Purification of Yeast Hexokinase and its Reaction with $\beta\beta'$-Dichlorodiethyl Sulphide*

By K. BAILEY (Imperial Chemical Industries Research Fellow) and E. C. WEBB
Biochemical Laboratory, Cambridge

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The enzyme theory of vesication, as developed by Peters and Dixon and their respective schools, provides that the primary attack of a vesicant is upon one or more specially sensitive enzymes whose destruction leads to the arrest of some essential metabolic processes of the cell. The vesicant action of compounds such as lewisite and mustard gas ($\beta\beta'$-dichlorodiethyl sulphide, described hereafter as *H*) have thus been studied in relation to their effect upon respiring tissue slices or *brei*, and also upon isolated enzyme systems concerned with intermediary carbohydrate metabolism. In this laboratory, we were more immediately concerned with the role of mustard gas in the inhibition of skin glycolysis, and attention became focused upon hexokinase when there was found to be a correlation between the fall of skin glycolysis, the onset of skin damage, the lowered hexokinase activity of extracts of such skin, and the sensitivity of hexokinase to mustard gas *in vitro* (Dixon, Needham & van Heyningen, 1942; see also review by Dixon & Needham, 1946).

At this stage it was thought desirable to study the action of the vesicant upon the pure enzyme, which could only be obtained in reasonable amount from yeast. The limited objective was to find whether a sensitive enzyme under comparable conditions of treatment reacts more readily than do other proteins whose amino-acid composition is approximately known; the further objective, not yet achieved, was to establish the nature of the sensitive group, assumed to be the active enzyme centre.

Part 1 of the present paper describes the purification, crystallization and properties of yeast hexokinase, and Part 2 describes the action of SH poisons and vesicants. The effect of SH poisons (including lachrymators) is only summarily reported, since the results with the pure enzyme confirm the earlier work of van Heyningen (1942) using crude hexokinase. The inhibition by *H* on the other hand, is described in some detail, and embraces two types of experiment; first, the relation between extent of inhibition and inhibitor concentration under varying conditions, and secondly, the quantitative assay of the number of vesicant groups combined with the enzyme at the point of complete, or almost complete, inactivation. With the small amount of crystalline enzyme at our disposal, this work was only possible by use of mustard gas containing radioactive sulphur (*H**), and by the collaboration of Drs Boursnell, Francis and Wormall, who kindly determined the radioactive S content (*S**) of hexokinase thus inactivated. Under these 'mild' conditions of inhibition, when the enzyme is subjected to a solution of vesicant (as distinct from the more drastic treatment with droplets of liquid vesicant, cf. Herriott, Anson & Northrop, 1946), the uptake of mustard gas by hexokinase was compared with that of other proteins of well defined amino-acid composition and containing a representative number of reactive side-chain groups, viz. ovalbumin and fibrinogen. Moreover, a comparison was made of the amount of vesicant combined with ovalbumin after both mild and drastic treatment, in order to obtain some estimate of the maximal number of vesicant groups which can combine with a protein as compared with the number necessary for enzymic inactivation.

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PART 1. PURIFICATION AND CRYSTALLIZATION OF YEAST HEXOKINASE

Yeast hexokinase was partially purified by Meyerhof (1927), van Heyningen (1942) and Dixon & van Heyningen (unpublished), the latter employing adsorption on calcium phosphate. The present authors then continued the research at a stage when American workers had also taken up the problem of vesication. The crystallization of hexokinase was first reported by Cori, Colowick, Berger & Slein (1942), using methods involving ethanol precipitations, and later by Northrop, Kunitz & McDonald (1942) (see also Kunitz & McDonald, 1945–6), using mainly ammonium sulphate fractionation. Our method was developed independently and differs from those of the American workers, but in the last stage of purification we made use of the Kunitz-McDonald newly reported and elegant method for the final crystallization. The activity and crystalline

form of our preparation were similar to those of the crystals which Dr Northrop kindly sent us. It is not clear whether the 'hexagonal plates' obtained by Cori and co-workers represent the same or a different crystalline form, but after crystallization by the Kunitz-McDonald method they are similar to their and our crystals (Berger, Slein, Colowick & Cori, 1945–6).

