CIII. OBSERVATIONS ON TISSUE GLYCOLYSIS: THE EFFECT OF FLUORIDE AND SOME OTHER SUBSTANCES.

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The effect of fluorides on enzyme action has been a special object of study since the observation of Kastle and Loevenhart [1900], that the action of lipases is inhibited even by dilutions of 1 in 5000 fluoride. Pierce [1913] extended these results in a quantitative investigation of the equilibria involved. He was able to show that in the case of the hydrolysis of ethyl butyrate by lipase the action of fluoride could be explained as a monomolecular combination of the enzyme with a portion of the sodium fluoride added. It was assumed by Pierce that the complex formed was inactive and that the rate of reaction was proportional to the concentration of free enzyme. Loevenhart and Pierce [1906] had previously shown that the action of fluoride was completely reversible by removal of the NaF by dialysis.

It may be remarked that similar considerations have been shown by Euler and Svanberg to apply to the inactivation of saccharase by mercuric chloride [1920], and by aniline [1921].

The effect of oxalate and fluoride on glycolysis in blood has been studied by several authors [Macleod, 1913; Evans, 1922], and it has been found that fluoride is the more efficient inhibitor. The experiments described in the present paper show that in tissue glycolysis also the effect of oxalate is less than that of fluoride.

Investigations of the action of fluoride on tissue glycolysis have been mainly concerned with the transformation of carbohydrate in muscle, and this point has been investigated in particular by Embden *et al.* [1924] who showed that the breakdown of hexosephosphate in minced muscle or muscle extract is inhibited by fluoride. In this connection Kay [1928] has shown that the rate of synthesis of esters of phosphoric acid in the presence of extracts of kidney or intestine is also retarded by sodium fluoride, although the final equilibrium is probably not affected. Recently Lipmann [1928, 1929], working with yeast and with minced muscle, has extended these observations. He has studied the action of fluoride on the hydrolysis of some esters of phosphoric acid and also in two examples of inorganic catalysis. In a discussion of these experiments from the point of view suggested by the theories of Meyerhof and Warburg, Lipmann comes to the conclusion that the inactivation by fluoride

is probably due to the formation of a complex compound of iron with the fluoride. Support for this view is adduced from a comparison of the above results with the dissociation-curve of fluoromethaemoglobin.

Apart from the work on muscle, apparently only two papers have appeared on the effect of fluoride on the formation of lactic acid by tissues *in vitro*. Tissue glycolysis is of importance not only as an energy-liberating reaction [Warburg, 1923] but also in view of the great interest connected with the relationship between anaerobic glycolysis, aerobic glycolysis, and respiration. Loebel [1925] has shown that in the cases of the skin and brain of the rat and of the spinal cord of the frog, the addition of 0·01 % of sodium fluoride has an appreciable effect in inhibiting the anaerobic production of lactic acid from glucose. The effect is much greater than that on respiration. Ewig [1929] has also recently made some experiments on this point. Neither of these papers deals with the type or nature of the relationship between lactic acid production and the amount of fluoride, the determinations being made for one or two concentrations of fluoride only.

In the present paper some experiments are described upon the effect of various salts and particularly of fluoride on the glycolysis of tissues.

The extreme position of fluoride in the Hofmeister series suggested that a comparison of its effect with that of other ions in this series would be of interest. Of the salts examined, however, only fluoride and oxalate have any appreciable action in inhibiting the glycolysis in low concentrations, but the influence of these two ions is very marked and the results are reproducible as nearly quantitatively as could be expected. It is also shown that the action in this case, as in that of fluoride upon lipase, is reversible.

The results of the following experiments are expressed in the form of the percentage inhibition of glycolysis, *i.e.*

$$\frac{\text{glycolysis (control)} - \text{glycolysis (inhibited)}}{\text{glycolysis (control)}} \times 100.$$

It is shown that in the case of rat testis, rat brain, and Jensen rat sarcoma, the action of fluoride under anaerobic conditions may be represented by an equation derived from the law of mass action. This equation is of the type:

$$\text{inhibition} = \frac{[F]^n}{k + [F]^n},$$

where $[F]$ is the concentration of fluoride in the Ringer solution, k is a constant having a fixed value for each tissue. From the experimental data it is found that the value of the exponent n is close to unity in the case of rat brain and Jensen rat sarcoma and is nearly equal to two in the case of rat testis.

Experimental.

In the following experiments, the technical details described by Warburg [1926] have been followed exactly. The Ringer solutions used were prepared from stock isotonic solutions of the various salts [Okamoto, 1925] and unless stated to the contrary had the following composition.

	Preparation of solution (parts by volume)				Composition of solution (m.-mols)			
	NaCl	KCl	$CaCl_2$	$NaHCO_3$	NaCl	KCl	$CaCl_2$	$NaHCO_3$
Ca-Ringer	100	2	2	20	125	2·5	1·7	25
Ca-free Ringer	102	2	0	20	127	2·5	0	25

Glucose was added in the form of a 10 % solution to make the final concentration 0·2 %. When the effect of any added substance was to be investigated, part of the NaCl was substituted by an equal volume of an isotonic solution of that substance. This method has the advantage that the other components, and especially the concentration of bicarbonate, remain unaltered. All such added solutions were neutralised before use and their osmotic pressure was controlled by determination of the freezing-point in all cases: this was − 0·56°. After saturating with the gas-mixtures used (5 % CO_2 in either N_2 or O_2 was supplied from a cylinder of the mixed gases, the percentage of CO_2 being checked by analysis), the p_H values of the solutions were determined by the use of the glass electrode[1]. The values of p_H found for these solutions did not differ by more than 0·10.

Method of expressing the results. The results are expressed in conformity with Warburg's nomenclature as mm.^{3} CO_2 at N.T.P. liberated from the bicarbonate by the lactic acid formed (glycolysis), or as mm.^{3} oxygen consumed from the gas-mixture (respiration) per mg. tissue per hour. The results were calculated on the basis of the weights of the tissues after drying at 110°. The values obtained are denoted by $Q_M^{N_2}$ (anaerobic glycolysis measured in nitrogen) or Q_{O_2} (respiration). The former multiplied by the factor 0·004 gives the lactic acid formed, in mg. per hour per mg. tissue. In the case of Ringer solutions containing the salts of weak acids, the whole of the carbon dioxide equivalent to the lactic acid formed is not liberated, and in these cases the "retention" [Warburg, 1926, p. 199] was determined, and where necessary a correction was applied for the CO_2 bound in this way. If this correction is not included in the results it may be taken that it was negligible.

Anaerobic glycolysis.

A. *The use of calcium-free Ringer solution.* The first point which had to be decided was whether reliable and consistent readings could be obtained in the calcium-free medium. The use of such a solution is necessary in order to avoid the complication introduced by the combination of part of the calcium with certain of the salts used. This subject has recently been investigated by Lasnitzki and Rosenthal [1929], but these authors used solutions which contained neither calcium nor potassium. Under these conditions they found that the values obtained for $Q_M^{N_2}$ were somewhat lower than in ordinary Ringer solution; their readings, however, were consistent. As this is the important point for the present purpose, it will be sufficient to quote a typical example from a series of readings obtained with 10 manometers using tissue from one and the same rat, and to compare it with other measurements taken with

[1] We wish to express our thanks to Mr N. F. Maclagan for these measurements.

material from a number of different animals. In the same table are included some other results in solutions with and without calcium.

