XXXIX. EFFECTS OF HYDROXYMALONATE ON THE METABOLISM OF BRAIN

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As a result of the earlier work of Meyerhof on muscle, the applicability of a similar theory of carbohydrate metabolism to other tissues, including brain, has been examined. According to the Meyerhof view, the following reactions take place:

$$\text{Carbohydrate} \underset{O_2\,(2)}{\overset{(1)}{\rightleftarrows}} \text{Lactic acid} \xrightarrow{O_2\,(3)} \text{Oxidation products}$$

Attempts to show production of carbohydrate from lactic acid by brain have been unsuccessful [Holmes & Ashford, 1930; Ashford & Holmes, 1931] and therefore reaction (2) of the scheme most probably does not take place in brain.

Granted this, the oxidation of lactic acid by brain (reaction (3)) does not proceed fast enough to account for the normal difference between the net aerobic production of lactic acid and the anaerobic production, as far as glucose is concerned.

It must therefore be concluded that reaction (1), when glucose is the carbohydrate in question, does not normally proceed in brain as rapidly aerobically as it does anaerobically. This conclusion [cf. Dixon, 1936] is independent of the particular mechanisms which have so far been suggested to account for the fact [Jowett & Quastel, 1933; 1934; Lipmann, 1933].

It has, however, been possible up to the present to believe that the only mode of breakdown of glucose in brain is to lactic acid. On this view, the oxidative metabolism of glucose may be resolved into two sets of processes, involving the formation and the oxidation of lactic acid.

The experiments reported here lead to the conclusion that glucose can be oxidized in brain tissue by a mechanism or mechanisms not involving lactic acid as an intermediary. It may be added that it has for some time been clear that lactic acid is not an intermediary in the oxidation of fructose by brain, for whilst fructose is oxidized at a rate similar to that of glucose it is not broken down to lactic acid at an appreciable rate.

The use of hydroxymalonate as an inhibitor of lactic dehydrogenase in brain was suggested by the work of Quastel and Wooldridge [1928], who found such an inhibiting action exerted with *B. coli*.

Experimental methods

The manometric methods of Warburg have been applied to the study of the metabolism of slices of the grey matter of the cerebral cortex of the rat and guinea-pig. About 8–15 mg. dry weight of brain have been used in each vessel, in 3 or 3·2 ml. solution. Media have been made up on the lines followed by Jowett & Quastel [1935], acids being added as sodium salts unless otherwise stated.

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Colorimetric method for pyruvic acid estimation

Use has been made of the fact that pyruvic 2:4-dinitrophenylhydrazone dissolves in aqueous alkali, giving a deep red colour. Typically, to 1 ml. of the solution to be estimated there is added 1 ml. of a freshly filtered saturated solution of 2:4-dinitrophenylhydrazine in *N* HCl. Half an hour later 2·5 ml. *N* NaOH are also added, whereupon a deep colour appears at once. This disappears rapidly, leaving a deep red colour where pyruvic acid has been present, and a pale yellow colour in the "blanks".[1] The red colour is approximately proportional to the amount of pyruvic acid. To eliminate the influence of the yellow "blank" colour in matching, we have thought it best to prepare each time a series of standard colours with known amounts of pyruvic acid, each unknown solution being compared with a known solution the colour of which is within 30% in depth. All solutions are diluted to an equal, convenient volume with water, before their colours are compared in a Klett colorimeter. The method appears satisfactory as far as it has been tested. It is an extremely sensitive test for pyruvic acid.

Other ketonic acids give similar colours, and the results must be interpreted with this fact in mind. Under the conditions described acetaldehyde and acetone give feeble colours. The method is therefore less specific for ketonic acids than the very elaborate method of Case [1932] based on the same principle.

