XLI. THE INTERMEDIARY METABOLISM OF CARBOHYDRATES. GLYOXALASE CONTENT OF RABBIT'S MUSCLE.

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THE mechanism of the production of lactic acid from carbohydrates in the animal body is a problem of fundamental importance, and the view that methylglyoxal may be the immediate precursor of lactic acid received strong support when it was demonstrated that an enzyme capable of converting methylglyoxal into lactic acid with great rapidity is present in animal tissues [Dakin and Dudley, 1913, 1, 2].

This enzyme, glyoxalase, was found in all the tissues examined, except the pancreas, of a variety of animals, as well as in yeast; it is therefore widely distributed, and it was a matter of considerable surprise when in a recent paper Foster [1925] reported its absence from rabbit's muscle, particularly as it had been detected in the muscle of several other animals. This finding involved, of course, a deduction of great moment, for, if glyoxalase be absent from rabbit's muscle, it is extremely unlikely that methylglyoxal may be a precursor of the lactic acid which is undoubtedly formed in quantity by this tissue. Foster suggests, therefore, that muscle may have a specialised carbohydrate metabolism of its own for the production of the lactic acid required in the processes concerned with muscular movement and different from that concerned in the "general metabolism" of muscle and other organs "where lactic acid is merely a stage in the complete combustion of glucose."

The importance of this deduction and the apparently anomalous behaviour of rabbit's muscle prompted a few experiments to test the accuracy of this interesting and unexpected experimental result.

On examination of Foster's actual experiments, in which she investigated the production of *l*-mandelic acid from phenylglyoxal by extracts of rabbit's muscle, using the technique described by Dakin and Dudley [1913, 2, 3] for studying the activity of glyoxalase, it will be seen that in two out of six experiments small but definite negative rotations (-0.21° and -0.19°) are recorded. It is true that they are only about one-tenth of those which are obtained when the equivalent experiment with rabbit's liver is made, but they might be interpreted as indicating a small formation of *l*-mandelic acid by the action of glyoxalase.

It should be borne in mind in this connection that the method of extraction of the mandelic acid from the reaction mixture would undoubtedly result in the concomitant extraction of a certain amount of *d*-lactic acid, and that the rotation of the latter would oppose that of the former, at any rate to a slight extent, and would thus tend to mask the presence of very small amounts of *l*-mandelic acid. The presence of lactic acid might also account for the failure of any small amount of mandelic acid, which may have been present, to crystallise in the characteristic way noted in experiments yielding larger quantities of this substance. It is clear, however, that relatively very little *l*-mandelic acid was formed in Foster's experiments with rabbit's muscle.

In the investigation here reported two experiments were made, by the original technique, exactly comparable with those of Foster, on the same sample of minced rabbit's (thigh) muscle, and the results were in accord with those which she obtained. The residues did not crystallise directly, although they actually contained mandelic acid (see experimental section), and the rotations were -0.15° and -0.18° respectively, whilst from an equivalent experiment using the animal's liver as the source of the enzyme, a rotation of -1.87° and typical crystals of mandelic acid were obtained. The rotations obtained in these experiments on muscle correspond to minute amounts of *l*-mandelic acid, and the further experiments, now to be described, warrant the supposition that they were due to this substance and that a definite, though very weak, glyoxalase action was indicated even under these conditions.

It was shown by Dakin and Dudley [1913, 2] that the action of glyoxalase in a 20 % extract of mixed rabbit's liver and muscle is almost completely inhibited by 0.1 % acetic acid and that this concentration of acid completely destroys the enzyme in less than $3\frac{1}{2}$ hours; and, further, that the conversion of methylglyoxal into lactic acid by glyoxalase ceases when the concentration of lactic acid in the digest reaches 0.085 %. The extracts used by Dakin and Dudley and by Foster were prepared by digesting the minced tissue with five times its weight of water at room temperature for an hour and then straining through muslin. Now minced rabbit's muscle is known rapidly to produce large amounts of lactic acid, as is pointed out by Foster herself. Assuming a potential lactic acid production of about 0.5 %, which is well within the range of yields reported in Foster's paper, the lactic acid content of a 20 % muscle extract might reach about 0.1 %. This, of course, would not all be free lactic acid on account of the buffering effect of the tissue, but it seemed reasonable to suspect that the failure of rabbit's muscle extracts to exhibit vigorous glyoxalase activity might be due, not to actual absence of the enzyme from the tissue, but to its destruction by the acids formed during the mincing and the period of extraction of the tissue with water.

