that formic esters of glycerol are metabolized to incorporation factor-like material more readily than glycerol itself; this could be due to substitution of other residues for the formyl groups, or to protection of glycerol from metabolic breakdown.

There is no point in attempting, at present, to interpret in detail what is occurring during the stimulation of incorporation of glycine carbon by incorporation factor or glycerol. The next stage is to follow the metabolism of glycerol under the experimental conditions described here and in previous work (Gale & Folkes, 1958*a*, *b*). We are now studying the incorporation of radioactivity from DL-[1-$^{14}$C]glycerol into the 'nucleic acid' fraction (hot-ethanol insoluble, cold-trichloroacetic acid insoluble, hot-trichloroacetic acid soluble) of both *E. coli* KI and disrupted staphylococcal cells, and evidence has been obtained for the presence in this 'nucleic acid' fraction of labelled components which readily break down to yield free glycerol.

## SUMMARY

1. The incorporation of glycine carbon into the trichloroacetic acid-precipitable fraction of *Escherichia coli* KI, harvested in the early exponential stage of growth and preincubated for 30 min. in buffered saline, is stimulated 10- to 13-fold by 'incorporation factor' preparation. The response is linear with time for at least 2 hr.

2. Glycerol has little or no initial effect under these conditions, but stimulation develops during incubation and, after a lag period of about 60 min., an initial concentration of 0·1 mM-glycerol gives a stimulation equivalent to that obtained with incorporation factor. Higher concentrations of glycerol are not more effective.

3. When the washed cells are incubated with glycerol for 60 min. before the addition of glycine, incorporation of the latter is stimulated immediately on addition and the response is linear.

4. Glucose gives an immediate stimulation of glycine incorporation but the effect decreases rapidly with time.

5. No breakdown product of glycerol has been found which has significant stimulatory action. A 'formin' preparation containing formic acid esters of glycerol is approximately three times as active as glycerol itself. Other glycerides are inactive.

6. Glycerol is markedly less effective than incorporation-factor preparation in promoting glutamic acid incorporation, under conditions of protein synthesis, in disrupted staphylococcal cells. Formin is again more effective than glycerol and, at a relatively high concentration, can replace incorporation-factor preparation.

## REFERENCES

Delaby, R. & Dubois, P. (1928). *C.R. Acad. Sci., Paris*, **187**, 767.

Demain, A. L., Newkirk, J. F., Barnes, E. C. & Hendlin, D. (1962). *Biochem. J.* **83**, 424.

Euler, H. von & Martius, C. (1933). *Liebigs Ann.* **505**, 73.

Evans, W. L. (1938). *J. Amer. chem. Soc.* **60**, 1628.

Gale, E. F. (1951). *Biochem. J.* **48**, 286.

Gale, E. F. (1959). *Synthesis and Organization in the Bacterial Cell.* New York: John Wiley and Sons Inc.

Gale, E. F. & Folkes, J. P. (1955). *Biochem. J.* **59**, 661.

Gale, E. F. & Folkes, J. P. (1958*a*). *Biochem. J.* **69**, 611.

Gale, E. F. & Folkes, J. P. (1958*b*). *Biochem. J.* **69**, 620.

Hibbert, H. & Carter, N. M. (1928). *J. Amer. chem. Soc.* **50**, 3120.

Koehler, A. (1913). *Bull. Soc. chim. Fr. 4th series*, **13**, 1103.

Kuehl, F. A., Demain, A. L. & Rickes, E. L. (1960). *J. Amer. chem. Soc.* **82**, 2079.

Perkin, W. H. & Simonsen, J. L. (1905). *J. chem. Soc.* **87**, 858.

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli. Publication 607, p. 13. Washington: Carnegie Institution of Washington.

Schuette, H. A. & Hale, J. T. (1930). *J. Amer. chem. Soc.* **52**, 1978.

---

*Biochem. J.* (1962) **83**, 438

# Inhibition of Glycolysis in Rat Skeletal Muscle by Malonate

BY E. N. FAWAZ AND G. FAWAZ

*Department of Pharmacology, School of Medicine, American University of Beirut, Beirut, Lebanon*

(*Received* 15 *August* 1961)

Fawaz, Tutunji & Fawaz (1958) studied the effect of sodium malonate on the isolated dog heart (heart–lung preparation). Although 0·03 M-malonate almost completely inhibited the Krebs cycle in pigeon-breast or sheep-heart mince, it had no deleterious effect on the performance of the heart; however, a substantial amount of succinate accumulated in the cardiac tissue. When the concentration of malonate was raised to 0·06 M, rapid failure ensued, accompanied by a decreased heart rate and

transient arrhythmias. We could not explain these results on the basis of the known action of malonate on succinate oxidation, since, with the double dose of malonate, succinate accumulation was not significantly increased and there was no correlation between succinate accumulation and degree of heart failure. Kuby, Noda & Lardy (1954) reported an inhibitory action of 0·12 M-malonate on creatine kinase. In the experiments described below we have studied the effect of malonate on the metabolism of phosphate esters in a rat-skeletal-muscle suspension. We found that 0·06 M-malonate inhibits glycolysis, as evidenced by decreased lactic acid formation and by accumulation of some intermediates of the glycolytic pathway. We can therefore offer an explanation for our previous results on the heart–lung preparation. This paper deals with the identification of the accumulating intermediates, as well as the location of the enzymic block produced by malonate.

