CCXXVIII. ENZYME SYSTEMS OF SARCOMA AND OF MUSCLE DEALING WITH HEXOSEPHOSPHATES.¹

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THE discovery of a peculiar carbohydrate metabolism in tumour tissue, first pointed out by Warburg *et al.* [1924; see also Warburg, 1931], has as yet led to no understanding of its cause. The chief obstacle to study of carbohydrate transformations in tumour lay in the failure of tumour extracts to produce lactic acid from glycogen or glucose. This impediment to progress has been commented upon by Boyland and Mawson [1934]. It has been found in this laboratory, however, that tumour extract will transform hexosephosphates into lactic acid [Scharles *et al.*, 1935]. In consequence it is now possible to compare in sarcoma and in muscle the enzyme systems which deal with hexosephosphates.

It has been observed [Scharles *et al.*, 1935] that in sarcoma extract the enzyme systems connected with hexosephosphate formation appear defective. Similarly, Matsuzaki [1933] has reported lack of "phosphatase" (*i.e.* phosphorylating enzyme) in minced tumour acting upon glycogen. It would be tempting to ascribe the peculiar carbohydrate metabolism of malignant tissue entirely to one such enzyme. First, however, one must be assured that all subsequent enzyme systems resemble those of a normal tissue like muscle. This paper reports observations which indicate that other enzymic differences actually exist, and that therefore the problem is complex.

In order to compare enzymic action in tumour extract with that in analogous extracts from normal tissue (like muscle), it is essential first to know the characteristic behaviours of both tissue enzyme systems. This paper reports optimum conditions for (1) the formation of phosphate esters from glycogen and inorganic phosphate, (2) the formation of hexosediphosphate from hexosemonophosphate, and (3) the breakdown of hexosephosphates into inorganic phosphate and lactic acid. Then, in order to separate the successive steps in this series of reactions, which normally merge smoothly one into another, use was made of toxic agents which check the progressive degradation at appropriate points. It has been shown, for example, that fluoride and iodoacetate inhibit formation of lactic acid from glycogen (or from hexosephosphate) at different stages in the breakdown of the substrate [Embden and Deuticke, 1934, 2]. This device has permitted the chemical dissection of the respective carbohydrate metabolisms of the normal and malignant tissues for purposes of comparison.

Discussion.

In studying the peculiarities in tumour metabolism which lead to anaerobic glycolysis it is necessary to have for control a knowledge of similar processes in normal tissue. Of all normal tissues, skeletal muscle has been most intensively

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studied along these lines. Muscle metabolism has therefore been used in these studies as a guide to the study of tumour.

As suggested in our previous communication, the behaviour of muscle extract can be described by the following schema: glycogen, after hydrolysis, is converted into (a) hexosemonophosphate, which, in turn, is converted by a second phosphorylating enzyme into (b) hexosediphosphate. By the action of appropriate enzymes the hexosediphosphate is split into trioses esterified with phosphate. These esters then undergo a series of chemical rearrangements in the three-carbon state [Embden and Deuticke, 1934, 1]. Ultimately lactic acid and free phosphate are produced.

This orderly series of reactions may be interrupted by "toxic" agents (like iodoacetate and fluoride) which interfere with enzymic activity. Iodoacetate appears to inhibit moderately the formation of monophosphate, and also moderately to check further esterification to diphosphate, as indicated in Tables I and III of this report. At any rate, it completely prevents lactic acid formation by muscle extract, as also described by Embden and Deuticke [1934, 2].

Likewise, in the presence of fluoride, even though glycogen is split and phosphate is esterified to a marked degree, the resulting ester, first described by Lohmann [1930] and identified as a mixture of phosphoglyceric and glycerophosphoric acids by Embden *et al.* [1933], is incapable of further change to lactic acid as long as fluoride is present (Table I).