Experiments on respiration

In Table I are shown the effects of sodium hydroxymalonate, at a concentration of about 0·07 *M*, on the oxidation of several substrates by slices of guinea-pig brain. The concentration of the inhibitor is as high as can be used in isotonic

Table I. *The effect of hydroxymalonate on oxidations of guinea-pig brain*

Composition of medium: K^+ 0·002 *M*; Ca^{++} 0·001 *M*; Mg^{++} 0·0008 *M*; phosphate 0·02 *M*; initial *p*H 7·2. O_2. Temp. 39°. Experimental period 40 min. Hydroxymalonate (when present) 0·067–0·075 *M*.

Exp.	Substrate	Q_{O_2} in absence of inhibitor	Q_{O_2} in presence of inhibitor	Effect of inhibitor %
1	Glucose 0·02 *M*	15·8	11·1	−30
	dl-Lactate 0·01 *M*	13·9	6·6	−52
2	Glucose 0·02 *M*	14·7	11·6	−21
	dl-Lactate 0·01 *M*	13·5	9·4	−30
3	Pyruvate 0·01 *M*	12·6	11·9	− 5
	dl-Lactate 0·01 *M*	14·0	8·8	−37
4	Pyruvate 0·01 *M*	13·5	12·5	− 7
5	Nil	6·5	4·3	−33
	Fructose	14·2	11·9	−16
6	Nil	5·3	3·5	−34
	Fructose	13·8	11·1	−20
7	*d*-Lactate 0·01 *M*	13·8	9·2	−33
	dl-Lactate 0·01 *M*	13·8	9·6	−30
8	*d*-Lactate 0·005 *M*	14·8	8·6	−42
	dl-Lactate 0·005 *M*	12·8	6·9	−46
9	*d*-Lactate 0·02 *M*	14·8	11·6	−22
	d-Lactate 0·005 *M*	13·2	8·7	−34
10	*d*-Lactate 0·02 *M*	16·2	11·9	−26
	d-Lactate 0·005 *M*	13·5	8·5	−37

[1] The saturated reagent should be kept for some days before use, or a turbidity may appear at this stage.

buffered solutions, if we take an isotonic solution of sodium hydroxymalonate to be 0·107 *M*. Lower concentrations of hydroxymalonate give smaller inhibitions.

It is evident that the oxidation of lactate is inhibited more than that of other substrates. The oxidations of glucose and of fructose are less inhibited, and that of pyruvate hardly at all.

The respiration in presence of *d*-lactate is inhibited to about the same extent as that in presence of *dl*-lactate. Also the inhibition of respiration in presence of lactate is greater at lower lactate concentrations.

Now lactate is believed on good grounds to be oxidized through pyruvate as an intermediary. Since the oxidation of lactate is considerably inhibited, and that of pyruvate almost unaffected, it follows that the inhibitory action of hydroxymalonate on the oxidation of lactate is exerted almost entirely on the first step, the oxidation of lactate to pyruvate. Further, the action is probably due to competition of hydroxymalonate with lactate for union with lactic dehydrogenase, since the inhibition varies with the lactate concentration.

An important conclusion appears to follow regarding the mechanism of oxidation of glucose. In the presence of hydroxymalonate the oxidation of glucose is definitely faster than that of lactate. Hence oxidation of glucose cannot proceed more than partially through lactate as an intermediary. Glucose need not necessarily pass at all through lactate as an intermediary: what is shown is that a path of oxidation, other than through lactate, must exist, which is quantitatively of some importance.

Indicator tests show that during the experiments the glucose solutions in presence of brain become acid, and it can be concluded that lactic acid is formed to an appreciable extent. Probably, therefore, some of the oxidation in the glucose solutions is oxidation of lactate. It is therefore possible that the inhibition of oxidation by hydroxymalonate in presence of glucose is actually an inhibition of lactate oxidation. The oxidation of fructose is, however, also somewhat inhibited by hydroxymalonate, although brain has practically no power of splitting fructose to lactic acid [Dickens & Greville, 1932]. By analogy, the inhibition of oxidation in presence of glucose is not necessarily an inhibition of lactate oxidation.