## METHODS AND MATERIALS

### *Preparation of muscle suspensions and extracts*

Adult non-starved albino rats were anaesthetized with pentobarbital (40 mg./kg., intraperitoneally). The thigh muscles were removed and immediately immersed in solid carbon dioxide–ether at −60°. The frozen muscle was quickly blotted with filter paper to remove the ether and weighed without delay in a closed weighing bottle. It was then ground in a mortar to a fine powder with frequent additions of solid carbon dioxide to prevent thawing. The powder was quantitatively transferred by means of dry ice-cooled spatula and wide-necked funnel to a flask containing an amount of 0·65 N-perchloric acid or incubation medium 9·2 times the volume of the tissue. This procedure is used in this Laboratory (Fawaz & Hawa, 1953) to estimate the steady-state concentrations of phosphorus compounds in tissues. Omission of the weighing of the tissue before powdering proved to be equally satisfactory. Samples of the powder were transferred to weighed flasks containing the medium and their weight was found by difference. The latter procedure is advantageous in that the same powder can be used for both control and malonate suspensions, as well as for the determination of steady-state concentrations.

The incubation medium consisted of 0·1 M-glycine buffer, pH 8·5, in the preliminary experiments, but was later replaced by 2·5 mN-potassium hydroxide. Malonate was dissolved in the medium before the tissue powder was added. The pH of the medium after incubation was 7·05 for the malonate experiments and 6·65 for the controls. The corresponding pH values with glycine buffer were 7·6 and 7·3. The accumulation of lactic acid and precursors occurred at the same rate in the two media.

The suspension was deproteinized with one-tenth volume of 6·6 N-perchloric acid (final tissue dilution 1:11). Malonic acid was removed by exhaustive ether extraction of the perchloric acid filtrate with a Kutscher–Steudel apparatus, a procedure later found to be unnecessary. The perchloric acid extract was neutralized with potassium hydroxide, cooled in ice–water and centrifuged.

### *Analytical methods*

Lactate was estimated by the method of Barker & Summerson (1941), and phosphocreatine and inorganic phosphate by the method of Fiske & Subbarow (1929). In preliminary experiments (Table 1), acid hydrolysis (N-hydrochloric acid at 100°) for different periods was used to identify some of the phosphate esters. For instance, the '7 min.' value ($H_7$) − (phosphocreatine + inorganic phosphate) is taken, at least in fresh muscle, as a measure of the 'labile' nucleotide phosphorus or two-thirds of the ATP phosphorus. In all other experiments enzymic methods were used to estimate the intermediates of glycolysis. These are based on the $DPN^+ \rightleftharpoons DPNH$ or $TPN^+ \rightleftharpoons TPNH$ reaction and the corresponding change in extinction at 340 mμ. Most of these methods have been described (Slater, 1953; Scholz, Schmitz, Bücher & Lampen, 1958; Hohorst, Kreutz & Bücher, 1959). We have followed directions given to us by the C. F. Boehringer Biochemical Laboratory (Bergmeyer, 1962). To shorten the reaction time and to counteract any possible inhibitory effect of malonate remaining in the test samples, the enzyme concentrations mentioned below are in some cases greater than those given by Bergmeyer. A Beckman spectrophotometer (Model B) was used in all enzymic tests. The semi-microcuvettes had a light path of 1 cm. and a width of 0·4 cm. All measurements were made at 25–26°. The molecular extinction coefficient, $\epsilon$, of DPNH or TPNH at 340 mμ was assumed to be $6{\cdot}22 \times 10^3$. In general, each enzymic reaction was completed in 5–10 min., except those for 3-phosphoglycerate and glycerol 1-phosphate where more time was allowed.

*Enzymes.* All enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim–Waldhof, Germany. They were transported, and subsequently stored, at 2–4°. The enzymes—mostly crystalline—were obtained as suspensions in ammonium sulphate soln. The enzyme-protein concentration in the suspension and the approximate activity/mg. of protein (specific activity) were given. Boehringer und Soehne express activity in terms of 'Bücher units' [for definition see Beisenherz *et al.* (1953)]. We regularly checked the activity of these enzymes and observed, as a rule, no significant loss even after several months. Professor Bücher kindly supplied a sample of glucose 6-phosphate dehydrogenase with very low glutathione-reductase activity.

