CCXLIX. THE RÔLE OF GLUTATHIONE IN MUSCLE GLYCOLYSIS.

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THE reactions by which carbohydrate is converted into lactic acid in muscle do not, according to the Embden-Meyerhof scheme, involve the intermediate production of methylglyoxal. The fundamental observation on which the exclusion of methylglyoxal is based is the production of lactic acid by dialysed muscle extracts to which magnesium ions and adenylypyrophosphate have been added. Such extracts are presumably free from glutathione [Meyerhof, 1933], and consequently contain no functioning glyoxalase. Meyerhof [1934] dismisses the fact that methylglyoxal has repeatedly been isolated from muscle extracts by assuming that in such cases it is either an artefact or a by-product formed spontaneously from triosephosphoric acids. It is known that in acid solution both synthetic glyceraldehydephosphoric acid and dihydroxyacetonephosphoric acid do yield methylglyoxal *in vitro* [Kiessling, 1934].

On the other hand it has been denied that methylglyoxal found in incubated muscle extracts is due to the presence of fixing agents [Auhagen and Auhagen, 1934]. The energy derived from the conversion of methylglyoxal into lactic acid has been shown to be utilised by cardiac muscle under anaerobic conditions [Gaddie and Stewart, 1934], and it therefore seems either that this reaction provides an alternative normal mode of breakdown of carbohydrate or that, even under anaerobic conditions, the muscle can use energy from abnormal reactions. According to Meyerhof's view of the position of methylglyoxal, glyoxalase must occupy a position analogous to that of catalase, except that in the latter case there is at least no evidence that the reaction catalysed serves any useful purpose other than the removal of a substance toxic when present in excessive amounts.

Even though the methylglyoxal be formed from its precursor without the intervention of enzymes, it cannot, on that account, be discarded as a by-product of no importance. The fact that the energy of its further breakdown to lactic acid is utilisable, in conjunction with the fact that it is formed under a variety of conditions, suggests that it is more than a by-product, and that, as has been suggested on several occasions [*e.g.* Gaddie and Stewart, 1934; Ashford, 1934; Jowett and Quastel, 1934], there exist alternative routes by which lactic acid may be formed from carbohydrate.

Recently we showed [Stewart *et al.*, 1934] that the Friedmann-Cotonio-Shaffer method for the estimation of lactic acid estimated methylglyoxal in addition, though other intermediates in carbohydrate breakdown did not interfere appreciably. We found that by distillation before removal of carbohydrate by copper sulphate and calcium hydroxide the methylglyoxal could be removed and the true lactic acid estimated in the residual solution. The interference of methylglyoxal has also been demonstrated by Giršavičius and Heyfetz [1934],

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who, however, removed the methylglyoxal by oxidation with hydrogen peroxide. Although Meyerhof and his collaborators had, presumably, used their manometric method for the determination of lactic acid, it seemed desirable to us to repeat the experiments with dialysed muscle extracts using the old and modified oxidation methods simultaneously, and to determine whether there was any marked accumulation of methylglyoxal in the absence of glutathione, and whether the presence of glutathione led to an increased production of lactic acid.

Methods.

We used, at first, a suspension in phosphate buffer (p_H 7.5) of muscle dried *in vacuo* after repeated washing with acetone. The dried material was ground, suspended in water and dialysed through thin collodion membranes against running water for 8–12 hours. At this time no glutathione (or occasionally only a mere trace) could be detected in the fluid by the nitroprusside reaction, though the powder itself still gave a strong reaction owing to the presence of “fixed SH”.

Later, we used a cell-free extract, prepared by freezing solid an aqueous suspension of the dried muscle powder and then centrifuging until the mass had thawed. The supernatant fluid was dialysed as before, but for a longer period—up to 16 hours. The shorter period was usually sufficient to yield a solution in which the nitroprusside reaction was negative.

Both frog and rabbit muscles were used, with essentially similar results. The experiments quoted in this paper were made with rabbit muscle.

