fractions. This radioactivity was separated from the fumaric and aconitic acids by paper chromatography with a butanol-formic acid-water solvent. Each spot was cut out, eluted with water, dried on a planchet, and counted. The radioactivity of the malic, lactic, and citric acid fractions, as measured after column chromatography, was corrected for small amounts of radioactivity found in the controls. Radioactivity was further identified with these acids by counting the spots obtained on paper chromatography.

In order to eliminate the possibility of bacterial action, 1.2 mg. of penicillin and 2 mg. of streptomycin were added to the digestion medium in one of the experiments. Small amounts of the suspension obtained after incubation were streaked on blood agar and tryptose agar plates. Both cultures were negative.

Results

Quantitative Analysis—The amounts of organic acids present in bone, egg shells, and blood (Table II) were determined by column chromatography and confirmed by paper chromatography in both alkaline and acidic solvents. About 0.2 mg. per cent of fumaric acid, an amount which cannot be determined accurately by column chromatography, is present in fresh bone. This acid and α-ketoglutaric acid are the only acids present in bone in smaller concentrations than those found in liver. Succinic acid is present in about the same concentration, and aconitic, malic, and citric acids are present in concentrations about 30, 3.5, and 230 times those found in liver. Liver has a greater concentration of these acids than muscle (13). In all of the samples studied, citric and aconitic acids were present in higher concentrations in compact than in cancellous bone. In the few samples of each species studied, the bones of dogs contained more citric acid than those of the rabbits. Only malic and citric acids were found in egg shells. Pyruvic acid, previously reported by Cartier (14), and a trace of α-ketoglutaric acid were found to be present in bone by paper chromatography of the 2,4-dinitrophenylhydrazones of these acids. Oxalacetate was not detected by this method.

Coprecipitation of Organic Acids with Bone Salt in Vitro—The amounts of organic acids coprecipitated with bone salt were measured with the acids present initially in concentrations of 2.0, 0.2, and 0.1 mg. per cent (Table III). At the relatively high initial concentrations of 2 mg. per cent, about 60 per cent of the citrate and 30 per cent of the aconitate were

precipitated. When the initial concentrations of the acids were low, the amounts coprecipitated were small, and their concentrations in the supernatant solution may be considered to be constant throughout the precipitation. Little or no fumaric acid was coprecipitated at the levels studied. A small amount of succinic acid was precipitated from a concentration of 2 mg. per cent, but at 0.2 mg. per cent the amount was negligible. Aconitic and malic acids were precipitated from initial concentrations of 0.1 mg. per cent and would probably be precipitated from still lower concentrations.

TABLE II

Organic Acids in Beef, Dog, and Rabbit Bones, Egg Shells, and Rat Liver

The values are given in mg. per 100 gm.

Tissue	Type of bone	Organic acid in fresh tissue*				
		Fumaric	Succinic	Aconitic	Malic	Citric
Bone, beef	Cancellous	0.2	1.8	2.7	5.2	475
" Dog A	Compact	0.2	2.7	3.6	6.6	852
	Cancellous	0.2	3.0	3.3	3.9	609
" " B	Compact	0.1	1.7	3.8	5.0	966
	Cancellous	0.2		1.6	4.9	553
" Rabbit A	Compact	0.2	3.6	2.6	6.3	565
	Cancellous	0.2	3.1	2.2	5.3	394
" " B	Compact	0.2	1.3	3.6	4.6	627
	Cancellous	0.2	2.6	2.0	8.0	509
Egg shell A		0.0	0.0	0.0	2.9	66
" " B		0.0	0.0	0.0	0.8	11
Rat liver		0.5	2.5	0.1	1.6	3.3

* Means of duplicate determinations, the values of which checked closely.

TABLE III

Coprecipitation of Organic Acids with Bone Salts in Vitro

Experiment No.	Organic acid present in	Acid				
		Fumaric	Succinic	Aconitic	Malic	Citric
1	Supernatant, *mg. per 100 ml.*	2	2	2	2	2
	Ppt., *mg. per gm. Ca*	0	0.5	12.9	4.4	26.7
2	Supernatant, *mg. per 100 ml.*	2	2	2	2	0
	Ppt., *mg. per gm. Ca*	0	0.7	16.0	5.2	0
3	Supernatant, *mg. per 100 ml.*	0.2	0.2	0.2	0.2	2
	Ppt., *mg. per gm. Ca*	0	0	0.9	0.3	26.0
4	Supernatant, *mg. per 100 ml.*	0.1	0.1	0.1	0.1	2
	Ppt., *mg. per gm. Ca*	0	0	0.2	0.1	23.5

al concentrations of the acids were low, the small, and their concentrations in the super- ered to be constant throughout the precipi-

TABLE II

and Rabbit Bones, Egg Shells, and Rat Liver r 100 gm.

of bone	Organic acid in fresh tissue*				
	Fumaric	Succinic	Aconitic	Malic	Citric
cellous	0.2	1.8	2.7	5.2	475
pact	0.2	2.7	3.6	6.6	852
cellous	0.2	3.0	3.3	3.9	609
pact	0.1	1.7	3.8	5.0	966
ellous	0.2		1.6	4.9	553
pact	0.2	3.6	2.6	6.3	565
ellous	0.2	3.1	2.2	5.3	394
pact	0.2	1.3	3.6	4.6	627
ellous	0.2	2.6	2.0	8.0	509
	0.0	0.0	0.0	2.0	66
	0.0	0.0	0.0	0.8	11
	0.5	2.5	0.1	1.6	3.3

ions, the values of which checked closely.

TABLE III

nic Acids with Bone Salts in Vitro

	Acid				
	Fumaric	Succinic	Aconitic	Malic	Citric
ml.	2	2	2	2	2
	0	0.5	12.9	4.4	26.7
ml.	2	2	2	2	0
	0	0.7	16.0	5.2	0
ml.	0.2	0.2	0.2	0.2	2
	0	0	0.9	0.3	26.0
ml.	0.1	0.1	0.1	0.1	2
	0	0	0.2	0.1	23.5

was coprecipitated at the levels studied. was precipitated from a concentration of er cent the amount was negligible. Aco- pitated from initial concentrations of 0.1 be precipitated from still lower concen-

Incorporation of Radioacetate—When finely divided bone was incubated with radioactive acetate, radioactivity was incorporated into all the acids studied (Table IV). Small amounts of radioactivity were taken up by acids present in bone in small concentrations (less than 0.02 mmole per 100 gm.), but large amounts were taken up by the acids present in relatively high concentrations. The specific activities of aconitic and citric acids were about equal and were much lower than the values for the other acids. Succinate is not listed in Table IV because a small amount of radioactive succinate present in the sodium acetate used for incubation interfered with measurements of radioactivity incorporated into this acid.

TABLE IV

Incorporation of Radioacetate into Organic Acids of Bone

Experiment No.	Acid	Concentration	Radioactivity in acid	
		mmoles per 100 gm. bone	*c.p.m. per 100 gm. bone*	*c.p.m. per mmole* $\times 10^{-3}$
1	Fumaric*	0.0015	880	590
	Aconitic	0.0188	440	23
	Malic	0.082	37,700	458
	Lactic	0.082	26,800	326
	Citric	4.09	84,800	21
2†	Fumaric	0.0007	1,800	2600
	Aconitic	0.0104	360	35
	Malic	0.0216	40,200	1860
	Lactic	0.104	22,100	213
	Citric	3.95	97,900	25

* Data for this acid are approximate because of its low concentration.

† 2 mg. of streptomycin and 1.2 mg. of penicillin were present in the incubation mixture.

DISCUSSION

Six of the acids of the tricarboxylic acid cycle, citric, aconitic, malic, succinic, fumaric, and α-ketoglutaric acids, have been identified in compact bone. Of these, only α-ketoglutaric and fumaric acids are found at a lower concentration in bone than in liver, a representative soft tissue. Succinic acid is present in bone at about the same concentration and the other acids at significantly greater concentrations than in liver.

The organic acids appear to be present in bone in two different forms: a metabolically active, soluble form which corresponds to that of soft tissue and an inactive, insoluble form which is incorporated into the bone salt. Fumaric and α-ketoglutaric acids appear to be present primarily as metabolic acids. They occur at a lower concentration in bone than in liver, and fumaric acid is not incorporated *in vitro* into bone salts. Furthermore,

when bone is incubated with radioactive acetate, the fumaric acid of the bone reaches a relatively high specific activity. The other acids occur in bone in both forms. They are coprecipitated *in vitro* in varying degrees with bone salt, and, when bone is incubated with radioacetate, the specific activities of those acids which have been studied vary inversely with the extent of coprecipitation. The low specific activities of aconitic and citric acids, as compared with the other acids, are due to dilution of the activity of the active portions of these acids by relatively large amounts of the inactive acids.

The data in Table IV are sufficiently complete to allow an estimation of the concentration of organic acids in the supernatant solutions required to give a precipitate containing the same concentration of acid as bone. When expressed in terms of mg. of acid present per gm. of calcium in bone, the concentrations of succinic, aconitic, malic, and citric acids in bone (Table II) are approximately 0.1, 0.12, 0.24, and 30 mg., respectively. Under the conditions of the experiments performed *in vitro* (Table III), these concentrations are reached when the initial concentrations of the acids are about 0.7, 0.06, 0.2, and 2.5 mg. per cent, respectively. These concentrations are similar to the concentrations of the same acids in rat liver and are within the rather wide range of values reported for soft tissues by other workers (13).

The experiments indicate that the members of the tricarboxylic acid cycle are metabolically active in bone. With the exception of isocitric acid and the unstable oxalacetic and oxalosuccinic acids, all of the acids of the cycle have been found in bone. Radioactivity is incorporated into fumaric, aconitic, malic, and citric acids on incubation of intact bone with radioactive acetate. Cartier (14) pointed out that bone has a histological structure favorable to aerobic oxidation. Dixon and Perkins (4) demonstrated isocitric acid dehydrogenase activity in cancellous bone. Their failure to find it in compact bone may have been due to the low aconitase activity, coupled with the use of citric instead of isocitric acid as a substrate. The incorporation of radioactivity is probably a more sensitive method, but it may be less specific than enzyme studies (4, 5) for demonstrating the activity of the oxidative cycle.

SUMMARY

Citric, malic, aconitic, succinic, and fumaric acids have been shown to be present in compact bone in concentrations of about 750, 5.5, 3, 2.5, and 0.2 mg. per cent, respectively. These amounts correspond to about 230, 3.5, 30, 1, and 0.4 times the concentrations of these acids present in liver. Lactic, pyruvic, and a trace of α-ketoglutaric acids are also present in bone.

Citric, malic, aconitic, and succinic acids are coprecipitated *in vitro* with

radioactive acetate, the fumaric acid of the specific activity. The other acids occur in coprecipitated *in vitro* in varying degrees is incubated with radioacetate, the specific have been studied vary inversely with the low specific activities of aconitic and citric ner acids, are due to dilution of the activity acids by relatively large amounts of the in-

ficiently complete to allow an estimation of cids in the supernatant solutions required g the same concentration of acid as bone. of acid present per gm. of calcium in bone, aconitic, malic, and citric acids in bone 0.1, 0.12, 0.24, and 30 mg., respectively. periments performed *in vitro* (Table III), ed when the initial concentrations of the nd 2.5 mg. per cent, respectively. These e concentrations of the same acids in rat id ge of values reported for soft tissues

at the members of the tricarboxylic acid in bone. With the exception of isocitric c and oxalosuccinic acids, all of the acids bone. Radioactivity is incorporated into ric acids on incubation of intact bone with 4) pointed out that bone has a histological xidation. Dixon and Perkins (4) demon- nase activity in cancellous bone. Their e may have been due to the low aconitase of citric instead of isocitric acid as a sub- radioactivity is probably a more sensitive ific than enzyme studies (4, 5) for demon- tive cycle.

SUMMARY

c, and fumaric acids have been shown to oncentrations of about 750, 5.5, 3, 2.5, and These amounts correspond to about 230, centrations of these acids present in liver. ketoglutaric acids are also present in bone. cinic acids are coprecipitated *in vitro* with

bone salts from inorganic salt solutions. The amounts of acids in the supernatant solution required for the coprecipitation of the amounts of acids present in bone are within the ranges of values reported for these acids for various tissues.

When finely divided bone is incubated with carboxyl-labeled acetate, radioactivity is incorporated into the organic acids. The specific activity of these acids varies inversely with the extent to which the acids are coprecipitated with bone salt.

BIBLIOGRAPHY

1. Dickens, F., *Biochem. J.*, **35**, 1011 (1941).
2. Thunberg, T., *Acta physiol. Scand.*, **15**, 38 (1948).
3. Kuyper, A. C., *J. Biol. Chem.*, **123**, 405 (1938).
4. Dixon, T. F., and Perkins, H. R., *Biochem. J.*, **52**, 260 (1952).
5. Neuman, W. F., Firschein, H., Chen, P. S., Jr., Mulryan, B. J., and DiStefano, V., *J. Am. Chem. Soc.*, **78**, 3863 (1956).
6. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **38**, 426 (1944).
7. Marvel, C. S., and Rands, R. D., Jr., *J. Am. Chem. Soc.*, **72**, 2642 (1950).
8. Kalyankar, G. D., Krishnaswamy, P. R., and Sreenivasaya, M., *Current Sc.*, India, **21**, 220 (1952).
9. Cheftel, R. I., Munier, R., and Macheboeuf, M., *Bull. Soc. chim. biol.*, **34**, 380 (1952).
10. Seligson, D., and Shapiro, B., *Anal. Chem.*, **24**, 754 (1952).
11. El Hawary, M. F. S., and Thompson, R. H. S., *Biochem. J.*, **53**, 340 (1953).
12. Metzler, D. E., and Snell, E. E., *J. Biol. Chem.*, **198**, 353 (1952).
13. Frohman, C. E., Orten, J. M., and Smith, A. H., *J. Biol. Chem.*, **193**, 277 (1951).
14. Cartier, P., *Bull. Soc. chim. biol.*, **33**, 161 (1951).

C^{14}-labeled glucose metabolism by bone from normal and parathyroid-treated rats[1,2]

HARIPADA CHATTOPADHYAY[3] AND SMITH FREEMAN
Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois

CHATTOPADHYAY, HARIPADA, AND SMITH FREEMAN. *C^{14}-labeled glucose metabolism by bone from normal and parathyroid-treated rats.* Am. J. Physiol. 208(5): 1036–1041. 1965.—Calvaria and the metaphyseal and epiphyseal sections of tibia and femora freed of bone marrow from young growing rats were incubated in vitro for 2 hr at 37 C in Krebs-Ringer bicarbonate buffer containing 2 mM glucose. The glucose was labeled either in the 1 position or in the 6 position or uniformly in all positions with C^{14}. The incorporation of C^{14} into citrate, lactate, and various tricarboxylic acid cycle intermediates was studied. The release of $C^{14}O_2$ and the incorporation of C^{14} into bone matrix were also measured. Results obtained from bones of untreated control, parathyroid extract-treated, and parathyroidectomized animals were compared. It was found that treatment of animals with parathyroid extract increased the total accumulation of labeled citrate and malate as well as the incorporation of these acids into bone mineral. Bones from extract-treated animals also exhibited a significant decrease in the total accumulation of radioactive fumarate, succinate, and pyruvate. Lactate was the major end product of glucose metabolism, but its accumulation was only slightly influenced by parathyroid extract.

citrate in bone lactate production by bone matrix of bone mineral fraction of bone pentose cycle in bone Krebs cycle in bone

BONE CONTAINS A LARGE AMOUNT of citrate and it has been suggested that there exists in bone a mechanism for accumulating this ion (15, 21). The relation between citrate, calcium, and parathyroid function is not clearly understood. There is considerable disagreement in recent findings concerning the effect of parathyroid hormone on citrate and lactate formation by bone. Firschein et al. (19) and Martin et al. (24) found increased levels of citrate in venous blood collected from bone after parathyroid extract had been administered to dogs. Borle et al. (5) found that lactate production by mice metaphyses was markedly increased after parathyroid extract administration but citrate production was not significantly increased. Raisz et al. (29), working with slices of young rat skull bones in vitro, did not find any difference in the citrate or lactate production in the buffer medium after incubation for 8 hr of bone from normal, parathyroidectomized, or parathyroid extract-treated animals. Firschein (20) has recently reviewed much of the literature concerned with the effect of parathyroid hormone on carbohydrate metabolism by bone. The citrate content and the citrate/calcium ratio of bone vary with the species, age, sex, and site from which the bone sample is taken. In general, this ratio is higher in the metabolically more active parts of the skeleton (22). Parathyroid extract (13), vitamin D (9, 32), and estrogen (30, 31) have been shown to influence the citrate content of bone. Though it has been held by some (1) that citrate in bone is of adventitious origin, this view does not satisfactorily explain how citrate is increased or decreased in bone by the different agents mentioned since the change sometimes occurs without a concomitant increase or decrease of bone calcium (26). It seems highly probable that the origin, circulation, and metabolism of citrate has some relation to calcium metabolism. It is now well established that bone and cartilage contain most of the enzymes required for carbohydrate metabolism and citrate formation (2, 15, 16, 36).

Because of the conflicting findings reported in the literature concerning citrate and lactate production by bone, a detailed study of the metabolic behavior of bone slices in vitro was made using glucose labeled in different positions as substrates. An attempt was made to evaluate the relative activities of the Embden-Meyerhof, the Krebs cycle, and the pentose cycle pathways in various areas of metabolically active bone in normal, parathyroid extract-treated, and parathyroidectomized animals.

MATERIALS AND METHODS

Animal procedures. Male Sprague-Dawley rats fed Rockland rat and mouse laboratory diet were used. All ani-

Received for publication 22 May 1964.
[1] This work was supported, in part, by Research Grant AM 00666 from the National Institute of Arthritis and Metabolic Diseases and, in part, by funds provided by The Schweppe Foundation.
[2] A preliminary report has been published (10).
[3] Present address: Dept. of Physiology, Presidency College, Calcutta 12, India.

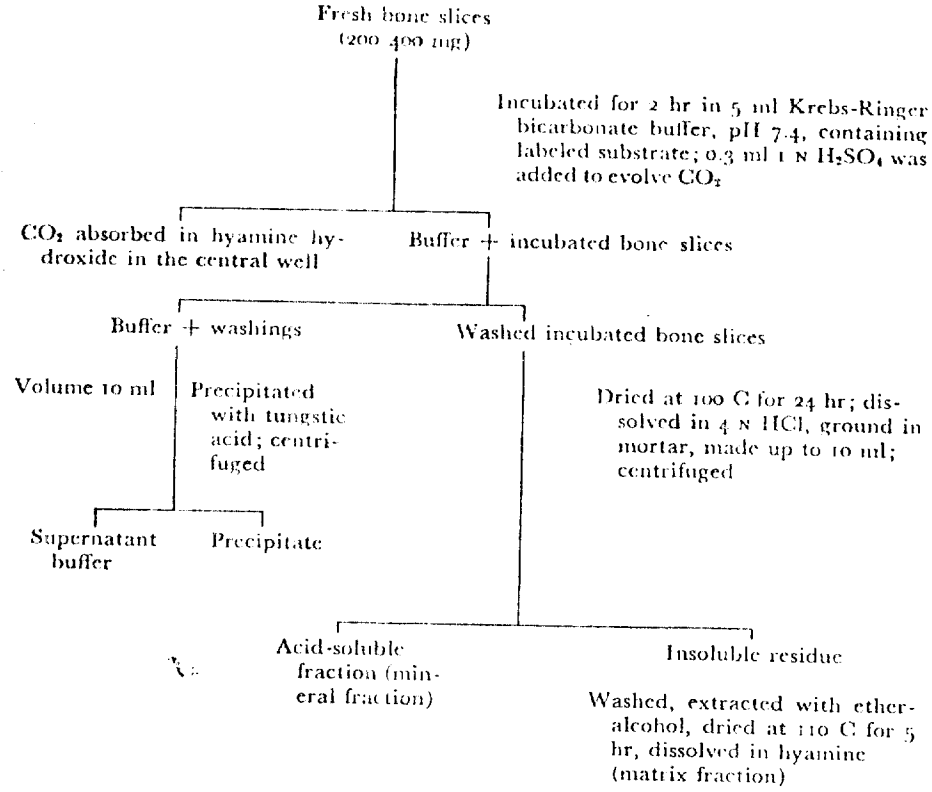


FIG. 1. General sequence of procedures used in separating bone fractions.

mals were fasted 12–14 hr prior to decapitation. Blood samples were drawn from the abdominal aorta for determination of plasma calcium, used as an index of the parathyroid state of the animal (see below).

Parathyroid extract (PTE, Eli Lilly) was administered. Rats weighing 120–150 g received 200 units subcutaneously for 2 days and 100 units intraperitoneally the 3rd day, 4 hr prior to sacrifice, for a total of 500 units. Control groups of equal size received corresponding injections of 0.85% NaCl.

Parathyroidectomy was performed by electric cautery under ether anesthesia on 120- to 150-g animals that were sacrificed 4 days later. Only those animals in which the plasma calcium dropped to 5–8 mg/100 ml were included in the data to be presented.

Preparation of bone specimens. Flat portions of the parietal bones were dissected, cleaned mechanically with gauze soaked in cold buffer solution, and cut into several small pieces. The epiphyses and the metaphyses of the distal end of the femur and the proximal end of the tibia were separated, cleaned as completely as possible of all connective tissue and cartilage, and the marrow of the metaphyses was removed with gauze soaked in buffer solution.[4] The bone was then cut into thin slices or fragments with a sharp scalpel or razor blade. The small pieces of calvaria, epiphyses, and metaphyses (mainly spongy trabecular bone with a little cortical bone) were stored from 1 to 3 hr in cold Krebs-Ringer bicarbonate buffer solution at 0–5 C (hereafter referred to as the buffer) until the actual start of incubation. In some experiments, especially in older parathyroidectomized animals, bone slices from individual animals were incubated separately, but in most of the experiments the slices from a particular group of animals were pooled and duplicate samples incubated. In general, each value reported was obtained by averaging the results of at least three experiments. In each experiment six or more animals were used in each of the control and treated groups. Significant differences between group means were determined by Student's *t* test (32).

Incubation of bone slices. The pooled slices were quickly dried on filter paper and weighed on a Roller-Smith balance. A 200- to 400-mg sample of bone fragments was placed in 3 ml Ca^{++}-free buffer solution at pH 7.4 kept in chilled 50-ml flasks fitted with a central well. Appropriate substrate was added and the flasks were equilibrated for 10 min with a mixture of 5% CO_2 and 95% O_2, then sealed with rubber caps and incubated at 37 C for 2 hr in a Dubnoff metabolic shaker moving at the rate of 100 oscillations/min. In initial experiments where radioactive precursors were not used, the incubated bone fragments were immediately separated and washed without the pretreatment with 1 N H_2SO_4, described below.

Preparation of radioactive materials for analysis. The general scheme employed in fractionation and analysis of bone slices incubated with glucose-C^{14} is shown in Fig. 1. After incubation the flasks were chilled in ice, 1.5 ml 1 M hyamine hydroxide (Packard Instrument Co.) was injected into the center well of the flask with a hypodermic needle inserted through the rubber cap, and 0.3 ml

[4] Epiphyseal and metaphyseal bone samples were separated from one another according to the location of the epiphyseal line.

1 N H_2SO_4 was injected into the buffer medium to stop the reactions and evolve CO_2. The final pH was 5.2. Using a greater amount of 1 N H_2SO_4 (2 ml) did not appreciably increase the amount of $C^{14}O_2$ recovered. The flasks were shaken for 1 hr to permit complete absorption of CO_2 into the hyamine hydroxide (28). The hyamine solution was quantitatively transferred with a syringe to the counting vial containing 10 ml of scintillator solution (details given under *Counting technique*). The central well was rinsed with two 2-ml portions of fresh toluene reagent, which were transferred to the vial.

The buffer in the flask was transferred quantitatively into a 15-ml centrifuge tube with the aid of two washings of 2 ml each of distilled water, precipitated with 5% tungstic acid, and centrifuged. The supernatant is termed the "buffer" fraction (Fig. 1) and may have contained intracellular components and acids weakly adsorbed on the bone mineral as well as components originally in the medium. The bone slices were rinsed repeatedly with cold distilled water until the washings were free of radioactivity (usually three washings of 20 ml of water each time were sufficient) and then dried in an oven at 110 C overnight. The washed, dried slices were weighed, dissolved in 3 ml 4 N HCl at room temperature for 2 hr, ground with a mortar and pestle, and made up to a volume of 10 ml with distilled water and centrifuged. The acid-soluble supernatant fluid has been termed the inorganic "mineral fraction" (Fig. 1). The precipitate, presumably consisting mostly of collagen and mucopolysaccharides, has been designated as the organic "matrix fraction." This fraction was twice resuspended in 10 ml 1 N HCl and centrifuged to free it from contaminating minerals.

Chemical methods. The incubated buffer was analyzed directly for citric acid (18) and lactic acid (3). After acidification of the buffer, the organic acids of the Krebs cycle were adsorbed on Celite and eluted with ether as described by Swim and Utter (33). These organic acids were subsequently separated by gradient elution chromatography on a Dowex-1-formate column by the method of Busch (8) as modified by Palmer (27). Pyruvic acid was separated as 2,4-dinitrophenylhydrazine derivatives from the citric acid fraction and was analyzed colorimetrically (4) in some experiments. The accuracy of all procedures was established by recovery of known compounds added to the buffer solution in the presence of unincubated bone and the probable error of these procedures is approximately 5%.

The acid-soluble portion of bone was analyzed for calcium by the Clark and Collip (11) modification of the method of Kramer and Tisdall (23), using Wang's wash (37). The citric and other organic acids were analyzed in the same way as described for the buffer. Calcium in the plasma was determined by the method of Diehl and Ellingboe (14).

Counting technique. Radioactivity was counted in a Tri-Carb liquid-scintillation spectrometer, model 314 X. For counting $C^{14}O_2$ in hyamine, toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) was used as the scintillator solution (28). The counting efficiency was about 65%. The matrix fraction was washed in cold water repeatedly until the washings were free from radioactivity, then washed in acetone and extracted with ethanol-ether (1:1) and finally dissolved in 3 ml 1.0 M hyamine hydroxide with the aid of heat at 60 C, prior to determination of its radioactivity. Sometimes the dried matrix was digested with a few drops of concentrated formic acid and after subsequent evaporation of this acid the matrix was dissolved in hyamine and its radioactivity determined. The efficiency of counting the matrix solution was approximately 55%. The total radioactivity in the mineral fraction was determined on 0.5 ml of the acid solution treated with 1 ml 1 M hyamine hydroxide and dissolved in 10 ml of a phosphor system containing xylene, dioxane, and Cellosolve (NDC) as described by Bruno and Christian (6). One-milliliter aliquots of the fractions eluted from the Dowex-1-formate column were also counted in 10 ml of this phosphor solution. This system can retain 16.1% water and has a counting efficiency of about 50% under the conditions of the present experiments.

Slight quenching due to formic acid and hyamine was routinely corrected by the count-rate ratio method (7). However, all radioactive counting data have been converted to counts per minute at 50% efficiency instead of absolute activity. Quenching in the counting of the matrix solution was further checked by internal standardization with standard toluene-C^{14} (Packard Instrument Co., Inc.). Uniformly labeled D-glucose-C^{14} was obtained from

TABLE 1. *Effect of parathyroid extract on conversion of glucose-C^{14} to carbon dioxide*

	$C^{14}O_2$, Counts/min in Thousands per g Fresh Bone		
	A Glucose-U-C^{14}	B Glucose-1-C^{14}	C Glucose-6-C^{14}
Calvaria			
Control	8.1±0.9	13.7±0.7	4.9±0.7
PTE	10.3±1.0*	15.8±0.9*	7.3±0.6†
Metaphyses			
Control	62.9±2.0	83.6±2.5	50.3±4.1
PTE	72.9±5.7*	96.2±2.9†	66.2±4.5†
Epiphyses			
Control	93.6±4.2	132.1±4.5	72.8±3.5
PTE	103.5±4.1*	149.3±4.2†	86.6±4.4†

Values are averages of six determinations, ± SD. In each flask 1 × 10^6 dpm (0.5 × 10^6 counts/min) of appropriately labeled glucose was added in the final concentration of 2 μmoles/ml. Activities were corrected to 50% counting efficiency. Two-hour incubation in Krebs bicarbonate buffer, pH 7.4 at 37 C. Gas phase: 5% CO_2, 95% O_2. Eighteen rats were used in each group. All values obtained from parathyroid extract (PTE)-treated animals have been compared to control animals of the same group. Probability that change from control is due to chance is indicated by symbols and unmarked differences are not significant. * $P < 0.05$. † $P < 0.01$.

Volk Radiochemical Company and D-glucose-1-C^{14} and D-glucose-6-C^{14} from New England Nuclear Corporation. Paper chromatography of these preparations revealed no contaminants.

RESULTS

Formation of $C^{14}O_2$ from labeled glucose. Table 1 compares the $C^{14}O_2$ released from glucose-1-C^{14}, glucose-6-C^{14}, and glucose-U-C^{14} by calvaria, metaphyses, and epiphyses. The initial concentration and specific radioactivity of glucose was the same in all experiments. Epiphyses generally exhibited the greatest release of $C^{14}O_2$. All three types of bone released more $C^{14}O_2$ from glucose-1-C^{14} than from glucose-6-C^{14}, which suggests the presence of hexosemonophosphate shunt activity. The greatest relative effect of PTE is evident in the increases in $C^{14}O_2$ from glucose-6-C^{14}, indicating that PTE stimulated oxidation of pyruvate by way of the Krebs cycle. The ratio of $C^{14}O_2$ from C-6 to the $C^{14}O_2$ from C-1 was significantly increased by PTE in the cases of calvaria (from 0.358 ± .055 to 0.461 ± .046; $P < .02$) and metaphyses (0.599 ± .052 to 0.689 ± .051; $P < .05$), although the increase was not significant in the case of epiphyses. Since the recovery of $C^{14}O_2$ may not have been complete, more quantitative statements would be unwarranted (see below).

