XLIV. STUDIES IN TISSUE METABOLISM

XI. THE ACTION OF TUMOUR SLICES AND EXTRACTS ON DIFFERENT CARBOHYDRATES

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WATER extracts of frozen tumour tissue will produce lactic acid for a short time if adenylpyrophosphate is added [Boyland & Boyland, 1935, 2], and for longer periods from glucose, glycogen, hexosediphosphate or hexosemonophosphate if cozymase is added in addition to a trace of hexosediphosphate [Boyland *et al.* 1937]. The transient glycolysis obtained when adenylpyrophosphate alone is added is dependent upon the cozymase originally contained in the tumour tissue. The rate of glycolysis by such extracts is increased by adding adenylic acid. Fructose is converted into lactic acid as rapidly as or more rapidly than glucose in extracts, but tissue slices always attack glucose more rapidly than fructose.

The inhibition of carbohydrate breakdown by tumour slices by *dl*-glyceraldehyde was described by Mendel [1929]. A similar inhibition is now shown to take place with tumour extract, dried yeast or dialysed muscle extract.

EXPERIMENTAL

Tumour extracts were made by freezing and grinding the tissue in a mortar cooled in freezing mixture, adding an equal volume of glass-distilled water and refreezing the mixture. The extract was separated from the tissue debris by centrifuging while the frozen mass thawed. Samples of the extract, usually 1 ml., were incubated with the various substrates and 0·2% $NaHCO_3$ in the Warburg apparatus in an atmosphere of 95% N_2+5% CO_2. After preliminary shaking, the coenzymes were tipped in from the side bulbs and manometer readings taken at 5 min. intervals. At the end of the incubation period trichloroacetic acid was added and lactic acid estimated by Lohmann's method [1928] in the deproteinized solutions. Precipitation with copper-lime was avoided. Figures for lactic acid production in this paper are taken from the chemical estimations except for a few experiments with tissue slices with glucose or fructose as substrates. The manometric readings were used to follow the speed of the reaction. Manometric readings are unreliable when hexosephosphates are present as it is impossible to compensate for CO_2 evolution or retention by the degradation products of the esters when it is not known what these products are. For this reason it is impossible to interpret the results of B. E. Holmes [1937] on hexosediphosphate breakdown in tumour tissue, as no indication is given when the chemical method of estimation was used in the experiments with hexosephosphates.

Glycolytic extracts from different tumours

Previously actively glycolysing extracts of the Crocker sarcoma 180 have been described but the same technique applied to other grafted tumours has given extracts with varying activity. The lactic acid production from glucose and fructose by different tumour extracts is shown in Table I.

Table I. *Lactic acid formation by extracts of tumours*

1 ml. extract with addition of 1 mg. cozymase, 1 mg. adenylic acid, hexosediphosphate (0·01 mg. P), 3 mg. $NaHCO_3$. Total volume 1·5 ml.

	mg. lactic acid formed in 30 min. in 2 ml. extract from	
Tumour extract	10 mg. glucose	10 mg. fructose
Mouse sarcoma Crocker 180	7·7	8·7
,, ,,	1·9	5·2
Mouse sarcoma 37	4·8	6·8
,, ,,	4·0	4·8
Mouse carcinoma 63	0·8	1·6
,, ,,	2·0	2·4
Walker rat carcinoma 256	0·6	1·0

The difference in activity is most probably due to differences in the relative strengths of the enzymes destroying the coenzymes and those producing lactic acid; for example it was shown [Boyland & Boyland, 1935, 1] that J.R.S. extracts rapidly attack nucleotides. In most cases in which the same extract was allowed to act on glucose and fructose more lactic acid was produced from fructose, but there was no constant relationship between fructolysis and glucolysis.