Table I.

Temperature 38°.

Tissue	Solution	$Q_M^{N_2}$	Mean value
Rat testis (Same animal)	Ca-free Ringer	8·0, 8·6, 8·6, 8·3, 6·9, 8·8, 7·2, 7·1, 7·2, 7·0	7·77
Rat testis (Different animals)	Ca-free Ringer	9·4, 6·8, 9·4, 6·2, 6·8, 6·6, 11·5, 12·0, 8·4, 9·2, 10·0, 6·4, 8·2, 7·9, 7·6, 6·7, 6·9	8·23
	Ca-Ringer	9·4, 6·7, 8·1, 5·9, 10·4, 9·6, 9·7, 11·0, 10·3	9·01
Rat brain grey-matter (Different animals)	Ca-free Ringer	9·5, 6·2, 6·2, 7·1, 7·2, 5·8, 7·3, 9·3, 8·1, 7·6, 5·9, 8·1, 7·8, 5·9, 6·7, 8·6, 12·4, 8·6, 9·0	7·75
	Ca-Ringer	8·5, 8·9, 6·8, 9·6	8·45
Jensen rat sarcoma (Different animals)	Ca-free Ringer	21·7, 22·6, 21·5, 17·2, 18·6, 19·0, 20·8, 20·7, 20·1, 30·8	21·30
	Ca-Ringer	27·7, 21·1, 19·7, 28·9, 38·0, 27·7, 28·9, 28·2, 29·9, 22·9	27·30
Retina of rat	Ca-free Ringer	54·0, 42·0, 41·5	45·80
	Ca-Ringer	68·0, 63·5, 61·0	64·20

It will be observed that the glycolysis is usually a little lower in the calcium-free Ringer solution than in that containing calcium—particularly in the case of tissues with high anaerobic glycolysis. As would be expected, more consistent duplicate values are given by tissues from the same animal. In the following experiments we have therefore used tissue from one animal for each whenever possible, with normal controls made at the same time in each case.

In order to obtain further evidence on the effect of calcium and potassium on anaerobic glycolysis, the whole of the sodium chloride was replaced by an equal volume of solutions of chlorides of calcium and potassium in making up the Ringer solution. The results of measurements of anaerobic glycolysis of rat testis are shown in the following table:

Exp.	Composition of Ringer solution (m.-mols) Ca	K	$Q_M^{N_2}$
1	0	2·5	+ 9·4
2	1·7	0	+ 9·1
3	85	2·5	+11·6
4	1·7	125	+ 7·6

In the above case the concentration of sodium ions is lowered to the same extent in Exps. 3 and 4 since the whole of the sodium chloride is replaced in both experiments, and the sodium ions present are derived solely from the bicarbonate. Even such very large concentrations of Ca and K have little effect; there is, however, again a slight increase in Exp. 3, containing an increased amount of calcium.

B. *Effect of anions.* From the above experiments it is safe to conclude that in comparison with the other effects studied in this paper, that of replacing calcium or potassium by sodium is slight, and in all the following experiments, unless the contrary is stated, the medium used was calcium-free Ringer solution.

Embden and Lenhartz [1924] have investigated the effect of various anions on minced muscle, and they find that, judged by the effects produced, the ions may be arranged in a Hofmeister series. Those ions which produce the greatest swelling of the tissue (CNS^- and I^-) cause the greatest splitting off of phosphoric acid from hexosephosphate, NO_3^- and Br^- have less effect, whilst $SO_4^=$ and tartrate cause scarcely any more hydrolysis than occurs in water. On the other hand, the action of citrate and fluoride is so powerful in diminishing free phosphoric acid that almost the whole of the organic phosphoric acid may be caused to disappear in the presence of these salts. Except for the action of fluoride, our experiments with tissue glycolysis are not parallel with these observations on muscle; in particular, citrate had no effect in our experiments. Table II gives the results of adding a series of sodium salts to the Ringer solution in place of the whole or part of the sodium chloride.

Table II.

		Rat testis			Jensen rat sarcoma	
Anion	Concn. in Ringer (m.-mols)	$Q_M^{N_2}$ mean of controls	$Q_M^{N_2}$ with anion	Concn. in Ringer (m.-mols)	$Q_M^{N_2}$ mean of controls	$Q_M^{N_2}$ with anion
Thiocyanate	125	8·2	12·0	12	19·0	16·0
Iodide	.	.	.	50	27·7	33·0
	.	.	.	125		26·5
Nitrate	125	8·2	11·4	.	.	.
Bromide	.	.	.	125	27·7	32·6
Oxalate*	10	8·4	7·0	10	20·7	17·5
	50		2·8	50		4·4
Tartrate*	50	8·2	8·4	.	.	.
	83	6·7	6·9	.	.	.
		6·2	7·0	.	.	.
Citrate†	20	6·8	6·7	.	.	.
	50		6·4	.	.	.
Fluoride	10	7·6	2·8	10	21·9	2·7
	18·6	11·5	1·9	20		1·3
	50	6·8	0·6	50	18·6	1·0
						0·75
Phosphate†	.	.	.	22	20·8	26·4
Pyrophosphate†	5	10·5	13·5	.	.	.
	20		9·7	.	.	.
Cyanide*	10	6·5	5·8	.	.	.
	50		2·9	.	.	.

* Retention determined and found to be negligible. † Retention allowed for.

The main fact apparent from Table II is that, of the salts examined, fluoride has the greatest inhibitory effect, whilst oxalate and cyanide, though they affect glycolysis, do so to a considerably less extent.

The action of these three salts is in contrast to that of the others quoted in

Table II which shows that little change results even from high concentrations of bromide, tartrate and citrate. In these results little importance can be attached to slight variations, but it seems probable that in the case of high concentrations of thiocyanate and possibly of nitrate glycolysis may be slightly increased.

Warburg [1926] states that bicarbonate can be substituted by other salts, *e.g.* phosphate. Since the effect of increased concentration of bicarbonate is to increase glycolysis, a slight increase in the value of $Q_M^{N_2}$ results from the addition of phosphate. The case of pyrophosphate is of interest in connection with the heavy metal theories of respiration and glycolysis: these will be referred to later. It will be seen that in concentrations up to 20 m.-mols its effect is slight. In the case of this salt it was necessary to neutralise the alkaline sodium pyrophosphate with hydrochloric acid before use, whereas the orthophosphates were made up from mixed mono- and di-sodium phosphate solutions. In both cases the p_H was accurately adjusted.

C. *Fluoride and oxalate.* The method used in these experiments consisted in setting up several (usually twelve) Barcroft manometers, of which two were thermobarometric control vessels without tissue. Each of the remaining ten vessels contained a section of tissue and a suitable volume of Ringer solution. Some of the vessels had Ringer solution containing varying amounts of sodium oxalate or sodium fluoride, and the remaining vessels were used for the controls in Ca-free Ringer solution. The concentrations of the salts were checked by analysis.

Several such series of experiments for each tissue were made, and in spite of the variations due to the use of different animals, when the results were compared, the percentage inhibition resulting from a given concentration of fluoride was constant for each tissue examined within the limits of experimental error. This can be seen from an examination of the curves and tables given below.