Lactate-C^{14} formation from glucose-C^{14}. From Table 2, it is evident that more labeled lactate was obtained from glucose-6-C^{14} than from glucose-1-C^{14}. Every glucose molecule progressing to lactate via glycolysis or the hexose monophosphate shunt will yield one lactate containing a C-6 from glucose; hence the total radioactivity from glucose-6-C^{14} gives a measure of the glucose starting to lactate by both routes. The lactate containing the C-1 from glucose may be assumed to arise from glycolysis only. However, the deficit in lactate containing C-1 is not necessarily all attributable to the C-1 lost via the shunt, since an incomplete equilibration between the dihydroxyacetone phosphate and glyceraldehyde 3-phosphate could lead to a loss of C-1 to products formed specifically from dihydroxyacetone phosphate such as α-glycerol phosphate. The deficit in radioactive lactate coming from glucose-1-C^{14}, or F-E (Table 2), is then a measure of the glucose starting to lactate via "asymmetric" routes but is not specifically a measure of glucose entering the shunt. The excess CO_2 produced from glucose-1-C^{14}, or B-C from Table 1, indicates that this excess amounts to about 8,000, 30,000, and 60,000 counts/min per g fresh bone for calvaria, metaphyses, and epiphyses, respectively, values which are substantially less than F-E. This discrepancy suggests two general alternatives: *a*) either the shunt represents only a fraction of the asymmetric pathway, the rest being attributable to incomplete equilibration between triose phosphates in glycolysis or to some other pathway; or *b*) the recovery of CO_2 was incomplete. This question is considered further in the DISCUSSION.

The specific activity of lactate released into the medium was determined in some experiments using glucose-U-C^{14} and metaphyseal bone. The specific activity of the initial glucose in the buffer was 1.0×10^5 counts/min per μmole and the average specific activity of isolated lactate was 0.48×10^5 counts/min per μmole. This indicates that dilution of the lactate from added glucose by lactate from endogenous sources was relatively slight.

C^{14}-labeled organic acids from glucose-C^{14}. The radioactivities of the organic acids isolated from the combined

TABLE 2. *Effect of parathyroid extract on conversion of glucose-C^{14} to lactate*

	Lactate-C^{14}, Counts/min in Thousands per g Fresh Bone			F-E
	D Glucose-U-C^{14}	E Glucose-1-C^{14}	F Glucose-6-C^{14}	
Calvaria				
Control	351±19	315±11	415±9	100±14
PTE	343±26	298±15	378±9†	80±18
PTX	315±10*	289±21	387±20	
Metaphyses				
Control	639±15	556±17	757±15	201±23
PTE	652±22	567±26	702±16†	135±31
PTX	618±13	558±16	746±18	
Epiphyses				
Control	613±21	525±18	714±24	189±30
PTE	610±16	547±16	693±29	146±33
PTX	630±23	536±20	725±28	

Values are averages of six determinations ± SD and represent the radioactivity recovered in the supernatant buffer after treatment with 1 N H_2SO_4 (cf. Fig. 1). In each flask 5×10^5 counts/min of appropriately labeled glucose was added in the final concentration of 2 μmoles/ml. Sixteen rats were used in each group. Probability that the change is due to chance is indicated by symbols. * $P < 0.05$. † $P < 0.01$.

TABLE 3. *Effect of parathyroid extract on total incorporation of C^{14} from glucose-C^{14} into organic acids of bone in vitro*

Organic Acid	Counts/min in Thousands per g Fresh Bone			
	Metaphyses incubated with glucose-1-C^{14}		Calvaria incubated with glucose-6-C^{14}	
	Control	PTE treated	Control	PTE treated
Lactic	562±17	573±19	418±9	381±9*
Pyruvic	37.5±4.9	20.2±2.9*	21.0±2.3	16.8±1.3†
Citric	9.1±1.6	12.0±1.6†	7.3±0.9	10.2±0.8*
Malic	0.84±0.14	1.4±0.13	0.94±0.06	1.25±0.16†
Glutamic-aspartic	0.68±0.06	0.34±0.04*	0.42±0.06	0.37±0.04
Fumaric	0.56±0.07	0.22±0.03*	0.37±0.03	0.19±0.02†
Succinic	0.34±0.04	0.11±0.02*	0.38±0.05	0.23±0.03†
α-Keto-glutaric	0.28±0.05	0.22±0.04	0.16±0.03	0.14±0.02
Aconitic	0.20±0.04	0.16±0.02	0.16±0.03	0.17±0.02

Values are averages of six determinations ± SD and represent the total radioactivity recovered in the bone and medium. 1×10^6 dpm of glucose was added to each flask (cf. Tables 1 and 2). * $P < 0.01$. † $P < 0.05$.

buffer and the mineral fractions of metaphyses and calvaria are shown in Table 3. Although metaphyses were incubated with glucose-1-C^{14} and calvaria with glucose-6-C^{14}, the results obtained from the two systems were very similar. It can be seen that there was a significant increase in citrate-C^{14} accumulation and a decrease in the accumulation of labeled pyruvate, fumarate, succinate, glutamate, and aspartate in metaphyseal bones from PTE-treated animals. In calvaria there was a significant increase in the total amount of both citrate-C^{14} and malate-C^{14} after PTE administration.

The partition of organic acids between the mineral fraction and the buffer fraction is of interest. Since the buffer fraction included components extracted by dilute acid treatment, it probably represents substances from the medium, from within the cells, and from weakly bound mineral components (cf. Fig. 1); hence it is impossible to estimate exactly the total amount of an acid originally present in the bone mineral immediately after incubation. However, distinct qualitative differences distinguish citric acid and malic acid from all the other acids measured. The relative amounts of labeled acids which remain in the mineral fraction, prepared as shown in Fig. 1, is given for metaphyses incubated with glucose-1-C^{14} in Table 4. Similar results were obtained with calvaria incubated with glucose-6-C^{14}, although the changes after PTE treatment were not quite so pronounced. First, it may be noted that the percent of acid in the mineral fraction was highest for citric and malic acids. After PTE treatment the percent mineral-bound acid increased in all cases except lactate (possibly pyruvate, but in the cases of pyruvic, fumaric, succinic, and α-ketoglutaric acids this increase was associated with a decrease in the total accumulation (Table 3), whereas in the cases of citric and malic acids it resulted from a substantial increase in the mineral-bound fraction (Table 4). Aconitic acid may belong to the citric-malic group, although the results obtained with calvaria indicated little change in the mineral-bound aconitic acid in this bone.

TABLE 4. *Effect of parathyroid extract on radioactive organic acids in mineral fraction of metaphyses*

	Mineral Fraction, Counts/min in Thousands per g Fresh Bone		% of Total Acid in Mineral Fraction	
	Control	PTE treated	Control	PTE treated
Lactic	6.9±1.7	5.8±1.4	1.2	1.0
Pyruvic	0.46±0.11	0.47±0.10	1.2	2.3
Citric	3.9±0.8	6.3±1.0*	43	53
Malic	0.43±.08	0.84±0.11†	51	81
Glutamic-aspartic	.09±.01	.08±.01	13	24
Fumaric	.08±.01	.07±.01	14	32
Succinic	.06±.01	.04±.01	18	36
α-Keto-glutaric	.04±.02	.05±.01	14	23
Aconitic	.04±.01	.09±.02*	20	56

The mineral fraction is defined in Fig. 1. Metaphyses were incubated with glucose-1-C^{14} as given in Table 3. * $P < .05$. † $P < 0.01$.

Incorporation of glucose-1-C^{14} into bone matrix. Under the conditions described in Tables 1 and 2, the total incorporation of C^{14} into the bone matrix from glucose-1-C^{14} was 4.5, 16.0, and 13.6 thousand counts/min per g fresh bone by calvaria, metaphyses, and epiphyses, respectively. Pretreatment of the rats with parathyroid extract increased this incorporation in all three bones, but the significance of the increase in any one bone type was uncertain ($0.01 < P < .05$).

DISCUSSION

Effect of parathyroid hormone on glucose catabolism. The finding that lactate is the principal product of glucose degradation in bone confirms the report of Borle et al. (5). The values for lactate-C^{14} from glucose-U-C^{14} in Table 2 indicate that pretreatment of the animals with PTE causes no significant change in the lactate coming from exogenous glucose. Since the values in Table 2 are corrected to 50% counting efficiency and the absolute radioactivity of the 6 μmoles of glucose added was 10^6 dpm, 1 μmole of lactate from this glucose would have a measured activity of 41.7×10^3 counts/min. With this conversion factor, it may be calculated that the control values of lactate from glucose-U-C^{14} are equivalent to 8.4, 15.3, and 14.7 μmoles/g fresh bone from calvaria, metaphyses, and epiphyses, respectively. Chemical determination of the lactate released into the medium in comparable experiments confirmed these results, indicating a release of 8.1, 12.3, and 11.0 μmoles lactate during incubation of the three bone types.

The only significant effect of parathyroid hormone in Table 2 is the depression of lactate formation from glucose-6-C^{14}, which implies that the hormone may depress the asymmetric pathways described above. The constancy in the excess $C^{14}O_2$ released from glucose-1-C^{14}, or B-C from Table 1, with any one type of bone suggests that the hexose monophosphate shunt is not the asymmetric pathway affected by the hormone.

Effect of parathyroid hormone on the Krebs cycle. The increase in $C^{14}O_2$ from glucose-U-C^{14} in all bones after PTE administration indicates that parathyroid hormone stimulates oxidation by way of the Krebs cycle. These findings agree with those obtained with rabbit femur recently reported by Cohn and Forscher (12). The increased conversion of glucose to CO_2 is also accompanied by histological observations of an increased number of osteoclasts in PTE-stimulated bone (34) and the recent demonstration of a larger number of mitochondria in osteoclasts (17) as compared to osteoblasts.

The pattern of labeling among the organic acids from bone also implies a change in Krebs cycle activity after parathyroid hormone treatment. The shift is most apparent in the study with metaphyses and glucose-1-C^{14} (Tables 3 and 4). Pretreatment of an animal with PTE depresses the total accumulations of radioactive pyruvic

and all radioactive acids associated with the Krebs cycle except for citric and malic acids. Total radioactive citric acid increases and malic acid remains constant. Moreover, the amount of labeled citrate and malate in the mineral fraction increases after PTE treatment whereas the amounts of other acids remain constant in this fraction. By itself, the increase in total citrate accumulation could be attributed to either depression of citrate oxidation or stimulation of citrate synthesis; however, the latter possibility is strongly favored by the decrease in pyruvate and the general increase in Krebs cycle oxidation as indicated by the increase in $C^{14}O_2$ release.

The authors acknowledge the constructive suggestions received from the following individuals during the preparation of this manuscript: Drs. Elmon L. Coe, Lucile R. Hac, Robert E. Ramey, and Franklin C. McLean. We are also indebted to Gouri Chattopadhyay for her technical assistance during the course of this work.

REFERENCES

1. Armstrong, W. D., and L. Singer. *In vitro* uptake and exchange of bone citrate. *Ciba Found. Symp. Bone Struct. Metab.*, 1956, p. 103–116.
2. Balogh, K., Jr., H. R. Dudley, and R. B. Cohen. Oxidative enzyme activity in skeletal cartilage and bone. *Lab. Invest.* 10: 839–845, 1961.
3. Barker, S. B., and W. H. Summerson. The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.* 138: 535–554, 1941.
4. Bonting, S. L. Colorimetric determination of pyruvic acid and other α-keto acids in submicrogram quantities. *Arch. Biochem. Biophys.* 58: 100–108, 1955.
5. Borle, A. B., N. Nichols, and G. Nichols, Jr. Metabolic studies of bone *in vitro*. II. The metabolic patterns of accretion and resorption. *J. Biol. Chem.* 235: 1211–1214, 1960.
6. Bruno, G. A., and J. E. Christian. Determination of carbon-14 in aqueous bicarbonate solutions by liquid scintillation counting techniques. *Anal. Chem.* 33: 1216–1218, 1961.
7. Bruno, G. A., and J. E. Christian. Correction for quenching associated with liquid scintillation counting. *Anal. Chem.* 33: 650–651, 1961.
8. Busch, H., R. B. Hurlbert, and V. R. Potter. Anion exchange chromatography of acids of the citric acid cycle. *J. Biol. Chem.* 196: 717–727, 1952.
9. Carlsson, A., and G. Hollunger. The effect of vitamin D on the citric acid metabolism. *Acta Physiol. Scand.* 31: 317–333, 1954.
10. Chattopadhyay, H. Studies on parathyroid hormone and organic acid turnover by rat bone. *Federation Proc.* 21: 206, 1962.
11. Clark, E. P., and J. B. Collip. A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. *J. Biol. Chem.* 63: 461–464, 1925.
12. Cohn, D. V., and B. K. Forscher. Effect of parathyroid extract on the oxidation *in vitro* of glucose and the production of $^{14}CO_2$ by bone and kidney. *Biochim. Biophys. Acta* 65: 20–26, 1962.
13. Dickens, F. The citric acid content of animal tissues with reference to its occurrence in bone and tumor. *Biochem. J.* 35: 1011–1023, 1941.
14. Diehl, H., and J. L. Ellingboe. Indicator for titration of calcium in presence of magnesium using disodium dihydrogen ethylenediamine tetraacetate. *Anal. Chem.* 28: 882–884, 1956.
15. Dixon, T. F., and H. R. Perkins. Citric acid and bone metabolism. *Biochem. J.* 52: 260–265, 1952.
16. Dixon, T. F., and H. R. Perkins. Citric acid and bone. In: *The Biochemistry and Physiology of Bone*, edited by G. H. Bourne. New York: Academic, 1956, p. 309–323.
17. Dudley, H. R., and D. Spiro. The fine structure of bone cells. *J. Biophys. Biochem. Cytol.* 11: 627–649, 1961.
18. Ettinger, R. H., L. R. Goldbaum, and L. H. Smith, Jr. A simplified photometric method for the determination of citric acid in biological fluids. *J. Biol. Chem.* 199: 531–536, 1952.
19. Firschein, H., G. Martin, B. J. Mulryan, B. Strates, and W. F. Neuman. Mechanism of action of parathyroid hormone. I. On ion gradients. *J. Am. Chem. Soc.* 80: 1619–1623, 1958.
20. Firschein, H. E. Parathyroid hormone and carbohydrate metabolism in bone. *Trans. N. Y. Acad. Sci.* 24: 262–264, 1961–1962.
21. Firschein, H. E., W. F. Neuman, G. R. Martin, and B. J. Mulryan. Studies on the mechanism of action of the parathyroid hormone. *Recent Progr. Hormone Res.* 15: 427–454, 1959.
22. Freeman, S. Citrate metabolism with special reference to calcium and bone. In: *Bone as a Tissue*, edited by K. Rodahl, J. T. Nicholson, and E. M. Brown. New York: McGraw, 1960, p. 314–329.
23. Kramer, B., and F. F. Tisdall. A simple technique for the determination of calcium and magnesium in small amounts of serum. *J. Biol. Chem.* 47: 475–481, 1921.
24. Martin, G. R., H. E. Firschein, B. J. Mulryan, and W. F. Neuman. Mechanism of action of parathyroid hormone. II. Metabolic effects. *J. Am. Chem. Soc.* 80: 6201–6204, 1958.
25. Neuman, W. F., and C. M. Dowse. Possible fundamental action of parathyroid hormone in bone. In: *The Parathyroids*, edited by R. O. Greep and R. V. Talmage. Springfield: Thomas, 1961, p. 310–326.
26. Nicolaysen, R., and R. Nardbo. Calcium metabolism and citric acid. *Acta Physiol. Scand.* 5: 212–214, 1943
27. Palmer, J. K. Chemical investigations of the tobacco plants. X. Determination of organic acids by ion exchange chromatography. *Conn. Agr. Exptl. Sta., New Haven, Bull.* 589: 1–31, 1955.
28. Passmann, J. M., N. S. Radin, and J. A. D. Cooper. Liquid scintillation technique for measuring carbon 14-dioxide activity. *Anal. Chem.* 28: 484–486, 1956.
29. Raisz, L. G., W. Y. W. Au, and J. Tepperman. Effect of changes in parathyroid activity on bone metabolism *in vitro*. *Endocrinology* 68: 783–794, 1961.
30. Ranny, R. E. The effect of estrone and parathyroid extract on bone citrate metabolism. *Endocrinology* 67: 166–169, 1960.
31. Snedecor, G. W. *Statistical Methods*. Ames, Iowa: Iowa State College Press, 1946, p. 38.
32. Steenbock, H., and S. A. Bellin. Vitamin D and tissue citrate. *J. Biol. Chem.* 205: 985–991, 1953.
33. Swim, H. E., and M. F. Utter. Isotopic experimentation with intermediates of the tricarboxylic acid cycles. In: *Methods in Enzymology*, edited by S. P. Colowick and N. O. Kaplan. New York: Academic, 1957, vol. IV, p. 584–609.
34. Toft, R. J., and R. V. Talmage. Quantitative relationship of osteoclasts to parathyroid function. *Proc. Soc. Exptl. Biol. Med.* 103: 611–613, 1960.
35. Vaes, G., and G. Nichols, Jr. Metabolic studies of bone *in vitro*. III. Citric acid metabolism and bone mineral solubility. Effects of parathyroid hormone and estradiol. *J. Biol. Chem.* 236: 3323–3329, 1961.
36. Walker, D. G. Citric acid cycle in osteoblasts and osteoclasts. A histochemical study of normal and parathormone-treated rats. *Bull. Johns Hopkins Hosp.* 106: 80–99, 1961.
37. Wang, C. C. Improvements in the methods for calcium determination in biological material. *J. Biol. Chem.* 111: 443–453, 1935.

J. Reprod. Fert. (1968) **15**, 103–111

THE PERMEABILITY OF TWO- AND EIGHT-CELL MOUSE EMBRYOS TO L-MALIC ACID

R. G. WALES* AND J. D. BIGGERS†

King Ranch Laboratory of Reproductive Physiology, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

(*Received* 30*th November* 1966)

Summary. The level of radio-isotope in 2- and 8-cell mouse embryos was measured following a 30-min incubation in isotopically labelled malate. Although there was very little accumulation of substrate carbon and no output of radio-active carbon dioxide by 2-cell embryos, 8-cell embryos accumulated substrate carbon during the incubation and oxidized some of the malate to carbon dioxide. A change in the concentration of malate in the medium and the addition of ouabain had little effect on the accumulation of substrate in 8-cell embryos. On the other hand, its accumulation was suppressed during incubations at 5° C.

The evidence suggests that there is little or no uptake of malate by 2-cell embryos, and that an active uptake of the compound occurs at a later stage of development.

INTRODUCTION

Biggers, Gwatkin & Brinster (1962) showed that complete development of the mouse zygote to a blastocyst *in vitro* only occurred in organ cultures of Fallopian tubes. At the time these organ culture studies were done, only late 2-cell mouse embryos would develop into blastocysts completely *in vitro*, provided lactate was incorporated in the medium (Whitten, 1957). These results provided indirect evidence that cleavage in the mouse is dependent on the maternal environment. More recent studies have shown that development of the late 2-cell mouse embryo to the blastocyst (Brinster, 1963), and the zygote to the early 2-cell stage (Whittingham & Biggers, 1967), can occur in a simple chemically-defined medium, only if an energy source is present. Only in the intermitotic period between the first and second cleavages is some unknown Fallopian tube factor involved. Thus, there is strong circumstantial evidence that Fallopian tube secretions supply at least an exogenous energy source to the embryo throughout cleavage.

The fact that cleaving mouse embryos require an exogenous energy source raises questions as to what energy sources can be used and how they are assimilated. Studies on the nutritional requirements of the cleaving mouse embryo

* Present address: Department of Veterinary Physiology, University of Sydney, Sydney, N.S.W. Australia.

† Present address: Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe Street, Baltimore, Md. 21205, U.S.A.

in vitro indicate that the energetics of early development are complex. The spectrum of compounds which can be utilized by the late 2-cell embryo is narrow, restricted to the four compounds: lactate, pyruvate, phosphoenolpyruvate and oxaloacetate (Brinster, 1963). By the 8-cell stage, however, several other compounds, such as glucose, malate, α-ketoglutarate and citrate can be used (Whitten, 1957; Brinster & Thomson, 1966). Two alternative mechanisms may be invoked to explain these findings. Either the energy source cannot enter the embryo until a certain stage of development is reached because of permeability barriers, or the energy source enters the early embryo, but the necessary metabolic pathways for its utilization do not develop until later. Studies of the uptake of $U^{14}C$-L-malate by 2- and 8-cell mouse embryos have shown that the cell membrane becomes permeable to the compound between the first and third cleavage divisions. The results are reported in this paper.

MATERIALS AND METHODS

Mouse embryos were obtained from random-bred Swiss mice superovulated by the intraperitoneal injection of 10 i.u. of serum gonadotrophin (Gestyl, Organon), followed 48 hr later by the intraperitoneal injection of 10 i.u. of chorionic gonadotrophin (Pregnyl, Organon) (Brinster, 1963). Two-cell embryos were flushed from the Fallopian tubes of mated females 44 to 48 hr after the second injection, while 8-cell embryos were recovered from the Fallopian tubes and upper third of the uterus approximately 24 hr later.

The basic medium used in the study was Krebs–Ringer bicarbonate containing 1 mg/ml of crystalline bovine serum albumen, 100 i.u./ml of penicillin and 50 μg/ml of streptomycin. Where substrates were added to the medium, isotonicity was maintained by decreasing the sodium chloride content of the medium. For recovery and preliminary handling of the embryos, 25 mM sodium lactate and 0·25 mM sodium pyruvate were added as an energy source (Brinster, 1965). Following collection, the embryos were washed twice by transferring in a minimum volume through two changes of substrate-free medium (3 ml). Following the second wash, the embryos were collected in a minimum of substrate-free medium, transferred to a medium containing U-^{14}C-L-malate (1·7 mM and specific activity 4·4 μc/mole) and incubated at 37° C for 30 min with air:carbon dioxide (95:5) as the gas phase. Between 150 and 250 2-cell embryos, and between ninety and 110 8-cell embryos, were incubated for each treatment. In the first tests embryological watch glasses containing 0·5 ml of the malate medium covered with 2 ml of light paraffin oil were used. However, in all subsequent experiments, incubations were carried out in tissue culture dishes containing 0·1 ml of malate medium as a droplet in 10 ml of light paraffin oil (Brinster, 1963). This latter method was more convenient and conserved isotope. A preliminary trial showed that embryos could be incubated 30 min in the malate medium without loss of viability, as gauged by subsequent development into blastocysts.

A method used by Wales & O'Shea (1966) for the measurement of intracellular constituents of spermatozoa was adopted for the rapid recovery of the embryos from the radio-active malate medium. A 2·6-ml Pichler-Spikes type centrifuge tube with a uniform bore of 2 mm in the capillary tip was used

(Owens-Illinois Glass Co.). The bottom 0·5 cm (approximately 16 μl) was filled with 10% w/v sucrose solution containing 1% (v/v) formalin, and the remaining narrow portion of the tube (2 cm) was filled with isotonic sucrose. Above this, approximately 1 ml of non-radio-active malate medium (1·7 mM malate) was pipetted into the wide portion of the tube. At the completion of incubation in the radio-active malate, the embryos were transferred in a minimum volume of medium to the top of the non-radio-active medium and immediately centrifuged at 500 *g* for 3 min. The non-radio-active medium thus acted as an initial dilutor of the highly active incubation medium before centrifuging the embryos through the sucrose rinse.

After centrifugation, the supernatant medium was removed, the inner surface of the wide portion of the centrifuge tube wiped with absorbent tissue, rinsed with 1 ml of non-radio-active diluent and again wiped dry. The sucrose column was then carefully removed to within 1 cm of the base of the tube. The next 0·5 cm of sucrose was transferred to a scintillation vial to act as a background count for the embryos. The bottom of the tube (0·5 cm) was then removed and

TABLE 1

RADIO-ACTIVITY IN SUCCEEDING 16 μl ALIQUOTS OF THE SUCROSE RINSE AND IN EQUIVALENT AMOUNTS OF THE SUPERNATANT AND INCUBATION MEDIUM AFTER CENTRIFUGING MOUSE EMBRYOS

Stage of development	*Radio-activity*							
	Medium	*Supernatant*	*Sucrose aliquots*					
			1	2	3	4	5	6
2-cell embryo	114,757	7,629	21	4	2	1	0	11
8-cell embryo	124,733	4,379	15	2	2	2	0	64

Radio-activity is expressed as counts/min/aliquot corrected for background (26·8 counts/min). Following centrifugation and removal of the supernatant, succeeding aliquots of the sucrose rinse were withdrawn from the capillary tip of the centrifuge tube and assayed for radio-activity. The sucrose aliquots are numbered 1 to 6 from supernatant interface to base and aliquot number 6 contains the embryos.

transferred with its contents to a scintillation vial. Radio-activity was assayed by liquid scintillation techniques using toluene containing 0·5% 2,5-diphenyl-oxazole (PPO) and 0·03% 1-4-bis-2(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) as scintillator. All samples were first dried, then incubated in 1 ml of 1 M methyl dodecyl benzyl trimethyl ammonium hydroxide (Hyamine hydroxide) before the addition of 10 ml of scintillator. Samples of low activity were counted for sufficient time to ensure accuracy. For each treatment a sample of incubation medium was assayed for radio-activity and the results used to determine the concentration of malate after the introduction of the embryos. The amount of intracellular substrate, expressed as μatoms of substrate carbon/embryo was calculated from the counts in the embryos, corrected for background and the specific activity of the incubation medium.

The distribution of isotope in the sucrose rinse, as shown in Table 1, indicates that the method effectively washes embryos free of radio-active medium. Little isotope was detected in the sucrose and the counts in the lower portion were

equal to those obtained with a standard background. Thus the mean count for the sucrose overlying the embryo fraction in all experiments was 26·8 (SE = 0·34, 32 observations) compared with a mean count of 26·8 (SE = 0·15, 8 observations) for a standard background.

To measure ^{14}C-carbon dioxide production, embryos were incubated in 0·1 ml of radio-active malate medium covered with a thin layer of light paraffin oil. Following incubation for 3 hr in sealed vials that had been flushed with air: carbon dioxide (95:5), metabolism was terminated by the injection of 0·1 ml of 0·2 N H_2SO_4 and the evolved carbon dioxide absorbed with 0·2 ml of 2 N NaOH. The absorbed carbon dioxide was precipitated as barium carbonate, collected on a filter disc, washed and quantitatively transferred to a scintillation vial in 2 ml of ethanol. The precipitate was dispersed in 10 ml of scintillator with the aid of an ultrasonic disintegrator and suspended with a thixotropic gel powder (Carb-O-Sil, Packard Instrument Company). As a consistent amount of radio-active carbon dioxide was collected from samples of the malate medium treated in this way, blanks containing no embryos were used as controls.

RESULTS

The level of intracellular substrate carbon in normal 2- and 8-cell mouse embryos incubated in $U^{14}C$-malate is shown in Table 2. There was little accumulation of substrate in 2-cell embryos following a 30-min incubation and the values represent counts only 57% higher than the corresponding background. On the other hand, significant amounts of substrate accumulated in 8-cell embryos and values were approximately eight times those found at the earlier stage in development.

TABLE 2

THE ACCUMULATION OF SUBSTRATE CARBON BY MOUSE EMBRYOS DURING A 30-MIN INCUBATION IN A DILUENT CONTAINING L-MALATE

Stage of development	*No. of observations*	*Substrate carbon accumulated* (μ*gatoms* $\times 10^{-8}$/*embryo*)
2-cell	4	7·3 ± 2·9
8-cell	7	56·2 ± 5·0

Mean values ± standard errors of the means are given.

The production of carbon dioxide from malate by 2- and 8-cell embryos was also measured. Two samples of 8-cell embryos produced 350×10^{-8} μmole and 420×10^{-8} μmole of carbon dioxide per embryo, respectively, during a 3-hr incubation. However, no carbon dioxide was produced by either of two samples of 2-cell embryos under similar conditions.

To examine the effect of removal of the zona pellucida on the accumulation of substrate carbon, 2- and 8-cell mouse embryos were collected. Before washing and incubation, half of each sample was incubated in a 0·25% solution of Pronase in phosphate buffered saline, containing 1% polyvinylpyrrolidone (pH 7·6) until the zona pelludica was digested (Mintz, 1962; Gwatkin, 1963).

The uptake of malate by these cells was then compared with that of the remaining untreated embryos (Table 3). Although the results are somewhat variable it seems that Pronase treatment had no effect on 2-cell embryos. In contrast, the accumulation of substrate by 8-cell embryos was significantly depressed by this treatment ($t_{(2)} = 7{\cdot}7$, $0{\cdot}01 < P < 0{\cdot}05$).