Action on different carbohydrates

The amounts of lactic acid formed by extracts and slices of the Crocker sarcoma 180 and of sarcoma 37 on incubation in the Warburg apparatus with different carbohydrates are shown in Table II. The lactic acid production by

Table II. *Lactic acid formation by slices and extracts from various substrates*

Incubated in 2 ml. Ringer, 1 ml. extract with additions as in Table I

	mg. lactic acid formed in 30 min. by			
	Crocker sarcoma 180		Sarcoma 37	
Substrate	1 g. slices in Ringer	2 ml. extract with additions	1 g. slices in Ringer	2 ml. extract with additions
None added	0·05	1·0	0·2	0·6
20 mg. glucose	6·2	7·7	6·5	4·0
20 mg. fructose	2·0	8·7	2·7	4·8
10 mg. glucose + 10 mg. fructose	6·0	7·6	—	3·9
20 mg. mannose	6·3	6·8	—	4·0
20 mg. glycogen	0·05	7·2	0·3	4·0
Hexosemonophosphate (≡20 mg. carbohydrate)	0·1	7·4	0·4	3·8
Hexosediphosphate (≡20 mg. carbohydrate)	0·2	7·0	0·7	3·7

extracts from glucose, mannose, glycogen, hexosemonophosphate and hexosediphosphate is the same within the limits of the experimental technique. Fructose is generally more rapidly broken down, but with a mixture of glucose and fructose lactic acid is produced at nearly the same rate as with glucose alone. Slices produce lactic acid rapidly from glucose and mannose or from a mixture of glucose and fructose. Glycogen is not glycolysed at all by slices presumably because it is non-diffusible. Hexosemonophosphate and hexose diphosphate are also not glycolysed and in the case of these phosphoric esters it can be shown that this is due to their inability to penetrate into the cells.

While fructose is the carbohydrate more rapidly glycolysed by extracts, it is attacked at less than a third as rapidly as glucose by tumour slices [cf. Dickens & Greville, 1932]. That this difference between glucolysis and fructolysis in slices is due to a relatively slow diffusion of fructose into the cells is indicated by experiments described below.

Zymohexase activity of slices and extract

Although tumour slices do not readily form lactic acid from hexosediphosphate they show zymohexase activity, i.e. they convert this ester into triosephosphate. If hexosediphosphate alone is incubated with tumour extract triosephosphate is formed and remains as such, but if cozymase is added some of the triosephosphate is further broken down. The rates of formation of triosephosphate as estimated by the amount of alkali-labile phosphate formed from hexosediphosphate by slices of Crocker sarcoma 180 and by different dilutions of an

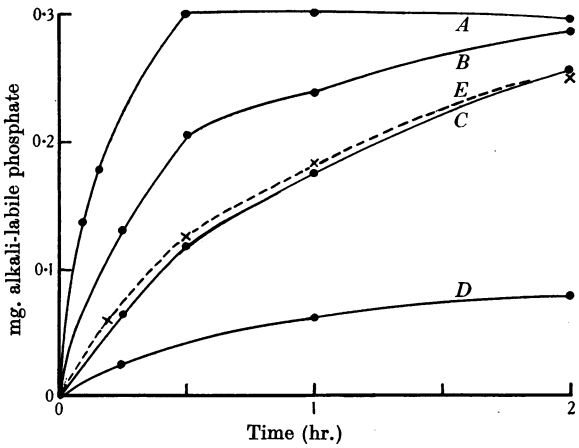

Fig. 1. Triosephosphate formation by Crocker 180. Total volume 2 ml. containing hexosediphosphate (1·5 mg. P). Curves: *A*, 0·20 ml. extract; *B*, 0·05 ml. extract; *C*, 0·012 ml. extract; *D*, 0·003 ml. extract; *E*, 200 mg. slices.

extract of the same tumour are shown in Fig. 1. It will be seen that the zymohexase activity of 200 mg. slices is almost identical with that of 1/16 of the equivalent amount (0·2 ml.) of extract diluted to the same volume (2 ml.) as that in which the slices were suspended (i.e. 0·006 ml. extract per ml.). The curve showing the reaction of triosephosphate with time is identical in form in the case of slices (curve *E*) and 16 × diluted extract (curve *C*).