Adenylpyrophosphate was added in the form of crude co-enzyme prepared from rabbit muscle as described by Lohmann [1931].

Lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer, and by the modification described by Stewart *et al.* [1934]. In one or two experiments the distillate from the modified method was treated with 2:4-dinitrophenylhydrazine and shown to contain methylglyoxal.

RESULTS AND DISCUSSION.

The first question to be decided was of course whether the presence of glutathione had any effect on the production of lactic acid from glycogen. To answer this question the dialysed muscle extract, mixed with half its volume of phosphate buffer (p_H 7.5), was activated by addition of glycogen, magnesium sulphate and adenylpyrophosphate. Reduced glutathione was added to an exactly similar mixture, and the extract with buffer and glycogen but no other addition was used as control. Air was excluded by a layer of paraffin and the reaction was allowed to continue overnight at room temperature.

Invariably the mixture containing glutathione yielded the most lactic acid and the control the least. The control was never absolutely negative, presumably owing to the difficulty of removing the last traces of glutathione and adenylpyrophosphate, but in most experiments it was very small. Its actual size depended *inter alia* upon the concentration of the muscle extract, but never masked the lactic acid production due to the presence of the co-enzymes. Comparison of the results obtained by using the old and modified forms of the method for lactic acid estimation showed (Table I) that in the absence of glutathione, but not in its presence, there was a considerable accumulation of some substance which was not lactic acid but was estimated as such in the unmodified procedure. The results of Stewart *et al.* [1934] suggest that this substance was methylglyoxal. It was never isolated in these experiments, but

Table I.

Each mixture contained 20 ml. of dialysed muscle extract and 10 ml. of phosphate buffer, p_H 7.5, with the following additions: (a) substrate only, (b) as (a) + magnesium sulphate and adenylypyrophosphate, (c) as (b) + reduced glutathione.

Substrate	Lactic acid mg. per ml. of muscle extract	
	Original method	Modified method
Glycogen (a)	0.87	0.84
(b)	2.73	1.97
(c)	2.82	2.60
Glycogen (a)	0.36	0.23
(b)	2.00	0.83
(c)	1.81	1.23
Hexosediphosphoric acid (a)	0.32	0.31
(b)	0.77	0.39
(c)	1.56	1.44
Hexosediphosphoric acid (a)	0.42	0.41
(b)	0.68	0.41
(c)	1.03	1.00

was, on several occasions, distilled into a solution of 2:4-dinitrophenylhydrazine, and formed a red compound which gave the colour reactions characteristic of the methylglyoxal derivative. The presence of glutathione did not always completely prevent the accumulation of this substance, but it invariably decreased it markedly and sometimes abolished it. It seemed as though the rates of production and removal of methylglyoxal did not necessarily bear a constant relationship to one another in different preparations.

In all these experiments glycogen was the substrate. They indicate quite clearly that inactivation of the glyoxalase system by removal of glutathione causes a decrease in lactic acid production. Consequently they support the view that, in addition to the route suggested by Embden and Meyerhof, lactic acid may be formed from glycogen by way of methylglyoxal. Similar results were obtained with magnesium hexosediphosphate or glucose as substrate (Table I). Glucose and hexosediphosphoric acid resembled each other and differed from glycogen in giving increased activity in presence of glutathione as indicated by an increase in the apparent lactic acid determined by the unmodified method. With glycogen as substrate the unmodified method only occasionally indicated increased activity in the presence of reduced glutathione, and usually the results were similar to those shown in Table I. Geiger [1935], using the unmodified method, has recently reported results very similar to those which we have obtained by the same method of analysis and has concluded that reduced glutathione is without effect on the production of lactic acid from glycogen, but increases the lactic acid production from glucose. He thus suggests a qualitative difference between the mono- and poly-saccharides. Our results with the modified method of analysis show clearly that this conclusion is unjustified, and that the different behaviours of the two substrates are due to quantitative differences in the choice of alternative routes. It is of course possible that glucose does behave differently from glycogen, and that some glycogen is hydrolysed to glucose which is then converted, at least in part, by way of methylglyoxal into lactic acid, and that glycogen, *qua* glycogen, passes exclusively through the stages postulated by Embden and Meyerhof. The increased lactic acid production from glycogen in the presence of glutathione (and the accumulation of methylglyoxal in its absence) would then be explained