Experiments were therefore made in which fresh rabbit's muscle, after being minced as quickly as possible, was extracted in sodium carbonate solutions of various strengths in order to ensure prompt neutralisation of the acid formed.

The presence of glyoxalase in such extracts was unmistakably demonstrable, rotations of -1° , due to *l*-mandelic acid formed by its activity, being observed, whilst the acid itself was isolated and identified.

There is no question, then, that rabbit's muscle, in common with other mammalian muscle, contains glyoxalase. The hypothesis that methylglyoxal may be the precursor of lactic acid in carbohydrate metabolism is, therefore, not less valid for rabbit's muscle than for other tissues, and the general findings of Dakin and Dudley concerning the distribution and function of glyoxalase require no modification. Since Foster's experiments failed to reveal definitely the glyoxalase actually present in rabbit's muscle they cannot be regarded as affording any experimental support for the hypothesis that muscle may have alternative metabolic paths for the production of lactic acid from carbohydrate, according to the purpose for which the lactic acid is needed.

The new and interesting point brought to light by Foster's experiments is that rabbit's muscle alone, of all muscle so far examined, fails to yield an active extract to water.

It is probably significant that, with the exception of the fowl, the activity of the glyoxalase of the livers of the animals examined, as judged by the formation of *l*-mandelic acid from phenylglyoxal, has always been considerably greater than that of corresponding amounts of muscle from the same animals. It is possible that a partial destruction of the glyoxalase present in muscle may always take place during simple extraction of the minced tissue with water, though it is surprising, to take the extreme cases, that the same procedure should give a practically inactive extract from rabbit's (thigh) muscle and a very active extract from fowl's (breast) muscle. The latter tissue is stated to contain a relatively large amount of lactacidogen [Lyding, 1921], and Lyding is of the opinion that in the muscle of birds this compound breaks down with greater rapidity *post mortem* than in mammalian muscle; presumably, therefore, lactic and phosphoric acids will be produced in extracts of the minced fowl's muscle as promptly and in quantities at any rate as large as in similar extracts of rabbit's muscle. The glyoxalase of rabbit's muscle may be more sensitive to acid than that of fowl's muscle, or the efficiency of the natural buffering during extraction may be different in the two cases, but the elucidation of this curious difference between the glyoxalases of rabbit's and fowl's muscle must form the subject of a separate investigation. The experiments so far made on the occurrence of glyoxalase in muscle do not bear quantitative interpretation, especially in view of the results reported in this communication, nor have they, as a rule, been made on particular muscles of the animals used. It is worthy of note that Emden and his collaborators [Emden and Adler, 1921; Lyding, 1921] have shown that, in rabbits and fowls, the amount of lactacidogen in the rapidly contractile and readily fatiguable muscle (white muscle) is considerably greater than in the relatively more slowly contractile and difficultly fatiguable muscle (red muscle) of the same animal, whilst the reverse relationship holds in the case of the "residual

phosphoric acid" of these muscles. It seems possible, therefore, that the glyoxalase of such muscles, which is probably intimately involved in the production of lactic acid in these organs, may differ in quantity or properties, and this point must be considered in any more detailed enquiry into the glyoxalase of muscle.

EXPERIMENTAL.

Glyoxalase content of aqueous extract of rabbit's (thigh) muscle.

A rabbit was killed and its thigh muscles were immediately removed, minced and suspended in five times their weight of distilled water. The liver was similarly treated. After occasional stirring for $1\frac{1}{2}$ hours the extracts were strained through cheese-cloth and the following experiments made:

(a) and (b). To 50 cc. of the muscle extract were added 10 cc. of a 2 % solution of phenylglyoxal, 10 cc. of a suspension of chalk in water and a few drops of toluene.

(c). The same additions were made to 50 cc. liver extract.

After incubation at 37° for 22 hours 25 g. solid ammonium sulphate were added to each of the three flasks which were then heated in a boiling water-bath until precipitation of the protein was complete (about 10 minutes). After cooling, 10 cc. syrupy phosphoric acid were added to each of the mixtures which, after standing for 10 minutes, were filtered on a Buchner funnel. The clear filtrates were extracted four times with ether and the ethereal solutions were evaporated.

Experiments (a) and (b) gave small syrupy residues, whilst (c) yielded a typical crystalline residue of mandelic acid. The residues were dissolved in 16 cc. water, clarified by means of a pinch of charcoal, filtered and examined in a 2 dm. polarimeter tube (Hg green light).