*Reagents and buffers.* The concentrations of nucleotides used for the estimation of substrates were as follows: 10 mM-DPNH (disodium salt), 60 mM-$DPN^+$, 13 mM-$TPN^+$ (monosodium salt), 20 mM-ADP (trisodium salt), 16 mM-ATP (disodium salt). All nucleotides were dissolved in water except DPNH which was dissolved in 0·05 M-triethanolamine buffer, pH 7·6. Boehringer und Soehne supplied the above reagents as well as sodium pyruvate, phosphoenolpyruvate (tricyclohexylammonium salt), 3-phosphoglycerate (tricyclohexylammonium salt) and 2-phosphoglycerate (barium salt). Triethanolamine buffer, pH 7·6 (9·3 g. of the hydrochloride + 22 ml. of N-sodium hydroxide, diluted to 1 l.), was used in all estimations of substrates except that of glycerol 1-phosphate. It was pipetted into the cuvette first (in amount such that the final volume was 1 ml.). For measuring pyruvate-kinase activity 0·5 ml. of 0·1 M-triethanolamine containing EDTA (5 mM) was used per test. Glycine–EDTA–hydrazine buffer,

pH 9·5, was used for the determination of glycerol 1-phosphate. A stock solution containing 1·89 g. of glycine, 1·3 g. of hydrazine sulphate and 46·8 mg. of EDTA (disodium salt) in a final volume of 20 ml. was stored in a refrigerator. Just before use a portion was brought to pH 9·5 with 5N-sodium hydroxide. The dilution factor due to pH adjustment was 1·4, water being added if necessary.

Glass-redistilled water was used throughout this study.

### *Estimation of metabolites*

In all photometric work, blank cuvettes contained the buffer used in the reaction cuvette.

*2-Phosphoglycerate, phosphoenolpyruvate and pyruvate.* These were estimated simultaneously by using the following reactions:

$$\text{2-Phosphoglycerate} \xrightarrow{\text{enolase}} \text{phosphoenolpyruvate} + H_2O$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{DPNH} + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{DPN}^+$$

To the cuvette were added triethanolamine buffer (to give a total volume without enzymes of 1 ml.), 0·05 ml. of 0·2M-$MgSO_4$ in 2M-KCl, 0·04 ml. of 20 mM-ADP, 0·012 ml. of 10 mM-DPNH and the test solution (volume such that the total change in extinction did not exceed 0·4). The addition of 0·005 ml. of lactate dehydrogenase (approx. 500 units) gave a change in extinction ($\Delta E$) which measured pyruvate. When $E$ became steady, 0·005 ml. of pyruvate kinase (50 units) was added, $\Delta E$ now measuring phosphoenolpyruvate. Finally, 0·01 ml. of enolase (20 units) was added, $\Delta E$ measuring 2-phosphoglycerate.

*3-Phosphoglycerate and 1,3-diphosphoglycerate.* These were estimated simultaneously by using the following reactions:

$$\text{3-Phosphoglycerate} + \text{ATP} \xrightarrow{\text{phosphoglycerate kinase}} \text{1,3-diphosphoglycerate} + \text{ADP}$$

$$\text{1,3-Diphosphoglycerate} + \text{DPNH} + H^+ \xrightarrow{\text{glyceraldehyde phosphate dehydrogenase}} \text{glyceraldehyde 3-phosphate} + \text{phosphate} + \text{DPN}^+$$

To the cuvette were added triethanolamine buffer as above, 0·008 ml. of 0·2M-$MgSO_4$, 0·1 ml. of 16 mM-ATP, 0·012 ml. of 10 mM-DPNH and test solution. Addition of 0·005 ml. of glyceraldehyde phosphate dehydrogenase (70 units) gave $\Delta E$ measuring 1,3-diphosphoglycerate. Finally, 0·01 ml. of phosphoglycerate kinase (200 units) was added, $\Delta E$ measuring 3-phosphoglycerate.

*Hexose 1,6-diphosphate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.* These were estimated simultaneously by using the following reactions:

$$\text{Hexose 1,6-diphosphate} \xrightarrow{\text{aldolase}} \text{glyceraldehyde 3-phosphate} + \text{dihydroxyacetone phosphate}$$

$$\text{Glyceraldehyde 3-phosphate} \xrightarrow{\text{triose phosphate isomerase}} \text{dihydroxyacetone phosphate}$$

$$\text{Dihydroxyacetone phosphate} + \text{DPNH} + H^+ \xrightarrow{\text{glycerol phosphate dehydrogenase}} \text{glycerol 1-phosphate} + \text{DPN}^+$$

To the cuvette were added triethanolamine buffer as above, 0·012 ml. of 10 mM-DPNH and test solution. Successive additions of 0·01 ml. of glycerol phosphate dehydrogenase (60 units), 0·005 ml. of triose phosphate isomerase (50 units) and 0·005 ml. of aldolase (50 units) gave $\Delta E$ values measuring respectively dihydroxyacetone phosphate, glyceraldehyde 3-phosphate and hexose 1,6-diphosphate.

*Glucose 6-phosphate.* This was estimated by using the following reaction:

$$\text{Glucose 6-phosphate} + \text{TPN}^+ \xrightarrow{\text{glucose 6-phosphate dehydrogenase}} \text{6-phosphogluconate} + \text{TPNH} + H^+$$

To the cuvette were added triethanolamine buffer as above, 0·008 ml. of 0·2M-$MgSO_4$, 0·02 ml. of 13 mM-$TPN^+$ and test solution. Addition of 0·01 ml. of glucose 6-phosphate dehydrogenase (40 units) gave $\Delta E$ measuring glucose 6-phosphate. If the enzyme preparation contained glutathione reductase, TPNH would be reoxidized when extracts contained much glutathione. This 'back run' could be detected by taking readings every 2 min., and could be prevented by addition of 0·01 ml. of 0·8 mM-$ZnSO_4$ which inhibits glutathione reductase.