Activity estimations

The decrease in easily hydrolyzable phosphate during the incubation of the enzyme with adenosinetriphosphate (ATP), glucose and Mg^{++} was determined by the Fiske-Subarrow method, after hydrolyzing the digest with N-HCl for 7 min. at 100°. The method in principle is essentially that given by van Heyningen (1942). The incubation mixture contained 0·5 ml. 0·1 M-$MgCl_2$, 0·6 ml. M-glucose, enzyme diluted to 0·5 ml., and 0·1 M-veronal-acetate buffer pH 7·9 to a total volume of 4·5 ml. Sodium-ATP solution (0·5 ml.) containing *c.* 1·2 mg. 7 min. P was added in the cold, and the mixture placed in the bath at 38°. The amount of enzyme taken was sufficiently small to give an approx. linear breakdown for 7–8 min. Samples of 1 ml. were removed at 1, 3, 5 and 7 min. and pipetted directly into 0·12 ml. 10N-HCl. After immersion in a boiling water bath for 7 min., each sample was washed into a 50 ml. flask, mixed with Fiske-Subarrow reagents, diluted to volume, and the orthophosphate determined colorimetrically against a standard containing 0·2 mg. P in 50 ml.

One hexokinase unit (HK unit) was defined as the amount of enzyme which transfers 1 mg. 7 min. P from ATP to glucose in 1 hr. under the conditions of the above test, the initial linear portion of the curve 7 min. P against time being used.

Purification of hexokinase

Stage 1. *Autolysis of baker's yeast.* Preliminary experiments showed that autolysis of yeast with toluene and water takes place in two stages. No hexokinase activity is given in the standard test by a suspension of fresh baker's yeast, but yeast treated with toluene for 30 min. at 38° shows a maximal activity soon after mixing with water if the whole suspension is used. At this point, very little of the enzyme is in true solution but is merely accessible to the substrate. On more prolonged incubation of the autolysate, the enzyme gradually passes into solution, and eventually the activity of the supernatant liquid equals that of the whole suspension. Prolonged autolysis at 38°, however, causes considerable destruction of enzyme (up to 50% in 2 days) before extraction is complete (Exps. 1*a*, *b*, Table 1). If, after the preliminary toluene treatment for a few hours at 38°, the autolysis is allowed to proceed at room temperature, destruction losses are smaller, especially in presence of 1% glucose, but the activity of the supernatant liquid usually attained its maximum only after several days. The general effect of glucose, whilst conferring stability, was to retard autolysis (Exps. 2*a*, *b*).

It was then found that addition of SH compounds (cysteine or neutralized Na_2S) had the combined effect of stabilization, accelerating hydrolysis and increasing the final yield of liberated hexokinase (Exps. 3*a*–4*b*). (These results suggest that English baker's yeast is much more

Table 1. *Liberation of 'fixed' hexokinase from yeast under varying autolysis conditions*

	Conditions of autolysis			Hexokinase content (units/ml.)	
Exp.*	Temp. (° C.)	Additions	Duration (hr.)	Whole suspension	Supernatant liquid
1*a*	38	None	18	34	14
			42	14	Nil
1*b*	38	1% glucose	18	21	8
			42	10	Nil
2*a*	20–25	None	18	62	18
			42	40	43
2*b*	20–25	1% glucose	18	45	13
			42	45	23
			66	45	35
			90	43	48
3*a*	20–25	1% glucose	42	38	26
			96	43	31
3*b*	20–25	0·1 M-cysteine	42	56	42
			96	53	53
3*c*	20–25	0·1 M-NaSH	42	42	46
			96	51	42
4*a*	20–25	None	18	51	12
			42	(39)	26
			66	50	34
4*b*	20–25	0·1 M-NaSH	18	49	41
			42	66	58

* Different yeast samples behave differently under standard conditions of autolysis. Since various samples were used, comparison can only be made between results of each individual numbered experiment.

resistant to autolysis than the yeasts used by the American workers. Similar differences between Delft and Manchester yeasts have been noted by Bach, Dixon & Zerfas (1942, 1946) in the preparation of lactic dehydrogenase. Prof. F. Dickens (private communication) has experienced similar difficulties in extracting hexokinase from Distillers Company Ltd. baker's yeast. The earlier work of van Heyningen (1942) suggests that the role of SH compounds in the stabilization of the enzyme may be the prevention of oxidative destruction; the acceleration of autolysis, on the other hand, may be due to their activating effect on proteolytic enzymes which liberate hexokinase from the particulate components of the cell.