Rate of diffusion of hexosediphosphate and hexosemonophosphate into tumour slices

Table III shows the results of some experiments in which tumour slices were incubated with hexosediphosphate and with glucose. At the end of the incubation period the slices were removed from the vessels, quickly rinsed in Ringer's solution and extracted with trichloroacetic acid. Trichloroacetic acid was also added to the Ringer's solution in which the slices had been suspended and estimations of free phosphate and alkali-labile (i.e. triose) phosphate were made in both extracts. It will be seen that the concentration of triosephosphate is much lower in the slices than in the surrounding medium, showing that the

Table III. *Distribution of triosephosphate*

200 mg. slices in 2 ml. Ringer containing 4 mg. $NaHCO_3$ incubated in $N_2 + 5\%$ CO_2 for 1 hr.

		Concentration of phosphate (mg. P per g. or ml.) after 1 hr.			
		In slices		In Ringer	
Tumour	Substrate	Free phosphate	Alkali-labile (triose)-phosphate	Free phosphate	Alkali-labile (triose)-phosphate
Crocker	None	0·19	0·02	0·021	0·000
	200 mg. glucose	0·10	0·00	0·015	0·000
	Hexosediphosphate (≡20 mg. carbohydrate)	0·31	0·01	0·030	0·132
	Unincubated	0·18	0·00	—	—
Carcinoma 63	None	0·08	0·00	0·002	0·001
	20 mg. glucose	0·07	0·00	0·001	0·000
	Hexosediphosphate (≡20 mg. carbohydrate)	0·14	0·01	0·001	0·060
	Unincubated	0·14	0·02	—	—

hexosediphosphate diffuses at most very slowly into the tissue. The triosephosphate found in the solution is therefore most probably formed by the injured cells on the surface of the tumour slices. The evidence that hexosemonophosphate does not diffuse into tissue slices is not quite so direct. We have shown [Boyland *et al.* 1937] that hexosemonophosphate is partially converted into triosephosphate by extracts in the presence of cozymase. There is no triosephosphate formation by slices from hexosemonophosphate. If there were any diffusion of hexosemonophosphate or triosephosphate into the intact cells some alkali-labile phosphate would be found after incubation. Even if cozymase is added to the Ringer no triosephosphate is formed from hexosemonophosphate (Table IV).

Table IV. *Lactic acid and triosephosphate formation by slices of Crocker 180*

100 mg. slices incubated in 2 ml. Ringer

	mg. lactic acid formed per g. in 1 hr.		Alkali-labile (triose)phosphate mg. P	
Substrate	No addition	1 mg. cozymase + 1 mg. adenylic acid	No addition	1 mg. cozymase + 1 mg. adenylic acid
None	0·2	—	0·02	—
Glucose	13·0	—	0·02	—
Hexosemonophosphate	0·3	0·4	0·01	0·01
Hexosediphosphate	0·4	0·4	0·22	0·19

The effect of dilution of extracts on their glycolysis

The enzyme exposed on the cut and injured cells of tumour slices converts hexosediphosphate into triosephosphate but not appreciably into lactic acid even if cozymase be added to the surrounding Ringer's solution (see Table V). This is due to dilution of the enzyme in the suspension so that it is no longer active. The results of two typical experiments showing the effect of diluting tumour extracts on their glycolysis are shown in Fig. 2; in all cases the concentrations of coenzymes and of hexosediphosphate are the same. At dilutions from 4 to 6× the extract produces more lactic acid than the undiluted extract because relatively more cozymase is available and the reaction goes on for a

Table V. *Lactic acid formation by slices of Crocker 180*

200 mg. slices incubated in 2 ml. Ringer (or 1·5 ml. Ringer + 0·5 ml. boiled muscle extract)

Substrate	mg. lactic acid formed per g. slices in 1 hr.		
	No addition	1 mg. cozymase + 1 mg. adenylic acid	0·5 ml. boiled muscle extract
None added	1·1	1·0	1·0
20 mg. glucose	17·6	—	16·5
Hexosediphosphate (≡20 mg. carbohydrate)	1·3	1·2	1·2
Hexosemonophosphate (≡20 mg. carbohydrate)	1·3	1·1	1·2