*Glycerol 1-phosphate.* This was estimated by using the following reaction:

$$\text{Glycerol 1-phosphate} + \text{DPN}^+ \xrightarrow{\text{glycerol phosphate dehydrogenase}} \text{dihydroxyacetone phosphate} + \text{DPNH} + H^+$$

To the cuvette were added 0·5 ml. of glycine–EDTA–hydrazine buffer (pH 9·5), water to give a final volume of 1 ml. before addition of enzyme, 0·05 ml. of 60 mM-$DPN^+$ and the test solution. Glycerol 1-phosphate was determined from the $\Delta E$ obtained after addition of 0·015 ml. of glycerol phosphate dehydrogenase (300 units) (reaction time about 15 min.). The reaction mixture should contain no phenolphthalein.

*Adenosine diphosphate.* This was estimated by using the following reactions:

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{DPNH} + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{DPN}^+$$

To the cuvette were added triethanolamine buffer as above, 0·04 ml. of 0·2M-$MgSO_4$, 0·05 ml. of 0·2M-KCl, 0·02 ml. of 0·02M-phosphoenolpyruvate, 0·008 ml. of 10 mM-DPNH and the test solution. When $E$ had reached a steady value after addition of 0·005 ml. of lactate dehydrogenase (200 units), 0·005 ml. of pyruvate kinase (50 units) was added, $\Delta E$ then measuring ADP.

*Adenosine triphosphate.* This was estimated by using the following reactions:

$$\text{ATP} + \text{3-phosphoglycerate} \xrightarrow{\text{phosphoglycerate kinase}} \text{ADP} + \text{1,3-diphosphoglycerate}$$

$$\text{1,3-Diphosphoglycerate} + \text{DPNH} + H^+ \xrightarrow{\text{glyceraldehyde phosphate dehydrogenase}} \text{glyceraldehyde 3-phosphate} + \text{DPN}^+ + \text{phosphate}$$

To the cuvette were added triethanolamine buffer as above, 0·04 ml. of 0·2M-$MgSO_4$, 0·07 ml. of 0·1M-3-

phosphoglycerate, 0·008 ml. of 10 mM-DPNH and the test solution. Addition of 0·01 ml. of glyceraldehyde phosphate dehydrogenase (40 units) followed immediately by 0·01 ml. of phosphoglycerate kinase (200 units) gave $\Delta E$ measuring ATP.

*Assay of pyruvate kinase*

To the cuvette were added 0·5 ml. of 0·1 M-triethanolamine buffer, pH 7·6, containing EDTA (10 mM), and water to give a final volume of 1 ml. after adding the following: 0·015 ml. of 0·5 M-$MgSO_4$, 0·04 ml. of 2 M-KCl, 0·02 ml. of 0·02 M-phosphoenolpyruvate, 0·025 ml. of 20 mM-ADP, 0·015 ml. of 10 mM-DPNH and 0·001 ml. of lactate dehydrogenase (100 units). A limiting amount (about 1 unit) of diluted pyruvate kinase was then introduced and when the reaction was well under way ($\Delta E$ approx. 0·05) the time for $E$ to change by 0·1 was measured. The same experiment was repeated in the presence of malonate or sodium chloride. Here solutions of malonate or sodium chloride were substituted for part of the water added to the cuvette to give final concentrations of 0·12 and 0·06 M with respect to malonate and 0·12 M with respect to sodium chloride. Blank cuvettes contained the buffer used in the reaction cuvettes.

## RESULTS

Table 1 shows the effect of malonate on a glycolysing muscle suspension. The usual methods of acid hydrolysis were applied to make a preliminary study of the phosphorus compounds. Phosphorus values for fresh muscle without incubation are also given. During incubation without malonate most of the 'labile phosphorus' appears as inorganic phosphate. In the malonate-treated suspension, however, about half of the total phosphorus appears in a bound form which is not hydrolysed after contact with N-hydrochloric acid for 3 hr. at 100°. Since the malonate-treated suspension formed much less lactate than did the control, it was concluded that the accumulating acid-resistant-phosphate fraction must be an intermediate in the glycolytic pathway. The nature of this fraction was determined enzymically after removal of malonate which, it was feared, might inhibit some of the enzymes. Table 2 shows that more than one phosphorus compound accumulates, 3-phosphoglycerate and glycerol 1-phosphate being formed in about equal quantities. In addition to these acid-resistant compounds, the more labile phosphoenolpyruvate is formed in smaller amount. The last-named must be responsible for the higher 'labile-phosphorus' value in the malonate-treated suspension than that in the control suspension (Table 1), since at the end of the incubation period both suspensions contained only traces of ATP and small amounts of ADP. Table 2 shows also that the presence of malonate in the test samples did not interfere with the enzymic tests. This is not surprising, as the inhibitor was diluted many-fold by the reaction medium and an excess of the necessary enzymes was used in each test. The ether-extraction step was thus abandoned in later experiments.