The effect of fluoride on anaerobic glycolysis. The tissues used for these experiments include Jensen rat sarcoma, rat brain and rat testis. The results are expressed in Tables III, IV and V and also in the curves given in Figs. 1, 2 and 3. The measurements are probably most accurate in the case of the sarcoma since the rate of glycolysis is higher in the case of this tissue, and consequently the pressure changes observed are greater. The curves and the last column in each of the tables represent the values calculated from the equations. The derivation of these will be described later. Details of a typical experiment are given in Protocol 1.

Retina. The case of the retina is of special interest, not only because it possesses the highest anaerobic glycolysis of any tissue yet studied, but also because it forms the most marked exception to the theories seeking to differentiate normal from malignant tissues on the basis of their carbohydrate metabolism [Warburg, 1926]. It was therefore of interest to investigate the action of fluoride in this case, particularly as the effect on retina of substances known to inhibit glycolysis in other tissues had not previously been studied.

Protocol 1.

Jensen rat sarcoma. V_F = 3 cc. Ca-free Ringer in all vessels. Temp. 38°. N_2 with 5 % CO_2.

Vessel	1	2	4	5 E	6	7	8	10	12
NaF (m.-mols)	0	0	1	2·5	4	0	7	10	20
Pressure change per 10 min. (mm.)	+22 +22·5 +22	+33 +32 +32	+18 +22·5 +21	+ 8 + 7·5 + 7·5	+ 6·5 + 7 + 6	+21 +20·5 +18·5	+ 0·5 + 1·5 + 1·5	+ 1 + 2 + 2	+ 1 + 0 + 1·5
Pressure change per 30 min. (h)	+66·5	+97	+61·5	+23	+19·5	+60	+ 3·5	+ 5	+ 2·5
V gas (cc.)	8·61	8·44	8·37	10·24	9·47	14·15	13·42	14·02	12·09
Vessel constant k_{CO_2}	0·918	0·902	0·896	1·060	0·993	1·402	1·366	1·392	1·222
Wt. of dried tissue (mg.)	5·65	7·72	9·20	5·63	10·70	7·74	7·20	4·82	4·45
$Q_M^{N_2}$	21·5	22·6	12·0	8·6	3·6	21·7	1·3	2·9	1·4
Inhibition (% of controls)	.	.	46	61	84	.	94	87	95

Table III.

Jensen rat sarcoma. Temp. 38°.

NaF (m.-mols)	$Q_M^{N_2}$ mean of controls	$Q_M^{N_2}$ with NaF	Inhibition observed %	$100 \times \frac{[F]}{1+[F]}$ calc.
0·1	20·1	18·3	9	9
1·0	21·9 20·1	12·0 12·0	46 40	50
2·5	21·9 20·1 19·0	8·6 7·5 4·2	61 63 78	71·5
4·0	21·9	3·6	84	80
4·7	30·8	4·7	85	82·5
7·0	21·9 21·1*	1·3 3·5*	94 83*	87·5
9·3	30·8	2·7	91	90
10·0	21·9	2·9	87	91
15·0	21·9	1·7	92	94
18·6	30·8	2·3	93	95
20·0	21·9 19·0	1·4 2·4	95 87	95·5
50·0	17·2 17·2	1·0 1·1	94 94	98
	18·6	1·0 0·8	95 96	.

* Calcium-containing Ringer.

Table IV.

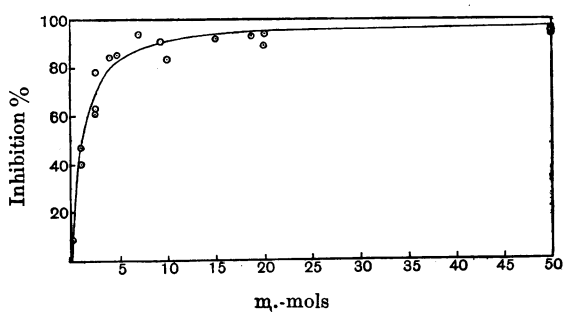
Rat brain cortex. Temp. 38°.

NaF (m.-mols)	$Q_M^{N_2}$ mean of controls	$Q_M^{N_2}$ with NaF	Inhibition observed %	$100 \times \frac{[F]}{1{\cdot}6+[F]}$ calc.
0·1	6·8	6·2	9	6
0·7	7·8	4·2	46	30
1·7	7·8	3·7	53	52
2·7	7·8 9·3	2·9 2·0	63 79	63
4·7	7·8 8·1	1·2 1·9	85 77	75
6·7	7·8	1·4	82	81
10·0	7·8 9·5	0·7 1·4	91 85	86
13·3	7·8	1·4	82	89
33·3	7·2	0·6	92	95

Table V.

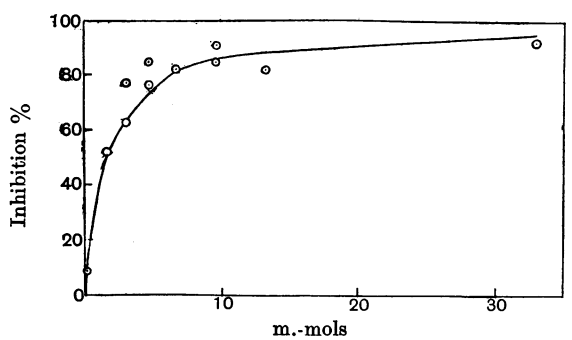
Rat testis. Temp. 38°.

NaF (m.-mols)	$Q_M^{N_2}$ mean of controls	$Q_M^{N_2}$ with NaF	Inhibition observed %	$100 \times \frac{[F]^2}{33+[F]^2}$ calc.
0·01	6·6	6·6	0	*ca.* 0
0·1	6·6	6·4	3	*ca.* 0
0·7	8·8	9·2	−5	2
1·0	6·6	7·0	−6	3
1·7	7·2	7·5	−4	8
2·7	7·1	5·9	17	18
4·7	7·2	3·4	53	40
	11·5	4·8	58	
5·0	9·1	5·0	45	43
6·7	7·0	3·1	56	58
9·3	11·5	3·3	71	72
10·0	7·8	2·8	64	75
	9·4	1·9	80	.
	6·4	1·8	72	.
	9·1	3·0	67	.
13·3	7·8	0·7	91	84
18·6	11·5	1·9	84	91



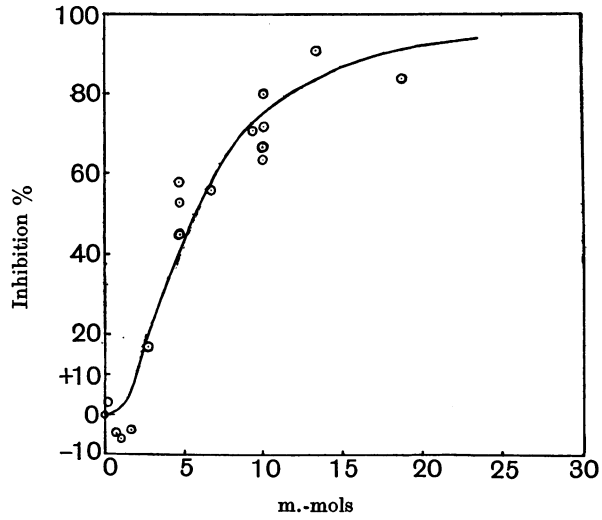
Sodium fluoride—m.-mols. Curve: $\frac{i}{100} = \frac{[F]}{1+[F]}$.