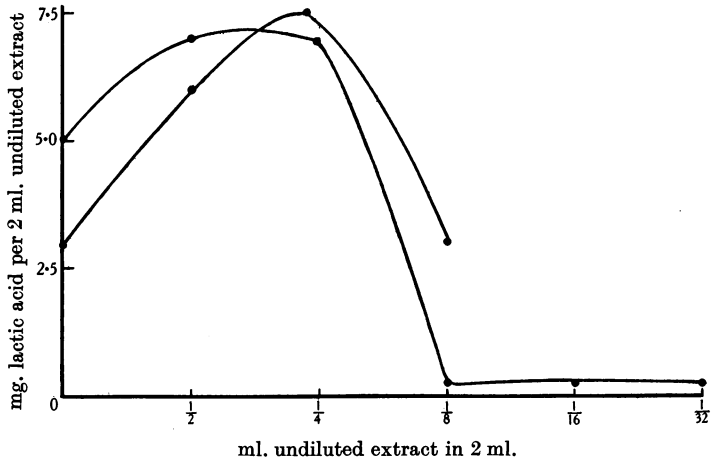

Fig. 2. Lactic acid formation by diluted extracts. 10 mg. glucose, hexosediphosphate (0·01 mg. P), 1 mg. cozymase, 1 mg. adenylic acid, 3 mg. $NaHCO_3$. Total volume 1·5 ml.

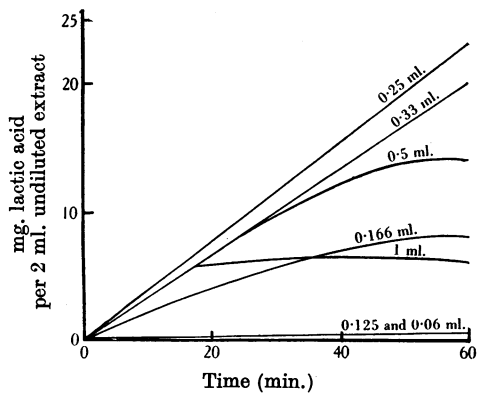

Fig. 3. Effect of dilution on lactic acid formation by extracts of Crocker 180. Exp. as Fig. 2. Figures refer to ml. undiluted extract in 1·5 ml. solution.

longer time (see Fig. 3). If the enzyme be diluted more than 12× (0·08 ml. original extract per ml.), however, the production of lactic acid ceases (Fig. 2). As it was shown that the zymohexase activity of tumour slices was approximately equivalent to an extract containing 0·006 ml. original extract per ml. it appears that slices produce no lactic acid because the exposed enzyme is diluted beyond its effective concentration.

If the extract is diluted with boiled muscle extract or boiled tumour extract the fall in activity on dilution does not occur (Table VI). This is similar to the

Table VI. *Effect of boiled tissue extracts on glycolysis of diluted tumour extracts*

Extract incubated with 20 mg. glucose, 1 mg. adenylic acid, hexosediphosphate (0·01 mg. P), 4 mg. $NaHCO_3$

		mg. lactic acid formed per ml. original extract					
Exp.	ml. original extract in 2 ml.	No addition	1 mg. cozymase	0·5 ml. boiled muscle extract	1 mg. cozymase + 0·5 ml. boiled muscle extract	0·5 ml. boiled tumour extract	1 mg. cozymase + 0·5 ml. boiled tumour extract
1	1	1·6	6·8	2·5	7·6	—	—
	0·25	0·2	12·6	5·6	13·4	—	—
	0·062	0·1	3·7	13·2	13·5	—	—
2	1	2·5	11·1	—	—	5·9	13·2
	0·25	3·5	7·3	—	—	22·0	20·5
	0·062	4·0	5·0	—	15·4	16·0	15·6

result obtained by Kendal & Stickland [1937] on dilution of muscle extract' and is another point of similarity in the glycolytic mechanisms of muscle and tumour tissue.