The autolysis was carried out in batches as follows: 28 lb. (12·7 kg.) of baker's yeast (Encore) were crumbled, mixed with 760 ml. toluene and maintained at 38° for 3–5 hr. To the liquid mass were added 12 l. of cold tap water, together with 100 g. $Na_2S.9H_2O$ and enough conc. HCl to restore the pH to 6–6·5 (*c.* 50 ml. 10 N-HCl). The mixture was centrifuged after standing for 42 hr. at 20–25°, and the supernatant liquid treated with solid $(NH_4)_2SO_4$ to 75% saturation. The protein was filtered off under suction and stored as a moist filter cake at 0°. Altogether, 185 lb. (84 kg.) of yeast were worked up in this way.

Stage 2. *Dialysis.* The collected filter cakes were dissolved in an equal volume of water. An attempt to fractionate with $(NH_4)_2SO_4$ was abandoned, as the precipitate could not be spun or filtered. The liquid (9·4 l. containing 4,350,000 HK units) was dialyzed against distilled water at 1° in 40 cellophan sacs. After changing the water daily, the $(NH_4)_2SO_4$ concentration fell after 7 days to 0·55 g./l.

Dialysate: 12·8 l.; 3,300,000 HK units; 56 units/mg. N.

Stage 3. *Adsorption.* (Here, as in subsequent adsorptions, small scale experiments were first carried out. A known volume of calcium phosphate gel was added to the enzyme solution and spun down; after determining the enzyme activity of the supernatant liquid, a further portion of gel was added and the whole process repeated until the hexokinase was entirely adsorbed. In the main solution this procedure was shortened by adding just the amount of gel to take down impurities, and after centrifuging, a subsequent portion to adsorb most of the enzyme.) The dialysate (pH 6) was stirred with 5 l. of calcium phosphate gel (4·4% dry wt.), which was spun off and discarded. The enzyme was adsorbed on a further 5 l. of gel, and after centrifuging, the precipitate was eluted six times with Na_2SO_4 solution (100 g. anhydrous salt/l.); 30 g. glucose were added to the eluates to stabilize the enzyme.

Eluates: 7·13 l.; 1,750,000 HK units; 113 units/mg. N.

Stage 4. *Ammonium sulphate fractionation.* Solid $(NH_4)_2SO_4$ was added to give 55% saturation. (Here again, the conditions for fractional salting out were determined in small-scale experiments.) The precipitate was filtered off under gravity and rejected. The salt concentration was then increased to 75% saturation, the precipitate filtered off, dissolved in 1% glucose solution and dialyzed against several changes of 1% glucose at 0° for 3 days.

Dialysate: 885 ml.; 860,000 HK units; 264 units/mg. N.

Stage 5. *Adsorption.* The solution was treated with 88 ml. calcium phosphate gel, spun, and the precipitate rejected. Another 550 ml. of gel were added, spun off, and the precipitate eluted with Na_2SO_4-glucose solution (100 g. anhydrous sodium sulphate, 10 g. glucose/l.).

Eluates: 925 ml.; 670,000 HK units; 550 units/mg. N.

Stage 6. Attempts to increase the activity by fractional salting-out, or to crystallize the enzyme were unsuccessful. Eventually all fractions were combined, salted out with $(NH_4)_2SO_4$ (85% saturation), filtered under suction, taken up in a small volume of water and dialyzed against 0·1 M-acetate buffer at pH 5·9 and containing 1% glucose. About 210,000 HK units at 300 units/mg. N resulted from these unsuccessful trials. In future preparations, these losses would be avoided by passing on to the next step, thereby increasing the eventual yield threefold.

Stage 7. *Ethanol precipitation.* Absolute ethanol at $-10°$ was added to the cooled solution (98 ml.) to give a final concentration of 34% (v/v). The precipitate was centrifuged down at 0° and discarded. The ethanol content of the supernatant liquid was raised to 53% (v/v), the precipitate centrifuged down, dispersed immediately in 1% glucose, and insoluble material separated by filtration through paper pulp. All operations were carried out at or near 0°.

Solution: 172 ml.; 115,000 HK units; 400 units/mg. N.