TABLE 3

EFFECT OF REMOVING THE ZONA PELLUCIDA WITH PRONASE ON THE AMOUNT OF SUBSTRATE CARBON IN MOUSE EMBRYOS INCUBATED IN L-MALATE

Stage of development	*Replicates*	*Substrate carbon accumulated (μgatoms $\times 10^{-8}$/embryo)*	
		Untreated control	*Pronase treated*
2-cell	1	3·5	4·1
	2	15·4	3·6
	3	5·2	4·8
8-cell	1	69·5	40·4
	2	53·3	21·8
	3	77·6	34·8

The effect of varying the concentration of malate on the accumulation of substrate carbon by 8-cell embryos is shown in Table 4. A decrease in the concentration of malate during incubation from 1·17 μmole/ml to 0·117 μmole/ml caused a small (25%) decrease in the accumulation of substrate carbon by the embryos.

The uptake of malate by 8-cell embryos at low temperature was examined in the next experiment. After collection, the embryos were divided into three equal groups in separate embryological watch glasses containing 1 ml of medium

TABLE 4

THE EFFECT OF A CHANGE IN MALATE CONCENTRATION ON THE ACCUMULATION OF SUBSTRATE CARBON BY 8-CELL MOUSE EMBRYOS

Malate concentration (μmole/ml)	*Substrate carbon accumulated (μgatoms $\times 10^{-8}$/embryo)*	
	Rep 1	*Rep* 2
1·17	35·9	40·8
0·117	24·0	32·0

covered with 2 ml of light paraffin oil. Two groups were slowly cooled to 5° C over 30 min by standing in a shallow dish of water in a refrigerated room while the third was maintained at 37° C. A preliminary trial indicated that embryos so cooled showed no loss of viability as gauged by their subsequent development into blastocysts. One of the cooled groups was re-warmed for washing and incubation with the control, uncooled group. The other cooled group was washed

twice, incubated 30 min in the presence of radio-active malate and separated from the incubation medium; all at 5° C. The results for two replicates (Table 5) showed that the accumulation of substrate carbon was greatly depressed by incubation in malate at 5° C and was similar to that found in 2-cell embryos. On the other hand, embryos incubated in malate at 37° C, after cooling, accumulated substrate carbon as readily as the controls.

TABLE 5

EFFECT OF INCUBATION IN L-MALATE AT 5° C ON THE AMOUNT OF SUBSTRATE CARBON IN 8-CELL MOUSE EMBRYOS

Pretreatment	*Temperature of incubation*	*Substrate carbon accumulated* (μ*gatoms* $\times 10^{-8}$/*embryo*)	
		Rep 1	*Rep* 2
Nil (control)	37° C	58·1	47·7
Cooled to 5° C	37° C	54·9	44·4
Cooled to 5° C	5° C	14·5	8·9

An attempt was made to block the uptake of malate by 8-cell mouse embryos with ouabain, an inhibitor of sodium–potassium dependent adenosine triphosphatase important in active transport of some substances across cell membranes (see Repke (1964) for a review).

The accumulation of substrate carbon by 8-cell mouse embryos in the presence of varying concentrations of ouabain was compared with that of control incubations containing no ouabain. The results (Table 6) indicate that

TABLE 6

EFFECT OF OUABAIN ON THE AMOUNT OF SUBSTRATE CARBON IN 8-CELL MOUSE EMBRYOS INCUBATED IN L-MALATE

Ouabain concentration (M)	*Substrate carbon accumulated* (μ*gatoms* $\times 10^{-8}$/*embryo*)		
	Rep 1	*Rep* 2	*Rep* 3
0	44	44	52
10^{-7}	28	37	50
10^{-6}	56	37	—
10^{-5}	36	36	—

there is no effect of ouabain on the amount of intracellular substrate carbon during the 30-min incubation period.

DISCUSSION

UTILIZATION OF L-MALATE

Brinster (1966) has shown that NAD-dependent malic dehydrogenase is present in both 2- and 8-cell embryos and that oxaloacetate is able to support development at both stages (Brinster, 1965; Brinster & Thomson, 1966). Thus, it would

be expected that, if malate could penetrate the cell wall of the 2-cell embryo, it would be utilized provided there was no inhibition of the dehydrogenase *in vivo*. However, it has been shown that malate will not support development at this stage (Brinster, 1965) and, in the present studies, oxidative utilization of malate by 2-cell embryos could not be demonstrated. In addition, the almost complete absence of substrate carbon in embryos at this stage indicates that there is no uptake of the substrate rather than an enzymic inhibition.

By comparison, there were consistently higher concentrations of isotopically labelled carbon in 8-cell embryos incubated in the presence of $U^{14}C$-malate, and a significant production of labelled carbon dioxide during incubation in its presence. These findings, plus the fact that a proportion of these embryos continue to develop with malate as sole substrate (Brinster & Thompson, 1966), indicate that this compound penetrates the 8-cell embryo and is utilized by oxidative pathways.

If 138,000 μ^3 is assumed to be the mean volume of the 8-cell mouse embryo (from data of Lewis & Wright, 1935), the values presented in Table 4 would give concentrations of intracellular substrate carbon of 2·78 and 2·02 μgatoms/ml when the extracellular values were 4·68 μgatoms/ml and 0·47 μgatoms/ml respectively. If the majority of substrate carbon in the cell was malate, the latter figure could indicate that uptake occurs against a concentration gradient. However, as very low levels of labelled substrate carbon were found even in 8-cell embryos, it was impossible to identify positively the intracellular constituent accumulating.

In the renal tubule it has been shown that three polycarboxylic acids of the tricarboxylic acid cycle, namely malate, α-ketoglutarate and citrate, are reabsorbed by active transport probably via a common mechanism (Vishwakarma & Miller, 1963). These three acids will not support the development of 2-cell mouse embryos but will support development of 8-cell stages (Brinster & Thomson, 1966). The present studies indicate that malate enters the 8-cell embryo by an active process. A change of substrate concentration in the incubation medium had a minor effect on the intracellular accumulation of substrate carbon whereas incubation at 5° C inhibited the accumulation. However, ouabain had no effect and it is unlikely therefore that the uptake of malate is linked to the sodium–potassium dependent adenosine triphosphatase as has been found for some non-electrolytes in other tissues (see Csaky, 1965).

The lack of accumulation of substrate carbon by 2-cell embryos during incubations in malate is not due to the impermeability of the zona pellucida at this stage. It has been shown that in the rabbit, rat and hamster the zona is permeable to substances with a molecular weight of at least 1200 (Austin & Lovelock, 1958) and, in the present experiments, removal of the zona by Pronase did not increase the uptake by 2-cell embryos. The effect of Pronase on the uptake of malate by 8-cell embryos is probably due to an effect of the non-specific proteolytic enzyme on cell constituents decreasing active uptake rather than to a direct effect of the removal of the zona pellucida freeing isotopic malate trapped in the perivitelline space. The tendency of some blastomeres to separate during treatment with this enzyme indicates that even with due precautions, there is some effect of the enzyme on the cell surface.

BIOCHEMICAL DIFFERENTIATION IN THE CLEAVING MOUSE EMBRYO

The work on the nutritional requirements of mouse embryos referred to in the introduction to this paper shows that the cleaving embryo is dependent on an exogenous supply of energy. Moreover, an increasing number of compounds can be used as development proceeds. The work described in the present paper has allowed us to identify more specifically one mechanism, namely a change in permeability, which may be involved in regulating development. Since active transport seems to be concerned in the uptake of malate by the mouse embryo the activation of a membrane-linked enzyme may be involved. The work of Fridhandler (1961) on the rabbit showed that initially the pentose phosphate pathway is the main route of energy metabolism, which is later replaced by the Emden-Myerhoff pathway. Thus, the joint evidence shows that more than one type of change in biochemical function may occur during cleavage in mammalian embryos. Cleavage, therefore, is not merely six to seven mitotic divisions in which a relatively undistinguished cytoplasm is shared out among the nuclei until some 64 to 128 cells are produced.

Although the ovum of a marine organism like *Arbacia* is very similar in size to the mouse ovum, there are at least two crucial differences in the mechanisms involved in cleavage of the two species. First, *Arbacia* readily cleave in artificial sea water which is devoid of an exogenous energy source (Shapiro, 1941), whereas a mouse requires an exogenous source of energy. Secondly, strong evidence exists that cleavage of the *Arbacia* egg until gastrulation is regulated by messenger RNA, which is performed in the oocyte (Gross & Cousineau, 1964), whereas, in the mouse, studies on the effect of actinomycin D on cleavage suggest that some synthesis of messenger RNA is necessary even at the 2-cell stage (Thomson & Biggers, 1966). Conclusive evidence of gene action during cleavage in the mouse is provided by two mutants (t^1 and t^{12}) which are lethal during cleavage in the homozygous state (Gluecksohn-Schoenheimer, 1938; Smith, 1956). In addition, the work of Fridhandler (1961) in the rabbit together with the demonstration that an active transport mechanism for the uptake of malate develops after the second cleavage may indicate that the differential reading of the genome begins early in cleavage.

ACKNOWLEDGMENTS

This work was aided by grants from the Population Council and the National Science Foundation (Grant No. GB 4465). One of the authors (R.G.W.) is also indebted to the Population Council for the provision of a fellowship while on sabbatical leave from the Department of Veterinary Physiology, University of Sydney, Australia.

REFERENCES

Austin, C. R. & Lovelock, J. E. (1958) Permeability of rabbit, rat and hamster egg membranes. *Expl Cell Res.* **15**, 260.

Biggers, J. D., Gwatkin, R. B. L. & Brinster, R. L. (1962) Development of mouse embryos in organ cultures of fallopian tubes on a chemically defined medium. *Nature, Lond.* **194**, 747.

Brinster, R. L. (1963) A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. *Expl Cell Res.* **32**, 205.

BRINSTER, R. L. (1965) Studies on the development of mouse embryos *in vitro*. IV. Interaction of energy sources. *J. Reprod. Fert.* **10**, 227.

BRINSTER, R. L. (1966) Malic dehydrogenase activity in the pre-implantation mouse embryo. *Expl Cell Res.* **43**, 131.

BRINSTER, R. L. & THOMSON, J. L. (1966) Development of eight-cell mouse embryos *in vitro*. *Expl Cell Res.* **42**, 308.

CSAKY, T. Z. (1965) Transport through biological membranes. *A. Rev. Physiol.* **27**, 415.

FRIDHANDLER, L. (1961) Pathways of glucose metabolism in fertilized rabbit ova at various pre-implantation stages. *Expl Cell Res.* **22**, 303.

GLUECKSOHN-SCHOENHEIMER, S. (1938) Time of death of lethal homozygotes in the T (Brachyury) series in the mouse. *Proc. Soc. exp. Biol. Med.* **39**, 267.

GROSS, P. R. & COUSINEAU, G. H. (1964) Macromolecule synthesis and the influence of actinomycin on early development. *Expl Cell Res.* **33**, 368.

GWATKIN, R. B. L. (1963) Effect of viruses on early mammalian development. I. Action of Mengo encephalitis virus on mouse ova cultivated *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **50**, 576.

LEWIS, W. H. & WRIGHT, E. S. (1935) On the development of the mouse. *Contr. Embryol.* **25**, 113.

MINTZ, B. (1962) Experimental study of the developing mammalian egg: removal of the zona pellucida. *Science, N.Y.* **138**, 594.

REPKE, K. (1964) Uber den biochemischen Wirkungsmodus von Digitalis. *Klin. Wschr.* **42**, 157.

SHAPIRO, H. (1941) Centrifugal elongation of cells, and some conditions governing the return to sphericity, and cleavage time. *J. cell. comp. Physiol.* **18**, 61.

SMITH, L. J. (1956) A morphological and histochemical investigation of a pre-implantation lethal (t^{12}) in the house mouse. *J. exp. Zool.* **132**, 51.

THOMSON, J. L. & BIGGERS, J. D. (1966) Effect of inhibitors of protein synthesis on the development of preimplantation mouse embryos. *Expl Cell Res.* **41**, 411.

VISHWAKARMA, P. & MILLER, T. (1963) Renal tubular transport of citrate: relations with calcium. *Am. J. Physiol.* **205**, 281.

WALES, R. G. & O'SHEA, T. (1966) The oxidative utilization of fructose and acetate by washed ram spermatozoa in the presence or absence of potassium and magnesium. *Aust. J. biol. Sci.* **19**, 167.

WHITTEN, W. K. (1957) Culture of tubal ova. *Nature, Lond.* **179**, 1081.

WHITTINGHAM, D. G. & BIGGERS, J. D. (1967) Fallopian tube and early cleavage in the mouse. *Nature, Lond.* **213**, 942.

.IS

Haven, Yale University

..li isolate negli embrioni

produced by a toxin oc-
-104.
..asplantation bei Amphi-
rch. f. Entw.-mech., Bd.

tationsversuche an pig-

RUMPLESSNESS OF CHICKEN EMBRYOS PRODUCED BY THE INJECTION OF INSULIN AND OTHER CHEMICALS

WALTER LANDAUER

Storrs Agricultural Experiment Station, University of Connecticut, Storrs, Connecticut

Incidental to other investigations it was observed that the injection of solutions of certain chemicals into unincubated chicken eggs led to the appearance of increased numbers of rumpless embryos and chicks. It was decided to make a systematic study of this problem, and the following is a first report on our work.

MATERIAL AND METHODS

The experiments were done chiefly with eggs from White Leghorn matings, but in one instance eggs from matings of Creeper fowl were used for a series of tests (insulin). Eggs were collected for periods of 1 week. During this time they were kept in an air-conditioned storage room. Eggs from the same mothers were distributed as evenly as possible into experimental and control groups. Prior to injecting the eggs they were allowed to stand in upright position for about 24 hours. After cleaning with alcohol a hole was drilled into the shell with a dental drill. This was done near the level of the short axis of the eggs. A tuberculin syringe was used for the injections, together with a no. 37, ¾-inch hypodermic needle. The amount of fluid injected was always 0.05 cc. The hole in the shell was sealed with Scotch tape. Treated and control eggs were incubated in the same forced-draft incubator.

The particular length of hypodermic needle used was chosen in order to be certain that the solutions would be injected into the yolk of the eggs. Was this actually accomplished and did the injected material not escape through the needle hole in the yolk membrane? Information in this respect was obtained by injecting eggs with 0.05 cc. of India ink, letting the eggs stand for 24 hours, hard-boiling them, and then opening them by a cut through their long axis. It was found that in each instance the major part of the ink was within the yolk, although traces were found along the path of the needle.

250

All embryos which had died between 14 and 18 days and between 18 and 22 days of incubation were examined for morphological abnormalities, as were the hatched chicks.

Standard errors are used throughout this report.

RESULTS

To start with, it was important to know if the damage done by insertion of the hypodermic needle or the injection of saline, which was in many instances used as solvent or diluent, has any effect on embryonic development. The results of such tests are given in table 1. Damage to the eggs, chiefly to the yolk membrane, is reflected in a greatly reduced percentage of hatched chicks. It is evident, however, that neither

TABLE 1

TREATMENT	NUMBER OF FERTILE EGGS	FERTILE EGGS HATCHED %	NUMBER OF EMBRYOS SURVIVING 17TH DAY	RUMPLESS %
Untreated control	253	76.7 ± 2.7	229	2.62 ± 1.05
Hypodermic needle inserted, but no injection	406	65.3 ± 2.4	337	4.45 ± 1.13
0.05 cc. saline injected	401	50.1 ± 2.5	241	2.70 ± 1.05

the insertion of a syringe needle nor the injection of saline into the egg yolk had any significant effect on the frequency with which rumplessness occurred. The same is true with regard to other developmental abnormalities.

Insulin

The insulin preparation used in our experiments was Iletin (Insulin, Lilly) in a concentration of 40 units per cubic centimeter. The amount injected was always 0.05 cc., i.e., 2 units. Table 2 shows the results of experiments with Leghorn eggs. Embryo mortality was greater than after the injection of saline, indicating a certain degree of toxicity of the insulin. The frequency of rumplessness among embryos which survived the seventeenth day of incubation was 41.6% as compared with 1.5% for the untreated controls, an increase of 2770%. The difference between treated and control lots was 40.1 ± 3.9%. Morphological abnormalities, other than rumplessness, were also more common in the insulin-treated material, but this difference was not statistically significant.

There are traces of zinc in Iletin, approximately 0.2 parts per million. Control experiments in which a much larger amount of zinc ions was injected (3.4γ $ZnCl_2$ per egg) did not result in a significant rise in the frequency of rumplessness.

The insulin preparation which we used contains 0.25% phenol and 1.6% glycerine and has a pH of between 2.5 and 3.5. In a search for possible sources of error a new series of experiments was undertaken in which, in addition to untreated controls, a third group of eggs was

TABLE 2

Results after injecting two units of insulin (Iletin) into Leghorn eggs.

DATE	TREATMENT	NUMBER OF FERTILE EGGS	FERTILE EGGS HATCHED %	NUMBER OF EMBRYOS SURVIVING 17TH DAY	RUMPLESS %	ABNORMALITIES OTHER THAN RUMPLESSNESS %
2/22/44	Iletin	93	20.4	39	46.2	10.3
	Control	112	75.0	104	0	0
3/7/44	Iletin	115	41.7	69	39.1	2.9
	Control	126	78.6	117	0.9	0.8
3/14/44	Iletin	118	40.7	65	41.5	1.5
	Control	132	71.2	111	3.6	1.8
Total	Iletin	326	35.3	173	41.6 ± 3.8	4.0 ± 1.5
	Control	370	74.9	332	1.5 ± 0.7	0.9 ± 0.5

injected with a solution which contained 0.25% phenol and 1.6% glycerine, and which was, with HCl, adjusted to a pH of 3. These experiments were done with eggs from matings of Creeper fowl. The results are given in table 3. It is evident that neither the phenol-glycerine admixtures, nor the hydrogen ion concentration were responsible for the high frequency of rumplessness which had been obtained in the earlier experiments. In fact, there is no significant difference between the phenol-glycerine group and the untreated controls. Insulin, on the other hand, again produced a high incidence of rumplessness. The difference in frequency of rumplessness among the insulin-injected group and the phenol-glycerine lot was 15.4 ± 2.2%, and that between the insulin group and the untreated controls amounted to 16.1 ± 2.2%.

It is evident, however, that fewer cases of rumplessness occurred after injecting Creeper eggs than had been obtained in the earlier experiments with Leghorn eggs. The difference in average frequency of rumplessness in these two groups amounted to 24.5 ± 4.3% and is highly significant. It will be noted that the experiments with Creeper

the phenol-glycerine controls, and 0.5 % 0.4% in the untreated controls. As in the Leghorn material, there is a suggestion that the incidence of all abnormalities is increased by the influence of insulin, but the differences are not significant.

The records of the insulin-injected Leghorn eggs have been analysed with regard to homogeneity of response among the mothers which produced the eggs. The data were arranged in an R × 2 table. A χ^2 of 685.8 was found, with 104 degrees of freedom. Since the 1% point is about 6334 and the 5% point is 253, the data definitely suggest heterogeneity i.e., the mother's genetic constitution appears to influence the chances whether or not her eggs will respond to insulin injection with the production of rumplessness.

TABLE 4

Results of the injection of cystine. Experiments with Leghorn eggs.

TREATMENT	NUMBER OF FERTILE EGGS	FERTILE EGGS HATCHED %	NUMBER OF SURVIVORS OF 17TH DAY	RUMPLESS %
0.2 mg. cystine per cc.	181	55.8	141	6.4 ± 2.1
46.4 mg. cystine per cc.	194	57.7	158	6.3 ± 1.9
Total	375	56.8	229	6.3 ± 1.4

In earlier experiments it had been found that the injection of cysteine hydrochloride led to a considerable rise in the frequency with which rumplessness occurred. The eggs were injected with 0.05 cc. of a solution of 100 mg. per cubic centimeter of cysteine hydrochloride in saline. Among 111 embryos which survived the seventeenth day 12.6 ± 3.2% were rumpless. The difference from the untreated control eggs was 10.1 ± 3.3%. This result raised the question whether the effect of insulin is possibly due to its cystine content. Experiments were made to test this. Leghorn eggs were used. Cystine was dissolved in saline by addition of just enough HCl to obtain solution. In a first series the concentration of cystine was 0.2 mg. per cubic centimeter. By injection of 0.05 cc. of this solution into each egg, the amount of cystine was equivalent to that of two units of insulin, the quantity which was used in our experiments. In a second series the concentration of cystine was 232 times that of the first series (46.4 mg. per cc.). The data of these experiments are shown in table 4. The results were nearly identical in the two tests, in spite of the great difference in cystine concentration. The pH of both solutions was about 2. As compared with untreated controls, cystine injection produced a significant increase in the

occurrence of rumplessness. As compared with the insulin experiments, the results demonstrate that cystine alone is not responsible for the effect of insulin.

Experiments were made with one other amino acid, viz., glutamic acid hydrochloride. Both l- and dl-glutamic acid hydrochloride were used. We injected 0.05 cc. of a solution of 547 mg. per cubic centimeter of l- or dl-glutamic acid hydrochloride dissolved in saline. The results of these tests are assembled in table 5. The increase in frequency of rumplessness, as compared with the untreated controls, was 3.1 ± 1.0% for the l-glutamic acid hydrochloride and 6.8 ± 1.3% for the dl-glutamic acid hydrochloride. Both forms of glutamic acid hydrochloride produce

TABLE 5

Results of the injection of l- or dl-glutamic acid hydrochloride. Experiments with Leghorn eggs.

TREATMENT	NUMBER OF FERTILE EGGS	FERTILE EGGS HATCHED %	NUMBER OF SURVIVORS OF 17TH DAY	RUMPLESS %
l-glutamic acid hydrochloride	776	48.2	545	4.6 ± 0.9
dl-glutamic acid hydrochloride	772	48.6	556	8.3 ± 1.2
Untreated	822	68.7	726	1.5 ± 0.5

a significant rise in the frequency of rumplessness. The data also suggest the possibility that dl-glutamic acid hydrochloride is more effective than l-glutamic acid hydrochloride, the difference amounting to 3.68 ± 1.47%. On the other hand, there is certainly no suggestion in these data that the glutamic acid of insulin plays an important role in the insulin effect on rump development. No significant increase in incidence of morphological abnormalities other than rumplessness was produced by either of the glutamic acid hydrochlorides.

Tests with other substances

A series of experiments was run with l-, dl- and d-malic acid. The l- and dl-malic acids were commercial C.P. products. The d-malic acid was prepared for our experiments by Prof. A. W. Ingersoll. We injected 0.05 cc. of solutions containing 20 mg. per cubic centimeter of the three types, respectively, of malic acid. The results are shown in table 6. As judged by embryonic mortality, none of the three preparations had a pronounced, if any, toxic effect. Injection of d-malic acid had no significant influence on the occurrence of rumplessness. In the

group which was injected with dl-malic acid the frequency of rumplessness among embryos and chicks was 2.6 ± 1.1% greater than in the untreated controls. This suggests a significant increase. Such a rise in the production of rumplessness definitely followed the injection of l-malic acid, the difference from the control material being 3.0 ± 1.1%. If the results with dl- and l-malic acid are combined, it is found that there is an increase in rumpless frequency of 2.8 ± 0.9%. Abnormalities other than rumplessness occurred with the following frequencies: 0.2% in the controls, 1.5% each in the l- and d-malic acid groups, and

TABLE 6

Results of the injection of l-, dl-, and d-malic acid. Experiments with Leghorn eggs.

TREATMENT	NUMBER OF FERTILE EGGS	FERTILE EGGS HATCHED %	NUMBER OF SURVIVORS OF 17TH DAY	RUMPLESS %
l-malic	523	58.7	413	4.3 ± 1.0
dl-malic	513	57.3	401	3.9 ± 1.0
d-malic	519	59.0	401	2.0 ± 0.7
Untreated	526	69.4	457	1.3 ± 0.5

TABLE 7

Results of the injection of thioglycollic acid, hydrochloric acid and saline. The thioglycollic acid and saline were 0.2 molar with a pH of 7; the hydrochloric acid had a pH of about 1. Leghorn eggs.

TREATMENT	NUMBER OF FERTILE EGGS	FERTILE EGGS HATCHED %	NUMBER OF SURVIVORS OF 17TH DAY	RUMPLESS %
Thioglycollic acid	648	41.4	374	6.7 ± 1.3
Hydrochloric acid	1141	60.0	708	3.5 ± 0.6
Sodium chloride	864	46.5	537	2.2 ± 0.6
Untreated	1072	73.5	930	1.9 ± 0.5

2.2% in the series injected with dl-malic acid. The differences are suggestive of some effect in all groups, but are not significant.

In another series of experiments we compared the effects of thioglycollic acid, hydrochloric acid and a sodium chloride solution. The thioglycollic acid and sodium chloride solutions were 0.2 molar and had a pH of 7. The hydrochloric acid was adjusted to the same pH (about 1) as the solutions of glutamic acid hydrochloride which were discussed above. The molarity of the hydrochloric acid was the same as that of the least concentrated cysteine hydrochloride solution used in our experiments (20 mg. cysteine hydrochloride per cc.). The amount injected of each solution was 0.05 cc. per egg. The results are shown in table 7.

As compared with the untreated controls, thioglycollic acid accounted for a significant rise in the frequency of rumplessness, the difference between the two groups amounting to 4.8 ± 1.3%. The treatment with hydrochloric acid and sodium chloride, on the other hand, had no effect in this respect. It should be noted, however, that hydrochloric acid, if injected in concentrated form (0.05 cc.), probably does produce a significant rise in production of rumpless embryos. In such a test 3.8 ± 1.1% rumpless embryos were found as against 0.8 ± 0.5% among the untreated controls, the difference between the two groups amounting to 3.0 ± 1.2%. No significant increase in the frequency of malformations other than rumplessness was observed in any of these tests.

DISCUSSION

The outstanding result of our experiments is the high percentage of rumplessness produced by the injection of insulin. This appears to be the first recorded instance of a morphogenetic effect of insulin.

The morphological appearance of the distal end of the spine in insulin-induced rumplessness does, in most cases, resemble the sporadically occurring cases. Those rumpless embryos, in the insulin experiments, which died shortly before hatching time or which at the end of the incubation period were found alive within the shell were nearly all completely rumpless. In many of these embryos some of the synsacro-caudal vertebrae were missing in addition to the free tail vertebrae. In certain of the most extreme cases the posterior end of the body was edematous. Among the rumpless chicks which hatched, on the other hand, there were some which had a partial tail skeleton. In this respect the material differs from that of sporadic rumplessness since in the latter no intermediate forms have ever been observed.

In earlier experiments it was shown that rumplessness can be produced by mechanical shaking of eggs (Landauer and Baumann, '43). As in these shaking experiments, it was found after insulin treatment that the genetic constitution of the mother plays a role in the extent of response obtained under experimental conditions. It does not seem likely that the genetic factors which protect embryos from becoming rumpless on account of shaking of the eggs or because of the injection of insulin are the same ones.

In the case of shaking the evidence suggested that the results are brought about by injury to the blastoderm. It was shown that these mechanical forces not only raise the incidence of rumplessness, but also, and to a similar extent, that of all other commonly occurring malformations. This is not so in the case of insulin treatment. It is true

that the experiments suggest a slight (though not statistically significant) increase in the frequency of all types of abnormalities. This may be due to mechanical disturbances produced by injection per se. At any rate, it is obvious that insulin has a selective effect on rump development. It seems reasonable to assume that the selective response of the caudal part of the vertebral column is in some way related to developmental peculiarities of this region. Such perculiarities do actually exist. Degeneration of presumptive tail tissue plays a role in normal development of the tail and it is also the principal feature in the origin of dominant rumplessness (Zwilling, '42). In later stages cartilage differentiation in the tail lags behind that of other parts of the skeleton (Blinov, '38). These developmental features are probably in some way related to the effect which insulin has on formation of the tail.

How this effect is brought about cannot at present be determined with certainty, but it may be well to discuss some aspects of the problem. The physiological properties of insulin suggest the possibility that an interference with the embryo's carbohydrate metabolism may occur after its injection. But even if this assumption is correct, it remains entirely unknown why development of the tail should be affected selectively. Corda ('32) found that the injection of insulin into pregnant rabbits was followed by the abortion of undersized fetuses and the birth of small young. The results were ascribed to interference with the carbohydrate exchange between mother and fetuses, but the author envisioned also the possibility of a more direct action of insulin on the fetus. There were no teratological effects. Gregg and Pomerat ('42), working with developing Rana pipiens embryos, found that insulin was inhibiting and toxic above a concentration of 0.02%, but no effect of any kind was observed at lower concentrations. These results do not shed any light on our observations.

The fact that two of the amino acids which contribute most to the composition of insulin (cystine and glutamic acid), if injected by themselves, do not produce a rise in the occurrence of rumplessness comparable to that obtained with insulin, does not exclude the possibility that the rumpless effect may be due to still other amino acids or the combined action of more than one of them. This must be settled by further experiments.

It is of interest to point out that some of our most significant results were obtained after the injection of substances containing sulphur (insulin, cysteine, cystine, thioglycollic acid). The possible role of sulphur in the production of rumplessness requires further investigation. Our data show, however, that other substances, e.g., d-glutamic

acid hydrochloride, may lead to similar results. Ancel and Lallemand ('42) reported that treatment of early chicken embryos with certain chemicals resulted in rumplessness, but gave no further details.

Our observations do not support the conclusion that acidification per se is instrumental in diverting development in the direction of rumplessness.

It is known that differences in toxicity exist between the optical isomeres of certain substances (Cushny, '26; Gause, '41). If the production of rumplessness were a consequence of toxic action, then our observations on glutamic and malic acid could be taken as another instance of this kind. The isomeres which do not occur naturally are, in general, the ones which are more toxic. In the case of glutamic acid hydrochloride no differences in embryonic mortality existed after the injection of the l- and dl- forms, but the dl-glutamic acid hydrochloride produced nearly twice as many cases of rumplessness as did the l-glutamic acid hydrochloride. As judged by embryonic mortality there was also no difference in the effect produced by the isomeres of malic acid, but here it was the naturally occurring form (l-malic) which was most effective in bringing about the development of rumplessness. It is clear, therefore, that our results cannot be accounted for by differences in toxicity, but must be due to specific reactions of the embryo to particular isomeres.