Fig. 1. Jensen rat sarcoma.



Sodium fluoride—m.-mols. Curve: $\frac{i}{100} = \frac{[F]}{1{\cdot}6+[F]}$.

Fig. 2. Rat brain cortex.



Sodium fluoride—m.-mols. Curve: $\frac{i}{100} = \frac{[F]^2}{33+[F]^2}$.

Fig. 3. Rat testis.

Owing to the large number of animals which would be required to construct a curve similar to that obtained for sarcoma and the other tissues investigated, in this case the effect was studied for two concentrations of fluoride only, namely, 1 and 10 m.-mols. The preparation of the retina was made in a darkened room using Ringer solution warmed to 38°.

Comparison of glycolysis of rat-retina in Ca-Ringer and Ca-free Ringer with and without fluoride. As has been stated in discussing the effect of absence of calcium, there is, in the case of retina, a considerable lowering of glycolysis in the calcium-free Ringer solution. A further difficulty is introduced by the fact that during the experiments a slight fall in the value of $Q_M^{N_2}$ occurs as the time of the observation is increased. To control this point we have used two methods of experiment.

(*a*) Five vessels *A*, *B*, *C*, *D* and another as thermo-barometer were used. Vessels *A* and *B* each contained 3 cc. Ca-Ringer, and *C* and *D* the same volume of Ca-free Ringer solution. The retinas from two rats were placed in each vessel and when temperature equilibrium was reached (15 min.) pressure readings were made at intervals of 5 min. for a period of 15 min. At the end of this time, 2 cc. of the solution were removed and were replaced in the case of vessels *A* and *C* by 2 cc. of calcium-free Ringer solution containing a concentration of 15 m.-mols NaF: the final concentration in vessels *C* and *D* is therefore 10 m.-mols NaF. By this technique, the Ca content is lowered to such an extent that precipitation of calcium fluoride does not occur. The results are summarised in Table VI.

(*b*) The two experiments (with and without fluoride) were made concurrently. The inhibition by 1 m.-mol was found to be 64 %.

Table VI.

Retina of rat. Temp. 38°.

	A	*B*	*C*	*D*
	With Ca		Without Ca	
Without NaF	.	.	.	.
$Q_M^{N_2}$ (1st 15 min.)	+68	+63·5	+42	+54
With NaF (m.-mols)	0	10	0	10
$Q_M^{N_2}$ (2nd 15 min.)	+61	+10·4	+37	+ 6·2
Fall in $Q_M^{N_2}$ %	10	.	12	.
Inhibition % (corrected for above fall)	.	82	.	87

It will be seen that the inhibition in both cases is of the same order as in the case of Jensen rat sarcoma.

Kidney. In contrast to the tissues of high glycolytic power such as retina and Jensen rat sarcoma, the effect of fluoride was next studied on a tissue with very low anaerobic glycolysis, namely rat kidney ($Q_M^{N_2} = +3$). In this case the accuracy of the results was limited by the small pressure changes obtained. A further difficulty arose from the progressive decline of the readings which in the case of this tissue was much greater than in any of the others studied and was so large that it could not be allowed for with accuracy by control experiments. The reason for this is not at present clear. The experiments showed, however, that a concentration of approximately 10 m.-mols NaF is required to inhibit the glycolysis by 50 %. Whilst fluoride thus has a definite inhibitory power in this case also, its action is less than in the case of tissues with a higher initial value of anaerobic glycolysis.

Human carcinoma. Two specimens of human carcinoma of size and uniformity suitable for these experiments were kindly placed at our disposal by Mr Handley: the numbers given are those of the records in the Bland-Sutton Institute of Pathology. These were a schirrus carcinoma of the breast and a sclerosing, spheroidal-celled carcinoma of the stomach. The following results were obtained (Table VII).

Table VII.

	Carc. breast No. SS 437/1929		Carc. stomach No. SS 533/1929							
NaF (m.-mols)	0	5	0	0	1	2·5	4	7	15	50
$Q_M^{N_2}$ ...	+14·3	+4·7	+4·9	+4·1	+4·5	+4·4	+5·0	+2·8	+2·3	+1·1
			Mean 4·5							
Inhibition %	.	67	.	.	.	.	.	38	49	76

The difference between these results is very marked. There is no demonstrable effect even with 4 m.-mols of fluoride in the second case, whereas the effect of 5 m.-mols of fluoride on the carcinoma of the breast has been to reduce its glycolytic power to one-third. Hence, as in the experiment with kidney, the effect of fluoride is least when the initial value of the glycolysis is low.

This result is surprising, since the glycolytic activity of these tissues is generally supposed to be almost entirely due to cancer cells, and the other adventitious material has usually been regarded merely as a diluent [Warburg, 1928]. Since on the basis of this assumption, for which there is a good deal of evidence, the actual cancer cells should show a high glycolysis, one might expect much the same lowering of this value by fluoride in the two cases.

Reversibility of the action of fluoride. Of the various reagents which inhibit glycolysis, those in the case of which the reaction is reversible are the most likely to throw some light on the mechanism of the process, and are therefore of greater theoretical importance. In order to determine to what extent the action of fluoride is reversible, the tissues, after measurement of their glycolytic activity in fluoride-Ringer solution, were repeatedly washed in fluoride-free Ringer solution saturated with the gas-mixture and maintained at 38°. The controls were treated similarly. The result is summarised in Table VIII.

Table VIII.

Jensen rat sarcoma. Temp. 38°.

Vessel ...	*A*	*B*	*C*	*D*
	With Ca	Without Ca	Without Ca	Without Ca
NaF (m.-mols) ...	0	0	50	50
$Q_M^{N_2}$	19·7	17·2	1·0	1·1
Inhibition % of controls	.	.	95	94
After repeated washing, *A* and *C* with Ca-Ringer, *B* and *D* with Ca-free Ringer solution, at 38°:				
$Q_M^{N_2}$	18·4	12·0	11·6	7·4
Recovery % of controls	.	.	63	62

Two points are clear from these results. Firstly the fall which we have often observed in the value of $Q_M^{N_2}$ on washing many times with Ca-free Ringer solution, whereas the control washed with Ca-Ringer solution maintained a steady value. This appears to be an exaggerated example of the lower results in a Ca-free Ringer solution which are recorded in Table II. Secondly, the action of fluoride is clearly reversible to a considerable extent under these conditions, but in our experiments about one-third of the initial glycolytic activity is lost. It is difficult to express an opinion as to whether this is due to some definite destruction or "killing" of the cells or whether it is merely due to inadequate removal of the fluoride from the cell-contents, as no reliable data are available on this subject.

In any case, however, the action of fluoride is quite distinct from that of, for example, mercuric chloride, which is almost completely irreversible [Jowett and Brooks, 1928].