The observed rotations were: (a) -0.15° , (b) -0.18° , (c) -1.87° .

Blank experiments from which phenylglyoxal is omitted, usually give very slight positive rotations.

The glyoxalase activity of the liver extract is rather more than 10 times that of the muscle extract.

After polarimetric examination solutions (a) and (b) were evaporated to dryness on the water-bath, when a crystalline residue was obtained. This was dissolved in water and the solution was shaken four times with ether. On evaporating the ethereal solution to dryness a crystalline residue, weighing 0.1467 g., was obtained, which, after recrystallisation from benzene, melted at $116-118^{\circ}$. This was obviously *r*-mandelic acid. It is evident, therefore, that mandelic acid can be present without necessarily crystallising in the residue obtained by the ordinary technique from experiments with rabbit's muscle.

On account of this rather large quantity of *r*-mandelic acid an experiment was made to determine whether incubation of phenylglyoxal solution with chalk alone resulted in the formation of *r*-mandelic acid. 120 cc. water containing 0.4 g. phenylglyoxal and 10 cc. chalk suspension with 2 cc. of toluene

were incubated at 37° for 23 hours. 50 g. ammonium sulphate were then added, the solution was heated for 10 minutes in a boiling water-bath, cooled, acidified with 20 cc. syrupy phosphoric acid, and filtered. The filtrate was extracted four times with ether from which 0.1054 g. *r*-mandelic acid (M.P. 118°) was obtained. The phenylglyoxal used throughout these experiments was an old commercial specimen and may have contained some mandelic acid. An attempt to determine whether this was the case was made by treating 0.4 g. with aqueous ammonia, which combines with phenylglyoxal to form a yellow, insoluble compound. After acidification and filtration 0.0664 g. mandelic acid was extracted from the filtrate.

It would be necessary to perform this experiment with pure phenylglyoxal to be certain whether no mandelic acid at all is formed by the action of ammonia, but apparently a small amount of inactive mandelic acid may be formed by the digestion of phenylglyoxal with a suspension of calcium carbonate. It is worthy of note in passing that the amount of mandelic acid obtained from the rabbit's muscle experiments was definitely larger than that from the simple digestion of an equal amount of the glyoxal with chalk suspension, but whether any of the former resulted from actual enzyme activity must remain for the present undecided [cf. Dakin and Dudley, 1913, 2].

*Glyoxalase content of alkaline suspensions and extracts
of rabbit's (thigh) muscle.*

A rabbit was killed and its thigh muscles were removed and minced as quickly as possible.

The following experiments were made:

(*d*) 20 g. of the minced muscle were suspended in 100 cc. 0.1 % Na_2CO_3 ; 10 cc. of a 2 % solution of phenylglyoxal, 10 cc. of a suspension of chalk in water and 0.3 cc. toluene were immediately added.

(*e*) As in (*d*), except that the minced tissue was suspended in 0.25 % Na_2CO_3 .

(*f*) As in (*d*), except that the minced tissue was suspended in 0.5 % Na_2CO_3 .

The flasks containing these mixtures were then placed in the hot room at 37°.

Two extracts were made, (1) by digesting a portion of the minced muscle with five times its weight of 0.1 % Na_2CO_3 solution and (2) by digesting a portion with five times its weight of 0.5 % Na_2CO_3 for 1 hour, after which the fluids were strained through muslin and the following experiments made:

(*g*) To 50 cc. of extract (1) phenylglyoxal, chalk and toluene were added as in the previous experiments.

(*h*) To 50 cc. of extract (2) phenylglyoxal, chalk and toluene were added as in the previous experiments.

The flasks containing these mixtures were then incubated at 37°. After 20 hours' incubation they were worked up in the manner described above.

The residues in all experiments, after removal of the ether, were syrupy, containing scanty amounts of crystalline material. They were each dissolved in 16 cc. water, clarified with a pinch of charcoal and filtered. The rotations of these solutions were examined in a 2 dm. tube with Hg green light.

The following rotations were observed:

(d) -1.01° , (e) -0.94° , (f) -0.59° , (g) -0.40° , (h) -1.06° .

The solutions, after polarimetric examination, were combined and evaporated to dryness on the water bath. A crystalline residue of mandelic acid resulted. This was dissolved in water and the solution extracted with ether. The mandelic acid, after removal of the ether, was recrystallised twice from benzene. 0.1770 g. of the recrystallised acid, dissolved in 16 cc. water and examined in a 2 dm. tube with Hg green light, displayed a rotation of -1.97° .