Table I. *Effects of iodoacetate and fluoride on muscle and tumour extracts with glycogen. Incubated 2 hours at 40°.*

Extract	Poison	Final concentration of poison <i>M</i>	Lactic acid production millimols. per 100 ml.	Phosphate esterified millimols. per 100 ml.	Phosphate esterified %
Muscle	—	0	1.81	2.77	24
"	Iodoacetate	0.01	0.02	1.18	29
"	"	0.001	0.09	1.42	35
"	"	0.0005	0.15	1.40	31
Tumour	—	0	0	0.23	7
"	Iodoacetate	0.005	0	0.21	7
Muscle	—	0	0.95	0.69	21
"	Fluoride	0.01	0.08	1.60	50
"	"	0.005	0.08	1.43	44
"	"	0.001	0.84	0.92	28
"	"	0.0005	1.00	0.60	19
Tumour	—	0	0	0.34	10
"	Fluoride	0.01	0.02	0.41	12

The esterified product however when formed in the presence of fluoride and then isolated free from fluoride can be used by fresh muscle extract as a source of lactic acid. By contrast, in the presence of fluoride lactic acid may still be formed in appreciable quantity (Tables II and III) from both hexosemono- and hexosediphosphates prepared with yeast; but phosphate-esterification still proceeds more rapidly than its liberation from hexosemonophosphate.

As is well known [Scharles and Salter, 1934], tumour extract fails to produce lactic acid from glycogen although the splitting of glycogen is vigorous. The resulting carbohydrate complex (of unknown composition) cannot undergo further degradation to lactic acid. The data to be presented in this paper show that so little phosphate is esterified (Table I) by tumour at this stage, in contrast to muscle, as to suggest either (a) that different carbohydrate-phosphate

Table II. *Effects of iodoacetate and fluoride on muscle and tumour extracts with hexosediphosphate. Incubated 2 hours at 50°.*

Extract	Poison	Final concentration of poison <i>M</i>	Lactic acid produced millimols. per 100 ml.	Phosphate hydrolysed millimols. per 100 ml.	Phosphate hydrolysed %
Muscle	—	0	0.92	1.50	21
"	Iodoacetate	0.05	0	0.67	9
"	"	0.01	0.17	0.69	10
"	"	0.001	0.36	0.83	12
Tumour	—	0	0.57	0.58	8
"	Iodoacetate	0.05	0.21	0.47	7
"	"	0.01	0.33	0.55	8
"	"	0.001	0.48	0.56	8
Muscle	—	0	0.92	1.50	21
"	Fluoride	0.05	0.38	0.44	6
"	"	0.01	0.44	0.94	13
"	"	0.001	0.83	1.41	20
Tumour	—	0	0.57	0.58	8
"	Fluoride	0.05	0.55	0.46	6
"	"	0.01	0.57	0.52	7
"	"	0.001	0.63	0.58	8

Table III. *Effects of iodoacetate and fluoride on muscle and tumour extracts with hexosemonophosphate. Incubated 2 hours at 50°.*

Extract	Poison	Final concentration of poison <i>M</i>	Lactic acid produced millimols. per 100 ml.	Phosphate hydrolysed millimols. per 100 ml.	Phosphate hydrolysed %
Muscle	—	0	1.24	0.69	25
"	Iodoacetate	0.05	0	0.57	21
"	"	0.01	0	0.60	22
"	"	0.001	0.24	0.60	22
Tumour	—	0	0.51	0.50	18
"	Iodoacetate	0.05	0.19	0.41	15
"	"	0.01	0.21	0.46	17
"	"	0.001	0.32	0.50	18
Muscle	—	0	1.24	0.69	25
"	Fluoride	0.05	0.48	—0.07	—3
"	"	0.01	0.54	0	0
"	"	0.001	0.64	0.30	11
Tumour	—	0	0.51	0.50	18
"	Fluoride	0.05	0.50	0.40	15
"	"	0.01	0.51	0.45	16
"	"	0.001	0.49	0.46	17

esters are formed by the respective tissue extracts, or (b) that tumour is deficient in phosphatase. Furthermore, what little ester is formed cannot be converted into lactic acid by tumour extract. Nevertheless, experiments now in progress show that the isolated phosphoglyceric acid and glycerophosphoric esters formed by muscle from glycogen in the presence of fluoride can be converted into lactic acid by tumour extract. This finding indicates that tumour contains an assortment of enzyme systems necessary for lactic acid production, as can also be demonstrated by artificially supplying hexosephosphates made by yeast (see Figs. 3-6). These hexosephosphates are also available sources of lactic acid when acted upon by muscle extract. Nevertheless, that distinct differences exist in the enzyme equipments of these respective tissue extracts may be demonstrated both by the course of hexose ester degradation and by the effects of toxic agents like iodoacetate and fluoride.