Stage 8. *Crystallization.* Although at stage 7 the preparation was less pure than at stage 5, it yielded readily to the crystallization procedure used by the Northrop school. It seems very probable that the ethanol solutions removed lipid material which prevents crystallization. The solution from stage 7 was salted out in 85% saturated $(NH_4)_2SO_4$ solution, and the protein obtained as a filter cake. It was dissolved in a small quantity of 0·1 M-phosphate buffer pH 7, saturated ammonium sulphate was added to a faint turbidity, and the liquid allowed to drain through a filter at 0°. After a week, crystals (A) appeared in the filtrate. The residue on the paper was again dissolved and treated as above, and yielded a second crop (B). The crystals were washed in 75% saturated $(NH_4)_2SO_4$ solution, and dissolved for activity tests in 3% Na_2SO_4 containing 1% glucose.

Crystals A: 4 mg. protein N; 6200 HK units; 1530 units/mg. N.

Crystals B: 13 mg. protein N; 15,600 HK units; 1200 units/mg. N.

From the mother liquors, further small crops were obtained which eventually were combined with the main crops and recrystallized.

The final product, reproduced in Pl. 1, contained 13 mg. protein N (i.e. 83 mg. protein) and 40,000 HK units at 2500–3000 units/mg. N. The crystals were stored moist in saturated ammonium sulphate solution at 0°.

Comparison of the activity of the crystals with the preparations of Cori and of Northrop. Northrop *et al.* (1942) reported an activity of 1440 Cori units/mg. protein for five times recrystallized hexokinase, i.e. 9230 Cori units/mg. protein N. Assuming a temperature coefficient for the enzyme reaction of $Q_{10} = 2$, calculation shows that 1 Cori unit (defined as the amount of enzyme catalyzing the formation of acid equivalent to 1 μl. CO_2/min. at 25°) is equal to 0·205 of our HK unit (defined as the amount causing transfer of 1 mg. P/hr. at 38°). Using this conversion factor, Northrop's reported activity is equal to 1890 HK units/mg. N.

Cori, Colowick, Berger & Slein (1943), however, later reported an activity of 2100 Cori units/mg. protein for a sample of hexokinase obtained from

Northrop. Converted to our units, this corresponds to an activity of 2750 HK units/mg. N. In agreement with this value, a sample of Northrop's crystalline hexokinase, kindly made available to us by Prof. Peters, gave an activity of 2850 HK units/mg. protein N under our test conditions. These figures are similar to those for our own preparation.

Summarization of purification data. The quantitative data on the purification process are brought together in Table 2. The early steps of alternate salting out and adsorption give a much more rapid purification than the repeated salt fractionation adopted by Northrop and co-workers. In the one case, the activity is roughly doubled at each stage, in the other, a 25 % increase in purity was achieved.

The pure, recrystallized preparation represents a recovery of *c.* 1·2 % enzyme and a 53-fold purification on the dialysate of stage 2. With our present procedure, the yield could possibly be increased to 3·6 % by passing from stage 5 to 7.

above pH 7: 5 min. at 38° and pH 7·9 sufficed to inactivate completely, whilst at the same pH and 20°, 90 % of the activity was lost in 2·5 hr. No evidence of protection from spontaneous inactivation was observed with glycine or with cyanide, although the latter salt was stated by van Heyningen (1942) to act as stabilizer.

The enzyme was inactive in absence of Mg ions, and these could not be replaced by Ca^{++}. The activity in presence of 5×10^{-3} M-Mg^{++} was not affected by the addition of an equal concentration of Ca^{++}; the Ca : Mg antagonism which is so pronounced in the case of the Mg-activated yeast pyrophosphatase (Bailey & Webb, 1944*b*) is thus absent in the case of hexokinase. It would be of interest to discover whether such antagonism as is found in yeast pyrophosphatase and myosin adenosinetriphosphatase is general for those enzymes producing free phosphate, but absent in the phosphate-transferring enzymes (phosphokinases). The proteins oval-

Table 2. *Purification of yeast hexokinase*

Stage	Volume (l.)	Total activity* (hexokinase units)	Purity (units/mg. N)	Total N* of fraction (g.)
2. Filter cake from 84 kg. yeast	10·9	$4·7 \times 10^6$	—	—
Same after dialysis	12·8	$3·3 \times 10^6$	56	59·0
3. Adsorption and elution	7·13	$1·75 \times 10^6$	113	15·5
4. $(NH_4)_2SO_4$ fractionation	0·885	860,000	264	3·25
5. Adsorption and elution	0·925	670,000	550	1·22
6. Unsuccessful fractionation; fractions combined	0·215	210,000	300	0·70
7. Ethanol precipitation	0·172	115,000	400	0·30
8. Crystals A	—	6,200	1530	0·004
Crystals B	—	15,600	1200	0·013
Recrystallized (A, B and other crops)	—	40,000	2500–3000	0·013

* Activity and N (micro-Kjeldahl) determined on dialyzed material; corrections for traces of NH_3-N applied after distilling undigested sample at pH 9·5 in the micro-Kjeldahl apparatus.