Inhibitory and toxic effects of certain amino acids (tyrosine, phenylaline, cysteine) on embryonic development of a beetle (Bruchus quadrimaculatus Fabricius) were reported by Brauer ('42). This author also found selective susceptibility of certain embryonic regions and it must be such a selective response which accounts for the origin of rumplessness in our experiments.

Our observations demonstrate that rumplessness can be produced by a variety of chemical substances. In earlier work it was shown that rumplessness can also be induced by mechanical forces. The possible relation of these phenocopies to the developmental aspects of hereditary rumplessness cannot be discussed until a study of the embryonic development of experimentally-produced rumplessness has been made. In a general way, however, conclusions at which Grüneberg ('43, p. 243) arrived in a discussion of x-ray induced phenocopies in mice may apply also to hereditary rumplessness. He says: "One cannot help getting the impression that these structural changes, at any rate, are rather unspecific results of gene action. Some more fundamental change in development seems to upset those developmental processes, which at a given time happen to be in a critical and hence susceptible stage." With

regard to details of developmental events we do not know at present if any experimentally-produced phenocopy is truly analogous to its hereditary counterpart. Yet, even if this were not true for the present material, it would still remain a fact of considerable interest that, as in the case of the mutations for rumplessness and their modifiers, insulin can bring about either complete rumplessness or varied modifications intermediate between this condition and a normal tail.

SUMMARY

Experiments in which various chemical substances were injected into the yolk of eggs prior to incubation, had the following results.

After the injection of two units of zinc-free Iletin (Insulin, Lilly) into eggs of White Leghorn fowl 41.6 ± 3.8% of all embryos surviving the seventeenth day were rumpless. Among the controls there were only 1.5 ± 0.7% of rumpless embryos and chicks.

Similar experiments with eggs of Creeper fowl resulted in 17.1 ± 2.1% rumpless embryos with 1.0 ± 0.5% rumplessness among the controls.

The results are highly significant in both stocks. The difference between the stocks is due in part to variations in response on account of genetic dissimilarity and in part to seasonal influences. Significant heterogeneity in response of the eggs to insulin treatment was found among the hens involved in the experiment with Leghorn fowl.

Significant increases in the frequency with which rumplessness occurred were obtained also after the injection of cysteine hydrochloride, cystine, dl-glutamic acid hydrochloride, thioglycollic acid and l-malic acid. The effect of l-glutamic acid hydrochloride was much less pronounced than that of the dl- form; d-malic acid had no effect. Concentrated hydrochloric acid probably produced some increase in rumpless frequency, but this was not true for more dilute hydrochloric acid (pH 1). Injection of saline or mere insertion of a hypodermic needle gave no results.

None of the experiments led to a significant rise in abnormalities other than rumplessness; not, at any rate, among the late embryonic stages which we examined.

ACKNOWLEDGMENTS

The author is under great obligation to Prof. A. W. Ingersoll, Vanderbilt University, Nashville, Tennessee for preparing the d-malic acid used in our experiments and to Dr. F. B. Peck and the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana for pro-

t know at present if
al us to its hered-
for the present ma-
interest that, as in
ir modifiers, insulin
varied modifications
tail.

ances were injected
ollowing results.
etin (Insulin, Lilly)
all embryos surviv-
controls there were

fowl resulted in
rumplessness among

. The difference be-
sponse on account of
fluences. Significant
treatment was found
eghorn fowl.
ch rumplessness oc-
ite hydrochloride,
llic acid and l-malic
was much less pro-
d no effect. Concen-
increase in rumpless
te hydrochloric acid
a hypodermic needle

rise in abnormalities
g the late embryonic

W. Ingersoll, Vander-
ing the d-malic acid
d the Lilly Research
lis, Indiana for pro-

viding the Hetin (Insulin, Lilly) as well as for information concerning this product.

The author is especially grateful to Dr. Heinrich Waelsch, New York State Psychiatric Institute and Hospital, New York City for advice and help in many ways.

The author is much obliged to Dr. E. M. Bailey, Department of Analytical Chemistry, Connecticut Agricultural Experiment Station, New Haven for a zinc determination in a sample of Hetin (Insulin, Lilly).

LITERATURE CITED

ANCEL, P. ET S. LALLEMAND 1942 Sur une malformation du bec et des membres obtenue chez l'embryon de poulet à l'aide de sulfamides. Comptes Rendus des Séances de la Société de Biologie, vol. 136, pp. 255–256.

BLINOV, V. A. 1938 Zur Frage nach der Zeit der Anlagenbildung und dem Differenzierungstempo des Knorpelgewebes in verschiedenen Organen des Vogelembryos. (Russian with German summary.) Biologicheskij Zhurnal, vol. 7, pp. 311–324.

BRAUER, A. 1942 Modification of development in Bruchid (Coleoptera) embryos by treatment with amino acids. Transactions, Kentucky Academy of Science, vol. 10, pp. 24–28.

CORDA, G. M. 1932 Ricerche sperimentali sulla influenza della iperglicemia e della ipoglicemia materna sul decorsa della gravidanza e sullo sviluppo dei feti. Annali di Ostetricia e Ginecologia, vol. 54, pp. 143–170.

CUSHNY, A. R. 1926 Biological Relations of Optically Isomeric Substances. Baltimore (Williams and Wilkins).

GAUSE, G. F. 1941 Analysis of various biological processes by the study of the differential action of optical isomeres. Biodynamica, vol. 3, pp. 217–246.

GREGG, J. R., AND C. M. POMERAT 1942 The glycogen content of the embryo of Rana pipiens during development. Growth, vol. 6, pp. 231–234.

GRÜNEBERG, H. 1943 The Genetics of the Mouse. Cambridge (University Press).

LANDAUER, W., AND L. BAUMANN 1943 Rumplessness of chicken embryos produced by mechanical shaking of eggs prior to incubation. J. Exp. Zool., vol. 93, pp. 51–74.

51–74.

ZWILLING, E. 1942 The development of dominant rumplessness in chick embryos. Genetics, vol. 27, pp. 641–656.

616,009.2: 612.018

An Experimental Study on Decadron Convulsion

Report 3. An Experimental Study on the Action Mechanism of Decadron Convulsion.

(The essence of this thesis was published before the general meeting of the 36th Biochemical Society of Japan).

First Surgical Department Directors: Professor Dennosuke Jinuchi and Professor Sanae Tanaka) College of Medicine, Okayama University.

By Shintaro Miyake, Doctor of Medicine

(The manuscript was received on September 10, 1963).

Chapter 1: Introduction:

In Report 1 of this thesis, the present author discovered the fact that, if a decadron injection solution (which may hereafter be abbreviated as "de" injection solution) is injected into the large vessel of an animal, peculiar convulsion is induced and it was decided to call this convulsion the decadron convulsion (which may hereafter be abbreviated as "de" convulsion)(1). In Report No. 2, further, it was pointed out that an effective substance for convulsion in the "de" injection solution is sodium citrate. It was further reported that its function is strengthened by dexamethasone-21-phosphate disodium (DM-P), thereby inducing the "de" convulsion.

In this case,the "de" convulsion is different from the convulsions which is induced by such a convulsion-inducing substance as sodium glutamate, etc. During the course of the experiment, the author found that, even when the "de" injection solution or sodium citrate was caused to act in the skin movement area, no convulsions was developed.

In the present report, therefore, an attempt was made to clarify the problem as to what area in the celebrum sodium citrate or the "de" injection solution should apply in order to induce the convulsion.

Among the components of the "de" injection solution, further, it is believed that sodium citrate is in the form of citrate at normal times and the concentration is different from the concentration at which it was used in the experi-

ment. Despite the fact that it exists as a component of the TCA cycle, why is it that convulsion takes place at a high concentration? As means for the solution of this problem, an attempt was made to clarify what substances would inhibit the convulsion. An effort was made to clarify the mechanism of the development of convulsion by finding but whether convulsion will take place by substances which are similar to citric acid.

Chapter 2: Experimental Method

Section 1: Experimental Animals.

Grownup CF_1 mice whose weight was around 25 grams, rats whose weight was around 300 grams and dogs, male or female, whose weight was 10 kilograms were used.

Section 2: Examination of the Part where "de" convulsion Appears.

The "de" injection solution was applied only to the skin motion range of the celebrum and injected deep into the substance of the brains for the purpose of determining whether there is convulsion or not, thereby examining the part where it appears.

Clause 1: Examination of the Action of Inducing Convulsion in the Skin Motion Range of the Celebrum by the "De" Injection Solution.

(i) Examination by "Mice":

A mouse was fixed to a fixing stable, the skin of its head was cut off in the shape of a spindle. On the line of the upper edge of the eye ball, skull piercing was carried out by using a "monteau" needle at a position 0.2 centimeters away from the central line and the skull was removed little by little from the said small hole toward the environment by using a fine surgical sewing needle. In this manner, the skin of the cerebrum starts swelling from the portion of trepanation. At this point, filter paper which was impregnated with the "de" injection solution was pasted for the first time.

It is mentioned in this connection that there is no fear of the "de" injection solution being diffused in the cerebral solution at the time when the filter paper was pasted since the skin of the cerebrum starts swelling from the trepanation portion.

(ii) Examination by means of rats.

A rat was fixed to a fixing stand and the skin of the head was cut off in the shape of a spindle. After peeling the muscle of the side head from the skull toward the outside, trepanation was carried out 0.3 centimeters outside from the central line on the upper edges of the eye ball by means of a mouse tooth drill. The hard film was cut open by an injection needle and 0.01 milliliter of the "de" injection solution was injected under the skin under direct observation.

(iii) Examination by dogs.

A dog was anesthetized by vein anesthesia and was fixed to a fixing stand. The skin of the head was cut open in the arrow-shaped direction and trepanation was carried out through a front hollow of the head from outside. 1.5 centimeters from the central line on the line of the upper edge of the eye ball and we waited for awakening from anesthesia. After awakening, the hard film was cut out and 0.02 milliliters of the "de" injection solution was injected under direct observation into the gyrus sigmoideus anterior and posterior.

Clause 2: Examination of the "De" Convulsion Inducement by Injection of the "de" Injection Solution into the cerebral cortex.

By using rats and dogs, the "de" injection solution was injected deep by letting the injection needle go deep into the cerebral cortex, for the purpose of examining the portions where rigid convulsion (which will hereafter be abbreviated as TK) takes place in four limbs.

(i) Examination by means of rats.

According to the experimental method which was described in Report No. 1, Chapter 2, Section 6, skull piercing was carried out at a point 0.4 centimeters outside from the arrow-shaped sewing joint and 1.0 centimeter on the side of the tail. Injection was carried out after causing the injection needle to proceed 0.7 centimeters from the same location.

Prior to injection, the small amounts of the "de" injection solution were colored in three colors including charcoal black, field blue and field red and 0.01 milliliter was injected by using a separate injection needle by chang-

ing the direction slightly to the front and back and to the right and left.

After each injection, the passage of five minutes was observed and, when TK was induced in four limbs, the head was immediately cut off and the cerebrum was taken out to be fixed with a 10 per cent formalin solution.

After the fixing of the cerebral structure, horizontal cut pieces whose width was approximately 0.2 centimeters were prepared and, fromthe colored portion, an examination was made as to where the center would be which created the TK.

(ii) Examination by means of Dogs.

After vein anesthesia, a dog was fixed to a fixing stand. After giving an approximately 15 centimeter skin opening at the central line of the head for removal to right and left and for fixation, the dog tooth muscle was peeled outwardly, a hole was created in the skull 4.0 centimeters from the upper edge of the eye ball and 2.0 centimeters from the arrow-shaped sewing. Through the said hole, three centimeters of the needle were caused to proceed in various directions. The "de" injection solution which was colored in the manner described earlier was injected in an amount of 0.02 milliliters. If TK was found to have been induced, the examination was carried out in the same manner as previously.

Section 3: Examination of Control on "de" Convulsion

Clause1: Examination of Chemicals showing Inhibitive Effect upon "de" Convulsion.

The following nine kinds of chemicals were examined in order to discover the mechanism of the "de" convulsion by examining the inhibitive effect of various kinds of convulsion inhibitive substances upon the "de" convulsions. Even though malonic acid is not originally a convulsions inhibitive substances, it was also examined with others on the assumption that malonic acid could also become an inhibitive substance since it stops the citric acid cycle, if "de" convulsion and citric acid convulsion are induced by the energy of citric acid decomposition.

1) $CaCl_2$. 2) $CaCO_3$. 3) $MgCl_2$. 4) (gamma)-aminobutyric acid (GABA).

5) (gamma)-guanidino-(beta)-hydroxybutyric acid-HCl. 6) (gamma)-amino-(beta)-hydroxybutyric acid (GABOB. 7. Phenobital. 8) (gamma)-aminobutyric-histidine-H_2SO_4 (homo-carnosine). 9) malonic acid.

Clause 2: Method of giving Convulsion Inhibitive Substances.

Among the chemicals described in Clause 1, phenobital was injected hypodermically. The other chemicals were injected directly into the medullary cavities. In the case of dogs, injection was carried out into the large vessel through a vinyl tube of a dog which was operated as stated in Report No. 1, Chapter 2, Section 2. In the case of rats and mice, an injection was carried out into a cavity below the hard film as described in Report No. 1, Chapter 2, Section 6.

As stated in Report No. 2, Chapter 3, because of the fact that a convulsion which was very similar to "de" convulsion was induced by using citric acid (which will hereafter be referred to as citric acid convulsion) and because of the convenience of comparing and examining the inhibitive amount of a convulsion inhibitive substance in terms of molar numbers (which will be abbreviated as M), the inhibitive effect upon citric acid convulsion was also examined at the same time.

The experimental methods of various chemicals are as shown in Table 1 on the following page.

(Insert Table 1 on p. 871. Method of giving chemicals for the examination of inhibition of "de" convulsion. 1. Inhibitive substance. 2. Experimental animal. 3. Method of feeding. 4. Pre-treatment feeding. 5. Feeding immediately after TK inducement. 6. Feeding by mixing a citric acid solution and a convulsion inhibitive substance. 7. Mouse. 8. One minute after the injection of 0.01 milliliters of a 0.05 M $CaCl_2$ solution, 0.01 milliliters of the "de" in injection solution was injected. 9. Immediately after the inducement of citric acid convulsion by 0.01 milliliter of 0.05M citric acid, 0.01 milliliter of a 0.05M $CaCl_2$ solution was injected. 10. 0.05M citric acid, 0.05M $CaCl_2$, amount of injection. 11. Mouse. 12. One minute after the injection of 0.01 milliliter of the 0.05M $CaCO_3$ solution, 0.01 milliliter of the "de"

injection solution was injected. 13. Immediately after the inducement of citric acid convulsion, 0.01 milliliter of a 0.05M $CaCO_3$ solution was injected. 14. Mouse. 15. After the injection of 0.01 milliliters of a 0.05M $MgCl_2$ solution, 0.01 milliliter of the "de" injection solution was injected. 15. 0.05M citric acid 0.05M $MgCO_3$, amount of injection. 17. Dog and mouse. 18. After the injection of 0.01 milliliter of a GABA, 1M, solution (in the case of a mouse), 0.01 milliliter of the "de" injection solution was injected. 19. (In the case of a dog) 0.05M citric acid, 0.05M GABA, amount of injection. 20. Mouse. 21. One minute after four milligrams was dissolved in 0.01 milliliter of distilled water and injected, 0.01 milliliter of the "de" injection solution was injected. 22. Dog and mouse. 23. One minute after the injection of 0.01 milliliter of the gamibetal (in the case of a dog), 0.01 milliliters of the "de" injection solution was injected. 24. (in the case of a dog), immediately after the inducement of "de" convulsion, 50 milligrams of gamibetal was injected. 25. Mourse 26. Thirty (30) minutes after the hypodermic injection of 0.01 milliliter of a 10 per cent phenobal injection solution, 0.01 milliliter of the "de" injection solution was injected. 27. Dog, rat and mouse. 28. (in the case of a dog) after the injection of 150 milligrams, 1.0 milliliter of the "de" injection solution was injected. (in the case of a rat), five minutes after the injection of 20 milligrams, 0.02 milliliters of the "de" injection solution. (In the case of a mouse), five minutes after the injection of two milligrams, 0.01 milliliters of the "de" injection solution was injected. 29. (In the case of a mouse) two milligrams was injected immediately after the inducement of the "de" convulsion. 30. (In the case of a mouse) Two milligrams of homocarnosine was dissolved in 0.01 milliliter of the "de" injection solution and this was injected. 31. Mouse. 32. Five minutes after the injection of 0.01 milliliter of one per cent malonic acid, 0.01 milliliter of the "de" injection solution was injected. 33. 0.05M citric acid, malonic acid, amount of injection.)

Section 4: Examination of Convulsion Inducement Action of Sodium Citrate Homologues.

In Report No. 2, it was clarified that the "de" convulsion is induced by the participation of DM-P and sodium citrate among the components of the "de" injection solution. Of these, an examination has already been made relative to the DM-P homologues affecting convulsion. In this report, therefore, an examination will be made regarding the following 10 sodium citrate homologues upon convulsion.

It is mentioned in this connection that mice of the CF_1 system were used and 0.01 milliliter of the one per cent solution which is the concentration of sodium citrate in the "de" injections solution was injected into the cavity under the hard film.

(Insert 1) through 5) at the bottom of the right-hand column on p. 870).

(Insert 6) through 10) at the top of p. 871.)

Chapter 3: Experimental Result

Section 1: On the part where "de" convulsion is developed.

As stated in Chapter 2, Section 2, the "de" injection solution was directly made to work in the skin motion range of the cerebrum and the substance of the cerebrum for the purpose of examining the parts where the "de" convulsion is developed. The following result was obtained:

Clause 1: Action upon the Skin Motion Range of the Cerebrum:

The skin motion parts of the cerebrum of a mouse, rat and dog was directly stimulated by the "de" injection solution. In all of the cases, however, no convulsion at all took place on the surface. In addition, no convulsion was observed when other skin stimuli were applied.

Clause 2: Action on Substances of Cerebrum:

An examination was carried out by using rats and dogs by the method described in Chapter 2, Section 2. There were some in both cases where convulsion was developed. In such cases, it was found that the injection part in rats was the portion from theoutside front of the anmon angle as shown in Figure 1; it is the portion which is considered to be the nucleus caudatus. In the case of dogs, the injection was clearly carried out in the nucleus caudatus as shown in Photograph 1.

(Insert Figure 1 on p. 872. Injection portion in the cerebral substance where "de" convulsion was developed. 1. Anmon angle. 2. Outer case. 3. Inner case. 4. Third cerebral chamber. 5. Vision floor outer nucleus. 6. Coating shell. 7. Injection part - nuleus caudatus. 8. Side cerebral chamber. 9. Vision floor inside nucleus. 10. Cerebral beam.)

On the other hand, simuli were given by injecting the same into the base nucleus aside from the nucleus caudatus; however, no convulsion was developed.

Next, the convulsion which was indicated at the time when an injection was given to the nucleus caudatus will be described in the case of dogs and rats.

(i) "De" Convulsion in Rats when Injection Was Given to Nuleus Caudatus.

In two out of five rats, the said convulsion was induced. There was no difference whatsoever between this convulsion and the "de" convulsion which was induced at the time when an injection was given into the cerebral cavity insofar as the type was concerned, except that the latent time period before the inducement of TK in four limbs in this case was shortened to two to five seconds.

(ii) "De" Convulsion in Dogs at the Time of Injection into Nucleus Caudatus.

Convulsion was caused in one out of the three cases (dogs). The latent period in this convulsion was found to be extremely short in the range between two and five seconds. Repetition was made after pauses. In other words, TK took place in four limbs for a period of three minutes immediately after the injection, this was followed by a pause of 10 minutes and then the TK appeared in the four limbs to last for a period of 10 minutes.

(Insert Photo. 1 on p. 872. Portion of the injection in the dog's cerebral substance where "de" convulsion took place.)

Chapter 2: Inhibition of "de" Convulsion

As shown in Table 1, the inhibition of "de" convulsion was examined from a number of angles and the results shown in Table 2 were attained. It was found that $CaCl_2$, $CaCO_3$ and $MgCl_2$ were the most effective in the inhibition of "de" convulsion and homocarnosine can also be considered to be effective inasmuch as it was effective in the case of mice and dogs, even though it induced TK in

the case of rats, while increasing the latent period. GABOB brought about a worsening of the general condition, with a result that no convulsion was induced in more than a half of the cases.

(Insert Table 2 on p. 873. 1. Inhibitive substance. 2. Presence or absence of convulsion by administering during the pre-treatment. 3. Effect of inhibiting the convulsion by administering immediately after the inducement of TK. 4. Presence or absence of convulsion by administering in mixed form with citric acid. 5. No convulsion was induced. 6. The convulsion was suddenly stopped 10 seconds after the injection. 7. 0.05M citric acid, $CaCl_2$, convulsion. 8. No convulsion was induced. 9. The convulsion was stopped suddenly 10 seconds after the injection. 10. No convulsion was induced. 11. 0.05M citric acid, $MgCl_2$, convulsion. 12. TK was induced. 13. TK was induced. 14. TK was induced. 15. The general condition deteriorated as soon as gamibetal was injected. No convulsion was induced in a half of the cases. In the other half of the cases, TK was induced throughout the body and they died. 16. TK continued. 17. TK was induced. 18. No convulsion was induced in the case of dogs and mice. In the case of rats, TK was induced after an extended latent period or after a period of three minutes. 19. TK continued. 20. No convulsion was induced. 21. TK was induced. 22. 0.05M citric acid, malonic acid, convulsion.)

Section 3: Effect of Sodium Citrate upon the Inducement of Convulsion

An examination of the convulsion-inducing effect of the 10 different kinds of sodium citrate homologues which were mentioned in Chapter 2, Section 4 was carried out. As shown in Table 3, convulsion was induced in the case of citric acid, L-malic acid and oxalic acid. In the case of malonic acid, fumaric acid, maleic acid, (alpha)-ketoglutaric acid, oxalacetic acid, succinic acid and acetic acid, no change whatsoever was observed even though they were injected at pH 3.8, which is the acidity of a 0.05M citric acid solution which would induce citric acid convulsion.

Next, the development of convulsion in the aforementioned three cases will be described.

(i) Convulsion by Citric Acid

This was the same as the citric acid convulsion which was described in Report No. 2, Chapter 3, Section 2.

(ii) Convulsion by L-malic acid.

Injection: As soon as an injection was given, a slow jerking movement developed on the face, the neck was rotated, the tail was shaken and alternating convulsion in four limbs was observed.

30 Seconds After Injection: TK was induced in the four limbs, then they bent like bows, the tail was raised, stool and urine were discharged.

Two minutes and 30 seconds after injection: Disappearance of convulsion,

(Insert Table 3 on p. 873. Convulsion-inducing effect of sodium citrate homologues. a. Sodium citrate homologues. b. Presence or absence of convulsion inducement. c. Remarks. d. Same as the citric acid convulsion. e. Considerably lighter than the citric acid convulsion. f. Convulsion was induced in a repeated fashion.)

(iii) Convulsion by Oxalic Acid.

10 seconds after injection: Breathing became rapid.

20 seconds after injection: A jerking movement developed on the face.

Three minutes after injection: Bending like a bow, TK induced in four limbs, alternating convulsion, tail shaking, and discharge of stool and urine.

Three minutes 10 seconds after injection: The above disappeared, the breathing became rapid, the jerking movement of facial muscle continued.

Four minutes after injection: Bending like a bow, TK and alternating convulsion in four limbs, and tail shaking.

Four minutes five seconds after injection: The above disappeared, breathing became rapid, and the jerking movement of the facial muscle continued.

Five minutes after injection: Bending like a bow, TK and alternating convulsion in four limbs, and shaking of the tail.

Five minutes and five seconds after injection: The above disappeared, the breathing became rapid, and the jerking motion of the facial nuscle continued.

Five minutes 30 seconds after injection: The jerking movement of the facial

muscle and rapid breathing disappeared and quietness returned.

Chapter 4: Observations

In order to clarify the portions where "de" convulsion would take place, a "de" injection solution was directly injected into the skin motion range of the cerebrum and the cerebral substance. As a result of this, it was found in the case of mice, rats and dogs that no convulsion was induced by an injection directly into the skin motion range but that a convulsion which was characteristic of the "de" injection solution was induced after an extremely short period in the range between two and five seconds when an injection was given into the nucleus caudatus. Accordingly, it is assumed that, when an injection is administered in the medullary cavity, the development of convulsion will take place only after it has reached the nucleus caudatus.

Regarding the above part, Takahashi (3) has already made public a report in which he stated that TK and alternating convulsion are induced when the nucleus caudatus is stimulated by pentamothylene tetrazol, sodium asparaguinate, sodium glutamate, sodium oxalate, hydrozide isonicotate, nicotine, sodium citrate, and sodium arabate. This is in satisfactory agreement with the result of my experiment.

Next, the mechanism for the inducement of convulsion will be described. Regarding this, it was already clarified in Report No. 2 that the substance which causes "de" convulsion was sodium citrate and that EM-P would strengthen the same. In this report, an examination was made as to what substance would inhibit the inducement of the "de" convulsion or citric acid convulsion. As a result, it was learned that Ca^{++}, Mg^{++} and homocarnosine were these substances.

The existence of homocarnosine in the brains was clarified by Pisano (4) in 1960. In a recent report published by Mori et al. (5), it was found that the same is capable of inhibiting the convulsion of Ep-mouse and it is considered to be an inhibitory substance against the development of convulsion stemming from a peripheral reason. Therefore, it seems to have some inhibitive effect upon the inducement of convulsion stemming from the nucleus cauda-

tus. However, there are some doubts about homocarnosine with regards its intensity.

The inhibitive action of Ca^{++} is quite powerful. When Ca^{++} is administered beforehand, no convulsion takes place even if an injection is made by using the "dc" injection solution or citric acid. When Ca^{++} is administered during the course of convulsion, it is possible to terminate the convulsion. The reason for this is believed to stem from the following: Even though Ca^{++} forms a complex with citric acid, the free Ca^{++} cannot be made to disappear. Exactly the same thing can be stated with regards the inhibitive effect of Mg^{++}.

If the above situation is observed from the standpoint of the effect of citric acid to induce convulsion, it is believed that the following is valid between excitability and inorganic ions (6):

(Insert (A) on p. 874.)

It is further assumed that the citric acid of a non-biologically high concentration which was administered formed a complex with Ca^{++} or Mg^{++} and removed the free Ca^{++} and Mg^{++}. As a result, the denominator approaches zero, with a consequence that the excitability which is shown in this formula is raised to an abnormal height.

Messrs. Yamazaki and Takigawa (7) established a relationship between the citric acid convulsion and the TCA cycle and stated that, because of the fact that citric acid inhibits succinic dehydrogenase, it stops the TCA cycle at the level of succinic acid, the said citric acid entering the GABA shunt from the stage of succinic acid and being converted into GABA through succinic semi-aldehyde and that the same is converted into an excitable substance. Even if a large amount of GABA may be administered in a direct fashion, no convulsion is necessarily induced but it rather serves as an inhibitor in many cases. However, convulsion takes place without fail by citric acid. Thus, it appears that the above theory may contain some unreasonableness.

In the examination of the convulsion-inducing effect of substances which are homologous to the citric acid or sodium citrate, it was learned that malic acid and oxalic acid induce convulsions which are similar to those which were

induced by sodium citrate or citric acid. It is considered true that the mechanism of the function of malic acid is the same as that of citric acid and, in the case of oxalic acid, an extremely insoluble Ca salt is formed. These experiments are further indicative of the correctness of the theory that the excitability of the cells of the motor nerves is raised by the formation of a complex between sodium citrate or citric acid with Ca^{++}, and that, because of this, convulsion is induced.

Chapter 5: Conclusion

The mechanism of the function of "de" convulsion was examined from the stand-points of the portion in the cerebrum where it is induced and its inhibition and the following conclusions have been obtained:

1) No "de" convulsion is induced even when a "de" injection solution may be injected into the skin motion range of the cerebrum and the characteristic "de" convulsions was induced only when the injection of the same was made into the nucleus caudatus.

2) "De" convulsion can be inhibited by using Ca^{++}, Mg^{++} and homocarnosine.

3) In sodium citrate homoluges, a convulsion which is similar to the "de" convulsion can be induced by citric acid. L-malic acid and oxalic acid. However, it cannot be induced by malonic acid, fumaric acid, maleic acid, (alpha)-ketohlutaric acid, succinis acid, oxalacetic acid and acetic acid.

4) From the above conclusions, the mechanism of the action of "de" convulsion is as follows: Citric acid in the components of the "de" injection solution forms a complex with Ca^{++} or Mg^{++}, with a reduction in the free Ca^{++} and Mg^{++} being achieved, thereby raising the excitability of the cells of motion nerves, coupled with the multiplicative effect of DM-P, with a high degree of TK being induced. It is the convulsion of skin peripheral nature and the portion where it is induced is the nucleus caudatus.

References:

1) Miyake: Journal of the Okayama Medical College, 75, 15.

2) Miyake: Jounral of the Okayama Medical College, 75, 15.

3) Takahashi: Conditioned Reflexes, 16, 153-166, 1961.

4) Pisano, J. J., Wilson J. D., Cohen L. C., Abraham D. and udenfriend, S.; J. Brol. chem., 236, 499, 1961.