The maximum yield of intermediates was obtained when the suspension was allowed to stand at room temperature for a short period (about 3 min.) and at a lower temperature (6°) for about 2 hr. (Table 3). When the incubation was carried out only at room temperature the yield was small, owing presumably to inactivation of some enzymes, and, when a low temperature was maintained throughout, at least 5 hr. of incubation was required for maximum effect. Under all conditions all intermediates appeared simultaneously. Addition of 3-phosphoglycerate, 2-phosphoglycerate or phosphoenolpyruvate to the control suspension resulted in their breakdown to inorganic phosphate and pyruvate or lactate. When, however, these esters were added to the malonate-treated suspen-

Table 1. *Effect of malonate on phosphorus compounds of rat-skeletal-muscle suspension and on lactate production*

Frozen skeletal muscle was powdered as described in the Methods section. A portion of powder was treated with $HClO_4$ to obtain 'resting' values. The remaining powder was used for preparing control and malonate suspensions. For maximum effect the suspensions were incubated for 5 hr. at 6°; protein-free extracts were then prepared and analysed for acid-soluble phosphorus. All values are expressed as mg. of phosphorus/100 g. of wet tissue except for lactate which is given in mg. of lactic acid/100 g. of wet tissue. The terms 'Hydrolysable P (7 min.)' and 'Hydrolysable P (180 min.)' denote inorganic phosphate liberated after treatment with N-HCl at 100° for 7 and 180 min. respectively in addition to inorganic phosphate found before hydrolysis.

| | Unincubated control muscle | Control suspension | Malonate suspension |
|---|---|---|---|
| Lactic acid | 42·2 | 617 | 96·7 |
| Phosphocreatine | 49·8 | 1·0 | 1·3 |
| Phosphocreatine + inorganic phosphate | 79·8 | 134·0 | 58·5 |
| Hydrolysable P (7 min.) ($H_7$) | 122·6 | 136·5 | 65·8 |
| Labile P | 42·8 | 2·5 | 7·3 |
| Total P (TP) | 163·4 | 167·5 | 170·5 |
| Non-labile P (TP − $H_7$) | 40·8 | 31·0 | 104·7 |
| Hydrolysable P (180 min.) | — | 145·0 | 85·0 |
| Resistant P (to hydrolysis for 180 min.) | — | 22·5 | 85·5 |

sion, the greater part of each was found as 3-phosphoglycerate after incubation. The fact that phosphoenolpyruvate gave rise to 3-phosphoglycerate, but not to lactate or pyruvate, implies that the block must lie at the pyruvate-kinase level. The inhibition of pyruvate kinase must have resulted in a reversal of the glycolytic reactions which could not proceed beyond 3-phosphoglycerate owing to lack of ATP.

Table 4 summarizes the results obtained after the addition of various substances to malonate-treated muscle suspensions. The figures in Expt. i show the effect of incubation for 15 min. with malonate in the cold. A significant amount of phosphate esters accumulates and the process continues, although at a lower rate, during the next hour (Expt. ii). The rate of accumulation of esters increases if inorganic phosphate is present (Expt. iii). When inorganic phosphate is added at the beginning together with malonate and the incubation period prolonged, its effect is more pronounced than in Expt. iii. The stimulating effect of inorganic phosphate can be reproduced by phosphocreatine, ATP and other esters that can yield inorganic phosphate notwithstanding the block. These findings suggest that in this system inorganic phosphate is a limiting factor since the inorganic phosphate, or its precursors originally present in muscle, are trapped in the form of the accumulating esters. When phosphoenolpyruvate is added (Expt. iv) there is an increased formation of 3-phosphoglycerate which accounts almost quantitatively for the decrease in phosphoenolpyruvate, but the increase in lactate is insignificant. When pyruvate is added (Expt. v) lactate is formed, the increase in the latter being equivalent to the decrease in the former; however, very little glycerol 1-phosphate is formed. It is clear that, in the malonate experiments without pyruvate, glycerol 1-phosphate is a secondary product. In other words, in the absence of pyruvate, the DPNH formed in the 'oxidation reaction of fermentation' was reoxidized through the reduction of triose phosphate. Since the lactate-dehydrogenase-catalysed reaction is more efficient in regenerating $DPN^+$, it is clear that addition of pyruvate to the malonate-treated suspension (Expt. v) results in a

Table 2. *Glycolytic intermediates in rat-skeletal-muscle extracts assayed before and after ether extraction*

Portions of perchloric acid extracts previously analysed for acid-soluble phosphorus compounds (see Table 1) were used directly for enzymic estimation of intermediates. Remaining portions were freed of malonate by ether extraction for 3 hr. and then analysed. In both cases extracts were neutralized before assay. The values are expressed as mg. of phosphorus/100 g. of tissue (wet wt.). Where no value is given the compound was absent or present in amounts too low for assay by the enzymic method.