Properties of hexokinase

An extensive examination of the properties of hexokinase was not undertaken, since the material was required for combination with $\beta\beta'$-dichlorodiethyl sulphide containing radioactive S. Moreover, the general properties of the enzyme (metal activation, stability, pH optimum, substitution of other sugar substrates for glucose) had already been determined for a partially purified preparation by van Heyningen (1942). Similar investigations have also been made by Berger *et al.* (1945–6) and by Kunitz & McDonald (1945–6) on the crystalline enzyme.

Our own preparation dissolved in water to give a colourless solution. Except in high salt concentration or in presence of glucose the enzyme was rapidly inactivated (without visible precipitation) bumin and insulin, found by Cori *et al.* (1943) to produce activation of the enzyme, were quite ineffective with our preparation. As compared with the theoretical value of 50 %, about 43 % of the easily hydrolyzable P of ATP was transferred to glucose in presence of the enzyme in accordance with the generally accepted view that hexokinase catalyzes the reaction:

Adenosinetriphosphate + glucose
$\rightleftharpoons$ Adenosinediphosphate + glucose-6-phosphate

Physical constants for the enzyme have been obtained by Kunitz & McDonald (1945–6). The protein was found to be electrophoretically pure, and to give a solubility independent of the amount of solid phase. Cataphoretic studies gave an isoelectric point between pH 4·5–4·8, and sedimentation-diffusion a molecular weight of 96,600.

PART 2. INHIBITION OF HEXOKINASE BY SH POISONS AND BY ββ′-DICHLORODIETHYL SULPHIDE (*H*)

SH *poisons including lachrymators*

All SH poisons, including lachrymators and the vesicant lewisite, were most powerful inhibitors, and much more effective than iodoacetate. Of inhibitors unrelated to vesicants and lachrymators, 0·04 M-NaF gave a 46 % inhibition under the standard test conditions.

In Table 3 are listed the inhibitions obtained with varying concentrations of SH poisons. Except where otherwise stated, the poisons were incubated with enzyme in veronal-acetate buffer pH 7·9 containing $1·1 \times 10^{-3}$ M-$MgCl_2$ and M/73 glucose. Additional glucose (to raise the concentration to that used in the standard activity assay) was added with the ATP after this preliminary incubation. The marked inhibition which occurs at very low concentration in the case of chloroacetophenone, bromobenzyl cyanide, ethyl iodoacetate and lewisite, known from the work of Mackworth (1941, 1947) to be SH-combining reagents, is in general agreement with the results of van Heyningen (1942) carried out with an impure preparation, and support her conclusion that the presence of SH groups in the hexokinase molecule is essential for its activity.

Table 3. *Effect of* SH *poisons on hexokinase*

Inhibitor	Concentration of inhibitor	Conditions of preliminary incubation: Time (min.)	Conditions of preliminary incubation: Temp. (° C.)	Inhibition (%)
Chloroacetophenone	M/880	15	38	33
	M/880	15	38	50
	M/880	5	16	Nil
Bromobenzyl cyanide	M/800	5	38	100
Ethyl iodoacetate	M/800	5	38	49
Na iodoacetate	M/63	5	38	100
	M/200	5	38	20
Lewisite (in M/8 glucose)	M/1000	15	38	100
	M/2000	15	38	70
	M/2500	15	38	43
	M/4000	15	38	20

The inactivation of crystalline hexokinase by radioactive H (H)*

It was our aim to treat the enzyme with *H* under the mildest conditions consistent with a high degree of inactivation, so as to reduce to a minimum any general attack which might conceivably accompany the inactivation process, although playing no essential part in its causation. Preliminary experiments were carried out with non-radioactive *H* in order to determine these conditions. The inherent instability of the enzyme, however, creates special difficulties. van Heyningen (1942) reported an 80 % inactivation by $2·5 \times 10^{-3}$ M-*H* (initial concentration) in 0·014 M-veronal-acetate buffer at pH 7·8 and 38° in presence of 0·007 M-glucose. Under these conditions, a considerable amount of spontaneous inactivation occurred in the control without *H*, and although this was allowed for in the calculation of percentage inhibition, it is essential for our present purpose that any inactivation other than that due to *H* should be eliminated as far as possible. Without this provision, any comparison between extent of inactivation and the number of *H* groups combining is meaningless.