5) Mori, Sugio, Masukawa and Uno: Conditioned Reflexes, 34, 1962.

6) Alfred T. Shehl, M. D., Mineral Metabolism, Reinhold Publishing Corp., U.S.A., 1939.

7) Yamazaki and Takikawa: Conditioned Reflexes 32, 1301-1303, 1962.

Translated by Carl Demrick Associates, Inc./RO/db

Malic Acid 305

616-009.2 : 612.018

デカドロン痙攣に関する実験的研究

第 3 編

デカドロン痙攣の作用機序に関する実験的研究

（本論文の要旨は第36回日本生化学会総会において発表した）

岡山大学医学部第1（田中）外科教室（指導：[illegible]）
医学士　三　宅　新　太　郎

〔昭和38年9月10日受稿〕

第1章　緒　言

私は本論文第1編[1]において，**デカドロン注射液**（以下これを「デ」注射液と略す）を動物の大槽内に注入すると特異な痙攣が惹起されることを見出し，**デカドロン痙攣**（以下これを「デ」痙攣と略す）と呼称することにし，さらに第2編[2]において，「デ」注射液中の痙攣に対する有効物質は Sodium Citrate であり，Dexamethasone-21-phosphate disodium (DM-P) がこの作用を増強して，特異の「デ」痙攣を生ぜしめることを明らかにしたが，その際「デ」痙攣はグルタミン酸 Na 等の痙攣誘発物質による痙攣とは異なり，皮質運動領に「デ」注射液或はクエン酸 Na を作用さしても痙攣が発現しないことを実験中に確認してきた．従つて，本編においては，「デ」注射液或はクエン酸 Na が脳内のいずれの場所に作用して痙攣を発現せしめるかの問題を明らかにせんと試みた．

また，「デ」注射液成分のうち，クエン酸 Na は正常時にもクエン酸の形で，実験に用いた濃度とはほるかに少いとは考えられるが，脳の TCA Cycle の成分として存在しているにもかかわらず，高濃度になると痙攣が起るのは何故かという問題を解決する手がかりとして，ますいかなる物質によつて痙攣が抑制されるかを明らかにし，またクエン酸類似の物質によつても痙攣がおこるかどうかを明らかにし，痙攣発現の機序を解明せんと試みた．

第2章　実験方法

第1節　実験動物

体重 25g 前後の成熟 CF_1 系マウス，体重 300g 前後のラット及び体重 10kg 前後のイヌを雄雄をとり使用した．

第2節　「デ」痙攣の作用部位の探究

「デ」注射液を大脳皮質運動領にのみ作用させ，また脳実質内に深く注入して，痙攣の有無を検し，もつてその作用部位を探究した．

第1項　「デ」注射液の大脳皮質運動領に対する痙攣誘発作用の検索

(i)　マウスによる検索

マウスを固定台に固定，頭部の皮膚を紡錘形に切除し，眼窩上縁の線上にて正中線より 0.2cm 外側の位置にマントー針にて頭蓋穿刺をおこない，同小孔より，細い外科用絶合針にて周囲に向つて少しずつ頭蓋を除去する．そうすれば，大脳皮質は Trepanation の部位より膨隆してくるので，ここにおいて予め「デ」注射液を吸収させておいた濾紙を貼布した．なお，大脳皮質は Trepanation の部分より膨隆してくるので，濾紙を貼布したとき脳液中に「デ」注射液が拡散される心配はない．

(ii)　ラットによる検索

ラットを固定台に固定，頭部皮膚を紡錘形に切除し，側頭筋を頭蓋より外側に向つて剥離後ネズミ歯鉗で眼窩上縁の線上，中心線より 0.3cm 外側において Trepanation をおこなう．注射針にて硬膜を切開し，直視下で皮質下に「デ」注射液 0.01 ml を注入した．

(iii)　イヌによる検索

静脈麻酔にて麻酔し固定台に固定，頭部皮膚を矢状方向に切開し，眼窩上縁の線上，中心線より 1.5cm 外側より前頭洞を通過して Trepanation をおこない，麻酔の覚醒を待つ．麻酔覚醒後硬膜を切除し，gyrus sigmoideus anterior 及び posterior に直視下で「デ」注射液 0.02 ml ずつ注入した．

第2項　「デ」注射液の脳実質内注入による「デ」痙攣誘発作用の検索

ラット及びイヌを使用し，その脳実質内に深く注

皮針を用いて「デ」注射液を注入し，もつて四肢に痙攣を誘発し，（以下これを TK と略す）を惹起する試験を行なうこととした．

(i) ラットによる検索

第1章，第2章，第6節にのべた実験方法により，頭部上縁より外側 0.4cm，頭蓋上縁より尾側1.0cm の部位に穿孔をおこない，同所より注射針を 0.7cm 深さに注入した．

注入に先立ち，「デ」注射液を小量の墨汁，Field blue 及び Field red 染色液にて3色に着色し，それぞれ別の注射針を用いて前後左右に少しずつ方向を変えて 0.01ml 注入した．

以上の注入ごとに5分間隔を観察し，四肢に TK を惹起したならば，ただちに開頭し，脳をとり出し10% Formalin 液にて固定した．

固定後冠状染，約 0.2cm 巾の水平截片をつくり，その着色された部分より TK を惹起した中枢はどこであるかを探究した．

(ii) イヌにおける検索

麻酔後検イヌを固定台に固定，頭部正中線に約 15cm の皮膚切開を加えて左右に骨膜剥離し，大前囟を骨鉗にて開放し，頭蓋上縁より 4.0cm，矢状縫合より 2.0cm の部位に穿孔した．その孔を通じて針を 3cm 種々の方向に進め，前記のごとく着色した「デ」注射液を 0.02ml 注入し，TK を惹起しえたならば前記同様にして検した．

第3節　「デ」痙攣の抑制の検索

第1項　「デ」痙攣に対して抑制効果を示す薬物についての検索

薬物学的に作用機序の不明な「デ」痙攣に対する抑制効果を検索し，ついでこれにより「デ」痙攣の作用機序を思いたすべく，下記のごとく9種の薬物について試験した．さて，malonic acid は元来痙攣抑制物質ではないが，「デ」痙攣，クエン酸痙攣がクエン酸分解のエネルギーにより惹起されるものであるならば，クエン酸サイクルを止める malonic acid は，抑制作用をもたらすであろうとの考えで検討した．

1) $CaCl_2$
2) $CaCO_3$
3) $MgCl_2$
4) γ-aminobutyric acid (GABA)
5) γ-guanidino-β-hydroxybutyric acid·HCl
6) γ-amino-β-hydroxybutyric acid (GABOB)
(gamibetal 1ml : 50mg 相当量)
7) Phenobital
8) γ-aminobutyric-L-histidine·H_2SO_4
(Homocarnosine)
9) malonic acid

第2項　痙攣抑制物質投与法

第1項の薬物のうち，phenobital は皮下へ注入したが，これ以外の薬物は直接腦室内に注入した．なおイヌでは第1編，第2章，第2節のごとく手術を施したイヌのビニールチューブを通じて大槽内に注入し，ラット及びマウスでは第1編，第2章，第6節のごとく硬膜下腔に注入した．また第2編，第3章にのべたごとく，クエン酸により「デ」痙攣に非常によく類似した痙攣（以下これをクエン酸痙攣と呼称する）が惹起されることと，痙攣抑制物質の抑制量をモル数（M と略す）で比較検討するに便利なため，クエン酸痙攣に対する抑制作用をも併せて検した．

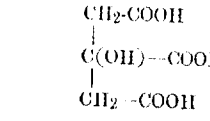
各薬物ごとの実験方法を列記すれば次頁第1表のごとくである．

第4節　クエン酸ソーダ類似物質の痙攣誘発作用の検索

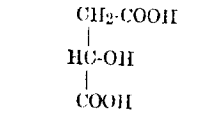
第2編において，「デ」痙攣は「デ」注射液成分中の DM-P 及びクエン酸ソーダが関与して惹起されるものであることを明らかにしたが，そのうち DM-P 類似物質の痙攣に関する研究は第1編においてすでに検索したので，本編においては下記10種のクエン酸ソーダ類似物質の痙攣に関する検索をおこなつた．

なお，CF_1 系マウスを使用し，その硬膜下腔にクエン酸ソーダが「デ」注射液中にしめる濃度，すなわち1%溶液として 0.01ml 注入してみた．

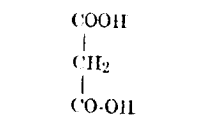
1) citric acid



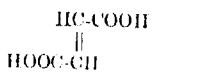
2) L-malic acid



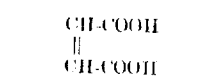
3) malonic acid



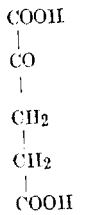
4) fumaric acid



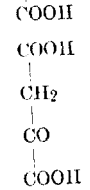
5) maleic acid



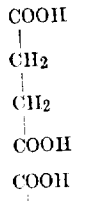
6) α-ketoglutaric acid



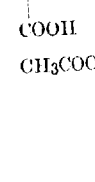
7) Oxalacetic acid



8) succinic acid



9) Oxalic acid



10) acetic acid

Table 1

第 1 表　「デ」痙攣抑制を検した薬物の投与法

1 抑制物質	2 実験動物	3 投与法 4 前処置投与	5 TK 誘発直後投与	6 クエン酸溶液と痙攣抑制物質を混合して投与
Ca Cl2	7 マウス	8 0.05M $CaCl_2$ 溶液 0.01ml 注入後1分，「デ」注射液 0.01ml 注入	9 0.05M クエン酸 0.01ml にてクエン酸痙攣誘発直後 0.05M $CaCl_2$ 溶液 0.01ml 注入	10 0.05M クエン酸 / 0.05M $CaCl_2$ / 注入量 1 : 1 / 0.02ml 1 : 1/2 / 0.015ml 1 : 1/4 / 0.015ml
$CaCO_3$	11 マウス	12 0.05M $CaCO_3$ 溶液0.01ml 注入後1分，「デ」注射液 0.01ml 注入	13 クエン酸痙攣誘発直後 0.05M $CaCO_3$ 溶液0.01ml 注入	
$MgCl_2$	14 マウス	15 0.05 M $MgCl_2$ 溶液 0.01ml 注入後1分，「デ」注射液 0.01ml 注入		16 0.05M クエン酸 / 0.05M $MgCO_3$ / 注入量 1 : 1/4 / 0.015ml 1 : 1/2 / 0.015ml 1 : 1 / 0.02ml 1 : 2 / 0.03ml
GABA	17 マウス及びイヌ	18 (マウス) GABA, 1M 溶液 0.01ml 注入後1分，「デ」注射液 0.01ml 注入		19 (イヌ) 0.05M クエン酸 / 0.05M GABA / 注入量 1 : 1 / 1.4ml
γ-guanidino β-hydroxybutyric acid-HCl	20 マウス	21 4mg/0.01ml 蒸留水に溶解し注入後1分「デ」注射液 0.01ml 注入		
GABOB	22 マウス及びイヌ	23 (マウス) gamibetal 0.01ml 注入後1分「デ」注射液 0.01ml 注入	24 (イヌ)「デ」痙攣誘発直後 gamibetal 50mg 注入	
Phenobarbital	25 マウス	26 10%フェノバール注射液 0.01ml 皮下注後30分に「デ」注射液0.01ml 注入		
Homocarnosine	27 マウス及びイヌ	28 (イヌ) 150mg 注入後10分「デ」1.0ml (ラット) 20mg 注入後5分「デ」0.02ml (マウス) 2mg注入後5分「デ」0.01ml	29 (マウス)「デ」痙攣誘発直後2mg 注入	30 (マウス) Homocarnosine 2mg を「デ」注射液 0.01ml にて溶解し注入
Malonic acid	31 マウス	32 1% malonic acid 0.01ml 注入後5分「デ」注射液 0.01ml 注入		33 0.05M クエン酸 / malonic acid / 注入量 1 : 1(0.05M) / 0.01ml 1 : 1(0.1M) / 0.01ml

第3章　実験成績

第1節　「デ」痙攣の作用部位について

第2章，第2節でのべたごとく，「デ」痙攣の作用部位を推定せんとし，大脳皮質運動領及び脳実質内に「デ」注射液を直接作用せしめたところ，下記のごとき結果をえた．

第1項　大脳皮質運動領に対する作用

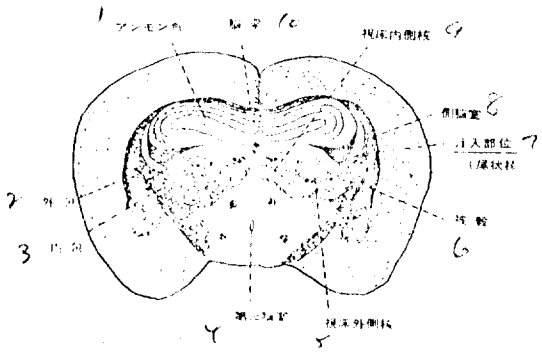
「デ」注射液で，マウス，ラット及びイヌの大脳皮質運動領を直接刺激したが，外観上全例において全く痙攣をみなかつた．またその他の皮質の刺激でも全く痙攣をみなかつた．

第2項　脳実質内に対する作用

第2章，第2項にのべたごとき方法により，ラット及びイヌを使用して検したが，両者において痙攣を惹起せしめたものがあつた．そしてその場合の注入部位は，ラットにおいては，第1図のごとくアンモン角の前方外側よりの部分で，尾状核とおぼしき部分であり，イヌにおいては，写真1のごとく明らかに尾状核に注入されていた．

また一方尾状核以外のすべての基底核にも注射して刺激してみたが全く痙攣は起らなかつた．

第1図　「デ」痙攣を惹起したラット脳実質内注入部位



つぎにイヌ及びラットの尾状核注入時に惹起される痙攣を記載する．

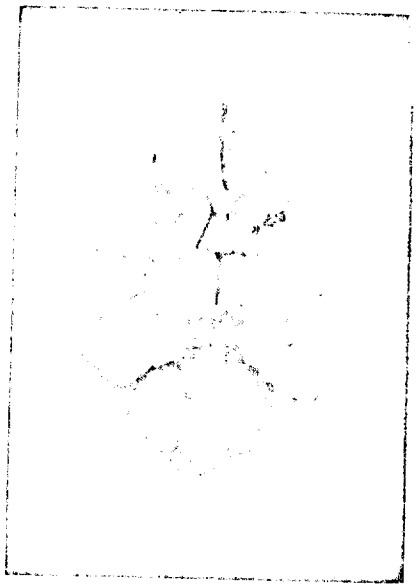
(i)　尾状核注入時ラットにおける「デ」痙攣

5例のラット1のうち2例に痙攣を起しえたが，その痙攣は，四肢の TK を生起するまでの潜時が2～5秒に短縮されている以外は，型において脳腔内注入により惹起される「デ」痙攣と全く同様で何らの差異を見出しえなかつた．

(ii)　尾状核注入時イヌにおける「デ」痙攣

3例のイヌのうち1例に痙攣を起しえた．その痙攣は非常に潜時が短かく2～5秒で，Pause をおいて反復した．すなわち注入直後四肢に TK 3分，10分 Pause をおいてふたたび四肢に TK が発現し約10分持続した．

写真1　「デ」痙攣を惹起したイヌ脳実質内注入部位



第2節　「デ」痙攣の抑制

第1表に示したように，「デ」痙攣の抑制を種々の面から検索し，その結果次頁第2表のごとき成績をえた．すなわち，$CaCl_2$，$CaCO_3$，$MgCl_2$ が「デ」痙攣阻止に最も効果的に働き，**Homocarnosine** も，ラットにおいて潜時の延長をみながら TK を誘発したが，イヌ及びマウスにおいては効果的であつたので，まず有効とみなしうる．GABOB は一般状態の悪化をきたし，そのためか半数において痙攣の発現をみなかつた．

Table 2 第 2 表　痙攣誘発物質の単独，痙攣発現の有無

	単独投与による痙攣発現の有無	TK 単独投与による痙攣発現の有無	クエン酸と単独投与による痙攣発現の有無
$CaCl_2$	痙攣発現なし	注入後10秒，急激に痙攣発現	0.05M クエン酸 : $CaCl_2$ 痙攣 1 : 1 (−) 1 : 1.2 (−) 1 : 1.4 (−)
$CaCO_3$	痙攣発現なし	注入後10秒，急激に痙攣発現	
$MgCl_2$	痙攣発現なし		0.05M クエン酸 : $MgCl_2$ 痙攣 1 : 1.4 (−) 1 : 1.2 (−) 1 : 1 (−) 1 : 2 (−)
GABA	TK 誘発		TK 誘発
γ-guanidino-β-hydroxybutyric acid HCl	TK 誘発		
GABOB	gamibetal 注入と同時にマウスはトーニック化，半数において，痙攣発現なし，半数において，全例 TK となり死亡	TK 誘発	
Phenobarbital	TK 誘発		
Homocarnosine	注入，マウスにおいて，痙攣発現なし，ラットにおいて注射の同時，すなわち，3分の経時ののちに TK 誘発	TK 誘発	痙攣発現なし
Malonic acid	TK 誘発		0.05 M クエン酸 : malonic A. 痙攣 1 : 1(0.05M) (−) 1 : 1(0.1M) (−)

第3節　クエン酸ソーダ類似物質の痙攣誘発作用

第2章，第4節で述べた10種類のクエン酸ソーダ類似物質について，痙攣誘発作用を検したところ，第3表に示すごとく citric acid, L-malic acid, oxalic acid の3種類は，malonic acid, fumaric acid, maleic acid, α-ketoglutaric acid, oxalacetic acid, succinic acid, acetic acid はクエン酸ソーダを溶解する 0.05 M クエン酸溶液の酸性，すなわち pH 3.8 まで注入されたが全く変化をみなかった．

以下に上記3者の実験経過を記載する．

(i) citric acid の痙攣

第2表，第3表，第2図に記載したクエン酸塩の注入による．

(ii) L-malic acid の痙攣

注入と同時に興奮，強直痙攣，振戦，間代性痙攣を経過する．

Table 3 第3表　クエン酸ソーダ類似物質の痙攣誘発作用

	a クエン酸ソーダ類似物質	b 痙攣誘発の有無	c 備考
1	citric acid	(+)	クエン酸痙攣と d 同一
2	L-malic acid	(+)	クエン酸痙攣より e 非常に軽度
3	malonic acid	(−)	
4	fumaric acid	(−)	
5	maleic acid	(−)	
6	α-ketoglutaric acid	(−)	
7	oxalacetic acid	(−)	
8	succinic acid	(−)	
9	oxalic acid	(±)	反応として痙攣が f 惹起される
10	acetic acid	(−)	

注入後30秒：四肢に TK．後弓反張．尾の挙上．脱糞．放尿．

注入後2分30秒：痙攣消失．

(iii) Oxalic acid の痙攣

注入後10秒：呼吸促迫．

注入後20秒：顔面ピクピク．

注入後3分：後弓反張．四肢に TK 及び交代性痙攣．振尾．脱糞．放尿．

注入後3分10秒：上記消失．呼吸促迫，顔面ピクピク持続．

注入後4分：後弓反張．四肢に TK 及び交代性痙攣．振尾．

注入後4分5秒：上記消失．呼吸促迫，顔面ピクピク持続．

注入後5分：後弓反張．四肢に TK 及び交代性痙攣．振尾．

注入後5分5秒：上記消失．呼吸促迫，顔面ピクピク持続．

注入後5分30秒：顔面ピクピク，呼吸促迫消失．安静に戻る．

第4章　考　按

「デ」痙攣の作用部位を明らかにするため，「デ」注射液を大脳皮質運動領及び脳実質内に直接注入した結果，マウス，ラット及びイヌのいずれの場合においても実質運動領への直接注入によつては痙攣が起らず，尾状核に注入された場合にのみ2～5秒という極めて短かい，潜時ののち，「デ」注射液特有の痙攣が発現することを明らかにした．従つて脳室内に注入した場合には，これが尾状核に到達してはじめて痙攣の発現となるものであろうと考えられる．この作用部位については，すでに高橋[3]は，尾状核をペンタメチレンテトラゾール，アスパラギン酸ソーダ，グルタミン酸ソーダ，酸酸ソーダ，イソニコチン酸ヒドラジット，ニコチン，クエン酸ソーダ，及びフラーデン酸ソーダで刺激すると，TK 及び交代性痙攣が起ることを報告しているが，これは私の実験の結果とよく一致している．

次に，痙攣発現の機序であるが，これに関しては，「デ」痙攣の原因となる物質は sodium citrate であり，DM-P がこれを増強することを第2章において明らかにしているので，本章においては「デ」痙攣は，クエン酸痙攣が如何なる物質により抑制されうるかを検索したところ，Ca^{++}，Mg^{++} 及び homocarnosine であることが明らかになつた．homocarnosine は，1960年 Pisano[4] により脳内存在が明らかにされた物質であるが，最近，森ら[5] によりこれが Ep-マウスの痙攣を阻止しうることが明らかにされ，辺縁系に起因する痙攣発作の抑制物質であると考えられている．従つて，尾状核に由来する痙攣発作に対しても抑制的に作用するのであろうと考えられるが，ただ homocarnosine の抑制作用はその全きの点でまだ問題が残つている．

Ca^{++} の抑制作用はきわめて強力であり，Ca^{++} を予め投与しておくと，「デ」注射液或は citric acid によつては痙攣が起らないし，痙攣中に Ca^{++} を投与すると痙攣を中絶せしめうるが，この原因としては，Ca^{++} が citric acid と complex を形成するが，free の Ca^{++} を消失せしめえないためと考えられる．このことは Mg^{++} の抑制効果についてもまつたく同一のことが言いうる．

以上の事実を逆に citric acid の痙攣誘発作用の立場から考えてみると，興奮性と無機イオンとの間には次式[6]，すなわち

$$\text{Irritabilitg} = \frac{[Na^+] + [K^+]}{[Ca^{++}] + [Mg^{++}] + [H^+]} \quad (A)$$

なる式が成立しているが，投与された非生理的高濃度の citric acid は Ca^{++} 或は Mg^{++} と Complex を形成し free の Ca^{++}，Mg^{++} を取り去るため上式の分母は0に近ずき，従つて本式に示される興奮性は異常に高まるものと考えられる．

山崎，淀川ら[7] はクエン酸痙攣を TCA Cycle と関係づけ，クエン酸は succinic dehydrogenase を阻害するため，TCA Cycle を succinic acid の level で止め，citric acid が succinic acid の段階から GABA shunt に入り，succinic semialdehyde を経て GABA になり，それが興奮物質に変化するというのであるが，GABA を大量に直接投与しても必ずしも痙攣は起らず，むしろ抑制的に作用する場合が多いにもかかわらず，citric acid によつては必ず痙攣が起るので，この説には若干の無理があると思われる．

citric acid 或は sodium citrate と近似した物質の痙攣作用についての検索では，malic acid と Oxalic acid が sodium citrate あるいは citric acid と同様な痙攣を惹起することがわかつたが，malic acid の作用機転も citric acid と同様であると考えられるし，Oxalic acid の場合は極めて不活性の Ca 塩を作成するので，これらの実験は sodium citrate 或は citric acid が Ca^{++} と complex を作ること

により運動性神経細胞の興奮性を高め，これが原因となつて痙攣を起すのであろうとする考えをさらに実証するものである．

第5章 結 論

「デ」痙攣の作用機序をその脳内作用部位及び抑制の面から探究し，次のごとき結論をえた．

1) 「デ」注射液を大脳皮質運動領に作用せしめても「デ」痙攣は起らず，尾状核に注入された時にのみ特有の「デ」痙攣を惹起せしめうる．

2) 「デ」痙攣は，Ca^{++}, Mg^{++}, Homocarnosine によつて抑制できる．

3) クエン酸ソーダ類似物質中では citric acid, L-malic acid, oxalic acid で「デ」痙攣と同様の痙攣を誘発しうるが，malonic acid, fumaric acid, maleic acid, α-ketoglutaric acid, succinic acid, Oxalacetic acid, acetic acid では誘発しえない．

4) 以上のことから，「デ」痙攣の作用機序は，「デ」注射液成分中のクエン酸が Ca^{++}, 或は Mg^{++} と Complex を形成し，free の Ca^{++}, Mg^{++} が減少して運動性神経細胞の興奮性を高め，それに DM-P が相乗的に作用して強度の TK を惹起するものであり，またそれは皮質辺縁性の痙攣であつて，その作用部位は尾状核である．

参 考 文 献

1) 三宅：同医誌，75, 15.
2) 三宅：同医誌，75, 15.
3) 高橋：条件反射，16, 153～166, 1961.
4) Pisano, J. J., Wilson, J. D., Cohen L. C, Abraham D, and udenfriend, S.,: J. Brol. chem. 236, 499, 1961.
5) 森，杉生，増川，宇野：条件反射34, 1962.
6) Alfred T. Shohl, M. D., Mineral Metabolism, Reinhold publishing corporation, U. S. A. 1939.
7) 山崎，滝川：条件反射，32, 1301～1303, 1962.

Studies of Convulsive Seizure Induced by Decadron

Part 3 Studies of the Mechanism of Inducing Convulsive Seizure by Decadron

By

Shintaro MIYAKE

Department of Surgery and Neurosurgery, Okayama Univ. Med. School

(Director: Prof. D. Jinnai
Prof. S. Tanaka)

The mechanism of inducing convulsive seizure by Decadron was investigated as followings:

1) No convulsive seizure was observed after apllying Decadron solution at the motor area of cerebral cortex, but the typical convulsive was observed only by administrating it directly at Nucleus caudatus.

2) The convulsion induced by Decadron could be inhibited by Ca. Mg and homocarnosine.

3) Amongst sodium citratum like substances, citric acid, L-malic acid and oxalic acid were found to elicit the convulsion as Decadron induced convulsion, but such as malonic acid, fumaric acid, maleic acid, α-ketoglutaric acid, succinic acid, oxalacetic acid and acetic acid were not.

4) Therefore, it was suggested that citric acid conbined in a non ion "complex" with Ca or Mg which inhibited irritabillty of the mortor neuron. And on the other hand, dexamethasone played the roll of an activator for inducing convulsion. These reactions in the brain are the most impotant factors for inducing the convulsive seizure.

Chemical Factors in the Excitation of Isolated Mammalian Cerebral Cortex

HENRY McILWAIN

Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London (Great Britain)

The nervous system which a biochemist studies is very frequently that of a laboratory animal, and in finding his way about it the biochemist is almost certain to appreciate the guidance of Professor C. U. Ariëns Kappers or his co-workers. Thus when some years ago I searched for a description of the guinea pig brain, my colleagues and Professor Ariëns Kapper's monograph[1] led me to the extensive paper of A. B. Droogleever Fortuyn[3] which appeared in the *Archives of Neurology and Psychiatry* published from the Central Pathological Laboratories of the London County Council in 1914. The Central Pathological Laboratories were the immediate precursor of the laboratories at the Maudsley Hospital, which now house much of this and other Departments of the Institute of Psychiatry; Dr. Fortuyn had come from Amsterdam to work with Sir Frederick Mott, their founder, and he returned in 1911 to the Institute whose Jubilee we are now celebrating. Thus we have, in the London *Archives of Neurology and Psychiatry*, the published version of Dr. Fortuyn's thesis for D. Sc. in the University of Amsterdam on the cerebral hemispheres of rodents. This is largely a histological study, in which tribute is paid to the guidance of Professor Kappers.

Biochemical and histological workers have a common interest in maintaining the nervous tissue which they are handling, in a condition as close as possible to its condition in the living animal. Our studies have given additional chemical and electrophysiological criteria of normal cerebral tissues, and the investigations which I will describe concern one of these criteria. This is the ability of isolated cerebral tissues to respond to applied electrical pulses by change in their level of metabolism, which is conveniently measured by increased rate of respiration and glycolysis[5,6] though these are only two of very many changes so induced. Such studies have shown the marked fashion in which cerebral tissues deteriorate *in vitro* if they are not provided with an adequate environment. These studies have now been developed further by taking tissues whose excitability has been impaired through exposure to adverse environments, and attempting to restore their excitability by the addition to them

of further materials. Our object in doing this was to learn more about chemical factors involved in the excitation of cerebral tissues, and also to identify materials which might have some practical value in restoring damaged cerebral tissues.

The situations which have been examined most thoroughly from this point of view are summarized in Table I[4,8,9]. The experimental arrangements which are involved

TABLE I

RESPONSE OF CEREBRAL CORTEX TO ELECTRIC PULSES: ITS LOSS AND RESTORATION

Loss caused by:	*Agents most effective in restoration or antagonism*
1. Pulses in absence of glucose	Fumaric and malic acids McIlwain and Gore[8]
2. Pulses in absence of oxygen	Adenine and nicotinamide McIlwain[9]
3. Keeping in saline at 0°C	Blood plasma constituents Marks and McIlwain[4]

are as follows. When cerebral cortex from man or laboratory animals is prepared promptly after its removal, as thin slices and incubated at 37° C in buffered saline solutions, oxygenated and with glucose, electrical pulses applied to electrodes around the tissue cause its respiratory rate to approximately double, and its glycolytic rate to increase threefold. If however the saline lacks glucose, respiratory rate is lower and diminishes with time, and the pulses do not increase respiration. Instead, they induce a defect in the tissue, for if glucose is now supplied it is incapable of supporting the normal metabolic response to pulses. However, by examining many substances for their ability to restore response to tissue treated in this way, it was found that the addition of malic or fumaric acids with glucose permitted some metabolic response[8].

Tissues treated similarly but in the presence of glucose and absence of oxygen again became non-responsive[9], and exhibited new interrelationships with adenine and nicotinamide.