The effect of temperature on the action of fluoride. In view of the fact that the action of fluoride seems to be due to some combination of the salt which is at least partially reversible, temperature may be expected to affect the equilibrium involved. In order to study the effect of temperature, the experiment detailed in Protocol 2 was carried out. For this purpose five manometer

Protocol 2.

Rat testis. $V_F = 3$ cc. Ringer in all vessels. N_2 with 5 % CO_2.

Vessel No.	1	4	5	6
NaF (m.-mols) ...	0	5	10	50
Pressure readings				
Temp. 25°				
10 min.	+ 3·5	+ 2·5	+ 1	+ 0·5
10 ,,	+ 4·5	+ 2	+ 1	− 0·5
10 ,,	+ 4	+ 2·5	+ 1	+ 0·5
Temp. 35°				
10 ,,	+15	+10·5	+ 4	+ 0·5
10 ,,	+16	+10·5	+ 5·5	+ 1
10 ,,	+15·5	+ 9·5	+ 4	+ 1
Temp. 45°				
5 ,,	+13·5	+ 9·5	+ 5·5	+ 3
5 ,,	+15	+ 8·5	+ 4·5	+ 2
5 ,,	+12·5	+ 9	+ 5	+ 2·5
5 ,,	+13	+ 9	+ 4	+ 3
Pressure change (h) for 30 min. (mm.)				
25°	12	7	3	0·5
35°	46·5	30·5	13·5	2·5
45°	81	54	28·5	16
V gas (cc.)	8·61	8·37	10·24	9·47
Vessel constants (k_{CO_2})				
25°	1·00	0·997	1·17	1·10
35°	0·941	0·922	1·09	1·02
45°	0·884	0·864	1·02	0·957
Dry wt. of tissue (mg.)	11·07	13·78	14·12	13·35
$Q_M^{N_2}$:				
25°	2·2	1·0	0·5	*ca.* 0·1
35°	7·9	4·1	2·1	0·4
45°	12·9	6·8	4·1	2·3

vessels were used; one of these was a thermo-barometer without tissue, and each of the remaining four vessels contained an approximately equal portion of rat testis in Ca-free Ringer solution. The experiment was begun with the vessels at 25° and afterwards the temperature of the thermostat was very quickly raised to 35°. After the readings had been taken the temperature was again raised to 45° and when thermal equilibrium was once more established the readings were continued. The results are summarised in Table IX.

Table IX.

Rat testis.					
NaF (m.-mols) ...		0	5	10	50
$Q_M^{N_2}$ at	25°	2·2	1·0	0·5	0·1
	35°	7·9	4·1	2·1	0·4
	45°	12·9	6·8	4·1	2·3
Inhibition by NaF % at	25°	0	56	77	96
	35°	0	48	73	95
	45°	0	46	68	82

From Table IX it is clear that the percentage inhibition caused by fluoride falls regularly as the temperature increases, suggesting a dissociation of the fluoride combination with rise of temperature.

In the second place the effect of temperature on the velocity of glycolysis approximates to the general van't Hoff temperature coefficient of chemical reactions, and in this respect rat testis resembles the specimen of carcinoma examined by Warburg [1926, p. 121]. This rule applies equally well whether fluoride is present or not, as is shown in Table X.

Table X.

Rat testis. Ratio of velocities of glycolysis at 25°, 35° and 45°.

NaF (m.-mols)	0	5	10
25° } 35°	3·6 : 1	4·1 : 1	4·2 : 1
35° } 45°	1·6 : 1	1·7 : 1	1·9 : 1

Effect of glucose concentration on fluoride inhibition. Further information on the mode of action of fluoride in inhibiting glycolysis is given from a study of the effect of concentration of the substrate. In the following experiment glucose solutions, made isotonic with NaCl and containing different percentages of glucose, were used and a similar series of solutions was prepared containing in addition sodium fluoride in a concentration of 50 m.-mols. The first part of the experiment was carried out in the solutions without fluoride; this was afterwards poured off and the Ringer solution containing fluoride was substituted for it. The results are shown in Table XI.

Table XI.

Rat testis.

Vessel	*A*	*B* (control)	*C*	*D*	*E*
Glucose %	0·1	0·2	0·5	1·0	2·0
$Q_M^{N_2}$ without NaF ...	7·0	6·2	10·0	10·3	12·7
Ringer solution replaced as follows:					
NaF (m.-mols) in Ringer	50	0	50	50	50
$Q_M^{N_2}$ with NaF	0·6	6·8 (control)	0·7	0·8	0·75

Two points are shown by the figures in this table. The increase of velocity of glycolysis with glucose concentration resembles that described for carcinoma sections by Warburg [1926, p. 121] except that it does not reach a maximum with 0·2 to 0·3 % of glucose, but there is a gradual increase with higher concentrations.

The effect of fluoride is to reduce the glycolysis to a low constant rate in all cases, whatever the glucose concentration.

Glucose consumption and lactic acid production in the presence of fluoride. It is conceivable, since the manometric method is non-specific, that the inhibition of the pressure changes in the presence of fluoride might not be due to a simple inhibitor of glycolysis, but that the glucose might be converted into some substance other than lactic acid, which does not liberate CO_2 from the bicarbonate. In several experiments, therefore, the concentration of glucose was determined before and after the experiment by Hagedorn and

Jensen's method, and in two experiments the lactic acid formed was determined by Friedemann, Cotonio and Shaffer's modification of Clausen's method: the details are given in an earlier paper [Baker, Dickens and Gallimore, 1929]. The results of one of these experiments are recorded in Table XII.

Table XII.

Jensen rat sarcoma, 38°. Time of exp. 1 hour.

Vessel	*A*	*B*	*C*	*D*
NaF (m.-mols)	0	50	0	2·5
$Q_M^{N_2}$	18·6	1·0	19·0	4·2
Calculated from pressure readings:				
Glucose consumed mg. ...	2·48	0·33	1·65	0·40
Observed (Hagedorn and Jensen method):				
Glucose consumed mg. ...	2·76	0·39	1·6	0·5
Observed (Clausen method):				
Lactic acid formed mg. ...	—	—	1·7	0·5

It will be seen that even in the presence of fluoride, the glucose consumption is quite parallel to the lactic acid formation as determined chemically and also by calculation from the observed pressure changes.

Since in many cases of glycolysis phosphate plays an important part, it was thought necessary to repeat the above experiment in a medium containing sodium phosphate, as well as bicarbonate, but no difference was observed in this case.

D. *Use of dihydroxyacetone in place of glucose.* Loebel [1925] has shown that tissues *in vitro* are able to bring about the conversion of dihydroxyacetone to lactic acid. This question is of importance in connection with the possibility of dihydroxyacetone being an intermediate in the metabolism of carbohydrate. Loebel used a concentration of 0·1 % of dihydroxyacetone, in which the lactic acid formation of rat brain was only 10 % of that of the control in 0·2 % glucose. With rat testis we have obtained much higher results, so much so that with the concentration used by Loebel the value of $Q_M^{N_2}$ was approximately equal to that in 0·2 % glucose, equimolecular concentrations of glucose and dihydroxyacetone giving the same value of $Q_M^{N_2}$. In higher concentrations of dihydroxyacetone the figures were much greater than are obtainable by the use of glucose. In all these experiments a control vessel containing dihydroxyacetone in Ringer solution was used as thermo-barometer; the results are thus corrected for changes due either to the spontaneous transformation of the dihydroxyacetone into lactic acid or to bacterial action. Such changes did in fact occur, as is shown by the slight positive pressures in the dihydroxyacetone control vessel, but they were small in relation to the pressure changes under consideration.