Glycolysis of mixtures of glucose and fructose

The lactic acid production by slices and extracts from glucose and fructose and mixtures of these are given in Table VII. The high rate of glycolysis occurring with fructose in extracts is reduced to the glucose level if one-quarter of the

Table VII. *Lactic acid formation by Crocker 180 slices and extract from glucose and fructose*

Conditions as in Table I

Tumour	Glucose mg.	Fructose mg.	mg. lactic acid formed per g. slices or 2 ml. extract
Extract	20	0	4·4
	15	5	4·6
	10	10	4·6
	5	15	4·9
	0	20	8·5
Slices	20	0	7·5
	10	10	6·9
	0	20	2·2

fructose is replaced by glucose. It is of interest to compare this effect with the fermentation of these sugars by yeast juice. Yeast juice with phosphate ferments fructose more quickly than glucose but an increase in the rate is obtained when

only 2·5 % of the glucose is replaced by fructose [Harden & Henley, 1921]. The effect of fructose in tumour glycolysis is therefore quantitatively different from that of fructose on fermentation by yeast juice.

Effect of change of sugar concentration on glycolysis by slices

If the difference between fructolysis and glycolysis in slices is due to difference in their rates of diffusion then the low fructolysis should increase with increasing fructose concentration. That this is the case is shown in Fig. 4 in

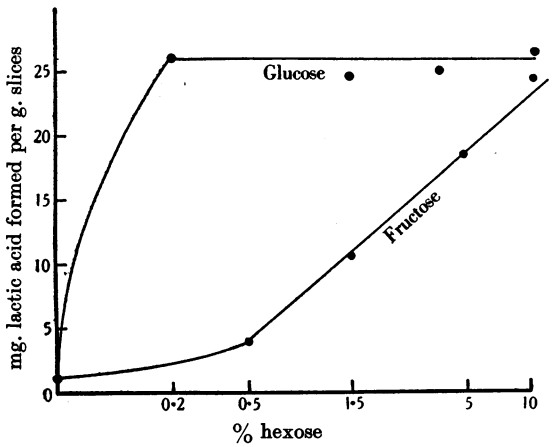

Fig. 4. Effect of concentration of substrate on the glycolysis of slices of Crocker 180. 150 mg. slices incubated in 2 ml. Ringer with 4 mg. $NaHCO_3$ and various concentrations of glucose and fructose. Lactic acid formation determined manometrically.

which lactic acid formation by tumour slices with different concentrations of glucose and fructose is shown. While the optimum concentration for glucolysis is about 0·2 %, fructose breakdown increases with increasing concentration up to 10 % and this appears to be due to the increased diffusion of fructose into the cells at the higher concentrations.

It therefore appears that all the differences in lactic acid production in tumour slices and extract from different carbohydrates shown in Table II are due to differences in rates of diffusion of these carbohydrates.

Effect of glyceraldehyde on glycolysis in muscle extract and on fermentation of dried yeast

Adler *et al.* [1937] have shown that *dl*-glyceraldehyde inhibits fermentation by washed dried yeast, and glycolysis by dialysed muscle and brain extracts and by tumour slices, of carbohydrates, but not of hexosediphosphate. The result with muscle extract is contrary to the findings of E. G. Holmes [1934] who has, however, published no details of his experiment. Table VIII shows the inhibitory effect of *dl*-glyceraldehyde on glycolysis of glycogen and starch by dialysed muscle extract. Undialysed muscle extract is only slightly inhibited even by high concentrations of glyceraldehyde. It is probable that some dialysable constituent of muscle extract combines with the glyceraldehyde and prevents its inhibitory action. Mendel *et al.* [1931] showed that the action of glyceraldehyde is reversed by pyruvate. The *dl*-glyceraldehyde inhibits the enzymes causing

Table VIII. *Effect of* dl-*glyceraldehyde on glycolysis of rat muscle extract*

1 ml. extract, 3 mg. adenylic acid, 3 mg. $NaHCO_3$—volume 1·5 ml.