DEFECT INDUCED IN MEDIA AT 0°C

I would like to describe in more detail the result of preparing mammalian cerebral cortex in the same fashion, but of keeping it subsequently in a complete, glucose-containing medium at 0° C. When, following this treatment, the tissue is incubated at 37° C its resting respiratory rate in absence of applied pulses falls, but is relatively little affected when compared with the respiratory rate reached with applied pulses.

299

Fig. 1 illustrates how, by these criteria, the tissue's excitability has fallen considerably in 5 h and is almost completely lost after 17 h. Incorporation of a wide variety of known substances in the incubating saline of the second part of the experiment, failed to restore normal response to the tissue. However, a large measure of response was restored by the incorporation of blood proteins[4]. Sera of several animal species were active in this respect, and of plasma proteins a fraction IV-4 of Cohn *et al.*[2] was most potent in restoring response (Fig. 2).

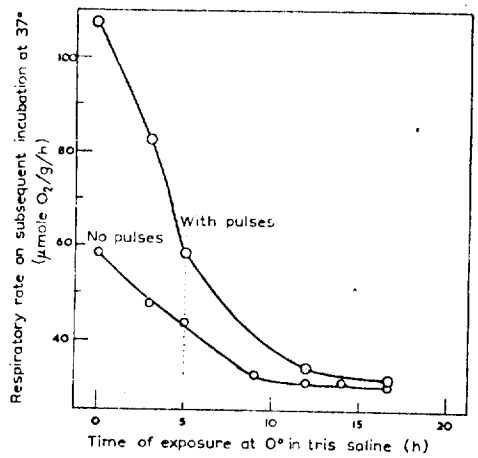


Fig. 1. Loss of response on keeping tissues at 0°. Pulses of exponential time-voltage relationships, peak potential gradient 3 V/mm, time constant 0.4 msec, were applied at 100/sec[4].

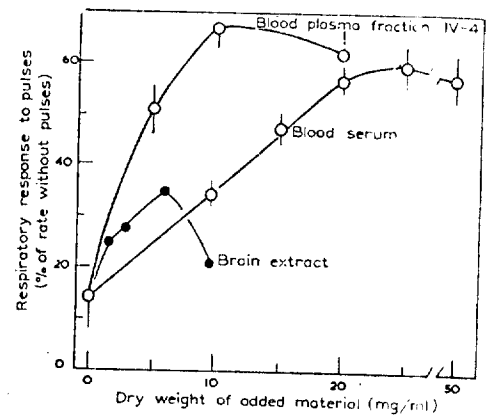


Fig. 2. Restoration of response to pulses. Guinea pig cerebral cortex after keeping 5 h in tris salines at 0°, was placed in electrodes and incubated in salines containing the added materials at 37°. Respiration was measured before and after applying electric pulses[4].

Curiously, blood serum did not prevent the development of the unresponsive state, which occurred on keeping the tissue in sera at 0°, just as it did on keeping it in salines. The serum or plasma fractions presumably undergo some interaction at a chemical level with a constituent of cerebral tissues, and an attempt was made to identify such a constituent by examining extracts of cerebral tissues[7]. To this end, the tissues were extracted in a variety of ways: by cold and hot solutions, neutral, acid, aqueous, non-aqueous, and of low and high tonicity. In this way a cold acid extract was found which formed a precipitate with the blood protein fraction IV-4. Moreover this extract, and not a number of others, caused loss of respiratory response to pulses when it was added to solutions bathing normal cerebral cortex. The inhibitory material did not easily dialyse and in view of this property and of its extraction by acids, a basic protein or lipid was suspected as responsible. A different method known to extract basic proteins was therefore applied: the salt-ethanol method for extracting histones, in which preparations are made 2.5 *M* in NaCl, and 2 vol. of ethanol added. This also produced an inhibitory extract which formed a precipitate with plasma fraction IV-4. Further, when the two methods of extraction were applied

to materials obtained by differentially centrifuging ground cerebral tissues, they yielded inhibitory extracts from nuclear preparations, and not from mitochondrial or the non-particulate portions.

BASIC PROTEINS AS INHIBITORS

Thus the search for a constituent of cerebral tissues capable of combining with plasma fraction IV-4 had shown that such constituents existed, and were probably histones; and indeed preparations of histones and protamines from other tissues are also capable of such combination. Moreover, the histone and protamine preparations were found to inhibit the metabolic response of cerebral tissues to electrical pulses (Fig. 3). Finally, tissues rendered unresponsive by added histones were, like those made unresponsive by keeping at 0° C, restored by plasma fraction IV-4 to a responsive state (Fig. 4).

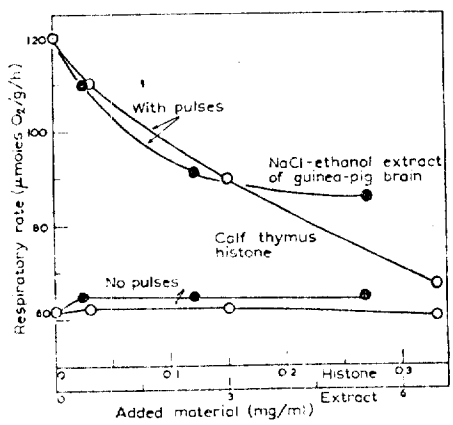


Fig. 3. Inhibition of response by a cerebral extract and by a histone. Extracts and histones were examined for action on guinea pig cerebral cortex incubated in glycylglycine – glucose media. No pulses: rates during the first 30 min incubation; pulses were then applied and the rates quoted are those during the last 30 min.

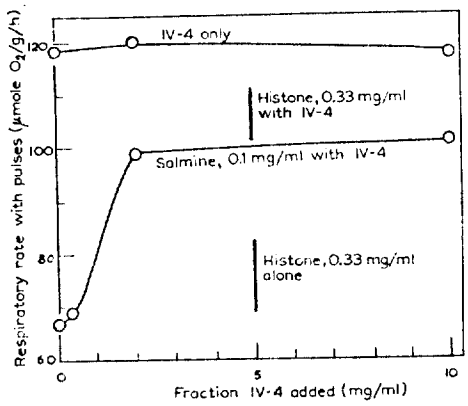


Fig. 4. Histone inhibition antagonised by a plasma protein. Guinea pig cerebral cortex slices were incubated with saline or histone, plasma protein fraction IV-4 was added 15 min later, and pulses applied at 40 min.

It is tempting, especially in the environment of the present meeting, to interpret these results in histochemical terms. Evidently cerebral tissues normally contain in the histone of cell nucleus a potent inhibitor of their response to pulses. The histone is sufficiently firmly bound to remain in the nuclei during their isolation from fresh tissue. Does the keeping at 0° permit its migration to other parts of the cell where it acts in the inhibitory fashion exhibited by externally added histone? In that case the keeping at 0° may cause the nucleus to become more basophilic, and acidophilic material may appear elsewhere in the cell. We hope also to approach such problems of migration by more directly chemical methods.

Reference p. 561

SUMMARY

Isolated mammalian cerebral cortex retains for some hours its ability to respond metabolically to electrical pulses, when maintained under appropriate metabolic conditions. However, at 37° C in media lacking either glucose or oxygen, or when kept at 0° C in otherwise adequate media, response to pulses is progressively lost. Maintenance or restoration of response under these adverse conditions has been achieved by incubation with media containing specific additional substances. In particular, the excitability of tissue rendered unresponsive by keeping at 0° C has been largely restored by a preparation of blood plasm proteins. In investigating their action, the protein preparation has been found to react with certain cerebral extracts which probably contained histones. These extracts, and also histones from other sources, are potent inhibitors of the response of cerebral tissues to electrical pulses. Inhibition by histone or protamine preparations can be antagonised by the plasma protein fraction. Histochemical mechanisms are proposed for these phenomena.

REFERENCES

1 ARIËNS KAPPERS, C. U., *Anatomie Comparée du Système Nerveux*, Bohn, Haarlem, 1947.
2 COHN, E. J., L. E. STRONG, W. L. HUGHES, D. J. MULFORD, J. N. ASHWORTH, M. MELIN AND H. L. TAYLOR, *J. Amer. chem. Soc.*, 68 (1946) 459.
3 DROOGLEVER FORTUYN, A. B., *Arch. Neurol. Psychiat. (Lond.)*, 6 (1914) 221.
4 MARKS, N. AND H. MCILWAIN, *Biochem. J.*, 73 (1959) 401.
5 MCILWAIN, H., *Biochem. J.*, 49 (1951) 382.
6 MCILWAIN, H., *Biochemistry and the Central Nervous System*, Churchill, London, 1959.
7 MARKS, N. AND H. MCILWAIN, *Biochem. J.*, 73 (1959) 401.
8 MCILWAIN, H. AND M. B. R. GORE, *Biochem. J.*, 54 (1953) 305.
9 MCILWAIN, H., *Biochem. J.*, 63 (1956) 257.

DISCUSSION

E. DE ROBERTIS: There are observations in other tissues such as muscle, that when immersed in plasm they do not gain sodium. It is not known whether there is a substance which prevents this or just th proteins in the plasma, but the effect is probably very similar to yours. In your case the sodium getting in and this lowers the metabolism.

H. MCILWAIN: I think it is very likely that ion movements may be involved in the unresponsive sta induced by keeping cerebral tissues in cold media and also in the unresponsive state induced b adding histones or protamines as such. We have not, however, any direct measurements at presen though that is one aspect which we hope to study further. The observations which you describe wit muscle differ from those made with cerebral tissues, in that the unresponsive state develops in cerebr tissues which have been kept cold even in the presence of blood plasma or plasma-containing fluid

J. FOLCH-PI: I wonder whether the membranes, being a part of the dynamic system of the tissue, not require an active metabolism for maintaining their selective permeability. You might be observi the change in permeability, a change brought about in a way not yet known.

H. WAELSCH: I wonder whether you have a real inhibition or just a breakdown of some kind of compartment.

H. MCILWAIN: I certainly agree with the relevance of Prof. Folch-Pi and Prof. Waelsch's commen The unresponsive condition induced in cerebral tissues in cold media does not come about only

its ability to respond
ppropriate metabolic
e or oxygen, or when
s is progressively lost.
conditions has been
tional substances. In
keeping at 0° C has
s. In investigating their
ertain cerebral extracts
histones from other
es to electrical pulses.
gonised by the plasma
r these phenomena.

. Haarlem, 1947.
ASHWORTH, M. MELIN AND

4) 221.

ll, London, 1959.

at when immersed in plasma
ich prevents this or just the
In your case the sodium is

ed in the unresponsive state
responsive state induced by
ct measurements at present,
ons which you describe with
ve state develops in cerebral
or plasma-containing fluids.

mic system of the tissue, do
lity. You might be observing
known.

eakdown of some kind of a

Prof. Waelsch's comments.
not come about only by

loss of material from tissue to fluid without further change, for response is not adequately restored by adding material leached from the tissue. External fluid is nevertheless required for development of the unresponsive state. The minimum volume needed, however, is no more than that of the tissue itself: enough, probably, to allow swelling and some of the structural changes which have been described by earlier contributors to this symposium.

E. ROBERTS: Do you use your usual saline? Is the one you do the measurements in subsequently the one you put the tissue in initially?

H. MCILWAIN: The unresponsive state develops in tissue kept in the cold in a variety of fluids, from balanced physiological salines with glucose to those with other substrates and with a variety of added materials. In ordinary glucose-containing media, the condition is exhibited when the tissue is examined in the batch of medium in which it has been kept in the cold (some 60 mg of tissue in 3.5 ml fluid) and also in fresh medium. The latter was the usual experimental arrangement.

344

EFFECT OF SOME METABOLIC AGENTS ON NERVOUS ACTIVITIES

YUTAKA OOMURA AND SABURO HASHIMURA*

Department of Physiology, Faculty of Medicine, Kyushu University, Fukuoka

In previous studies energy-rich phosphate compounds were found to be immediately effective in enhancing nervous activities indicated in such measurements as those of the resting potential, threshold, accommodation constant, excitatory state and oxygen consumption (15 *a*), and moreover, adding pyruvate was found to be capable of furnishing energy by its oxidation in enhancement of these activities (15 *b*). The actions of these substances seem to indicate that the oxidative metabolism of nerve fiber might be closely related to nervous activities, and that the occurrence of the Krebs cycle in the metabolism of this tissue might be promoted. The elevation of the excitability of a nerve fiber, therefore, might run parallel with the increment of oxygen consumption, although only pyruvate was tested in that case.

After the first attempt of Gerard (9 *a*, 9 *b*), Myerhof and Schulz (13) reported on the oxygen consumption of nerve tissue, and the problem of the relation between the oxidative metabolism of nervous tissue and its function seems to have attracted more and more attention. The importance of the Krebs cycle in considering nervous activities in their resting and acting states, has been suggested by many authors (4, 12, 15, 16 *a*, 16 *b*).

The present investigation was undertaken with the purpose of revealing the effect of substances having well-defined relations with the metabolism on nerve fibers, indeed, of investigating the ability of the compounds belonging to the Krebs cycle to serve as useful agents on nervous functions.

METHODS

As described in the previous reports (15 *a*, 15 *b*) a single motor nerve fiber in the sciatic nerve of the *Bufo vulgaris Japonica* was separated from other nerves and mounted on two separate glass plates filled with Ringer solution ("bridge-insulator" Tasaki (17)). Over the gap (*ca* 0.5 mm.) between the glass plates an internodal stretch of the fiber was mounted and connected in Zn-$ZnSO_4$-Ringer agar type, to the stimulating- and recording circuit or to the resting-potential measurement circuit. Action currents from a single nerve fiber were amplified with a three stage R-C amplifier leading to a cathode-ray oscillograph.

The electrical stimulation used was a brief condenser-discharge current which was produced by thyratron synchronizing with the voltage of the sweeping circuit.

Received for publication August 31, 1953.

* 大村 裕 橋村 [illegible]

The resting potential was measured by the vibrating capacity method (the sensitivity was ±0.3 mV) with a modified surface-potential measuring circuit (fig. 1). The liquid junction potential and electrode potential can be eliminated when this apparatus is used, for we can obtain the net resting potential by deduction of the above-mentioned potentials when the two pools are bridged with cotton wool containing Ringer solution.

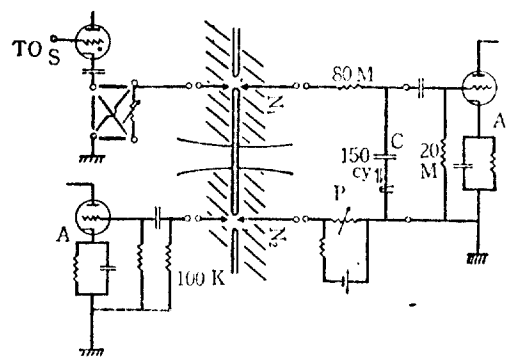


FIG. 1. Arrangements for recording the action current and the resting potential of single motor nerve fiber. The resting potential is measured by the circuit shown in the right diagram, *P* is a Leeds and Northrop type potentiometer, *C* consists of two copper disks of 3 cm. diameter, one of which vibrates with a frequency of 150 cycles per second, *A* is a 3 stage R-C coupled amplifier. The threshold is measured with the left circuit in this figure, and this circuit applying a condenser discharging current which is produced by thyratron synchronizing with the voltage of the sweeping curcuit. The Ranvier nods are denoted as N_1 and N_2.

By changing the circuit connecting with the input of the amplifier, the threshold and the resting potential are alternately measurable.

The measurement of the oxygen consumption of nerve fibers was made by the same procedure reported in the preceding paper (15 *b*).

Succinate, fumarate, malate, aspartate, malonate and monoiodacetate, are diluted with Ringer solution to proper concentrations before using and we added also to the solution $NaHCO_3$ in order that the *p*H is 7.2–7.4. α-ketoglutarate, citrate and glutamate as sodium salts are also used, dissolved in Ringer solution of *p*H 7.2–7.4. *p*H determination was carried out by the qinchdron electrode.

In using the compounds belonging to Krebs cycle, the formation of the Ca (citrate)- and other Ca-complexes must be expected because of the appreciable amounts of Ca ion removal by these compounds and the effect of the loss of Ca-ion from the medium on the nervous activities. Therefor we have always succeeded by the addition of $CaCl_2$ solution in maintaining the Ca ion concentration within 15 % of the original level. Owing to the idea of Furchgott and co-workers (8) the required amounts of $CaCl_2$ were calculated, and the *p*K and *p*H values of each test solutions also.

The experiments were made under temperatures ranging from 25° (in autumn) to 8° C. (in winter).

RESULTS

Because the threshold of either side of the Ranvier node in the Ringer pool was kept constant for 30–40 minutes, we could compare the threshold in the test node with a normal one, when the replacement of Ringer solution in one pool by the test solution has taken place.

In general, the threshold of the test node decreased to a certain minimum value within a few minutes after the replacement of the Ringer pool by one of the compounds of the krebs cycle, and this minimal threshold was maintained for the following 40–60 minutes; the resting potential of the same node became also positive with the change of the threshold.

In order to ascertain whether these decreases in the threshold were effected by an increase of ionic concentration, especially in sodium ion concentration, a series of control experiments were made in which 0.1 M NaCl solution was added to the Ringer solution so as to keep the concentration at a degree ranging from 10^{-3} to 2×10^{-2} M per liter. In these experiments the threshold were first unchanged by the increase of ionic concentration, producing no observable effects upon the resting potential. These results indicate that the above-mentioned phenomena were unlikely to be due to the rise in the ionic concentration of the medium.

a) The effects of Krebs cycle compounds

Succinate, fumarate and malate: An addition of succinate caused a significant drop in the threshold. Fig. 2, shows the decrease of the threshold of the nerve fiber obtained in a series of measurements, and it may be noticed in this figure a threshold decrease of 22.5% within a few minutes after the replacement of one pool with 2×10^{-2} M succinate-Ringer solution; it attained a minimum afterwards. This minimum was a 45% decrease and was maintained for at least the succeeding 60 minutes.

The concentration of 2×10^{-2} M was usually used except in a few cases, in order to obtain the maximal effects on the threshold and the resting potential.

The latency, the height and form of action currents in the test node were observed to be unchanged during the treatment with succinate.

On the other hand, the result in the contrast Ringer node was a decrease by 14.5% in the threshold. The threshold in the contrast node was, as a rule, not affected at all as reported in our previous paper (15 *a*).

As we shall find in the resting potential a negative potential, namely the anelectrotonus, is produced in this contrasted node in the course of time, and therefore the noticed fall of the threshold cannot be explained in terms of the electrotonus, but we suppose that either the enhancement of metabolism at the test node has affected in some way the controlling node or the diffusion of succinate molecules to this node.

It cannot be doubted that the resting potential is affected by the test solution, *i.e.* succinate in the same concentration produces in this node a positive potential of about 3 mV, which gradually attains its values within several minutes and goes with a change in the threshold. A like phenomenon was noticed in the case of the effect of pyruvate, as shown in our preceding study (15 *b*).

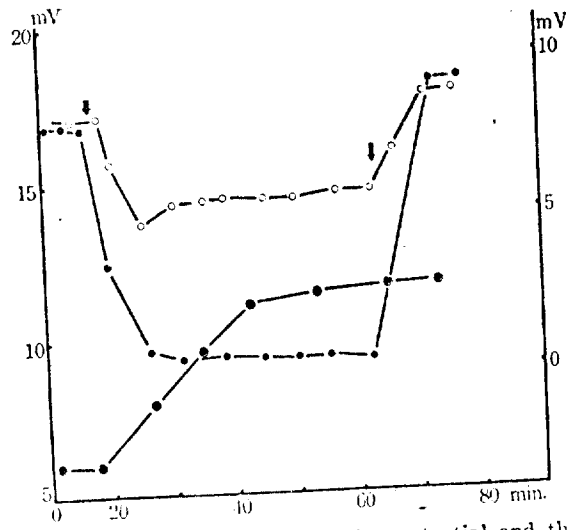


FIG. 2. A series of measurements of the resting potential and the threshold during a certain period after the addition of the 2×10^{-2} M succinate-Ringer solution to N_1 Ranvier node. The test solution was applied at the first arrow, and removed at the second one. The filled or empty circles show the threshold values in the test node or the normal Ringer node. The double circles indicate the resting potential. The right and left ordinates indicate the resting potential and the threshold. 24° C.

When the test solution was removed the threshold value overshot the original one by about 10%.

Further addition of inhibitors such as 2-4 dinitrophenol and monoiodacetate in the same concentration as accelerators after removal of accelerators, is followed by an increase in the threshold.

The ratio of change between the threshold and the resting potential, being almost constant, is 15 : 1.

With an equal concentration in the medium, fumarate is roughly about 80% as effective as succinate in decreasing the threshold of the fumarate treated node. The double response given at the test node by a brief condenser shock is recognized in a few cases 20 or 25 minutes after addition of fumarate. This double response ceases with elevation of the threshold, due to replacement of the test solution with Ringer solution.

Fumarate has the same type of effect as succinate on the threshold in the neighbouring node, but its effectiveness was half that of succinate.

Fumarate produces a positive potential of the mean value of 2.3 mV in the test node in the same manner as succinate. In this case the ratio in change of the threshold to the resting potential is 15 : 1.

When malate in the same 2×10^{-2} concentration was added to the test node, the responses caused by this compound were similar to those given by succinate and fumarate except that the former brought about a marked enlargement of the form of the spike. The mean decrease of the threshold was about 83%

and the mean drift to positive in the resting potential was 7.3 mV. The ratio of change in the threshold to the resting potential is 12:1. In the case of malate which has a somewhat higher value of pK_2 an appropriate amount of $CaCl_2$ (see Methods) was added to the test solution to keep the Ca ion concentration constant.

α-Ketoglutarate and citrate: α-ketoglutarate and citrate were also examined regarding their effectiveness, they were found to be less effective than succinate, fumarate and malate; however, it was found with α-ketoglutarate that the acceleration, if it took place, was greater at a lower concentration than at 10^{-2} M. In the case of α-ketoglutarate, the mean degree of threshold lowering which caused an enlargement of the spike height was 16.7 % and positive resting potential 6 mV; the concentration, in the test node in this case was 10^{-3} M, and the ratio of change between the threshold and the resting potential was 2.8:1. Further addition of the substance in 10^{-2} M concentration induced an appreciate 72 % supplementary increase of the threshold and a fall of the resting potential (about −18 mV).

As was investigated in the previous study (15 *b*) the threshold and the resting potential were closely related to the oxygen consumption of nerve fibers. Measurement of the oxygen consumption was also made to find whether a like phenomenon could be detected as in the foregoing cases. The results are shown in table 1. Each number represents the mean value obtained in three experiments. With α-ketoglutarate a relationship may be found between the oxygen consumption and the concentration, but the concentration cannot have a like relation to the electrical activities, because an increase of the threshold occurs in 10^{-2} M concentration.

TABLE 1. The Oxygen Consumption of the Sciatic Nerve Fiber of Toad in α-Ketoglutarate-Ringer Solution
Temperature in thermostat 22° C.

(min.)	10^{-2} M (cc/g.)	Contrast (cc/g.)	10^{-2} M (cc/g.)	Contrast (cc/g.)
5	32.5	4.6	65.0	5.0
10	12.5	2.8	35.9	5.7
15	7.2	2.8	16.9	2.3
20	7.2	8.6	9.5	8.5
25	4.0	2.9	5.2	3.5
30	4.9	6.5	7.9	7.5
40	5.4	7.1	2.1	7.3
50	5.7	7.9	4.7	6.4
60	10.3	17.4	6.3	12.1
80	10.3	8.9	11.6	12.1
100	11.5	11.5	9.5	12.8
120	8.9	8.9	5.2	8.4
Total	120.4	89.9	180.2	91.6

Citrate in 2×10^{-2} M concentration with excess Ca ions (see Method) had an effect on the threshold, decreasing it to 19 %. The resting potential was also

1.5 mV positive at this node. The ratio of change between the threshold and the resting potential was 13 : 1.

The contrast node was not affected by α-ketoglutarate or citrate, but its threshold was kept constant. The above-described phenomena were all reversible when the test solution was removed.

b) The effects of some other compounds related to the Krebs cycle

Among amino acids produced oxidatively through the Krebs cycle may be found aspartic acid and glutamic acid, and the relation between glutamic acid and the action of the brain tissue has been well studied by Gerard and collaborators (1, 2, 5), reporting oxidation of glutamate in axoplasma contained in the white matter of the rat spinal cord.

The effect of glutamic acid and aspartic acid on the threshold and the resting potential of Ranvier's node was demonstrated in our experiments. In this case, the concentration of glutamic acid was from 10^{-4} M to 10^{-2} M. The threshold values were decreased by 8.5-17% within a few minutes, after the addition of the test solution in 5×10^{-4} M–10^{-4} M concentration, but with more increase in the concentration, the threshold rose, on the contrary, *viz.*, when the concentration rose to 10^{-3} M the threshold was unchanged or increased by 8%, and increased by 15-20% when it mounted to 10^{-2} M. On the other hand, the threshold in the normal node side was kept constant or increased slightly in course of time.

Aspartic acid was more effective on the threshold and on the resting potential than glutamic acid. A 30-40% decrease in the threshold occurred when the concentration of this acid was 10 M, and the increase of the resting potential was 10 mV in the same concentration.

c) The effect of metabolic inhibitors

Monoiodacetate, 2-4 Dinitrophenol, sodium azide and malonate: It is practically clear that these substances attack the enzyme system and that they have also inhibitatory actions on nervous activities (4, 12). Our experiments have made it practically certain that these substances acted on the threshold and the resting potential in the opposite direction compared with the action of accelerators. As shown in fig. 3 *A*, in which a most typical one of the numerous measurements is shown, the threshold in the test node greatly rose with a precipitous slope and the resting potential drifted negativeward in 2×10^{-2} M concentration. Monoiodacetate, 2-4 dinitrophenol and sodium azide were included in this group. The thresholds were elevated to over 100% by monoidacetate or 2-4 dinitrophenol in 2×10^{-2} M, but the resting potential drifted toward the negative side by −10 and −5 mV. Sodium azide was included in this class in action, though its effects were rather smaller than those of monoiodacetate and dinitrophenol, that is, the rise of the threshold was about 60% and the resting potential drifting about −3 mV negativeward. These inhibitors did not affect the contrast node. In the case of malonate the action, as shown in fig. 3 *B*, was somewhat different from those of the abovementioned type, namely, the threshold was not affected or slightly decreased by its invasion despite a prolonged latency. Moreover the positive resting potential of about 10 mV was

also produced. Now we cannot as yet tell directly from the results of our experiment why malonate is different from other inhibitors in its effect on nervous activities, and further study is needed of this compound. On the other hand, at the neighbouring node immersed in the Ringer solution, the threshold was about 10% raised when malonate was applied to the test node.

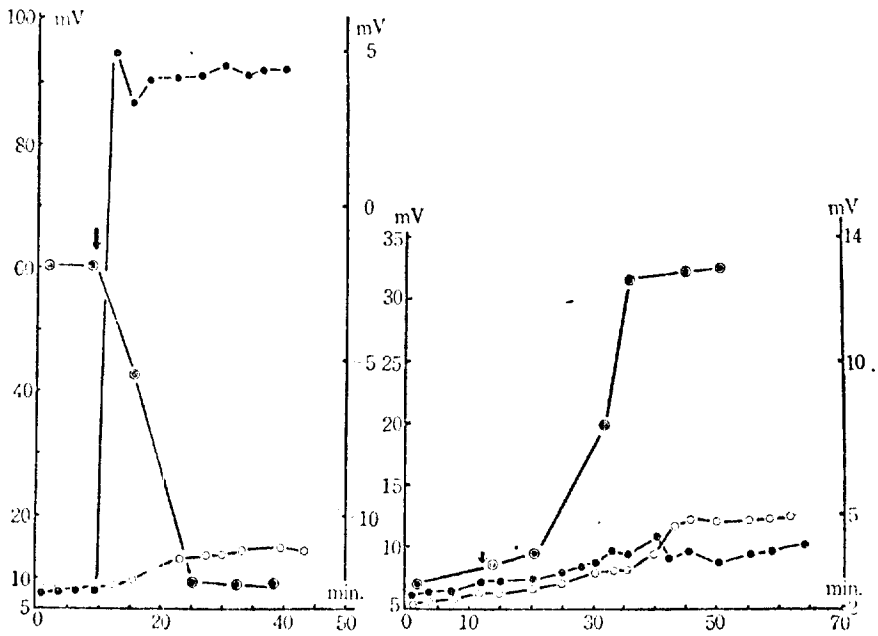


FIG. 3. A series of measurements of the resting potential and the threshold resulting form inhibitors. The test solution was added at the arrow. The right and left ordinates indicate the resting potential and the threshold.

A: In the case of 2×10^{-2} M 2-4 dinitrophenol. 23° C.

B: In the case of 2×10^{-2} M malonate. 23° C.

DISCUSSION

The present research has made it clear that, under experimental conditions, succinate, α-ketoglutarate, fumarate, malate, citrate, glutamate or aspartate may be utilized by a nerve fiber for the production of energy for its activities. These findings constitute strong evidence that the Krebs cycle plays a major rôle in nervous activities. It is already clear that all compounds of the Krebs cycle used in the present experiments have an ability to promote the rate of aerobic metabolism just as the substances metabolized through the Warburg-Keilin system from which the energy-rich phosphate compounds are produced in other tissues. It is thus manifest that various hydrogenases and other enzymes needed for tissue metabolism are contained in axoplasm (14, 1, 2, 5).