by hydrolysis of glycogen and hexosediphosphoric acid to glucose. Whether, in such a case, one describes the breakdown of glycogen to lactic acid by muscle as following two routes or only one, is really a matter of hair-splitting. This explanation however is less likely than that the difference between glycogen and glucose is merely quantitative, since the analytical differences are quantitative (as shown by application of the modified method) and since, moreover, hexosediphosphoric acid is an intermediate in the Embden-Meyerhof scheme for the production of lactic acid from glycogen and, by the unmodified method, gives results very like those given by glucose.

Our next object was to test Meyerhof's statement that any methylglyoxal formed in muscle preparations is an artefact or by-product. If this is the case, incubation of dialysed muscle extracts with glycogen and a magnesium salt alone should yield methylglyoxal, since this system is capable of forming triose-phosphoric acids [Meyerhof and Lohmann, 1934] which may then yield methylglyoxal spontaneously as they do *in vitro* in acid solution [Meyerhof, 1934]. Addition of reduced glutathione to such a system should then yield an equivalent amount of lactic acid. Experiments on these lines indicated (Table II) that the

Table II.

Each mixture contained 20 ml. of dialysed muscle extract and 10 ml. of phosphate buffer, p_H 7.5, with the following additions: (a) glycogen, (b) as (a) + magnesium sulphate, (c) as (b) + reduced glutathione, (d) as (c) + adenylypyrophosphate.

	Lactic acid mg. per ml. of muscle extract	
	Original method	Modified method
(a)	0.84	0.72
(b)	1.12	0.60
(c)	1.48	1.21
(d)	2.53	1.46
(a)	0.77	0.60
(b)	0.88	0.58
(c)	1.87	1.63
(d)	3.01	2.54

addition of magnesium alone to dialysed muscle extracts *plus* glycogen yielded only very small amounts of methylglyoxal, far smaller than those which were present in the more complete system containing adenylic acid pyrophosphate which we had previously studied. This at once suggested that the methylglyoxal which accumulated in the mixtures deficient only in glutathione had been produced by some process in which adenylypyrophosphate was concerned and which was therefore probably enzymic. When however glutathione was added to the mixture of dialysed muscle extract, glycogen and a magnesium salt, lactic acid was produced in quantities far greater than could be accounted for by the methylglyoxal produced in the absence of glutathione. The relatively small amount of methylglyoxal found in the absence of adenylypyrophosphate and glutathione negatives the supposition that formation of methylglyoxal had ceased owing to accumulation of that substance (a supposition hardly justified in any case if the reaction were non-enzymic), and that glutathione, by removing the excess of methylglyoxal, allowed its formation to continue. It therefore seems that glutathione, like adenylypyrophosphate, can, in some as yet unknown way, stimulate the production of methylglyoxal (see Table II). It is of course conceivable that glutathione, though not an essential component of the Embden-Meyerhof system, exercises an activating effect at one or more of the stages of

lactic acid production *via* pyruvic acid. Though there is no evidence to support this, there is equally none to refute it, and it remains a possible explanation of these results. It does not however affect the existence of an alternative route *via* methylglyoxal.

The last stage in the formation of lactic acid according to the Embden-Meyerhof scheme consists in the simultaneous reduction of pyruvic acid and oxidation of glycerophosphoric acid (to triosephosphoric acid). We have tested the influence of glutathione on this reaction. When magnesium ions and adenylypyrophosphate are added to the mixture of buffered dialysed muscle extract, sodium pyruvate and sodium glycerophosphate, lactic acid is formed, but the amount is considerably increased by the further addition of reduced glutathione. This is to be expected on the view that the triosephosphoric acid formed from glycerophosphoric acid may be transformed, in part at least, into methylglyoxal. The amount of extra lactic acid formed in presence of glutathione is quite considerable (Table III), and is definitely produced from the added substrate, but

Table III.