| | Unincubated control muscle | Control suspension | | Malonate suspension | |
|---|---|---|---|---|---|
| | | Before ether extraction | After ether extraction | Before ether extraction | After ether extraction |
| 3-Phosphoglycerate | — | — | — | 36·30 | 36·00 |
| 2-Phosphoglycerate | — | — | — | 3·59 | 3·32 |
| Glycerol 1-phosphate | 0·73 | 1·81 | 1·95 | 30·30 | 29·40 |
| Glucose 6-phosphate | 6·33 | 4·93 | 4·72 | 2·54 | 2·60 |
| Hexose 1,6-diphosphate | 0·25 | — | — | — | — |
| Dihydroxyacetone phosphate | — | — | — | 1·71 | 1·04 |
| Phosphoenolpyruvate | — | — | — | 12·07 | 10·25 |
| ATP (2 phosphate residues hydrolysed/mol.) | 39·2 | — | — | — | — |
| ADP (1 phosphate residue hydrolysed/mol.) | 2·25 | 1·76 | 1·62 | 1·60 | 1·16 |

Table 3. *Accumulation of glycolytic intermediates*

Frozen-muscle powder was added to weighed malonate medium kept at room temperature. After shaking and reweighing, which took 3 min., the suspension was cooled in ice–water. The volume was adjusted to obtain 1:10 dilution of muscle, and 10 min. from the time of powder transfer the suspension was placed in a refrigerator (6°). At various intervals portions were withdrawn, deproteinized and analysed enzymically for intermediates. All values are expressed as mg. of phosphorus/100 g. of tissue (wet wt.).

| Time of incubation with malonate (min.) | Phosphoenol-pyruvate | 2-Phospho-glycerate | 3-Phospho-glycerate | Glycerol 1-phosphate |
|---|---|---|---|---|
| 6 | 6·12 | 1·84 | 19·00 | 22·30 |
| 30 | 8·60 | 2·45 | 29·10 | 30·80 |
| 120 | 10·90 | 3·18 | 45·60 | 33·40 |

Table 4. *Effect of addition of phosphate, pyruvate, phosphate + pyruvate, and phosphoenolpyruvate on accumulation of glycolytic intermediates in a malonate-blocked system*

Malonate-treated muscle suspension (1:10 dilution) was prepared as in Table 3. Six portions (10 ml. each) were pipetted into separate flasks and kept on ice. At 15 min. (from time of powder transfer) one portion was deproteinized (Expt. i). To other portions different solutions were added in a total volume of 1 ml. All flasks were then kept at 6° for 1 hr. After deproteinization the neutralized filtrates were analysed. Values, including those for added compounds, are expressed as mg. of phosphorus/100 g. of tissue (wet wt.), except lactate and pyruvate values which are given in mg. of the corresponding acids/100 g. of tissue (wet wt.).

| Expt. | Time of incubation (hr.) | Addition | Amount added | Lactate | Pyruvate | Phosphoenol-pyruvate | 2-Phospho-glycerate | 3-Phospho-glycerate | Glycerol 1-phosphate |
|---|---|---|---|---|---|---|---|---|---|
| i | 0 | None | — | 84 | — | 6·8 | 2·1 | 23·1 | 24·4 |
| ii | 1 | None | — | 95·7 | — | 10·6 | 2·8 | 33·6 | 31·6 |
| iii | 1 | Inorganic phosphate | 101 | 85·2 | — | 12·5 | 3·2 | 38·2 | 38·6 |
| iv | 1 | Phosphoenolpyruvate | 92·2 | 91·0 | — | 34·4 | 8·5 | 106·2 | 33·9 |
| v | 1 | Pyruvate | 285 | 176 | 191 | 14·3 | 4·7 | 43·2 | 27·1 |
| vi | 1 | Inorganic phosphate + pyruvate | 101<br>285 | 233 | 136 | 15·7 | 6·4 | 62·2 | 26·6 |

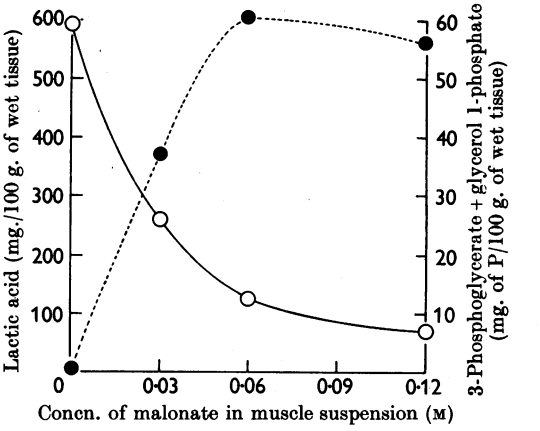


Fig. 1. Effect of malonate concn. on lactate production and on accumulation of acid-resistant phosphate esters. ○, Lactate; ●, phosphate esters. Rat-muscle suspensions were prepared as in Table 3. They were kept at room temperature for 10 min., then in ice–water for 5 min., then at 6° for 105 min.

greater formation of total esterified phosphorus than when no pyruvate is added (Expt. ii). Simultaneous addition of pyruvate and inorganic phosphate (Expt. vi) produces an additive effect. Here again lactate is formed at the expense of pyruvate.