As shown in the previous papers (15 *a*, 15 *b*) and examined in the present research, the enhanced oxygen consumption, the positive resting potential and

a decrease of the threshold are caused by the action of the substances in question; denoting a parallelism between oxygen consumption and electrical activities of nerve fibers, only in the case that there exists not only an increase in oxygen consumption but the production of energy-rich phosphate compounds through the Krebs and Warburg-Keilin system.

The compounds used were found to have differences in their effects on the resting potential and threshold in the same concentration, and the fact may be due to the dissimilar rate of penetration of these substances into the nerve fiber at the concentration used. However, the ratio of change between the threshold and the resting potential was constant at 12–15 when some accelerators, *viz.*, succinate, fumarate and malate were added to act on the test node.

The nature of the resting potential of nerve fibers has been studied using various metabolic substances, in order to discover the relationship between nervous activities and nervous metabolism (4, 6, 12, 16 *a*, 16 *b*, 16 *c*). In our experiments, for the analysis of the resting potential, we used a method devised after the idea of Joseph and coworkers (11). The potentials of the Ranvier node on either side (N_1, N_2) are denoted as E_{N_1} and E_{N_2} respectively, E_{N_1} evidently varying under different conditions of experiments caused by the influence of inhibitors or accelerators on one node N_1. The resting potential measured was given in the fomula:

$$E_R = E_{N_1} - E_{N_2}$$

The resting potential measured under the action of accelerators was in this case drifted by several mV to the postive, and likewise to the negative due to the influence of inhibitors. Taking the redox potential in the Warburg-Keilin system into acount, E_N means differences of potential between the source and terminal in the cytochrome system, and the effect of accelerators seems to be represented as the source potential of partially reduced cytochrome *b*, the terminal potential remaining at or near its normal level. If the value of E_{N_1} is enlarged, then E_R becomes positive. The action of inhibitors, in general, is assumed to decrease the terminal potential, *viz.*, E_{N_1} under the effects of inhibitors is reduced, and E_R is made negative.

On the other hand, the resting potential is explicable as diffusion potential induced by the shift of potassium ions in and out of the fiber (10 *a*, 10 *b*, 3). As is known (7, 18), the metabolic substance affects the accumulation of potassium inside the nerve fiber, and the accelerators probably stimulate its accumulation. It is thus presumable that its diffusion potential effected by accelerators becomes somewhat high in consequence of the increase of the inside potassium, and E_{N_1} also becomes higher than E_{N_2}.

However, we are not yet in a position to decide at once which is the better explanation of the result. We have to make many more experiments before reaching any final decision on the subject.

SUMMARY

The threshold and the resting potential of a node of a single motor nerve fiber under the action of various agents were compared in a toad with those of

a neighbouring node immersed in Ringer solution.

The following are the results of the experiment:

1) The decrease of threshold and the positive resting potential is produced in the test node when one of such compounds of the Krebs cycle as succinate, fumarate, malate, α-ketoglutarate and citrate (0.02 M) are introduced to the test node; the contrast node being also somewhat affected with a decrease of the threshold in the case of succinate, fumarate and malate. Of these compounds, α-ketoglutarate was also examined as to its effects on the oxygen consumption of nerve fibers. The oxygen consumption is found to be enhanced and the results obtained seem to indicate that nervous activities are closely related to oxidative metabolism.

2) Besides the compounds of the Krebs cycle, such amino acids as glutamic and aspartic acid, have also effects as accelerators on nervous activities.

3) All metabolic inhibitors used, except malonate, have the reverse action compared with that of accelerators, *viz.*, increase of the threshold and a negative potential is produced in the test node by the action of the inhibitors, and the contrast node immersed in Ringer solution is not affected.

4) It is conceivable that the resting potential measured under various metabolic conditions may be taken as the results of oxidoreduction of nerve fibers when the resting potential represents the balance of potentials between the two neighbouring nodes.

The results of the present experiments constitute strong evidence that the Krebs cycle plays a major rôle in the intermediary energy metabolism of nerve fibers.

Grateful acknowledgment is due to Professor A. Seo and Professor N. Toida for their helpful advice and criticism and their reading of this paper in manuscript.

REFERENCES

1. ABOOD, L. G., R. W. GERARD, J. BANKS AND R. O. TSCHIRGI. *Am. J. Physiol.* 168: 728, 1952.
2. ABOOD, L. G. AND R. W. GERARD. *Am. J. Physiol.* 168: 739, 1952.
3. *Annual Review of Physiol.* 9: 477. Annual Reviews, Inc. and the American Physiological Society, 1947.
4. BOYARSKY, L. L., A. D. ROSENBLATT, S. POSTEL AND R. W. GERARD. *Am. J. Physiol.* 157: 291, 1949.
5. BROOKS, V. B., R. E. RANSMEIER AND R. W. GERARD. *Am. J. Physiol.* 157: 299, 1949.
6. CONWAY, E. J. *Science* 113: 270, 1951.
7. FENN, W. O. AND R. GERSCHMAN. *J. Cell. Comp. Physiol.* 33: 195, 1950.
8. FURCHGOTT, R. F. AND M. R. WALES. *Am. J. Physiol.* 169: 326, 1952.
9. *a*: GERARD, R. W. *J. Physiol.* 62: 349, 1927.
   b: GERARD, R. W. *J. Physiol.* 63: 280, 1927.
10. *a*: HUXLEY, A. F. AND R. STÄMPFLI. *J. Physiol.* 112: 476, 1951.
    b: HUXLEY, A. F. AND R. STÄMPFLI. *J. Physiol.* 112: 496, 1951.
11. JOSEPH, N. R., C. I. REED, E. KAPÜAN AND I. E. STECK. *Am. J. Physiol.* 157: 63, 1949.
12. LORENTE DE NÓ, R. *A Study of Nerve Physiology* I, II. The Rockefeller Institute for Medical Research. N.Y., 1947.
13. MYERHOF, O. AND W. SCHULZ. *Biochem. Z.* 206: 158, 1929.

14. NACHMANSOHN, D. *Metabolism and Function.* Houston: Elsever Press, IAC, 1950.
15. *a*: OOMURA, Y. AND S. HASHIMURA. *Jap. J. Physiol.* 3: 107, 1953.
b: OOMURA, Y. AND S. HASHIMURA. *Jap. J. Physiol.* in press, 1953.
16. *a*: SHANES, A. M. *J. Cell. Comp. Physiol.* 19: 1, 1942.
b: SHANES, A. M. *J. Cell. Comp. Physiol.* 19: 249, 1942.
c: SHANES, A. M. *J. Cell. Comp. Physiol.* 38: 17, 1951.
17. TASAKI, I. *Am. J. Physiol.* 125: 367, 1939.
18. VAN HARREVELD, A. *J. Cell. Comp. Physiol.* 38: 199, 1951.

SUMMARY

ibino rats appear to be less susceptible to
mococcus than litter mates receiving the

is observation and our earlier demonstration
enic acid by sulfapyridine in normal rats (1)
ction of this drug lies in its ability to inhibit
ic acid in the tissues and body fluids of the
er available for the growth of the micro-
ite is essential for the pneumococcus of the
ts, the growth is retarded giving the familiar

REFERENCES

., AND RIVERA, R. E., *J. Nutrition* 25, 471 (1943).
J. Biol. Chem. 134, 455 (1940).
ND SAMPSON, W. L., *J. Nutrition* 22, 553 (1941).

288

The Influence of Various Chemicals and Vitamin Deficiencies on the Excretion of Glucuronic Acid in the Rat

Gustav J. Martin and Wolfram Stenzel

From the Warner Institute for Therapeutic Research
New York City

Received August 28, 1943

INTRODUCTION

Certain aliphatic alcohols, aldehydes, ketones, terpenes, phenols, hydrocarbons, certain amines, aromatic acids, and many heterocyclic nitrogen compounds are detoxified by conjugation with glucuronic acid (1, 2, 3, 4). Many of the compounds thus detoxified are medicinal agents (5, 6, 7). It has been suggested that glucuronic acid might be used clinically to decrease the toxicity of sulfonamides (8) but the difficulty of preparing glucuronic acid in any degree of purity precludes the practical application of this procedure. It was therefore deemed advisable to seek a simpler chemical which would increase the formation of glucuronic acid in the body.

PROCEDURE

Glucuronic acid (GA) content of the urine was determined by the use of naphthoresorcinol according to the technique of Maughan, *et al.* (9). As pointed out by Bueding (10) limited accuracy is achieved in applying any naphthoresorcinol method for GA. Some substances (glucose, etc.) inhibit the color development; others (mucic acid) tend to increase it. To circumvent this lack of specificity in method, the positive experiments were checked using the method of Quick (11) and the newer method of Bueding (12). It is felt by the present authors that the napthoresorcinol method can be safely applied to a dilute medium such as urine. This is in accord with the reports of other groups (13, 14, 15, 10). Meyer, *et al.* (15) stated, "None of the methods available gave accurate results for uronic acids in native fluids, with the possible exception of normal urine, since carbohydrates and other compounds containing carbonyl groups as well as proteins and their decomposition products interfered." In the experimental studies here reported, if, for example, mucic acid was being tested for its effect on GA excretion, a check was made using a solution containing an amount of the mucic acid equal

to the amount of GA determined. If at this concentration, the mucic acid gave a significant color, the series was discarded. Glucurone, prepared by the method of Williams (16) was used as the standard for all determinations.

All other chemicals unless specified were chemically pure reagent grade. The animals were housed in metabolism cages and urine samples collected under toluene for 24 hour periods after the oral administration of the chemical under test. Glass wool and special filtering devices prevented contamination of the urine samples.

TABLE I

The Effect of Various Compounds on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hr. period)

Compound	Dosage	Glucuronic acid excretion					
Control	*g./kg.*	*80 mg./kg. Av. value for 15 sets of 12 rats each. Max. 95.2 mg./kg., Min. 73.4 mg./kg.*					
Dihydroxyacetone	4	129.0	104.5				
Glycerine	4	168.0	153.0	111.0	135.0		
Lactic Acid	2	117.0	98.0	119.0	140.0	127.0	91.0
Ca Glycerophosphate	4	109.0	60.0	44.0			
Pyruvic Acid	2	24	37	58	127	27	
Acetic Acid	2	55	105	81	118	58	
Glycolic Acid	2	65	101	85			
Na Ethyl Oxalacetate	2	62	92	73	11	96	
Tartaric Acid	2	129	53	41			
Succinic Acid	4	104	156				
Malic Acid	4	231	178				
Fumaric Acid	4	69	83				
Citric Acid	6	86	99	80	98		
Saccharic Acid	4	132	125				
Glucurone	4	94	105				
Ca Phytate	4	58					
Glycerine + Malic Acid	4 ea.	106					
Adenylic Acid + Malic Acid	0.5 & 4	210					
Adenylic Acid	0.5	134	158				
Nucleic Acid	4.0	92	108				

Each value represents the average for 10 sets of 2 rats in each set.

Results

Table I presents the results obtained with carbon compounds. Included in this table are adenylic acid and nucleic acid.

Dihydroxyacetone, glycerine, and lactic, succinic, saccharic, malic, and adenylic acids increase GA excretion in the urine. Pyruvic acid decreased excretion of GA. It is believed that acetic acid, calcium gly-

If at this concentration, the mucic acid gave a
arded. Glucurone, prepared by the method of
dard for all determinations.
ied were chemically pure reagent grade. The
ages and urine samples collected under toluene
ministration of the chemical under test. Glass
revented contamination of the urine samples

TABLE I

ds on Glucuronic Acid Excretion in the Rat
g. of rat for a 24 hr. period)

	Glucuronic acid excretion					
	80 mg./kg. Av. value for 15 sets of 12 rats each. Max. 95.2 mg./kg., Min. 73.4 mg./kg					
	129.0	104.5				
	168.0	153.0	111.0	135.0		
	117.0	98.0	119.0	140.0	127.0	91.0
	109.0	60.0	44.0			
	24	37	58	127	27	
	55	105	81	118	58	
	[illegible]	101	85			
		92	73	11	96	
	129	53	41			
	104	156				
	231	178				
	69	83				
	86	99	80	98		
	132	125				
	94	105				
	58					
a.	106					
4	210					
	134	158				
	92	108				

erage for 10 sets of 2 rats in each set.

Results

its obtained with carbon compounds. In-
rlic acid and nucleic acid.
rine, and lactic, succinic, saccharic, malic,
GA excretion in the urine. Pyruvic acid
It is believed that acetic acid, calcium gly

erophosphate, glycolic acid, sodium ethyl oxalacetate, tartaric acid, and calcium phytate also decreased GA excretion, but variability of results prevents a final statement. Fumaric acid, citric acid, glucurone, and nucleic acid had little effect.

Of the amino acids tested, histidine, glycine, and alanine decreased GA excretion. Tyrosine and cysteine increased the excretion of GA. Cystine had no effect. Table II presents the results obtained. The results with ammonium chloride and sodium bicarbonate are included to show the effect of disturbance in alkaline acid balance.

TABLE II

The Effect of Various Amino Acids on Glucuronic Acid Excretion in the Rat
(Values in mg./kg. of rat for a 24 hour period)

Compound	Dosage	Glucuronic acid excretion				
	g./kg.					
Histidine	2	56	76	54	74	
Cystine	4	94	63	94		
Glycine	8	73	35	60	48	51
Tyrosine	4	159*	130	151	190	141
Cysteine Hydrochloride	4	128	123			
Alanine	4	48	61			
Ammonium Chloride	2	46	61	55		
Sodium Bicarbonate	2	51	80	58	51	

* Rats receiving stock diet + 10% *l*-tyrosine.
Each value represents the average for 10 sets of 2 rats in each set.

With the exception of riboflavin deficiency, all the vitamin deficiency states resulted in a marked decrease in the excretion of glucuronic acid. Table III lists the results and includes a value showing the effect of 48 hour starvation on the excretion of the acid. All values presented are for rats taken in a stage of a given vitamin deficiency before they became moribund and while their food consumption was still adequate to prevent the intervention of the starvation factor.

With the sulfonamides, the results were variable. In acute experiments all tended to increased GA excretion excepting sulfapyridine. Sulfaguanidine was tried in a chronic experiment in which it constituted 2% of the diet. Under these conditions the rats after two months on the diet showed a marked drop in the excretion of the organic sugar acid.

TABLE III

The Effect of Various Vitamin Deficiencies on Glucuronic Acid Excretion

(Values expressed in mg./kg. of rat for a 24 hr. period)

Deficiency	Urinary glucuronic acid content					
Pantothenate	92	24	54			
B_1 Thiamine	29.9	45.8				
B_2 Riboflavin	114	39	77	107	103	127
D	21.9	33.8	48	105		
A	43.3	80	27			
E	2.3	17.7	15.2	44		
B_6 Pyridoxin	38	33				
Starved 48 hrs	20					

Each value represents the average of 10 sets of 2 rats each.

TABLE IV

The Effect of Various Sulfonamides on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hour period)

Sulfonamide	Dosage	Added compound	Dosage	Glucuronic acid in urine				
	g./kg.		*g./kg.*					
Sulfanilamide	2				83	118	84	110
Sulfanilamide	2	Pyruvic Acid	4	76				
Sulfanilamide	1	Glucurone	2	80	198			
Sulfanilamide	2	Lactic Acid	4	75				
Sulfanilamide	2	Sodium Acetate	4	37				
Sulfanilamide	2	Malic Acid	4	94				
Sulfapyridine	4			59	31	28	34	
Na Sulfathiazole	1			210	134			
Sulfadiazine	6			97	60			
Sulfaguanidine	2% diet, 2 months			11.5	28.8			

Each value represents average for 10 sets of 2 rats each.

Tests conducted with sulfanilamide plus a compound known to produce an effect, positive or negative, on the excretion of GA showed that the sulfonamide tended to return all values to a more nearly normal figure.

TABLE III

:. Deficiencies on Glucuronic Acid Excretion

:. mg./kg. of rat for a 24 hr. period)

	Urinary glucuronic acid content					
	92	24	54			
	29.9	45.8				
	114	39	77	107	103	127
	21.9	33.8	48	105		
	43.3	80	27			
	2.3	17.7	15.2	44		
	38	33				
	20					

:erage of 10 sets of 2 rats each.

TABLE IV

:nid :n Glucuronic Acid Excretion in the Rat

kg. at for a 24 hour period)

Added compound	Dosage	Glucuronic acid in urine				
	g./kg.					
			83	118	84	110
Pyruvic Acid	4	76				
Glucurone	2	80	198			
Lactic Acid	4	75				
Sodium Acetate	4	37				
Malic Acid	4	94				
		59	31	28	34	
		210	134			
		97	60			
		11.5	28.8			

:age for 10 sets of 2 rats each.

:anilamide plus a compound known to produce :ive, on the excretion of GA showed that the :rn all values to a more nearly normal figure.

Discussion

The objective of this work and of a companion effort (17) was to determine methods by means of which decreased acetylation and increased glucuronic acid formation could be brought about. The hope was that certain compounds might force the detoxication, particularly of sulfanilamide, away from acetylation and into combination with glucuronic acid. The literature contains ample evidence of the probability of this concept.

Our results *in vivo* in rats confirm those of Lipschitz and Bueding (18) *in vitro* using rat liver, in that dihydroxyacetone and lactic acid increased the formation of GA; but they differ in that we did not observe an increase following the administration of pyruvic acid, actually finding decreased excretion of GA. Quick (19) reported that lactic acid decreased GA formation in dogs, a point of dissimilarity with our findings in the rat. His result with glycolic acid is confirmed by our work. Another point of dissimilarity is in our observation that alanine decreased GA excretion, which would be expected if alanine were converted into pyruvic acid by oxidative deamination. Here, our results are in disagreement with both Lipschitz and Bueding (18) and Quick (19). Adeline (20) found that glycine, alanine, arginine, tyrosine, and histidine increased the output of glucuronic acid. In our experience alanine, glycine, and histidine decreased glucuronic acid formation, while tyrosine and cysteine increased its formation. It is probable that all seeming discrepancies observed are merely the result of dosage and species differences.

The sequence of potency with regard to stimulation of GA formation is in the order of decreasing effect: glycerine, dihydroxyacetone, and lactic acid. It is therefore suggested that the three carbon unit precursor sequence is from dihydroxyacetone phosphate to glycerophosphate and finally to phosphoglyceric aldehyde, which then condenses to a six carbon unit.

The phosphorylation of the precursors of GA was suggested by Lipschitz and Bueding (18). This work is corroborated by our observation that adenylic acid increased GA excretion. It is suggested that the high phosphate potential of adenylic acid aids in the phosphorylation of the three carbon unit precursor of GA. Further, this concept is strengthened by our observations that certain dicarboxylic acids, particularly succinic and malic, markedly increase the excretion of GA.

Kalckar (21) demonstrated in experiments with kidney extracts that the oxidation of dicarboxylic acids such as succinic, fumaric, and malic, gives rise to vigorous phosphorylation of various substrates. From the more recent work of Colowick, *et al.* (22) the conclusion is drawn that the generation of energy-rich phosphate originates with the dehydrogenation step from succinic to fumaric acid. It is therefore suggested that succinic and malic acids increase GA excretion in the rat by facilitating the phosphorylation of its three carbon unit precursor.

In riboflavin-deficient rats, the GA excretion is somewhat increased, this constituting the exception to the general rule of decreased excretion in various vitamin deficiency states. Riboflavin is involved in many enzyme systems, among these the dehydrogenation of malic acid in muscle, of lactic acid in muscle, etc. This might lead to increased concentrations of certain four carbon dicarboxylic acids and of certain three carbon units which lead to increased GA formation. The lactic acid oxidase system would be defective and prevent lactic acid, a compound stimulating GA production, from being oxidized to pyruvic acid, a compound inhibiting its formation.

It is much more difficult to explain the results seen with the other vitamin states. Liver pathology observed in rats in some deficiencies may explain defective GA synthesis.

Scudi and Robinson (23) have reported that sulfanilamide, in contrast to sulfapyridine and sulfathiazole, does not stimulate GA output. The work emphasizes the high percentage of sulfapyridine, some 40% of which is found in the form of a highly soluble glucuronide. This work and that of Weber, *et al.* (24) conclusively established the fact that sulfapyridine in the dog is followed by the appearance in the urine of a glucuronide of a hydroxy derivative of sulfapyridine. In our experiments, sulfapyridine clearly depressed GA excretion. It should be noted again that our experimental animals were rats, whereas Scudi and Robinson (23) and Weber, *et al.* (24) used dogs. Sulfanilamide, in our experience, increased to a slight degree the excretion of GA; sulfathiazole increased it to a marked degree; sulfadiazine had little if any effect; and sulfaguanidine fed chronically resulted in a profound decrease.

Summary

Dihydroxyacetone, glycerine, lactic acid, succinic acid, malic acid, and adenylic acid markedly increase glucuronic acid excretion in the rat. Of the amino acids tested, only tyrosine and cysteine had a positive

xperiments with kidney extracts that the uch as succinic, fumaric, and malic, gives n of various substrates. From the more 22) the conclusion is drawn that the gen- te originates with the dehydrogenation cid. It is therefore suggested that suc- A excretion in the rat by facilitating the rbon unit precursor.

he GA excretion is somewhat increased, to the general rule of decreased excretion states. Riboflavin is involved in many the dehydrogenation of malic acid in cle, etc. This might lead to increased carbon dicarboxylic acids and of certain to increased GA formation. The lactic defective and prevent lactic acid, a com- ion, from being oxidized to pyruvic acid, ation.

in the results seen with the other erved in rats in some deficiencies thesis.

e reported that sulfanilamide, in contrast ole, does not stimulate GA output. The ercentage of sulfapyridine, some 40% of a highly soluble glucuronide. This work 4) conclusively established the fact that owed by the appearance in the urine of a ivative of sulfapyridine. In our experi- pressed GA excretion. It should be noted imals were rats, whereas Scudi and Rob- (24) used dogs. Sulfanilamide, in our t degree the excretion of GA; sulfathiazole ree; sulfadiazine had little if any effect; ally resulted in a profound decrease.

Summary

e, lactic acid, succinic acid, malic acid, and ase glucuronic acid excretion in the rat. nly tyrosine and cysteine had a positive

effect. With the exception of riboflavin deficiency in which there is an increase in glucuronic acid excretion, all the other deficiency states showed decreased excretion of this acid. The effect of sulfonamides on the excretion of glucuronic acid by the rat was studied.

References

1. Sherwin, C. P., *Physiol. Revs.* **2**, 238, (1922).
2. Quick, A. J., *Ann. Rev. Biochem.* **6**, 291 (1937).
3. Young, L., *Physiol. Revs.* **19**, 323 (1939).
4. Stekol, J. A., *Ann. Rev. Biochem.* **10**, 265 (1941).
5. Scudi, J. V., and Robinson, H. J., *Am. J. Med. Sci.* **310**, 201 (1941).
6. Di Somma, A. A., *J. Biol. Chem.* **133**, 277 (1940).
7. Mazur, A., and Shorr, E., *J. Biol. Chem.* **144**, 283 (1942).
8. Martin, G. J., Fisher, C. V., and Thompson, M. R., *Arch. Intern. Med.* **69**, 662 (1942).
9. Maughan, G. B., Evelyn, K. A., and Browne, J. L. S., *J. Biol. Chem.* **126**, 567 (1938).
10. Bueding, E., in press.
11. Quick, A. J., *J. Biol. Chem.* **61**, 667 (1924).
12. Bueding, E., Personal Communication.
13. Mosolowski, W., *Biochem. J.* **34**, 823 (1940).
14. Wagreich, H., Kamin, H., and Harrow, B., *Proc. Soc. Exptl. Biol. Med.* **43**, 468 (1940).
15. Meyer, K., Block, H. S., and Chaffee, E., *Federation Proceedings* **1**, 125 (1942).
16. Williams, R. T., *Biochem. J.* **34**, 272, 1940.
17. Martin, G. J., and Rennebaum, E. H., in press.
18. Lipschitz, W. L., and Bueding, E., *J. Biol. Chem.* **129**, 333 (1939).
19. Quick, A. J., *J. Biol. Chem.* **98**, 537 (1932).
20. Adeline, S. M., *Proc. Soc. Exptl. Biol. Med.* **25**, 8 (1927).
21. Kalckar, H., *Biochem. J.* **33**, 631 (1939).
22. Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.* **133**, 359 (1940).
23. Scudi, J. V., and Robinson, H. J., *Am. J. Med. Sci.* **201**, 711 (1941).
24. Weber, C. J., Lalich, J. J., and Major, R. H., *Proc. Soc. Exptl. Biol. Med.* **53**, 190 (1943).

402

Biochem. J. (1970) 116, 483–491
Printed in Great Britain

Gluconeogenesis in the Kidney Cortex

EFFECTS OF D-MALATE AND AMINO-OXYACETATE

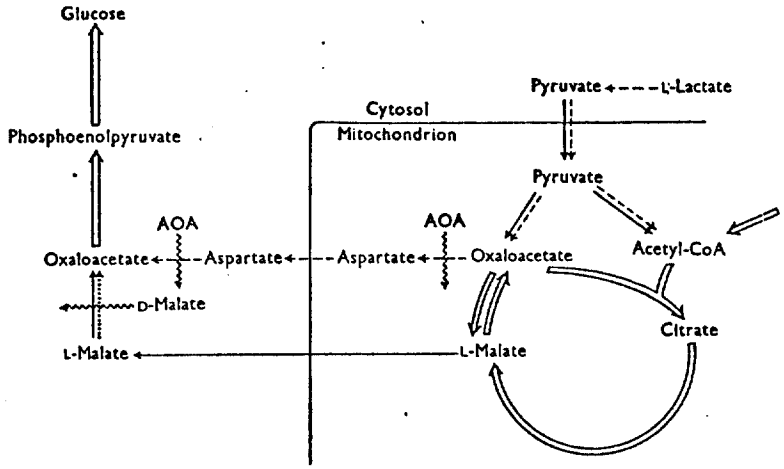
By R. ROGNSTAD and J. KATZ
Medical Research Institute, Cedars-Sinai Medical Center, Los Angeles, Calif. 90029, *U.S.A.*

(*Received* 8 *August* 1969)

1. Rat kidney-cortex slices incubated with D-malate alone formed very little glucose. D-Malate, however, augmented gluconeogenesis from L-lactate and inhibited gluconeogenesis from pyruvate and L-malate. 2. D-Malate had little effect on the rate of the tricarboxylic acid cycle with or without other substrates added. 3. D-Malate inhibited the activity of the L-malate dehydrogenase in a high-speed-supernatant fraction from kidney cortex. 4. It was concluded that D-malate inhibited either the operation of the cytoplasmic L-malate dehydrogenase or malate outflow from the mitochondria in the intact kidney-cortex cell. This supports the hypothesis of Lardy, Paetkau & Walter (1965) and Krebs, Gascoyne & Notton (1967) on the role of malate as carrier for carbon and reducing equivalents in gluconeogenesis. 5. Gluconeogenesis from L-lactate in kidney-cortex slices was strongly inhibited by a low concentration (0.1mM) of amino-oxyacetate, whereas glucose formation from pyruvate, malate, aspartate and several other compounds was only slightly affected. 6. High concentrations of L-aspartate largely reversed the inhibition of gluconeogenesis from L-lactate caused by amino-oxyacetate. 7. Amino-oxyacetate inhibited strongly the glutamate–oxaloacetate transaminase in the 30000*g* supernatant fraction of a kidney-cortex homogenate. The presence of L-aspartate decreased the inhibition of the transaminase by amino-oxyacetate. 8. Detritiation of L-[2-^{3}H]aspartate was inhibited by 90% during an incubation of kidney-cortex slices with L-lactate and amino-oxyacetate. 9. Low concentrations (10μM) of artificial electron acceptors such as Methylene Blue and phenazine methosulphate abolished most of the inhibition of gluconeogenesis from L-lactate by amino-oxyacetate. This is interpreted as an activation of net malate outflow from the mitochondria by-passing the inhibited transfer of oxaloacetate. 10. These findings support the concept that transamination to aspartate is involved in the transfer of oxaloacetate from mitochondria to cytosol required in gluconeogenesis from L-lactate.

It is generally believed that, in gluconeogenesis from L-lactate, oxaloacetate is produced in the mitochondria by the pyruvate carboxylase reaction and then must be transferred to the cytosol (Mehlman, Walter & Lardy, 1967; Shrago & Lardy, 1966). Oxaloacetate, however, apparently does not penetrate the mitochondrial membrane (Borst, 1963). Lardy, Paetkau & Walter (1965) have produced evidence that a dual transaminase system may operate in this situation. In the mitochondria, oxaloacetate is transaminated to aspartate, which passes into the cytosol and is reconverted into oxaloacetate by the soluble glutamate–oxaloacetate transaminase. The system also requires free flow of glutamate and α-oxoglutarate between the compartments.

L-Lactate and L-malate are on the same oxidation level as glucose, and dehydrogenases for these substrates are located in the cytosol. Thus no mitochondrially produced reducing equivalents are required when glucose is formed from either substrate. However, gluconeogenesis from pyruvate requires an additional source of reducing power. Lardy *et al.* (1965), Haynes (1965) and Krebs, Gascoyne & Notton (1967) have proposed that mitochondrial NADH is used in this case: oxaloacetate in the mitochondria is reduced to malate, which leaves the mitochondria and then is reoxidized to oxaloacetate. Here malate is a carrier for both the carbon skeleton and the reducing equivalents used in glucose synthesis. The proposed pathways of gluconeogenesis from pyruvate and L-lactate thus differ in this region, with L-malate and L-aspartate being the respective carriers in the



Scheme 1. Pathways of gluconeogenesis from pyruvate, L-lactate and L-malate. The pathways shown are those proposed by Lardy *et al.* (1965) and Krebs *et al.* (1967). The proposed gluconeogenic pathways from different substrates are shown by different arrows: solid arrows, pyruvate; dashed arrows, L-lactate; dotted arrows, L-malate. Common steps are shown by hollow arrows.

flow from mitochondria to cytosol. Gluconeogenesis from L-malate is strictly a cytoplasmic process.