Exp.	Type of extract	Substrate	mg. lactic acid formed per ml. in 1 hr. with added glyceraldehyde mg. 0	6	12	Free phosphate present after 1 hr. mg. P	
1	Undialysed	None added	0·4	0·5	0·9	—	—
		10 mg. glycogen	5·7	—	3·4	—	—
	Dialysed 1 hr. + *M*/30 phosphate buffer	None added	0·2	0·3	0·8	—	—
		10 mg. glycogen	2·6	1·6	0·8	—	—
		10 mg. starch	2·0	0·3	0·7	—	—
						Without glyceraldehyde	6 mg. glyceraldehyde
2	Dialysed 30 min.	None added	0·1	—	—	0·135	—
		10 mg. glycogen	2·5	0·6	—	0·100	0·155

esterification of carbohydrates; it does not act only on hexokinase, for it inhibits glycogen breakdown in muscle and tumour extracts, which do not require addition of hexokinase to activate them. Phosphate estimations on muscle extracts incubated with glycogen with and without *dl*-glyceraldehyde (Table VIII), show that more free phosphate is present when glycolysis is inhibited by glyceraldehyde, i.e. less glycogen is esterified.

The fermentation of glucose by unwashed dried yeast and the esterification of phosphatase are completely inhibited by 20 mg. glyceraldehyde per ml. (Table IX). Adler *et al.* [1937], using washed dried yeast, obtained about 70 % inhibition with 8·7 mg. glyceraldehyde per ml., and 50 % inhibition with 5·6 mg. per ml.

Table IX. *Effect of* dl-*glyceraldehyde on the fermentation of dried yeast*

30 mg. dried brewer's yeast in 0·5 ml. *M*/30 phosphate buffer

Substrate	μl. CO_2 evolved in 60 min. Without glyceraldehyde	10 mg. glyceraldehyde	Change in free phosphate (mg. P) Without glyceraldehyde	10 mg. glyceraldehyde
Glucose	+3600	−4	−0·72	+0·06
Hexosediphosphate	+ 240	+242	—	—

Table X. *Effect of* dl-*glyceraldehyde on glycolysis by Crocker 180*

Addition as in Table I

Material	Substrate	mg. lactic acid formed in 30 min. per 2 ml. extract or 1 g. slices Without glyceraldehyde	4 mg. glyceraldehyde
Extract	None added	0·1	0·0
	10 mg. glucose	3·2	0·2
	10 mg. fructose	4·0	0·9
	10 mg. glycogen	3·3	0·2
	Hexosemonophosphate (1·5 mg. P)	3·6	3·8
	Hexosediphosphate (3·5 mg. P)	2·8	3·1
Slices (200 mg.)	20 mg. glucose in 2 ml.	7·3	1·6

Effect of glyceraldehyde on glycolysis of tumours

Lactic acid formation from glucose, glycogen and fructose in extracts of Crocker 180 is inhibited by *dl*-glyceraldehyde, but that from hexosemono-

phosphate and hexosediphosphate is unaffected. This is in agreement with the findings of Adler *et al.* [1937] with extracts of other tissues. Glycolysis by tumour slices is also inhibited (see Table X).

Discussion

The experimental results described in this paper show that the intermediate processes in glycolysis by tumour tissue are exactly analogous to those of muscle. Tumour extract differs from muscle extract in glycolysing both glucose and glycogen and in containing nucleotidases which destroy necessary coenzymes. The high rate of glycolysis found in the tumour extracts is due in part to the presence of phosphatases which liberate free phosphate from substances present in the extracts.

Needham & Lehmann [1937] put "forward evidence that the glucose breakdown in embryo cannot go through the phosphorylation route" and "suggest that glucose breakdown in brain and tumour cannot to any great extent go that way either". These workers could not demonstrate the existence of cozymase in embryo but they did not use apozymase for its detection. As cozymase is the coenzyme for apozymase its estimation is more conclusive when this method is used. They also used methods of isolation which would be expected to give very small amounts of cozymase in the final solution used for the test. Tumours contain adenylpyrophosphate [Boyland, 1932] and cozymase [Boyland *et al.* 1937] but both these are destroyed by tumour extracts.