One such difference is exemplified by the esterification of free phosphate when fresh muscle extract acts on hexosemonophosphate, provided that time and temperature are appropriately controlled. Tumour extracts, by way of contrast, failed under any observed conditions to show this effect.

Another difference is disclosed by experiments with "rigor" muscle extract. The depletion of coenzyme (during rigor) renders the extract incapable of producing lactic acid from hexosemonophosphate—a defect which is remedied by the addition of pure adenosinetriphosphate. Tumour extract, on the other hand, when depleted (by dialysis) of coenzyme, is still capable of producing lactic acid from monophosphate [*cf.* Scharles *et al.*, 1935].

A third difference is noted on the addition of iodoacetate to muscle extract acting on hexosemonophosphates. This poison completely inhibits the formation of lactic acid from hexosemonophosphate. A similar concentration of iodoacetate added to tumour extract however fails to abolish glycolysis, although some inhibition was noted (Table III).

It seems clear therefore that disruption of muscle or tumour cells in the process of extraction does not destroy the complete complex of enzymes requisite to lactic acid production. Nevertheless, the failure of tumour extracts to produce lactic acid contrasts strikingly with the ability of intact tumour cells to utilise glucose as an external source of lactic acid, a fact which has been pointed out by Barr *et al.* [1928] and by Boyland and Mawson [1934]. It has been suspected that this paradox was in essence an artefact, due to biochemical manipulation. Our results suggest that, in extraction, an enzyme system which is needed to produce an appropriate hexosephosphate is either destroyed or masked. More important is the finding that, given the necessary missing state, the subsequent carbohydrate breakdown in the two tissue extracts follows different paths.

EXPERIMENTAL.

I. Procedure.

Extracts were made from rat muscle and from mouse sarcoma No. 180 as described in a previous paper [Scharles *et al.*, 1935]. The extracts were incubated either with glycogen or with hexosephosphates, and the extent of phosphate esterification or of hydrolysis was measured in comparison with the accompanying formation of lactic acid. Conditions of optimum temperature and acidity were determined and maintained throughout these experiments except where specially indicated.

Glycogen was prepared from rabbit liver according to the method of Sahyun and Alsberg [1930], and a 2% solution in *M*/15 phosphate buffer at appropriate p_H value was made up as stock substrate solution.

Hexosediphosphate and hexosemonophosphate were prepared as described in the previous paper [1935]. The sodium salts of these esters in solution were used as substrate after appropriate adjustment of the acidity without the use of phosphate buffer.

Glycogen determinations were made by the method of Good *et al.* [1933]. Phosphate, both inorganic and total, was estimated by the method of Fiske and Subbarow [1925]. Lactic acid was measured by the procedure of Friedemann *et al.* [1927]. Sodium iodoacetate and sodium fluoride in appropriate concentrations were used as enzyme poisons.

To conserve space, only typical examples of three or more experiments are reported in each instance.

II. Optimum conditions.

Glycogen. When glycogen was incubated for 2 hours with muscle extract, the optimum temperature for lactic acid production was found to be about 37–45°. For esterification of the phosphate the optimum temperature was

Fig. 1. Optimum temperature for production of lactic acid and esterification of phosphate for muscle and tumour extract, respectively. Glycogen as substrate in inorganic phosphate solution. o--o Muscle-lactic acid production. Δ--Δ Muscle-phosphate esterified. ▲--▲ Tumour-phosphate esterified.

Fig. 2. Optimum acidity for production of lactic acid and esterification of phosphate for muscle and tumour extract, respectively. Glycogen as substrate in inorganic phosphate solution. Δ--Δ Muscle-phosphate esterified. o--o Muscle-lactic acid. ▲--▲ Tumour-phosphate esterified.

sometimes 37°, sometimes lower, *i.e.* 25°, because at higher temperatures the ester formed may be concurrently hydrolysed to a greater extent. (This latter condition is noticed most when lactic acid formation is not optimum because of insufficient coenzyme.)