In view of the differences between our results with rat testis and those of Loebel with brain, the effect of varying the concentration of dihydroxyacetone was determined and is shown in Table XIII.

Table XIII.

Rat testis 38°. N_2 with 5 % CO_2.

Dihydroxyacetone % ...	0·1	0·3	0·5
$Q_M^{N_2}$	7·0	17·6	19·8
Control with glucose ...	(0·2 %)	$Q_M^{N_2}=8·1$	

The rapid change which occurs in the value of $Q_M^{N_2}$ as the dihydroxyacetone concentration is diminished is in marked contrast to the results obtained with glucose in similar concentrations.

Effect of fluoride in dihydroxyacetone-Ringer solution. The conversion of hexose to triose has often been regarded as an intermediate stage in the metabolism of carbohydrate. Whether similar intermediate compounds occur in glycolysis is uncertain, but in any case it is of interest to determine the action of fluoride on the transformation of dihydroxyacetone described in the last experiment. Jensen rat sarcoma and rat testis were the experimental materials used and the results are given in Table XIV.

Table XIV.

Tissue	Jensen rat sarcoma		Rat testis		
Dihydroxyacetone % ...	0·2	0·2	0·3	0·3	0·3
NaF (m.-mols)	0	50	0	0	120
$Q_M^{N_2}$	37·5	37·5	22·9	16·9	16·7
			Mean 19·9		

Table XV.

(1) *Rat testis.*

Oxalate (m.-mols)	0	5	10	50
$Q_M^{N_2}$	8·4	8·5	7	2·8
Inhibition %	0	0	17	67
Inhibition % by same concentration of NaF (see p. 943)	0	45	75	95

It will be seen that the inhibitory effect of oxalate is much less than that of fluoride particularly in the lower concentrations. This result is in accordance with the effect, already mentioned, of these two salts on glycolysis in the blood.

(2) *Jensen rat sarcoma.* Calcium-free Ringer solution at 38°.

Oxalate (m.-mols)	0	10	20	50
$Q_M^{N_2}$	20·7	5·8	5·4	4·4
Inhibition %	.	72	74	79

The reversibility of the action of oxalate was also investigated in the above exp. by the method described in connection with fluorides. Evidence of partial reversibility was obtained but the recovery after rinsing was less than in the case of fluoride.

(3) *Slow-growing rat sarcoma.* The specimens of sarcoma used, like those of Jensen rat sarcoma previously mentioned, were kindly supplied by Prof. Russ and belonged to a strain propagated in the Barnato-Joel Laboratories of the Middlesex Hospital. The following figures were obtained with this material.

Oxalate (m.-mols)	0	0	3	5	10
$Q_M^{N_2}$	18·1	16·8	18·8	11·7	10·4
	Mean 17·5				

This experiment shows that fluoride has little effect even when the whole of the sodium chloride in the Ringer solution is replaced by sodium fluoride

as in the last column of Table XIV. The action is thus quite distinct from that on glucose.

E. *Effect of oxalate with glucose.* The experiments were carried out exactly as in the case of fluoride, the only difference being the use of isotonic sodium oxalate instead of sodium fluoride in the preparation of the solution (Table XV).

F. *Effect of cyanide.* It is stated that concentrations of cyanide of the order of 1 m.-mol completely inhibit tissue respiration [Warburg, 1926], but this concentration does not affect glycolysis. In the following experiment the effect of higher concentrations of cyanide was studied. The experimental material used was rat testis. The sodium cyanide was previously neutralised with hydrochloric acid and was diluted so that the solution froze at − 0·56°.

Concentration of NaCN (m.-mols)	0	0	1	4	10	50	80
$Q_M^{N_2}$	6·7	6·2	7·6	6·5	5·8	2·9	2·1
	Mean 6·45						
Inhibition %	.	.	.	.	10	55	67

From this it will be seen that very high concentrations of cyanide definitely inhibit glycolysis also, but that the amount required is about one hundred times as great as for respiration.

Respiration and aerobic glycolysis.

In the preceding section the action on glycolysis of a substance—sodium cyanide—which is known to inhibit respiration is described. The following experiments were made to determine the effect on respiration of sodium fluoride, an agent already shown to reduce glycolysis. The improved method described by Warburg [1926, p. 102] was used in all experiments.

Protocol 3. *Effect of NaF on respiration and aerobic glycolysis.*

Rat testis. Temp. 38°. Ca-free Ringer solution.

Vessel No.	1	2	3	4	5 E	6
NaF (m.-mols)	0	0	10	10	20	20
V_F	3 cc.	7 cc.	3 cc.	7 cc.	3 cc.	7 cc.
Pressure change per 10 min. (mm.)	+ 4	−17·5	+ 4	−10	+ 4	− 6
	+ 4·5	−20·5	+ 4·5	−10·5	+ 3	− 5
	+ 4·5	−18·5	+ 5·5	− 9	+ 5	− 6
Pressure change per 30 min.	$h = +13$	$H = -56·5$	$h = +14$	$H = -29·5$	$h = +12$	$H = -17$
Wt. of dried tissue (mg.)	12·49	15·10	8·76	6·60	7·54	9·17
V gas (cc.)	8·61	4·44	9·51	4·37	10·24	5·47
Vessel constants	k_{CO_2} 0·916 K_{CO_2} 0·767		k_{CO_2} 0·996 K_{CO_2} 0·761		k_{CO_2} 1·06 K_{CO_2} 0·858	
	k_{O_2} 0·761 K_{O_2} 0·406		k_{O_2} 0·841 K_{O_2} 0·400		k_{O_2} 0·908 K_{O_2} 0·497	
Q_{O_2} (respiration)	−11·9		−13·9		−11·9	
Q_S (total acid produced)	+15·7		+19·7		+17·2	
$Q_M^{O_2}$ (aerobic glycolysis)	+ 4·6		+ 5·8		+ 5·3	

Effect of fluoride on rat testis. The details of the experiment are given in Protocol 3 and the results are summarised below.

NaF (m.-mols)	...	0	10	20
Q_{O_2} (respiration)	...	−11·9	−13·9	−11·9
$Q_M^{O_2}$ (aerobic glycolysis)	...	+ 4·6	+ 5·8	+ 5·3

Two points are clear from this experiment. Firstly, concentrations of fluoride which inhibit glycolysis strongly do not affect respiration to any measurable extent. This result is not in accordance with that of Loebel [1925], who, in the cases of rat brain and skin of a new-born mouse, found that 2·4 to 4·8 m.-mols sodium fluoride inhibit the respiration by about 30 or 40 %. In some of our other experiments with rat testis about 50 to 100 m.-mols sodium fluoride were needed for this effect. Secondly, it will be seen that fluoride, even in concentrations which inhibit the anaerobic glycolysis to the extent of 80 to 90 %, has little effect on the aerobic glycolysis. This surprising result is in marked contrast to that obtained with malignant tissues described below.