Needham & Lehmann [1937] found that hexosediphosphate was converted into triosephosphate by embryo and concluded that the hexosediphosphate must diffuse into the cell and there be acted on by zymohexase. In the analogous case of tumour slices hexosediphosphate is changed into triosephosphate, but this must be due to enzymes attached to the cut cells on the surface of the slices. In the case of tumour slices the slow glycolysis of hexosemonophosphate and hexosediphosphate is due to the inability of the phosphoric esters to penetrate into the tissue. Needham & Lehmann occasionally obtained glycolysis of hexosediphosphate and glycogen with embryos. Recent estimates of the molecular weight of glycogen indicate that it is of the order of 3,000,000 so that glycogen would not be expected to pass through the cell wall. It is therefore probable that the breakdown of glycogen occurred outside the cells and it is possible that this also occurred with hexosediphosphate.

Glycolysis in embryo, tumour, and other tissues is inhibited by NaF and by *dl*-glyceraldehyde. NaF is known to inhibit the conversion of phosphoglyceric acid into phosphopyruvic acid and results in the present paper show that *dl*-glyceraldehyde inhibits esterification of glycogen and glucose with phosphate. Needham & Lehmann [1937] found that $M/200$ NaF completely inhibited the action of minced embryo on phosphoglyceric acid while only inhibiting the glycolysis by 50 %. It seems most probable that the phosphoglyceric acid would be attacked outside the cells by such enzyme as was available because of cell injury, in which condition the enzyme would be more exposed to the action of NaF. The glycolysis probably proceeded within the cells where the concentration of NaF would be lower and consequently the inhibition of glycolysis only partial. Inhibition by these reagents, which act on different parts of the normal glycolytic mechanism, indicates that the glycolysis involves esterification with phosphate.

The nature of the intermediate processes in glycolysis is almost impossible to determine by examination of the whole cells because the substances attacked

depend not only upon the enzymes present but also on the diffusibility of the substances into the cell. This is illustrated by the difference in the actions of tumour slices and tumour extract on glycogen, fructose and glucose. All these substrates are rapidly glycolysed by extracts, but glycogen gives no lactic acid with tumour slices because of its non-diffusibility. Fructose is only slowly attacked by slices because it diffuses slowly while glucose is apparently readily diffusible. The different rates of diffusion of glucose and fructose are analogous to those found by Hopkins [1931] for Sauterne yeast. The diffusion rates of different carbohydrates and other substances into tumour tissue are being further investigated.

Summary

1. Extracts from the mouse sarcomas Crocker 180 and Sarcoma 37, mouse carcinoma 63 and rat carcinoma Walker 256, produce lactic acid from glucose and fructose if cozymase, adenylic acid and a trace of hexosediphosphate are added.

2. Extracts of these tumours rapidly glycolyse glycogen and hexosephosphates while slices of the same tumours do not, because of the non-diffusibility of these substances.

3. Slices of tumour tissue convert hexosediphosphate into triosephosphate. The distribution of triosephosphate in tumour slices and in the surrounding fluid indicates that the triosephosphate is formed outside the cells, presumably by the action of such enzyme as is exposed in cut and injured cells of the slices.

4. Tumour extracts diluted so as to show the zymohexase activity equivalent to the suspended slices do not form lactic acid either from glucose or hexosediphosphate.

5. Tumour extracts produce lactic acid more rapidly from fructose than from glucose while tumour slices produce lactic acid about 3–4 times as rapidly from glucose as fructose when the sugar concentration is below 0·5%.

6. The optimum glucose concentration for glycolysis in tumour slices is 0·2% while the optimum fructose concentration is 10% indicating that the difference in glycolysis is due to different rates of diffusion into the cells.

7. The fermentation of dried yeast and the glycolysis of starch and glycogen in muscle extract are inhibited by *dl*-glyceraldehyde. The inhibition is much greater in dialysed preparations.

8. The inhibition of glycolysis in muscle extract by glyceraldehyde is accompanied by inhibition of phosphate esterification. The glycolyses of hexosediphosphate and hexosemonophosphate are not inhibited.

9. The glycolyses of glycogen, glucose and fructose in tumour extracts are inhibited by glyceraldehyde but lactic acid formation from hexosephosphates is not affected. The action of glyceraldehyde is probably due to inhibition of esterification processes.

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