Unfortunately, the conditions for greatest sensitivity to *H* are also those of least stability. Enzymes in general seem to become more sensitive to *H* as the pH is raised (see Dixon (1943) and Table 4), but with hexokinase, pH values above 7 cannot be employed at temperatures higher than 20° because of the spontaneous inactivation which then occurs. The enzyme is more stable at lower temperatures, but these necessitate a long incubation with *H* to allow of complete reaction. Again, the presence of glucose, whilst effecting stabilization, reduces the sensitivity of the enzyme to *H* (Dixon, 1943). With reasonably small amounts of glucose (*c.* 0·01 M) the inactivation by *H* could be measured without serious spontaneous inactivation, either by incubation at 38° in buffer at pH 5·5, or by treatment at room temperature in buffer at pH 7·4–7·9. A few results with crystalline hexokinase are shown in Table 4; similar results were obtained with a crude yeast autolysate and with a partially purified preparation. In each case, a solution of *H* in ethylene glycol monoethyl ether was added to a dilution of enzyme sufficient for one test, to give a solution of concentration $2·5 \times 10^{-3}$ M. The activities after incubation were measured at pH 7·5 or 7·9 and compared with a control treated similarly with *H*-solvent in absence of *H*.

Table 4. *Inhibition of crystalline hexokinase by H*

pH	Temp. (° C.)	Time of incubation with $2·5 \times 10^{-3}$ M-*H* (min.)	Composition of incubation mixture: Buffer	Glucose (M)	Mg^{++} (M)	Enzyme N/ml. (μg.)	Inhibition (%)
7·5	16	135	0·08 M-veronal-acetate	0·011	0	2·4	25
7·9	20	15	0·09 M-veronal-acetate	0·0001	0·013	1·2	44
5·5	38	15	0·1 M-acetate	0·0056	0	2·8	13
5·5	38	15	0·1 M-acetate	0	0	1·4	10

From Table 4 it will be seen that hexokinase is comparatively insensitive to *H* at pH 5·5, even in the absence of glucose. At the higher pH values and lower temperature, the inactivation is much more marked, and the protection by glucose is in evidence. It was clear from these results that it was not possible both to obtain full sensitivity to *H* and freedom from spontaneous inactivation, and that a compromise must be sought. It was finally decided to sacrifice sensitivity to some extent in order to reduce the spontaneous inactivation to a reasonably small value, and to attain the necessary degree of inhibition by treating the enzyme several times in succession with small amounts of *H*.

Treatment of hexokinase with radioactive H. The compromise conditions for obtaining a reasonable sensitivity to *H* and low spontaneous inactivation were realized by treating with *H* at room temperature at pH 7·4 in presence of M/180 glucose. Additions of radioactive *H* in an organic solvent were made at intervals of about 45 min. until a sufficient degree of inactivation had been obtained, each addition in the concentration of $2·5 \times 10^{-3}$ M. A control without *H* gave a measure of the spontaneous inactivation. With the amount of crystalline enzyme available (80 mg.) only two experiments could be carried out. These are described below.

Exp. 1. Crystalline hexokinase containing 5·1 mg. protein N was dissolved in 5 ml. 0·1 M-phosphate buffer pH 7·4 containing M/180 glucose. For activity measurements, 0·01 ml. samples were pipetted into 1 ml. of ice-cold water and 0·1–0·3 ml. of this solution was used for each test. At various intervals, shown in Table 5, 0·05 ml. of 0·25 M-*H* in ethylene glycol monoethyl ether was added, until the activity fell to a fairly low value. No strictly parallel control to determine the extent of spontaneous inactivation was made in this experiment, but the first diluted sample, taken before *H* addition, was incubated under the conditions of the main experiment and used as a diluted control; this procedure is open to criticism since dilution may have the effect of decreasing stability, the control thus giving too high a value for spontaneous inactivation. In Exp. 2, therefore, this defect was remedied by incubating a control under the same conditions as those of the experimental solution.