It has been shown that a certain amount of *r*-mandelic acid was obtained by simple incubation of the phenylglyoxal with a suspension of chalk, and it is well known that alkalis readily convert glyoxals into the corresponding hydroxy-acids.

Sir F. G. Hopkins, to whom I am very grateful for his friendly criticism, suggested that it was conceivable that the *l*-mandelic acid found in these experiments might have arisen, not from the action of a glyoxalase on the phenylglyoxal, but by virtue of a preferential oxidation of the *dextro*-form in racemic acid produced by the direct action of the alkali on the phenylglyoxal, at any rate in the most strongly alkaline of the above mixtures. The following experiments were accordingly made to investigate this possibility.

*Comparative effects of suspensions and extracts of rabbit's muscle in 0.1 % and 0.5 % Na_2CO_3 on phenylglyoxal and *r*-mandelic acid.*

Suspensions. The thigh muscles of a freshly killed rabbit were used.

(i) Exactly as (d).

(j) Exactly as (d), except that, instead of 0.2 g. phenylglyoxal, 0.2 g. *r*-mandelic acid was added as neutral Na salt in 15 cc. water.

(k) Exactly as (f).

(l) Exactly as (f), except that, instead of 0.2 g. phenylglyoxal, 0.2 g. *r*-mandelic acid was added as neutral Na salt in 15 cc. water.

The extracts were incubated at 37° for 20 hours.

Extracts. The thigh muscles of another rabbit were used.

(m) Exactly as (g) except that 100 cc. extract was used.

(n) Exactly as (g), except that 100 cc. extract was used and 0.2 g. *r*-mandelic acid was added as neutral Na salt in 15 cc. water.

(o) Exactly as in (h), except that 100 cc. extract was used.

(p) Exactly as in (h), except that 100 cc. extract was used and 0.2 g. *r*-mandelic acid was added as neutral Na salt in 15 cc. water.

The mixtures were incubated at 37° for 23 hours.

The experiments were worked up in the way previously described. The combined ether extracts in each experiment were washed once with a small

quantity of water before the removal of the ether. Mandelic acid then crystallised in each residue in the normal manner.

The rotations of the residues, dissolved in 16 cc. water and observed in a 2 dm. tube in Hg green light, were as follows:

$$\begin{array}{llll} (i) & -1.03^{\circ}; & (j) & +0.01^{\circ}; & (k) & -0.45^{\circ}; & (l) & +0.05^{\circ}; \\ (m) & -0.57^{\circ}; & (n) & 0^{\circ}; & (o) & -0.85^{\circ}; & (p) & 0^{\circ}. \end{array}$$

Thus it is seen that no *l*-mandelic acid was produced in those experiments where *r*-mandelic acid was added, whilst it was present in every case where phenylglyoxal was the substrate. Under these conditions, therefore, there is no preferential oxidation of one optical isomeride of mandelic acid by alkaline muscle extracts or tissue suspensions.

After polarimetric examination the solutions of active mandelic acid from the experiments with 0.1 % Na_2CO_3 (*i* and *m*) were combined and evaporated to dryness and those from the experiments with 0.5 % Na_2CO_3 (*k* and *o*) were similarly treated. The residues were examined to ascertain whether there was any significant difference in the proportion of *l*-acid in them. That from the 0.1 % Na_2CO_3 experiments weighed 0.2378 g. and had a rotation of -1.24° when dissolved in 16 cc. water and examined in a 2 dm. tube with Na yellow light, indicating the presence of 19.6 % *l*-mandelic acid. The residue from the 0.5 % Na_2CO_3 experiments weighed 0.1407 g. and, under the same conditions, had a rotation of -0.91° , indicating the presence of 24.3 % *l*-mandelic acid.

It appears, therefore, that under the conditions of these experiments the more strongly alkaline solutions had no effect in increasing the amount of *r*-mandelic acid, which might have been expected to be formed by direct chemical action, relative to the *l*-acid arising undoubtedly from enzyme action.

SUMMARY.

Foster's [1925] observation, that rabbit's muscle, unlike all other muscle so far examined, fails to yield an aqueous extract in which the enzyme glyoxalase is clearly demonstrable, is confirmed. Her conclusion, however, that glyoxalase is absent from this tissue is shown to be erroneous, since it can be detected in alkaline extracts.

The hypothesis that in muscle there may be two distinct metabolic paths for the production of lactic acid from carbohydrate is thus deprived of experimental support.

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