The optimum p_H for muscle acting on glycogen was 6.8–7.2 for both lactic acid production and esterification (during 2 hours at 45°).

When tumour extract was incubated with glycogen no lactic acid was produced and there was only slight esterification of phosphate. Variations in temperature and in acidity had very little effect upon the amount of phosphate esterified. These results are illustrated in Figs. 1 and 2.

Hexosediphosphate. In Fig. 3, the optimum temperature for production of lactic acid and splitting of phosphate from hexosediphosphate by both muscle and tumour extracts is shown to be in the vicinity of 55°. The optimum p_H was very difficult to determine with hexosediphosphate substrate because the addition of phosphate buffer inhibited the reaction to some extent. The most favourable reaction seemed to be the p_H of the muscle extract itself, approximately 6.5. Therefore, in using hexosediphosphate as the substrate, it was neutralised to p_H 6.8 before adding tissue extract.

Fig. 3. Optimum temperature for production of lactic acid and hydrolysis of phosphate for muscle and tumour extract, respectively. Hexosediphosphate as substrate. o--o Muscle-lactic acid production. •--• Tumour-lactic acid production. Δ--Δ Muscle-phosphate hydrolysis. ▲--▲ Tumour-phosphate hydrolysis.

As illustrated by Fig. 4, the appearance of lactic acid was paralleled by the appearance of free phosphate in nearly equivalent quantity for the first 2 hours of incubation at 50°. Thereafter the rate of lactic acid production dropped off

whilst phosphate hydrolysis continued at the same rate for 2 hours further. This was true of both muscle and sarcoma extracts.

Fig. 4. Production of lactic acid and liberation of inorganic phosphate from hexosediphosphate by muscle and tumour extract, respectively. o-o Muscle-lactic acid production. ●-● Tumour-lactic acid production. Δ-Δ Muscle-phosphate hydrolysis. ▲-▲ Tumour-phosphate hydrolysis.

Fig. 5. Production of lactic acid and esterification (or liberation) of inorganic phosphate from hexosemonophosphate by muscle and tumour extract, respectively. o-o Muscle-lactic acid production. ●-● Tumour-lactic acid production. Δ-Δ Muscle-phosphate hydrolysis. ▲-▲ Tumour-phosphate hydrolysis.

Hexosemonophosphate. When fresh muscle extract was incubated at 50° with hexosemonophosphate solution for various periods of time there was a progressive increase in lactic acid. On the other hand, free phosphate decreased during the early period of incubation, to return eventually to its original con-

centration, and then progressively to increase. Similar experiments with tumour extract however failed to reveal any reversal of phosphate balance although

Fig. 6. Optimum temperature for production of lactic acid and esterification (or liberation) of inorganic phosphate by muscle and tumour extract, respectively. Hexosemonophosphate as substrate. o-o Muscle-lactic acid production. •-• Tumour-lactic acid production. Δ-Δ Muscle-phosphate hydrolysis. ▲-▲ Tumour-phosphate hydrolysis.

Fig. 7. Esterification of phosphate by muscle extract (but not by tumour extract) when incubated with hexosemonophosphate at varying temperatures.

lactic acid accumulated at approximately the same rate as with muscle. These results are shown in Fig. 5.

When muscle extract was incubated for 2 hours at various temperatures with hexosemonophosphate, lactic acid reached maximum concentration between 50° and 55°. With this long period of incubation however the amount of free phosphate showed the same reversal at lower temperatures as was encountered at 50° for a shorter incubation time. As shown in Fig. 6, maximum esterification of phosphate was found at room temperature, but maximum hydrolysis of phosphate was at 60°.

With tumour extract, the optimum temperature for lactic acid production was likewise 50° to 55°. At no temperature was any esterification observed. The optimum for hydrolysis of phosphate was similar to that of muscle.

Accordingly, the concentration of free phosphate may be regarded as a function both of time and of temperature. This relationship is given in diagrammatic form in Fig. 7. Comparison of the data for muscle with those for tumour discloses the absence of reversal in the latter case.

III. *Effect of poisons.*

The effects of iodoacetate and fluoride upon the activity of muscle or tumour extracts were studied with glycogen, hexosediphosphate and hexosemonophosphate as substrates. These results are shown in Tables I, II and III respectively.