Exp. 2. The enzyme (6·8 mg. protein N) was dissolved in 5·5 ml. 0·1 M-phosphate buffer pH 7·4 containing M/180 glucose. A 5 ml. sample was used for *H* treatment and 0·5 ml. incubated similarly as a control without *H*, the same concentration of *H* solvent (in this case ethanol) being added. The results are shown in Table 5.

In both experiments, the *H*-treated solutions remained clear and gave no visible evidence of denaturation.

Table 5. *Treatment of crystalline hexokinase with radioactive H*

(0·05 ml. 0·25 M-*H** added to final concentration of $2·5 \times 10^{-3}$ M at times marked →.)

Exp. no.	Time of activity determination (hr.)	Activity of *H**-treated enzyme as % of original (HK units/ml.)	Activity of control as % of original (HK units/ml.)
1	→ 0	100	100
	→ 0·75	95	—
	→ 1·75	—	—
	→ 2·5	40	—
	3·25	23	77*
2	→ 0	100	100
	→ 0·5	—	—
	→ 1·25	—	—
	→ 1·5	—	—
	2·0	31	97
	→ 3·0	—	—
	3·75	29	91

* 'Diluted' control, see text.

Determination of combined H. The *H*-treated hexokinase from each experiment was precipitated with an equal volume of ethanol and allowed to settle overnight. It was washed thrice with 50 % (v/v) ethanol and twice with absolute ethanol. After Soxhlet extraction with acetone and subsequently with ether to remove any *H** and thiodiglycol containing *S**, it was dried over $CaCl_2$. Protein N was determined by the micro-Kjeldahl method on a sample of the dried powder after removing non-protein N with 5 % trichloroacetic acid, and the remainder was used for *S** determinations. These latter were carried out by Drs Boursnell and Francis (cf. Boursnell, Francis & Wormall, 1946) using the Geiger counter method as adapted by Banks (1946). From the N and *S** determinations the number of *H* molecules combining with each molecule of enzyme was calculated, taking the N content of ash-free and moisture-free hexokinase as 15·6 %, and the molecular weight as 97,000 (Kunitz & McDonald, 1945–6). The results are shown in Table 6. For reasons

given above, the control in Exp. 2 has been used in the calculations for Exp. 1, rather than the 'diluted' control. Since both experiments were carried out under very comparable conditions, the rates of spontaneous inactivation could have differed only slightly. The number of *H* groups/molecule of enzyme inactivated by *H* is calculated on the assumption that the degree of inactivation is proportional to the *H* groups combined. In both experiments this number corresponds to 6–7 groups/molecule enzyme, or, assuming the average amino-acid residue weight to be comparable to that of a polar

Table 6. *Estimation of H* groups combined with hexokinase*

	Exp. 1	Exp. 2
Protein-N content of powder (%)	10·62	11·5
Wt. taken for *S** determination (mg.)	35·5	(*a*) 20·75 (*b*) 17·45
*S** found (as mg. *H**)	0·169	(*a*) 0·105 (*b*) 0·094
Molecules *H**/molecule hexokinase	4·3	(*a*) 4·2 (*b*) 4·4 Mean 4·3
Total enzyme inactivation (%)	77	71
Inactivation in control (%)	(9)	9
Inactivation due to *H* (%)	68	62
Estimated no. *H* groups/molecule hexokinase for 100% inactivation by *H*	6·3	7·0

protein such as fibrinogen, i.e. 114, to the combination of less than 1 *H* group in 100 amino-acid residues. By comparison, the extent of substitution when the enzyme is stirred with liquid *H* (at pH 5·5–6·0) can be gauged from the work of Herriott *et al.* (1946) who found that 30–35 *H* groups combined with the enzyme. This latter figure suggests a general attack upon the enzyme, whilst our own much lower figure is scarcely more than might be expected from a specific combination of *H* with the active enzyme centres.

The reaction of other soluble proteins with H

An enzyme may be particularly sensitive to *H* either because (*a*) it contains specially reactive groups, and is capable of substitution under conditions which are without effect on insensitive enzymes and presumably on proteins in general, or because (*b*) although the reactivity may be similar to that of other proteins, the reacting groups are specially connected with enzyme activity. To distinguish between these possibilities in the case of hexokinase it seemed desirable to compare the behaviour of other proteins, using the same mild conditions of *H* treatment. The proteins selected were thrice recrystallized ovalbumin, and six times precipitated ox fibrinogen.