Each mixture contained 20 ml. of dialysed muscle extract, 10 ml. of phosphate buffer, p_H 7.5, equimolecular mixture of pyruvate and glycerophosphate, (a) alone, (b) + magnesium sulphate and adenylypyrophosphate, (c) as (b) + reduced glutathione. In each case a control without added substrate was used, and the values obtained from these controls have been subtracted to give the figures shown.

	Lactic acid mg. per ml. of muscle extract	
	Original method	Modified method
(a)	1.20	0.90
(b)	2.60	1.60
(c)	2.75	1.85
(a)	1.17	1.00
(b)	2.78	1.93
(c)	3.39	2.68

the quantitative examination of the results in detail is a matter of some difficulty since the controls may not be entirely adequate owing to the possibility of some carbohydrate remaining in the dialysed muscle extract. As controls we used dialysed muscle extract with the same additions of co-enzyme, glutathione *etc.*, and only the substrate mixture omitted, but even so, it is hardly fair to found any further conclusions on the basis of the exact amount of lactic acid produced.

In the early experiments on glycogen, the amounts of methylglyoxal which accumulated in the absence of glutathione varied considerably, and even in the presence of glutathione similar amounts occasionally remained. Since this suggested a difference in the rates of production and removal of methylglyoxal so that the time and conditions of incubation might seriously influence the results of analysis at the end of the experiment, we carried out a series of parallel experiments with and without glutathione, in which samples were periodically removed for analysis. Table IV gives the results of two such experiments. Throughout, the true lactic acid production was greater in the presence of glutathione than in its absence. A curious feature however was the early production of a large amount of methylglyoxal (or some substance which, like methylglyoxal, was estimated as lactic acid by the unmodified method but was removed by distillation), which later disappeared and was not accounted for as lactic acid. This phenomenon, of which we can offer no explanation, certainly

Table IV.

Each mixture contained dialysed muscle extract, buffer, glycogen, magnesium sulphate and adenylypyrophosphate. In addition, series B contained reduced glutathione.

Time of incubation hours	Lactic acid, mg. per ml. of muscle extract			
	Original method		Modified method	
	A	B	A	B
0	0.15	0.15	0.11	0.11
1	1.05	0.53	0.18	0.34
2	0.78	0.76	0.25	0.38
4.5	0.45	0.73	0.26	0.41
24	0.43	0.75	0.30	0.45
0	0.36	0.36	0.23	0.23
1	0.76	1.37	0.33	0.54
2	1.01	1.16	0.41	—
4	2.26	1.84	0.56	0.88
24	2.00	1.81	0.83	1.23

helps to explain the variable methylglyoxal production which we had previously observed. It has been reported by Arayama [1934], who actually estimated the methylglyoxal, as its 2:4-dinitrophenylhydrazone.

SUMMARY.

Dialysed muscle extracts produce lactic acid from glycogen, glucose, hexose-diphosphoric acid or an equimolecular mixture of pyruvic and glycerophosphoric acids in greater amounts in the presence of magnesium ions, adenylypyrophosphate and reduced glutathione, than in the presence of the first two of these co-enzymes only.

In the absence of glutathione, methylglyoxal tends to accumulate; addition of glutathione diminishes and may prevent this accumulation.

In the absence of adenylypyrophosphate, magnesium ions and glutathione allow the production of lactic acid from glycogen by dialysed muscle extracts, but omission of the glutathione does not result in a corresponding accumulation of methylglyoxal.

During the formation of lactic acid, methylglyoxal accumulates and then disappears in part, the amount disappearing not being fully accounted for by conversion to lactic acid.

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