In principle, if there are sufficiently specific inhibitors of the various steps at which the proposed gluconeogenic pathways from lactate, pyruvate and malate differ, it should be possible to test the hypotheses in the intact cell. Scheme 1 shows the pathways proposed by the workers above, the inhibitors used and the sites at which we believe the major inhibition to occur. We show in the present paper that D-malate inhibits gluconeogenesis from pyruvate but not from L-lactate. On the other hand, AOA* diminishes glucose formation strongly from L-lactate but only slightly from pyruvate or L-malate. These results are consistent with Scheme 1.

EXPERIMENTAL

D-Malate was obtained from Calbiochem, Los Angeles, Calif., U.S.A. AOA was from Eastman Chemicals, Rochester, N.Y., U.S.A. L-[1-^{14}C]Lactate was from New England Nuclear Corp., Boston, Mass., U.S.A. L-[2-^{3}H]-Aspartate was synthesized by incubating 500μmol of L-aspartate with 100 units of glutamate–oxaloacetate transaminase and 0.5μmol of pyridoxal phosphate in 1ml of tritiated water (1Ci/ml) for 24h. The aspartate was purified by elution with 2M-acetic acid from a column (1cm × 30cm) of Dowex 1 (acetate form).

Male Wistar rats were starved for 18–48h and killed by decapitation. Slices of kidney cortex were cut with a Mickle Chopper (Brinkman Instruments, Westbury, N.Y., U.S.A.). Portions (125mg) of slices were incubated for 2h at 37°C under 100% O_2 in 2ml of the buffer described by Krebs, Hems & Gascoyne (1963), containing no bicarbonate. Incubations were carried out in 25ml Erlenmeyer flasks stoppered with rubber serum caps from which a plastic centre well was suspended. At the end of the incubation, 0.5ml of 0.5M-H_2SO_4 was injected into the medium and 0.3ml of 4M-NaOH (CO_2-free) was injected into the centre well. After collection of CO_2, the flasks were opened and samples of the NaOH solution were taken for manometric CO_2 determination. The aqueous phase was removed and the slices were washed, with the total volume of washings made to 10ml. Samples were taken for glucose analysis (Slein, 1965).

To determine D-malate inhibition of the soluble L-malate dehydrogenase and AOA inhibition of glutamate–oxaloacetate transaminase, 4g of kidney-cortex slices was homogenized in 36ml of 0.25M-sucrose containing 10mM-tris–HCl buffer, pH7.6, and 5mM-EDTA. The homogenate was centrifuged at 1000*g* for 10min and then at 30000*g* for 10min and the final supernatant used.

The experiments in which O_2 uptake was determined were carried out in Warburg flasks, with 0.3ml of 4M-NaOH in the centre well. Slices (125mg) in 2ml of buffer were in the main compartment, with substrates and inhibitors in 0.5ml of buffer in a side arm. The flasks were gassed with O_2. Samples of the initial and final medium were taken for enzymic determination of pyruvate (Bücher, Czok, Lamprecht & Latzko, 1965), L-lactate and L-malate (Hohorst, 1965).

RESULTS

Effects of D-malate on tricarboxylic acid-cycle metabolism. D-Malate is metabolized by kidney-cortex slices, probably via the mitochondrial D-α-

* Abbreviation: AOA, amino-oxyacetate ($H_2N{\cdot}O{\cdot}CH_2{\cdot}CO_2H$).

Table 1. *Effect of* D-*malate on net carbon dioxide production, oxygen uptake and substrate utilization in kidney-cortex slices*

The amount of substrate added was 10 μmol in all experiments except Expt. 7, where 20 μmol was added; 20 μmol of acetate also was added in Expt. 7 only.

Expt. no.	Substrate	Concn. of D-malate (mM)	Gas exchange (μmol/2h per 125mg) CO_2 formed	O_2 used	Substrate utilization (%)
6	Endogenous	0	17.3		
		50	19.6		
	Pyruvate	0	23.0		
		50	25.0		
	L-Lactate	0	25.9		
		50	24.5		
	L-Malate	0	33.8		
		50	23.7		
7	Endogenous	0	38.8		
		10	39.1		
		20	38.0		
	Pyruvate	0	54.9		
		10	49.4		
		20	52.2		
	L-Lactate	0	42.9		
		10	39.0		
		20	41.0		
	L-Malate	0	48.7		
		10	46.8		
		20	41.1		
8	Endogenous	0		16.9	—
		50		18.0	—
	Pyruvate	0		27.8	95
		50		26.4	90
	L-Lactate	0		28.1	80
		50		27.5	82
	L-Malate	0		29.4	90
		50		27.2	48
9	Endogenous	0		17.3	—
		50		17.7	—
	Pyruvate	0		26.0	92
		50		24.8	86
	L-Lactate	0		25.3	77
		50		26.1	78
	L-Malate	0		30.1	92
		50		28.5	54

hydroxy acid dehydrogenase described by Tubbs & Greville (1961). However, the rate of D-malate disappearance is only about one-fifth that of L-malate (R. Rognstad & J. Katz, unpublished work). The effects of D-malate on net carbon dioxide production and oxygen uptake are shown in Table 1. In the absence of added substrate the material oxidized is probably endogenous lipid (Weidemann & Krebs, 1969). Most of the carbon dioxide production is from tricarboxylic acid-cycle metabolism, and this was little affected by 50mM-D-malate, the highest concentration used. Oxygen uptake was also unimpaired. 50mM-D-Malate had little effect on net pyruvate utilization, indicating that entrance of pyruvate into the cell was not restricted. When L-malate was the substrate, carbon dioxide production was definitely depressed at the highest concentration (50mM) of D-malate, but this can be related to a rather strong inhibition of L-malate disappearance and consequent decrease in decarboxylation at C-4 of malate. This inhibition of malate utilization could be explained by an inhibition of the cytoplasmic L-malate dehydrogenase, but inhibition of entry of L-malate into the cell cannot be ruled out. The lack of any other marked effect of D-malate on oxygen uptake and carbon

Table 2. *Inhibition of kidney-cortex soluble* L-*malate dehydrogenase by* D-*malate*

The 30000*g* supernatant from kidney-cortex homogenate was used as a source of the enzyme. The following assay system was used: 200 μmol of tris–HCl buffer, pH 7.6, 10 μmol of acetyl phosphate, 1 μmol of CoA, 5 μmol of NAD^+, 12 units of transacetylase, 3 units of citrate synthase, 1 μmol of L-malate and 10 μl of kidney-cortex extract in a total volume of 3 ml. The reaction was followed in a Gilford recording spectrophotometer.

Concn. of D-malate (mM)	Initial rate (% of uninhibited rate)
0	100
0.3	80
1.6	63
8.0	27

dioxide production suggests that none of the tricarboxylic acid-cycle enzymes was affected sufficiently to change the net flow.

Effect of D-*malate on the cytoplasmic* L-*malate dehydrogenase from a homogenate of rat kidney cortex.* The inhibition by D-malate of the oxidation of L-malate by the soluble L-malate dehydrogenase in a cell-free preparation is shown in Table 2. At a high D-malate/L-malate ratio the inhibition became pronounced. Chance & Hagihara (1961) have reported that D-malate is a competitive inhibitor of L-malate dehydrogenase. Since malate dehydrogenase has been considered to be far from rate-limiting under normal conditions (Hohorst, Freutz & Bücher, 1959; Krebs, 1967), a rather strong effect on this enzyme would be necessary to alter net flow in the cell. The results in Table 2 indicate that cytoplasmic L-malate dehydrogenase is a possible site of the effects of D-malate shown below.

Effect of D-*malate on gluconeogenesis from* L-*lactate, pyruvate and* L-*malate.* When added by itself, D-malate formed very little more glucose than that formed from endogenous sources in the kidney-cortex slice (Table 3). D-Malate at low concentrations increased gluconeogenesis from L-lactate and at higher concentrations had a variable effect. D-Malate inhibited gluconeogenesis from pyruvate at all concentrations tested. There was some inhibition by D-malate of glucose formation from L-malate, but the effect was less than that on gluconeogenesis from pyruvate.

Effect of AOA on glutamate–oxaloacetate transaminase. AOA has been shown to inhibit a number of transaminases, including glutamate–oxaloacetate transaminase (Roberts, Wein & Simonsen, 1964; Braunstein, 1964; Hotta, 1968). The effect of AOA on the glutamate–oxaloacetate transaminase in a high-speed-supernatant fraction from a rat

Table 3. *Effects of* D-*malate on gluconeogenesis from* L-*lactate, pyruvate and* L-*malate by kidney-cortex slices*

Substrates were added at 10 μmol in a total volume of 2 ml in Expts. 1, 2, 3 and 5 and at 20 μmol in Expt. 4; D-malate concentrations were varied as shown.

Expt. no.	Substrate	Concn. of D-malate (mM)	Glucose formed (μmol/2 h per 125 mg wet wt)
1	Endogenous	0	0.62
		5	0.80
2	Endogenous	0	0.43
	Pyruvate	0	2.39
		5	1.82
	L-Lactate	0	2.49
		5	2.67
3	Endogenous	0	0.48
	Pyruvate	0	2.67
		5	2.18
		10	1.86
	L-Lactate	0	2.91
		5	3.51
		10	3.67
	L-Malate	0	3.38
		5	3.35
		10	3.17
4	Endogenous	0	0.44
		10	0.45
		20	0.28
	Pyruvate	0	3.61
		10	2.64
		20	1.71
	L-Lactate	0	2.20
		10	2.57
		20	2.15
	L-Malate	0	2.71
		10	2.18
		20	1.78
5	Endogenous	0	0.50
	Pyruvate	0	3.47
		50	1.90
	L-Lactate	0	2.34
		50	3.08
	L-Malate	0	2.84
		50	2.17

kidney-cortex homogenate is shown in Table 4. At low concentrations of L-aspartate, AOA caused an immediate and strong inhibition of transaminase activity. At high concentrations of L-aspartate, inhibition of the initial rate by AOA was much less. However, the inhibition did increase with time.

Similar effects were found with a highly purified commercial glutamate–oxaloacetate transaminase from pig heart. With this purified enzyme, protection against AOA inhibition was also shown by high concentrations of L-glutamate. Hopper & Segal (1962) have shown that AOA acts as a competitive

Table 4. *Effect of AOA on the soluble glutamate–oxaloacetate transaminase of the kidney cortex*

Each cuvette contained 300 μmol of triethanolamine–HCl buffer, pH 7.4, 0.3 μmol of NADH, L-aspartate as shown, 0.1 ml of purified malate dehydrogenase (5 mg/ml; Boehringer) and 20 μl of the high-speed supernatant fraction from the rat kidney cortex in a final volume of 1.0 ml. The reaction was started with 2 μmol of α-oxoglutarate. A control cuvette without aspartate run in each series gave a low rate of NADH oxidation. This was subtracted to give the rates shown.

Concn. of aspartate (mM)	Concn. of AOA (mM)	Initial rate (ΔE_{340}/2 min)	Rate (% of uninhibited rate)
1.0	0	0.159	100
1.0	0.1	0.011	7
50.0	0	0.468	100
50.0	0.1	0.270	54

Table 5. *Effect of AOA on production of* ^{3}H *in water from* L-[2-^{3}H]*aspartate during gluconeogenesis from* L-*lactate*

In these experiments 20 μmol of L-lactate and 20 μmol of acetate were incubated with 125 mg of kidney-cortex slices for 2 h. The final volume was 2 ml. L-[2-^{3}H]Aspartate (4×10^6 c.p.m.; 1.1 μmol) was added to separate replicate flasks at 0, 30, 60 and 90 min and the amount of ^{3}H produced in water was determined by taking samples 15 min after addition of labelled substrate. During a 5 min incubation with L-[2-^{3}H]-aspartate, about 60% of the ^{3}H was transferred to water in the absence of AOA.

Expt. no.	Concn. of AOA (mM)	^{3}H in H_2O (%) 0–15 min	30–45 min	60–75 min	90–105 min
15	0	98	99	100	100
	0.1	9	8	9	9
16	0	93	98	99	99
	0.1	11	8	10	11

inhibitor of the amino acid substrates of glutamate–pyruvate transaminase.

Table 5 provides evidence of the inhibition by AOA of glutamate–oxaloacetate transaminase in the intact cell. When L-aspartate was incubated in tritiated water with glutamate–oxaloacetate transaminase, ^{3}H was incorporated into aspartate at C-2; this ^{3}H was again released to water on subsequent incubation with the enzyme in unlabelled water. When L-[2-^{3}H]aspartate was incubated with kidney-cortex slices, detritiation occurred (Table 5), with nearly all of the isotope appearing in water after 15 min. The presence of 0.1 mM-AOA resulted in only 10% detritiation. This is a minimal value for inhibition of the transaminase, since the 10% detritiation that did occur could possibly be the result of enzymes other than glutamate–oxaloacetate transaminase metabolizing L-aspartate.

Effect of AOA on gluconeogenesis. Gluconeogenesis from L-lactate (if one accepts a strictly mitochondrial site for pyruvate carboxylase) requires a transfer of oxaloacetate from the mitochondria to the cytosol. Lardy *et al.* (1965) have proposed a dual transaminase mechanism for this transfer. Thus a specific and potent inhibitor of glutamate–oxaloacetate transaminase might be expected to depress gluconeogenesis from lactate if this hypothesis is correct. The effect of AOA on gluconeogenesis in the rat kidney cortex is shown in Table 6. At an AOA concentration of 0.1 mM, glucose formation from lactate was inhibited by 80–90%. Glucose synthesis from all other substrates tested was inhibited by less than 15% at this concentration of AOA.

Reversal by L-*aspartate of the AOA inhibition of gluconeogenesis from* L-*lactate.* At a concentration of 50 mM L-aspartate alone formed little glucose in the kidney-cortex slice (Table 7). When added to 10 mM-L-lactate, this high concentration caused a moderate inhibition of gluconeogenesis. However, when added to L-lactate plus AOA, the strong inhibition of glucose synthesis caused by AOA was largely overcome. When L-[1-^{14}C]lactate was used as substrate, 50 mM-L-aspartate abolished the inhibitory effect on the formation of [^{14}C]glucose caused by AOA. The presence of aspartate decreased the yield of ^{14}C in glucose both in the absence and presence of AOA because of exchange by transaminase of labelled oxaloacetate with the large unlabelled aspartate pool. The results with L-[1-^{14}C]lactate show that aspartate caused a true reversal of the inhibition of gluconeogenesis caused

by AOA. An alternative explanation might have been that glucose was synthesized from oxaloacetate formed from aspartate in the cytosol and NADH formed from L-lactate, in which case no transport of oxaloacetate from the mitochondria would have been required. However, the results with L-[1-^{14}C]lactate indicate that aspartate removed the block in the flow of carbon from lactate to glucose. Presumably aspartate acted to prevent or decrease the inhibition by AOA of glutamate–oxaloacetate transaminase.

Effect of artificial electron acceptors on the AOA inhibition of gluconeogenesis from lactate. The evidence presented above suggests that the inhibition of gluconeogenesis by AOA is caused by depression of oxaloacetate transfer as a result of inhibition of the glutamate–oxaloacetate transaminase either in the cytosol or in the mitochondria, or both. Since gluconeogenesis from pyruvate involves transfer of malate rather than oxaloacetate, no inhibition by AOA occurs. It seemed feasible that addition of a low concentration of an artificial electron acceptor such as Methylene Blue or phenazine methosulphate would alter the pathway of gluconeogenesis from lactate to be more similar to the pathway from pyruvate. These electron acceptors could react non-enzymically with the NADH generated from lactate in the cytosol, thus inducing malate outflow from the mitochondria to provide an additional source of cytoplasmic NADH. As shown in Table 8, low concentrations of the electron acceptors caused a moderate inhibition of gluconeogenesis from L-lactate (and pyruvate). However, in this same concentration range these compounds brought gluconeogenesis from L-lactate plus AOA back to nearly the rate found in the absence of AOA.

Table 6. *Effect of AOA on gluconeogenesis in the rat kidney cortex*

Each flask contained 20 μmol of the substrate together with 20 μmol of acetate. The volume was 2 ml. Glucose formation from endogenous sources was 0.4–0.5 μmol/2 h per 125 mg and has been subtracted to give the values in the table.

Expt. no.	Substrate	Concn. of AOA (mM)	Glucose formation (μmol/2 h per 125 mg)
10	L-Lactate	0	2.72
		0.1	0.31
	Pyruvate	0	3.05
		0.1	2.86
	L-Malate	0	3.12
		0.1	2.98
	L-Aspartate	0	1.10
		0.1	1.15
11	L-Lactate	0	2.72
		0.1	0.53
	L-Glutamate	0	1.45
		0.1	1.25
	L-Glutamine	0	1.11
		0.1	1.03
12	L-Lactate	0	2.84
		0.1	0.43
	L-Malate	0	4.24
		0.1	3.93
	α-Oxoglutarate	0	4.55
		0.1	4.53

Table 7. *Reversal of AOA inhibition of gluconeogenesis by high concentrations of L-aspartate*

Conditions were the same as in Table 6. In Expts. 14 and 15 L-[1-^{14}C]lactate was used as substrate. Values in the last column are percentages of the added ^{14}C recovered in glucose.

Expt. no.	Concn. of L-lactate (mM)	Concn. of AOA (mM)	Concn. of L-aspartate (mM)	Glucose formed (μmol/2 h per 125 mg)	^{14}C in glucose (%)
13	10	0	0	2.92	
	10	0.1	0	0.52	
	10	0	50	2.06	
	10	0.1	50	1.78	
14	10	0	0	2.88	9.3
	10	0.1	0	0.52	1.6
	0	0	50	0.20	—
	0	0.1	50	0.42	—
	10	0	50	2.06	3.4
	10	0.1	50	2.26	3.7
	10	0	0	2.69	10.7
	10	0.1	0	0.65	1.9
	0	0	50	0.42	—
	0	0.1	50	0.62	—
15	10	0	50	2.27	3.9
	10	0.1	50	2.11	3.8

Table 8. *Effect of Methylene Blue or phenazine methosulphate on gluconeogenesis from* L-*lactate in the absence and presence of AOA*

Conditions were the same as in Table 6.

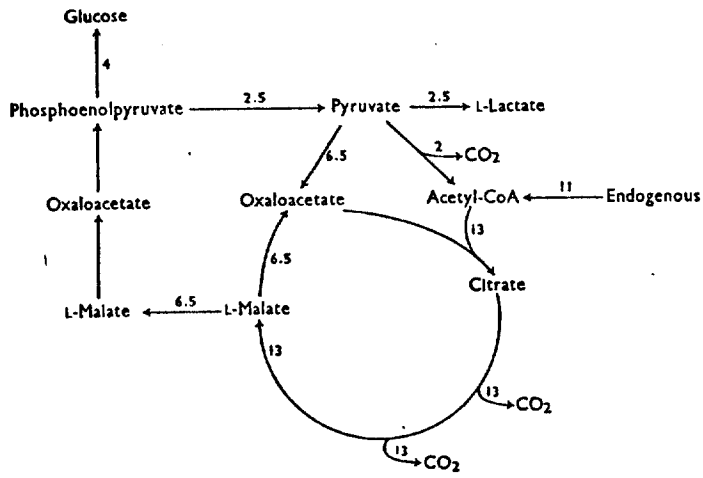
Expt. no.	Substrate	Concn. of AOA (mM)	Concn. of Methylene Blue (mM)	Concn. of phenazine methosulphate (mM)	Glucose formed (μmol/2h per 125mg)
1	Lactate	—	—	—	3.11
		—	0.01	—	2.63
		—	0.02	—	2.45
		0.1	—	—	0.54
		0.1	0.01	—	1.89
		0.1	0.02	—	2.19
2	Lactate	—	—	—	3.61
		—	0.005	—	3.19
		—	0.02	—	2.53
		—	—	0.005	2.63
		—	—	0.02	1.75
	Lactate	0.1	—	—	0.45
		0.1	0.005	—	1.03
		0.1	0.02	—	1.87
		0.1	—	0.005	2.33
		0.1	—	0.02	2.21
	Pyruvate	—	—	—	3.69
		—	0.005	—	3.17
		—	0.02	—	2.02
		—	—	0.005	2.78
		—	—	0.02	1.81

DISCUSSION

Direction of net flow in the mitochondrial L-*malate dehydrogenase reaction.* We have found little effect of D-malate on tricarboxylic acid-cycle metabolism (Table 1). Our results possibly might not rule out an effect on the mitochondrial L-malate dehydrogenase, since according to the hypotheses of Lardy *et al.* (1965) and Krebs *et al.* (1967) this enzyme functions 'backwards' in gluconeogenesis from pyruvate. However, in our experiments on kidney-cortex slices we do not confirm this speculation. Malate is formed by the forward reactions of the cycle and not by a net reduction of oxaloacetate with NADH. This can be shown as follows: analytical measurements in an experiment with pyruvate (10μmol) and kidney-cortex slices indicated that 8.5μmol of pyruvate disappeared, with 4μmol being converted into glucose and 2.5μmol into lactate, the remainder (2μmol) being presumably oxidized to acetyl-CoA and thence to carbon dioxide. Carbon dioxide formation was 28μmol. A balance can be constructed from these results (Scheme 2). The results clearly show that under our conditions net flow in the mitochondrial malate dehydrogenase is in the forward direction, although a rapid reversible exchange reaction between oxaloacetate and malate may occur. Schmidt & Katz (1969) in this laboratory have found that in adipose tissue the mitochondrial malate dehydrogenase may function (net flow) in a reverse direction during active lipogenesis from pyruvate.

Mechanism of the inhibitory effect of D-*malate.* The only steps in the proposed (Lardy *et al.* 1965) pathway of gluconeogenesis from pyruvate that do not occur in the pathway from L-lactate are thus outflow of malate from the mitochondria and the cytoplasmic malate dehydrogenase reaction. D-Malate inhibits gluconeogenesis from pyruvate and not (or only slightly) from L-lactate. We have shown (Table 2) that D-malate inhibition of malate dehydrogenase is possible. Haslam & Griffiths (1968) have shown an effect of D-malate on penetration of L-malate into liver mitochondria and a similar effect on outflow could occur. Inhibition at either site is consistent with malate transfer being involved in gluconeogenesis from pyruvate. D-Malate depresses gluconeogenesis from and utilization of L-malate. However, these effects require high concentrations of D-malate, and a possible effect on L-malate uptake by the cell might also be considered.

Site of pyruvate carboxylase. The results with D-malate suggest that malate outflow is indeed involved in the utilization of mitochondrial reducing power required in glucose formation from pyruvate.



Scheme 2. Carbon flow in gluconeogenesis from pyruvate. Numbers represent μmol/2h per 125 mg wet wt.

This has some relevance to the controversy over the site of pyruvate carboxylase (see Scrutton & Utter, 1968). Seubert & Huth (1965) have provided evidence that in rat liver pyruvate carboxylase also occurs extensively in the cytosol. However, if malate is the carrier of reducing power, only a mitochondrial pyruvate carboxylase would suffice to maintain the dicarboxylic acid pool in the tricarboxylic acid cycle out of which malate flows. Bottger, Wieland, Brdiczka & Pette (1969) have found nearly all of the pyruvate carboxylase activity in rat liver to be mitochondrial.

Mechanism of AOA inhibition of gluconeogenesis. AOA has been shown to inhibit glutamate–oxaloacetate transaminase in a cell-free preparation (Table 4) and also to inhibit these enzymes in the intact cell (Table 5). Gluconeogenesis from L-lactate in the kidney cortex is strongly inhibited by AOA, whereas glucose formation from pyruvate and L-malate is only slightly affected. These results provide strong confirmatory evidence for the role of the two transaminases in oxaloacetate transfer, as proposed by Lardy *et al.* (1965). High concentrations of aspartate tend to relieve both the inhibition of the transaminase in a cell-free system and the inhibition of gluconeogenesis. The reason for the complete lack of effect of AOA on glucose formation from L-aspartate is not clear (Table 6). One cause may be the protective effect of L-aspartate. However, much higher concentrations of L-aspartate (50mM) were required to reverse effectively the AOA inhibition of gluconeogenesis from L-lactate. Another possibility is that other enzymes that metabolize L-aspartate may be present in kidney (e.g. an amino acid oxidase).

AOA has a strong inhibitory effect on gluconeogenesis from lactate but not on that from pyruvate or that from lactate in the presence of Methylene Blue or phenazine methosulphate. The last results show that AOA does not inhibit lactate dehydrogenase in the kidney cortex. We have found (R. Rognstad & J. Katz, unpublished work) no inhibition of a purified pig heart lactate dehydrogenase at an AOA concentration of 1mM.

Energy requirement for oxaloacetate transfer. Although these studies support the dual transaminase mechanism of oxaloacetate transfer, other considerations indicate that the mechanism may be more involved. Thus Krebs (1967) has estimated that in the liver of starved rats the oxaloacetate concentration in the mitochondria is nearly 100-fold lower than that in the cytosol. Transfer of oxaloacetate from the mitochondria to the cytosol thus should require energy, and the dual transaminase system by itself would not be adequate for the transfer. A possible solution to this dilemma is found in the hypothesis of de Haan & Oestreicher (1968) that oxaloacetate is transferred between two compartments in the mitochondria by an energy-requiring process. One compartment includes malate dehydrogenase, and in this compartment the oxaloacetate concentration is low according to the calculations of Krebs (1967). The other compartment, to which oxaloacetate can be pumped and thus concentrated, includes glutamate–oxaloacetate transaminase. By a combination of the mechanism proposed by Lardy *et al.* (1965) and de Haan & Oestreicher (1968), oxaloacetate could be transferred from the mitochondria to the cytosol.

Limit of the α-glycerophosphate shuttle. Cytoplasmic and mitochondrial α-glycerophosphate

dehydrogenase have been reported in the kidney (Lee & Lardy, 1965). Thus one might envisage a pathway of net malate outflow from the mitochondria even in gluconeogenesis from lactate, with the excess of reducing equivalents being returned to the mitochondria by an α-glycerophosphate shuttle. The fact that AOA caused a pronounced inhibition in gluconeogenesis from lactate indicates that such an alternate pathway, if present at all, is severely restricted.

This work was supported by U.S. Public Health Service Grant no. AM-12604-01 and General Research Support Grant no. 5-S01FR05468-06.

REFERENCES

Borst, P. (1963). In *Funktionelle und Morphologische Organisation der Zelle*, p. 137. Ed. by Karlson, P. Berlin: Springer-Verlag.

Bottger, I., Wieland, O., Brdiczka, D. & Pette, D. (1969). *Eur. J. Biochem.* 8, 113.

Braunstein, A. E. (1964). *Vitams Horm.* 22, 451.

Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1965). In *Methods of Enzymatic Analysis*, p. 253. Ed. by Bergmeyer, H. New York: Academic Press Inc.

Chance, B. & Hagihara, B. (1961). *Proc. 5th int. Congr. Biochem., Moscow*, vol. 5, p. 3. New York: Pergamon Press Inc.

de Haan, E. J. & Oestreicher, A. B. (1968). *Abstr. FEBS 5th Meet., Prague*, p. 52.

Haslam, J. M. & Griffiths, D. E. (1968). *Biochem. J.* **109**, 921.

Haynes, R. C. (1965). *J. biol. Chem.* **240**, 4103.

Hohorst, H. J. (1965). In *Methods of Enzymatic Analysis*, p. 266. Ed. by Bergmeyer, H. New York: Academic Press Inc.

Hohorst, H. J., Freutz, F. H. & Bücher, T. (1959). *Biochem. Z.* **332**, 18.

Hopper, S. & Segal, H. L. (1962). *J. biol. Chem.* **237**, 3189.

Hotta, S. S. (1968). *Archs Biochem. Biophys.* **127**, 132.

Krebs, H. A. (1967). In *Advances in Enzyme Regulation*, vol. 5, p. 409. Ed. by Weber, G. London: Pergamon Press Ltd.

Krebs, H. A., Gascoyne, T. & Notton, B. M. (1967). *Biochem. J.* **102**, 275.

Krebs, H. A., Hems, R. & Gascoyne, T. (1963). *Acta biol. med. ger.* **11**, 607.

Lardy, H. A., Paetkau, V. & Walter, P. (1965). *Proc. natn. Acad. Sci. U.S.A.* **53**, 1410.

Lee, Y. & Lardy, H. A. (1965). *J. biol. Chem.* **240**, 1427.

Mehlman, H. A., Walter, P. & Lardy, H. A. (1967). *J. biol. Chem.* **242**, 4594.

Roberts, E., Wein, J. & Simonsen, D. G. (1964). *Vitams Horm.* **22**, 503.

Schmidt, K. & Katz, J. (1969). *J. biol. Chem.* **244**, 2125.

Scrutton, M. C. & Utter, M. F. (1968). *A. Rev. Biochem.* **37**, 249.

Seubert, W. & Huth, W. (1965). *Biochem. Z.* **343**, 176.

Shrago, E. & Lardy, H. A. (1966). *J. biol. Chem.* **241**, 663.

Slein, M. W. (1965). In *Methods of Enzymatic Analysis*, p. 117. Ed. by Bergmeyer, H. New York: Academic Press Inc.

Tubbs, P. K. & Greville, G. D. (1961). *Biochem. J.* **81**, 104.

Weidemann, M. J. & Krebs, H. A. (1969). *Biochem. J.* **112**, 149.