Glycogen. With glycogen, lactic acid production by muscle was almost completely inhibited by 0.0005 *M* iodoacetate or 0.005 *M* fluoride. Phosphate esterification was somewhat inhibited by iodoacetate, and greatly increased with fluoride. According to Meyerhof and Kiessling [1933] and Embden and Deuticke [1934, 1, 2] the esters formed under the influence of fluoride are the three-carbon esters, phosphoglyceric acid, and glycerophosphoric acid (see Table I).

When tumour extracts and glycogen were similarly treated, iodoacetate or fluoride in a concentration of 0.01 *M* showed almost no effect on the slight amount of esterification already present.

Hexosediphosphate. With hexosediphosphate stronger concentrations of poisons were necessary to affect the reaction. 0.05 *M* iodoacetate caused complete suppression of lactic acid formation with muscle extract, but only about 50 % inhibition with tumour extract. The effect on phosphate hydrolysis was about 50 % with muscle and hardly noticeable with tumour (see Table II).

A fluoride concentration of 0.05 *M* showed about 30 % inhibition of lactic acid production and phosphate hydrolysis with muscle and almost no effect upon tumour's action on hexosediphosphate.

Hexosemonophosphate. With the hexosemonophosphate mixture [Robison and Morgan 1930], iodoacetate in concentration of 0.01 *M* completely inhibited lactic acid production with muscle, and in concentration of 0.001 *M* markedly inhibited it. The effect on phosphate hydrolysis was slight. Again the action of iodoacetate on tumour activity with hexosemonophosphate was not marked; 0.05 *M* concentration reduced lactic acid production to 40 % but scarcely affected phosphate hydrolysis (see Table III).

Fluoride had almost no effect on the results with tumour extract acting on hexosemonophosphate. With muscle however 0.05 *M* fluoride caused some inhibition of lactic acid production and some accumulation of esters with hexosemonophosphate (as occurred also with glycogen).

IV. *Effect of coenzyme.*

As is well known from the work of Meyerhof *et al.* [1931] and of Milroy *et al.* [1933] "rigor muscle" extract, in which the coenzyme adenosinetriphosphate has been broken down by the body enzymes before extraction, is incapable of

producing lactic acid from glycogen. The extract however can be reactivated by addition of small amounts of adenosinetriphosphate.

As reported by us previously [1935], tumour extract is also inactive with glycogen. It already contains small amounts of adenosinetriphosphate by analysis however and cannot be made to produce lactic acid by addition of more coenzyme.

"Rigor muscle" extract was still active towards hexosediphosphate, though to a less extent than fresh muscle extract. Tumour extract, which was dialysed 24 hours to remove the coenzyme, was still active with hexosediphosphate without any reinforcement.

"Rigor muscle" extract is also inactive with hexosemonophosphate and can be reactivated by addition of coenzyme. Dialysed tumour extract however was as capable of producing lactic acid from hexosemonophosphate as fresh tumour extract; and the addition of adenosinetriphosphate was without effect.

SUMMARY.

Enzymes which control carbohydrate metabolism have been examined as a possible cause for the peculiar carbohydrate metabolism of tumour tissue. To this end lactic acid production was studied, coincidentally with phosphate esterification or hydrolysis of organic phosphates. When extracts of tumour and of skeletal muscle respectively were compared in their actions upon glycogen and hexosephosphate substrates, several differences were noted. These differences involved the effects of fluoride and iodoacetate, and of coenzyme deficiency.

Both tumour and muscle extracts produced lactic acid from hexosephosphates. Nevertheless tumour extract contrasted with muscle extract in the following features: (a) tumour extract remained unaffected by fluoride in its ability to produce lactic acid or to liberate phosphate from hexosephosphate esters; (b) tumour extract was much less susceptible to inhibition by iodoacetate; (c) tumour extract failed to esterify phosphate with hexosemonophosphate; (d) tumour extract produced lactic acid from hexosemonophosphate despite the depletion of coenzyme.

These findings suggest that, given an appropriate hexosephosphate ester from which both muscle and tumour extracts may produce lactic acid, nevertheless carbohydrate breakdown in the two tissue extracts proceeds along different paths.

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