Experiments with rat brain, reported in Table II, show similar results to those with guinea-pig brain. The inhibition of lactate oxidation is greater than

Table II. *The effect of hydroxymalonate on oxidations of rat brain*

Medium: phosphate 0·02 *M*, initial *p*H 7·2. O_2. Temp. 39°. Experimental period 30 min. Hydroxymalonate (when present) 0·070 *M*.

Exp.	Substrate	Q_{O_2} in absence of inhibitor	Q_{O_2} in presence of inhibitor	Effect of inhibitor %
	Medium *A*: $K^+ = 0{\cdot}002\,M$,	$Ca^{++} = 0{\cdot}001\,M$,	$Mg^{++} = 0{\cdot}0008\,M$.	
1	Glucose 0·02 *M*	13·8	12·5	− 9
	d-Lactate 0·01 *M*	15·25	11·5	−25
2	*dl*-Lactate 0·01 *M*	13·1	9·2	−30
	Medium *B*: $K^+ = 0{\cdot}0128\,M$,	$Ca^{++} = Mg^{++} = 0$.		
2	*dl*-Lactate 0·01 *M*	21·7	9·8	−55
3	*dl*-Lactate 0·01 *M*	22·6	9·7	−57
	Glucose 0·01 *M*	23·7	16·8	−29
4	*dl*-Lactate 0·01 *M*	25·7	11·9	−54
	Glucose 0·01 *M*	23·3	16·9	−28
5	*dl*-Lactate 0·01 *M*	21·9	9·5	−57
	Pyruvate 0·01 *M*	20·7	18·6	−10

that of glucose, while again pyruvate oxidation is little affected. In a medium rich in potassium and containing no calcium, it is particularly clear that glucose oxidation proceeds much faster than lactate oxidation in presence of hydroxymalonate. It may be noted that the data show an effect of the ionic composition of the medium on the sensitivity of brain oxidations to inhibitors. Other inhibitors show similar effects, which will be reported later.

After these experiments were made we found that Himwich and Fazikas have claimed in a brief note [1935] that nicotine has a similar action on the respiration of minced brain. Nicotine is stated to inhibit the respiration in the presence of lactate considerably under conditions where it does not inhibit oxidation of glucose or pyruvate. Nicotine may therefore be a more specific inhibitor than hydroxymalonate.

Experiments on the anaerobic breakdown of pyruvate

It has been shown that brain tissue reduces pyruvate to lactate under anaerobic conditions [Haarmann, 1932]. Krebs has claimed in a brief note [1936] that oxidation of part of the pyruvate accompanies the reduction.

Since hydroxymalonate inhibits the action of lactic dehydrogenase, which activates pyruvate as a hydrogen acceptor, it follows that hydroxymalonate should inhibit the reduction of pyruvate to lactate in brain, and should therefore inhibit the anaerobic breakdown of pyruvate by brain.

This expectation has been realized, as is shown in Table III. The inhibition of pyruvate breakdown which is observed is of the same order as the inhibition of lactate oxidation found aerobically. It will be seen that the carbon dioxide output is inhibited to an approximately equal extent, so that this output is perhaps, as Krebs suggests, coupled with the reduction of pyruvate to lactate.

In the experiments of Table III each part is set up in duplicate, one vessel being taken from the thermostat 15 min. after the beginning, the other vessel being shaken in the thermostat 2 hours longer. The first vessel serves as a control

Table III. *The effect of hydroxymalonate on the anaerobic breakdown of pyruvate by rat brain*

Composition of medium: $K^+ = 0{\cdot}006\,M$, $Ca^{++} = 0{\cdot}001\,M$, $Mg^{++} = 0{\cdot}0008\,M$, $HCO_3^- = 0{\cdot}0025\,M$. $N_2 + 5\%$ CO_2. Temp. 39°. Hydroxymalonate (when present) $= 0{\cdot}07\,M$. Experimental period = 2 hours. Chromous chloride usually present in side-tube. Q_p = rate of formation of pyruvic acid in the same units as for $Q_{CO_2}^{N_2}$.