Rat kidney. In view of the difference referred to above between our results with testis and that of Loebel, the effect of fluoride on the respiration of kidney was examined. The case of rat kidney is simpler than that of rat testis since this tissue has no aerobic glycolysis in Ringer solution. This is true even in calcium-free Ringer solution, as in the following experiment, which showed in addition that the presence of fluoride does not diminish the respiration in this case also.

NaF (m.-mols)	...	0	10	20
Q_{O_2}	... ...	−15·4	−16·9	−18·3

Jensen rat sarcoma. The effect of fluoride on malignant tissue under aerobic conditions is of great interest in view of the fact that the respiration of such tissues is unable to inhibit the aerobic production of lactic acid. Moreover, according to Warburg [1926] such tissues derive a large part of their energy from aerobic glycolysis. From the following experiment it is evident that in the case of Jensen rat sarcoma, whilst the respiration is unaffected by fluoride, the aerobic glycolysis is much reduced. In this experiment calcium-containing Ringer solution was used, but the amount of fluoride was insufficient to cause the formation of a precipitate of calcium fluoride.

NaF (m.-mols)	Q_{O_2} respiration	$Q_M^{O_2}$	Inhibition aerobic glycolysis
0	−11·9	+13·4	*ca.* 50 %
6	−10·1	+ 6·0	

Effect of oxalate under aerobic conditions. Rat testis was the material used for these experiments. The effect of varying concentrations of oxalate is shown in the following experiment.

Oxalate (m.-mols)	Q_{O_2}	$Q_M^{O_2}$
5	−8·4	+3·9
10	−7·2	+3·2
50	−7·5	+2·7

Thus in the case of oxalate also, there is no evidence of any definite change.

Discussion.

In this discussion the term "glycolytic enzyme" will be used for the sake of clearness for the substance essential for glycolysis, the action of which is inhibited by fluoride.

The study of irreversible reactions, such as destruction by heat and by mercuric chloride, does not yield any information as to the nature of the equilibria between the glycolytic enzyme and the metabolites and other substances concerned. Reversible processes are, therefore, of much greater theoretical interest. The effect of nitric oxide [Warburg, 1928] is an example of this type of inhibition.

In the present paper it is shown that fluoride inhibits glycolysis without destruction of the glycolytic power; the reaction is reversed merely by placing the tissue in a fluoride-free medium. Hence fluoride belongs to the category of specific inactivators, and is therefore to be distinguished from the large group of substances which inactivate enzymes by destruction. Several possible explanations of the action of fluoride suggest themselves.

Lyotropic action. The action of the various salts might be due to a direct action on the colloidal state, possibly by affecting the extent of dispersion of the colloid. In the case of the salts examined, the results, and particularly the fact that citrate and tartrate are without effect, do not support this view.

Adsorption. The action of strongly adsorbable, chemically indifferent substances on glycolysis and respiration has been much studied by Warburg [1926, 1928], and has been explained by him as being due to displacement of the substrate from the active centres in the surface of the adsorbent. The salts studied in the present paper, however, bear no resemblance to the capillary-active substances (*e.g.* urethanes and heptyl alcohol) of which this type of behaviour is characteristic. It is therefore very improbable that the action of fluoride and oxalate can be due to this type of adsorption.

Chemical action. All our experiments lend support to the view that the inhibition of tissue glycolysis in the cases studied can best be explained from the point of view that it is associated with the formation of a chemical compound of the fluoride with some unknown substance in the cell, and that the extent of glycolysis is proportional to the amount of this substance which is not combined with fluoride. The evidence in favour of this view will now be discussed.

Fluoride concentration and inhibition. Many points in the experiments which have been described suggest that the action of fluoride might be explained by the application of the laws of mass action. If we denote the substance which is responsible for glycolysis by X and make the simplest possible assumption that the velocity of glycolysis of the tissue is proportional to the concentration of this substance we may write:

$$\text{Initial velocity of glycolysis} = Q_{\mathrm{M}}^{\mathrm{N}_2}\,(\text{without NaF}) = c\,[X_0],$$

where $[X_0]$ is the concentration of the enzyme originally present in the tissues.

There are many familiar examples of this type of relationship between enzyme concentration and velocity of reaction.

It is now necessary to make some assumption as to the manner in which fluoride combines with the free enzyme X to form an inactive complex. This will be denoted by XF_n, where n molecules of fluoride combine with one molecule of enzyme. Then for any given concentration $[F]$ of fluoride, the total concentration of X (free and combined), is given by

$$[X] + [XF_n] = [X_0].$$

Since the velocity of glycolysis is proportional to the concentration of free enzyme, in this case we obtain

$$Q_M^{N_2} \text{ (with NaF)} = c\,[X],$$

hence

$$\frac{Q_M^{N_2} \text{ (with NaF)}}{Q_M^{N_2} \text{ (without NaF)}} = \frac{c\,[X]}{c\,[X_0]} = \frac{[X]}{[X]+[XF_n]};$$

or if i denotes the percentage of inhibition, then

$$\frac{i}{100} = \frac{Q_M^{N_2} \text{ (without NaF)} - Q_M^{N_2} \text{ (with NaF)}}{Q_M^{N_2} \text{ (without NaF)}} = \frac{[XF_n]}{[X]+[XF_n]}$$

In view of the experiments on the reversibility of the action of fluoride, we may now consider the relationship

$$X + nF \rightleftharpoons X.Fn$$

on the above assumption, and write

$$[X] \times [F]^n = k\,[XF_n].$$

Substituting for $[XF_n]$ we obtain

$$\frac{i}{100} = \frac{[F]^n}{k+[F]^n}.$$

On applying this equation to the results obtained by experiment in the case of rat brain and rat sarcoma, the observed and calculated values were in good agreement if the value of n was unity. From the above reasoning this might well indicate that the combination of X with fluoride is a simple monomolecular reaction: $X + F = XF$. It is doubtful, however, if it is justifiable on our present evidence to state that the reaction is monomolecular, since it is well known that slight variations of the exponent n cause only a small alteration in the shape of the curve. Thus, for example, the value n might lie between 0·9 and 1·1. However, as a better agreement is not obtained by the use of other values of n, the monomolecular type of reaction is the simplest and most reasonable hypothesis in this case. The experiments showed, however, that, whilst this type of equation holds for rat brain and rat sarcoma, it is not generally applicable in this simple form. In the case of rat testis a different type of curve was obtained (Fig. 3) and the values calculated from the monomolecular type of equation deviated much more from the observed values than in the preceding cases. This was true whatever value is assigned to k.

It was found that if the value of n is taken to be approximately equal to 2, the equation then represents the observations with about the same accuracy as does the monomolecular type in the other cases studied. This might be interpreted as evidence that the reaction in this case is of the type

$$X + 2F = XF_2.$$

In this connection it is necessary to repeat that the value of n may not be exactly equal to 2, but until some more accurate method of experiment is available it is not possible to decide its exact value.

In the above equations k is the constant of the dissociation of the (XF_n) complex into its components $(X) + (F_n)$, thus the value $\frac{1}{\sqrt[n]{k}}$ gives an approximate measure of the affinity of fluoride for X. In the tissues examined, this affinity is of the following order[1]: rat sarcoma, 1000; rat brain, 600; rat testis, 170. This shows that the affinity of X in the various tissues may be variable, and is probably nearly six times as great in the case of sarcoma as in rat testis.