Hitherto, malonate was used at a concentration of 0·12 M. However, 0·06 M-malonate is as effective as 0·12 M if the accumulation of phosphate esters is taken as a criterion, although there is incomplete (90 %) inhibition of lactate formation. With 0·03 M-malonate the inhibition of lactate production is about 60 % and the accumulation of phosphate esters is about 60 % of that observed with the higher concentrations (Fig. 1).

Finally, the effect of malonate on crystalline pyruvate kinase was tested. In a system where the enzyme was limiting, a decrease in $E_{340}$ of 0·1 required 51 sec. With 0·12 M-malonate the reaction was too slow for accurate measurement, with 0·06 M-malonate the reaction time was 365 sec. and with 0·03 M it was 153 sec. In the presence of 0·12 M-sodium chloride the reaction time was 83 sec., indicating that the inhibition by malonate is predominantly a malonate and not a $Na^+$-ion effect. Thus the effect of malonate on the pure enzyme system is not unlike that on the muscle suspension.

## DISCUSSION

Glycolysis in muscle has so far been studied with press juice, extracts from mince, slices and homogenates. The preparation described in this paper has certain advantages over these. First, samples obtained from a single frozen-muscle powder are

more likely to be uniform than if they were removed from various parts of a muscle or group of muscles. Secondly, the frozen powder is especially suitable for time studies. This is not the case with a mince or homogenate, since when the homogenization is completed the glycolytic process is well under way, and labile phosphorus compounds will have decomposed. Glycolysis can be studied in our system without the addition of substrates and 'fortifying' substances. Freezing and thawing may liberate some of the oxidative mitochondrial enzymes. However, this did not seem to influence the glycolysis in our system, for similar results were obtained when the suspension was incubated under either aerobic or strictly anaerobic conditions. When homogenates were prepared in a medium containing malonate, the same products accumulated as in our system, but it was not possible to perform quantitative studies owing to the difficulty in uniformly homogenizing striated muscle.

The results reported in this paper support the view that malonate in a concentration exceeding 0·06M almost completely inhibits the reaction involving pyruvate kinase. The formation of phosphoglycerate (from diphosphoglycerate) requires the presence of ADP and is accompanied by ATP formation. Table 2 shows that ADP is available throughout, its concentration after incubation with malonate not differing markedly from that found in the unincubated control. The fact that ATP is found only in traces implies that it is hydrolysed as soon as it is formed.

Malonate at a concentration of 0·3M causes only partial blockage of glycolysis in our muscle suspension, and only partly inhibits pyruvate kinase in a pure system. It has no action on the performance of the dog heart–lung preparation. At a concentration of 0·06M, malonate almost completely blocks glycolysis and causes nearly complete inhibition of the pure enzyme system. It also affects the isolated heart adversely in its force of contraction and rhythm. The parallelism of malonate action in all three preparations suggests that the deleterious action of 0·06M-malonate on the isolated heart is due, as in the muscle suspension and pure enzyme system, to an inhibition of pyruvate kinase rather than an inhibition of succinate oxidation.

Krebs (1960) considers that the accumulation of succinate in tissues (Krebs, Salvin & Johnson, 1938; Busch & Potter, 1952*a*) after injection of malonate into intact animals is evidence that the citric acid cycle operates *in vivo*. Our experiments with malonate on the heart–lung preparation may also be considered as evidence for the operation *in vivo* of the cycle, since here succinate also accumulates. The question, however, arises whether the citric acid cycle is the only oxidative pathway in the isolated dog heart. According to Krebs's experiments on muscle mince, 0·03M-malonate should inhibit succinate oxidation completely. There is no reason to believe that malonate added to a muscle-mince suspension would diffuse more readily into the cells than if it were added to the blood in a heart–lung preparation. Busch & Potter (1952*b*) injected malonate into intact rats in a dose roughly equivalent to ours (0·03M) in the isolated heart. The accumulation of succinate in the intact rat heart (5 $\mu$equiv./g.) was of the same order of magnitude as that found by us in the isolated heart. Busch & Potter (1952*b*) found in the rat heart about 18·4 $\mu$-equiv. of malonate/g., which suggests that malonate diffused into the heart cells as it did into liver and kidney cells. According to Krebs & Eggleston (1940) a succinate:malonate ratio of 0·3 should almost completely inhibit succinate oxidation. In our experiments on the heart–lung preparation the accumulation of succinate with 0·06M-malonate was insignificantly higher than with half the dose, yet heart failure was observed only with the higher dose, which in the muscle suspension and the pure enzyme system inhibited pyruvate kinase almost completely. In the light of these findings, we feel justified in asking whether the oxidation of pyruvate in the isolated dog heart proceeds solely via the citric acid cycle.

## SUMMARY

1. A rat-muscle suspension from powdered frozen muscle is described.

2. Malonate at concentrations above 0·06M inhibits lactate formation almost completely and causes accumulation of 3-phosphoglycerate, glycerol 1-phosphate and phosphoenolpyruvate.