Exp.	Initial conc. pyruvate	$Q_{CO_2}^{N_2}$ in absence of inhibitor	Q_p in absence of inhibitor	$Q_{CO_2}^{N_2}$ in presence of inhibitor	Q_p in presence of inhibitor	% effect of inhibitor on $Q_{CO_2}^{N_2}$	% effect of inhibitor on Q_p
1	$0{\cdot}0033\,M$	+1·69	?	+0·96	?	−43	?
2	$0{\cdot}004\,M$	+1·97	−3·8	+0·89	−2·0	−55	−47
3	0	+0·89	+0·0	+0·54	+0·1	—	—
4	$0{\cdot}004\,M$	+1·99	−3·2	+0·98	−1·85	−51	−42

on the taking up of pyruvate by the tissue from the medium and on the decomposition in the first 15 min. Pyruvate estimations have been made, as already explained, by a colorimetric method, the reagents being added to a portion of the medium directly after removal of the tissue slices.

The colorimetric method will probably respond to keto-acids (and to a less extent to other ketonic substances) which may be formed from pyruvate. The figures given for the rate of disappearance of pyruvate may therefore be too low, but the reality of the inhibition by hydroxymalonate can hardly be questioned.

Experiments on anaerobic glycolysis

Experiment shows (Table IV) that hydroxymalonate inhibits anaerobic glycolysis in brain. Hydroxymalonate likewise inhibits the enhanced rate of glycolysis which, as already shown [Mendel *et al.* 1931; Bumm *et al.* 1933], is brought about by the addition of pyruvate.

Table IV. *The effect of hydroxymalonate on anaerobic glycolysis of brain*

Composition of medium: $K^+ = 0{\cdot}006\,M$, $Ca^{++} = 0{\cdot}001\,M$, $Mg^{++} = 0{\cdot}0008\,M$, $HCO_3^- = 0{\cdot}025\,M$, glucose $= 0{\cdot}01\,M$. $N_2 + 5\%$ CO_2. Temp. 39°. Hydroxymalonate (when present) $= 0{\cdot}070\,M$. Experimental period 30 min.

Exp.	Animal	Conc. pyruvate	$Q_{CO_2}^{N_2}$ in absence of inhibitor	$Q_{CO_2}^{N_2}$ in presence of inhibitor	Effect of inhibitor on $Q_{CO_2}^{N_2}$ %
1	Rat	0	+ 5·65	+2·55	−55
2	Rat	0	+ 5·0	+2·35	−53
3	Guinea-pig	0	+10·6	+1·0	−91
4	Guinea-pig	0	+ 8·5	+1·15	−86
1	Rat	$0{\cdot}01\,M$	+16·5	+6·4	−61
2	Rat	$0{\cdot}001\,M$	+15·45	+5·7	−63
3	Guinea-pig	$0{\cdot}01\,M$	+16·4	+8·75	−47
4	Guinea-pig	$0{\cdot}001\,M$	+18·4	+8·5	−54

Examining the effects in detail, we see (*a*) that the hydroxymalonate inhibition of glycolysis in guinea-pig brain is very great and is relatively less in the presence of pyruvate, (*b*) that with rat brain the inhibition is smaller and little affected by the presence of pyruvate and (*c*) that the inhibitions are little affected by a tenfold change in the pyruvate concentration.