The experiments on the effect of temperature described in the experimental part may be interpreted as affecting the equilibrium discussed above. This effect is clearly shown by Table IX to result in a lessened inhibition of glycolysis as the temperature is raised. The probable explanation must be looked for in a dissociation of the fluoride-enzyme compound, yielding a higher concentration of the free enzyme. In other words there is an increase of the equilibrium constant k with rise of temperature. This case may be compared with the effect of temperature on the dissociation constant of oxyhaemoglobin [Barcroft and King, 1909; Barcroft, 1928] except that in the case of oxyhaemoglobin the effect of temperature is greater.

It is evident that the heat of reaction between fluoride and the enzyme could be calculated from further measurements of this type, and experiments on this point are at present in progress.

Further information as to the nature of the reaction of fluoride is given by the experiments on the effect of increased concentration of substrate on inhibition by fluoride. High concentration of glucose did not increase the glycolysis in the presence of fluoride. Michaelis and Rona [1914] distinguish between two types of inhibition according to whether a variation of the concentration of substrate affects the percentage inhibition or not. Since in the experiments described above there was no evidence of any such change, the conclusion seems to be that there is no evidence of any competitive action of fluoride and glucose for the enzyme.

In the light of the above result we can now consider, as far as these observations permit, what may be the probable nature of the substance to which anaerobic glycolysis of tissues is due. In the first place, the following points are evident from the preceding experiments.

(1) The substance has a definite and measurable affinity for fluoride with which it forms an inactive compound.

[1] Using molar concentrations in this case.

(2) The affinity for fluoride has a different value in different tissues.

(3) It also probably forms a similar inactive compound with oxalate. Its affinity for oxalate in the tissues examined was less than that for fluoride.

(4) Both fluoride and oxalate form many complex compounds with heavy metals. Warburg's view appears to be that both respiration and glycolysis are primarily due to the catalytic properties of some heavy metal, probably iron. If this is indeed the mechanism of glycolysis, and the inhibitory action of nitric oxide, for example, may be explained as a combination with iron, then a similar explanation might be applied to the action of fluoride and oxalate.

(5) An inactive compound is formed with cyanide also, but in this case the affinity is even less than for oxalate.

(6) No inactive compounds are formed with citrate or tartrate or with the other ions studied.

Aerobic glycolysis. In the case of aerobic glycolysis the action of fluoride seems to be more complicated. Warburg [1928] has shown that oxygen pressure has no effect on cyanide inhibition of respiration, from which he concludes that cyanide does not react with the reduced form of the respiratory enzyme since the combination is not affected by varying concentrations of oxygen. This does not apply to the action of fluoride on aerobic glycolysis, since the inhibition in this case is definitely less in the presence of oxygen. If it be assumed that the glycolytic enzyme is quite distinct from the respiratory enzyme, then this result might be explained by the assumption that in the presence of oxygen only a variable fraction of the glycolytic activity has been inhibited by oxygen, but that the inactivated enzyme still retains its power to combine with added fluoride. Consequently combination with fluoride might occur (as it may be supposed to do in the case of rat testis) without diminution of glycolysis. In this case also, the affinity for fluoride is small. In the case of rat sarcoma, with a larger affinity, there is a definite action of fluoride even on aerobic glycolysis.

There is some evidence, however, that the respiratory and glycolytic enzymes, if not identical, may at least be very closely related. If we imagine that this is the case, and that a definite amount of oxygen is able to remove a definite amount of lactic acid, either by conversion to carbohydrate or by direct oxidation, and that this is the explanation of the effect of oxygen in inhibiting or reducing anaerobic glycolysis, then the effect of fluoride on aerobic glycolysis is more difficult to explain. If the action of fluoride is a pure inhibition of glycolysis in this case, as apparently it is anaerobically, then the respired oxygen in the presence of fluoride might be expected to reduce the glycolysis still further. A possible explanation of this difficulty is available, if it is permissible to consider the fluoride as interfering with the "Pasteur reaction" [Warburg, 1926, p. 238]. Warburg's view is that in most normal tissues the oxidative reactions have a limited ability to prevent the appearance of lactic acid. The relationship is a quantitative one in the sense that when this reaction is operating under optimum conditions, one molecule

of respired oxygen can prevent the appearance of exactly two molecules of lactic acid. This relationship is called by Warburg the "Pasteur reaction." Various agents, however, have the property of interfering with the efficiency of this reaction in such a way that one molecule of respired oxygen is now no longer able to inhibit the appearance of two of lactic acid. Consequently in many tissues lactic acid may be made to appear aerobically under certain artificial conditions, such as in the presence of ethyl *iso*nitrile [Warburg, 1926], or even by slight mechanical or other damage, without the respiration being affected. Rat testis is an example of the latter case, and the fact that in Ringer solution (but not in serum) it causes aerobic glycolysis is attributed to this cause.

If this is the way in which the fluoride acts in this instance, the case of rat testis is an exceptionally complicated one. It is proposed to study this aspect of the problem further.

Effect on respiration. The fact that in very low concentration cyanide inhibits respiration but does not affect glycolysis, whereas fluoride has exactly the reverse effect, appears at first sight to suggest that respiration and glycolysis are due to entirely different substances. This is of course one possibility, but another explanation is that the reason for this difference might be connected with some other factor, for instance the state of oxygen combination in the substance necessary for respiration and glycolysis [Keilin, 1929].

As nothing is at present known of the action of fluoride on any compounds which have been considered to take part in cell respiration (*e.g.* haematin, cytochrome), it is not possible to go into this question further at this stage.

Summary.

1. The effect of various salts on anaerobic glycolysis in tissues has been studied; of these, fluoride and oxalate inhibit glycolysis very strongly in all the tissues examined.

2. The relation between concentration of fluoride and the percentage inhibition has been studied in the case of three tissues, namely Jensen rat sarcoma, rat brain, and rat testis.

3. It was found that the action of fluoride may be represented by a simple relationship, based on the law of mass action. This relationship, though of a similar type, is not identical in the tissues used. There is a variation which is considered to be due to a difference in (*a*) the order of the reaction, and (*b*) the affinity of fluoride for the glycolytic enzyme.

4. The approximate values of the affinity constant of the fluoride-enzyme complex have been determined in the case of the above-mentioned tissues.

5. The general inference from the above conclusions is that fluoride forms an inactive compound with some substance essential for glycolysis. This reaction follows the ordinary laws of chemical equilibria.

6. The effect of temperature on this equilibrium is described, and it is concluded that the enzyme-fluoride compound is exothermic.

7. Experiments have also been made on the action of fluoride on a number of other tissues. In the case of rat retina, rat kidney and two specimens of human carcinoma, fluoride also had a strongly inhibitory action on glycolysis.

8. The effect of oxalate has been studied and in all cases was less than that of fluoride, whilst cyanide only inhibits glycolysis when present in very high concentration.

9. Fluoride does not inhibit the conversion by tissues of dihydroxyacetone into lactic acid.

10. Fluoride and oxalate do not appreciably influence tissue respiration in the concentrations used.

11. Aerobic glycolysis is inhibited by fluoride in the case of Jensen rat sarcoma, but not in that of rat testis.

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