3. Addition of phosphoenolpyruvate to the malonate-treated suspension gives rise to 3-phosphoglycerate but not to pyruvate and lactate, as in the untreated suspension. The inhibition of pyruvate kinase results in a reversal of the glycolytic reactions which cannot proceed beyond 3-phosphoglycerate owing to lack of ATP.

4. Addition of pyruvate to the malonate-blocked system results in a stoicheiometric formation of lactate. 3-Phosphoglycerate production is increased, but no glycerol 1-phosphate is formed.

5. Malonate at a concentration of 0·03M only partly inhibits glycolysis in our system. Experiments on the pure enzyme system also showed that, though 0·06M-malonate strongly inhibits pyruvate kinase, 0·03M-malonate is only partially effective.

6. These results are discussed in the light of previous experiments on dog heart–lung preparations in which 0·03M-malonate did not interfere with heart action but 0·06M malonate caused marked failure, in spite of the fact that the extent of succinate accumulation with both concentrations was the same.

This work was supported by a grant from the American Heart Association. We wish to thank Dr H. U. Bergmeyer, Director of the Boehringer Biochemical Laboratory, Tutzing, Germany, for his advice and for his co-operation in delivering the enzymes used in this study.

## REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Beisenherz, G., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K. H., Meyer-Arendt, E. & Pfleiderer, G. (1953). *Z. Naturf.* **8b**, 555.
Bergmeyer, H. U. (1962). *Methoden der enzymatischen Analyse.* Weinheim: Verlag Chemie (in the Press).
Busch, H. & Potter, V. R. (1952*a*). *J. biol. Chem.* **198**, 71.
Busch, H. & Potter, V. R. (1952*b*). *Cancer Res.* **12**, 660.
Fawaz, G. & Hawa, E. S. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 277.
Fawaz, G., Tutunji, B. & Fawaz, E. N. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 770.
Fiske, C. H. & Subbarow, Y. (1929). *J. biol. Chem.* **81**, 629.
Hohorst, H. J., Kreutz, F. H. & Bücher, Th. (1959). *Biochem. Z.* **332**, 18.
Krebs, H. A. (1960). *Metabolic Pathways*, vol. I, p. 151. New York and London: Academic Press.
Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
Krebs, H. A., Salvin, E. & Johnson, W. A. (1938). *Biochem. J.* **32**, 113.
Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 65.
Scholz, R., Schmitz, H., Bücher, Th. & Lampen, J. O. (1958). *Biochem. Z.* **331**, 71.
Slater, E. C. (1953). *Biochem. J.* **53**, 157.

---

*Biochem. J.* (1962) **83**, 445

# The Oxidation of the Lipids of the Erythrocyte

BY B. COWLISHAW
*Biochemistry Department, University of Sydney, Sydney, N.S.W., Australia*

(*Received* 19 *September* 1961)

It was found by Rose & György (1952) and confirmed by later workers (Christensen, Dam, Gortner & Søndergaard, 1956; Horwitt, Harvey, Duncan & Wilson, 1956) that, in vitamin E deficiency, erythrocytes are very susceptible to haemolysis under oxidizing conditions. Tocopherol and other lipid antioxidants afford protection to the cells under these conditions and this has led to the hypothesis that in the absence of such protection oxidation of unsaturated-lipid components of the cell membrane occurs, and this leads to the observed haemolysis either directly as a result of structural changes or indirectly through the release of a haemolytic agent.

Evidence supporting this hypothesis has come from studies with the thiobarbituric acid test used as a measure of lipid oxidation (Horwitt *et al.* 1956; Bunyan, Green, Edwin & Diplock, 1960; Tsen & Collier, 1960). This reagent forms a coloured complex with malonaldehyde or epihydrin aldehyde (Glavind & Hartmann, 1951; Weissbach & Hurwitz, 1959) which is released under acid conditions from oxidized lipids. However, the positive results obtained by these workers are not sufficient to prove the hypothesis. The possibility that non-lipid components may be involved has not been eliminated, and this is particularly important in studies on erythrocytes since it has recently been shown that under some conditions haem compounds give a positive reaction (Cowlishaw, 1961). Moreover, it is known that linolenic acid is one of the most reactive fatty acids in the thiobarbituric acid test (Wilbur, Bernheim & Shapiro, 1949) and this acid is absent from erythrocytes (Evans, Waldron, Oleksyshyn & Riemenschneider, 1956). Hence direct evidence that erythrocyte lipids are sufficiently reactive to account for the observed malonaldehyde production in erythrocytes is desirable.

For this purpose it is necessary to use a lipid preparation that is free from endogenous antioxidants. Preliminary experiments showed that the most convenient preparation for this purpose is an aqueous emulsion of erythrocyte fatty acids from which all unsaponifiable material had been removed. The present study is concerned with the oxidative changes observed in a preparation of this kind. The behaviour of malonaldehyde and tocopherol have also been investigated.

## EXPERIMENTAL

*Preparation of erythrocyte-fatty acid suspension.* Erythrocytes from normal human blood were separated and washed five times with 0·15M-NaCl. The haematocrit of the final suspension was calculated from its haemoglobin content, and a portion of the suspension containing 7 ml. of erythrocytes was used for lipid extraction. This was diluted to give