The inhibiting effect of hydroxymalonate on anaerobic glycolysis might be taken as evidence in favour of the formation of pyruvate as an intermediary in anaerobic breakdown of glucose, hydroxymalonate exerting its inhibitory action on lactic dehydrogenase, which is known to activate pyruvate as a hydrogen acceptor. The accelerating effect of pyruvate on glycolysis can be explained if pyruvate acts as a hydrogen acceptor, as in the Embden-Meyerhof mechanism of glycolysis for muscle. If, however, hydroxymalonate inhibits glycolysis because it inhibits lactic dehydrogenase, the addition of pyruvate should decrease the hydroxymalonate inhibition, for it should displace hydroxymalonate from the enzyme. Such a decrease in inhibition occurs with guinea-pig brain, but not with rat brain. The fact that no decrease occurs with rat brain suggests strongly that pyruvic acid is not an intermediary in anaerobic glycolysis in this organ. It is suggested that hydroxymalonate in rat brain may inhibit a keto-hydroxy-acid system similar to, but not identical with, the pyruvic-lactic acid system. Pyruvic acid exerts its accelerating effect on glycolysis in rat brain by acting as a hydrogen acceptor. Even at low concentrations, and in presence of hydroxymalonate, pyruvate is capable of being reduced fast enough to show its full accelerating action on glycolysis.

The results as a whole suggest that in anaerobic glycolysis a keto-hydroxy-acid system is involved, similar to the pyruvic-lactic acid system postulated by the Embden-Meyerhof theory, and that hydroxymalonate decreases anaerobic glycolysis by inhibiting this system. This view will also account for the inhibitory effect of lactate on anaerobic glycolysis in brain [Meyerhof & Lohmann, 1926; Dickens & Greville, 1933], since lactate may be presumed to act like hydroxymalonate. Whether the keto-hydroxy-acid system postulated is identical with

the pyruvic-lactic acid system cannot be decided yet with certainty, but with rat brain the evidence does not support such an identity.

There is indeed at present little evidence that pyruvate is an intermediary in brain glycolysis. It has been claimed [Mazza & Valeri, 1935] that in brain extracts some of the reactions of the Meyerhof-Embden scheme take place. On the other hand in intact brain cells the rate of production of lactic acid anaerobically from pyruvate and α-glycerophosphate is very small [Ashford, 1934; cf. also Johnson, 1936].

To this example of failure of a reaction of the Meyerhof-Embden scheme in brain we can add another. In Table V are shown the effects of α-glycerophosphate

Table V. *Guinea-pig brain*

Medium as in Table I (*p*H 7·4 in Exp. 2). Experimental period = 1 hour.

Substrate	Q_{O_2} Exp. 1	Q_{O_2} Exp. 2
Nil	5·2	4·4
Phosphoglycerate 0·02 *M*	5·6	6·4
α-Glycerophosphate 0·02 *M*	10·7	12·3
Phosphoglycerate 0·02 *M* + α-glycerophosphate 0·02 *M*	9·7	10·6

and of phosphoglycerate (for a sample of which we are indebted to Prof. C. Neuberg) on the respiration of guinea-pig brain. Now from the *p*H change when glucose is present under these aerobic conditions it is probable that glucose is split appreciably into lactic acid. Hence, if the Meyerhof-Embden scheme applies phosphoglycerate should break down to pyruvic acid, and pyruvic acid should react with α-glycerophosphate to produce lactic acid. Therefore phosphoglycerate should raise the respiration appreciably both in the absence and presence of α-glycerophosphate. It is clear that the effect predicted is absent.

Summary

1. Sodium hydroxymalonate inhibits the oxidation of lactate by slices of rat and guinea-pig brains to a greater extent than it inhibits the oxidation of glucose and pyruvate. It is concluded that glucose can be oxidized in brain by a mechanism that does not involve lactate as an intermediary.

2. Hydroxymalonate inhibits the anaerobic breakdown of pyruvate by brain. A simple colorimetric method for estimating pyruvic acid is used.

3. Hydroxymalonate inhibits the anaerobic glycolysis of brain in the absence or presence of pyruvate. Phosphoglycerate does not raise the respiration of brain appreciably either in the absence or presence of α-glycerophosphate. These results are discussed with reference to the Embden-Meyerhof scheme.

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