Conditions of H treatment. The proteins were dissolved in phosphate buffer at pH 7·5 to give a concentration of 7–8 mg./ml. A solution of *H* in ethanol (0·25M) was added in five successive portions at intervals of 0·75 hr., each portion being 1/100 of the volume of the original solution. The temperature was maintained at 20°. Under these conditions, the *H* was in clear solution, and throughout the reaction the sols remained free of precipitated protein. After treatment the sols were dialyzed, precipitated with an equal volume of acetone after adjusting to pH 5·5, washed four times with acetone, four times with ethanol, and finally with ether. Samples of untreated protein were similarly precipitated and washed, and finally dried over $CaCl_2$ for N and S determinations.

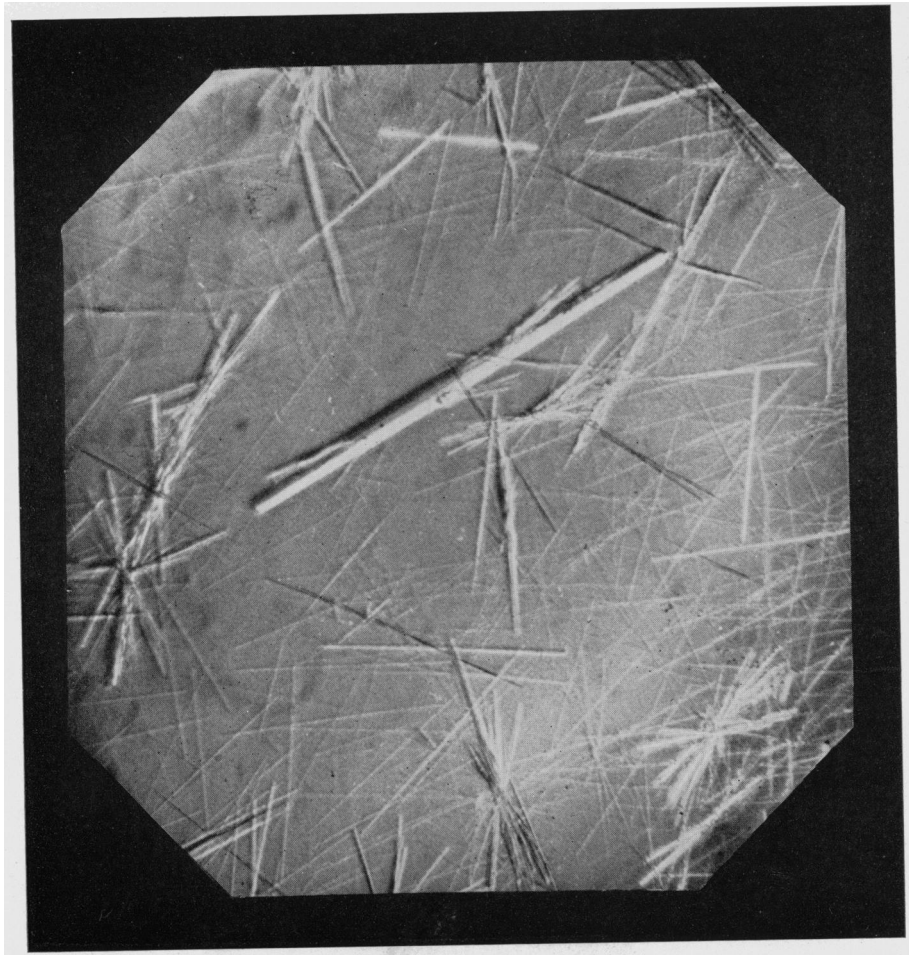
For comparison, ovalbumin was also treated with liquid *H*. The protein (4·5 g.) in 80 ml. water was stirred with 1 ml. of liquid *H*, dispersed in fine droplets, for 10 hr. at 30°; solid $NaHCO_3$ was added at intervals to maintain the pH at 7. The *H*-treated sol was then decanted from residual *H*, dialyzed against water, adjusted to pH 5·5, precipitated with 10 vol. acetone, and Soxhlet-extracted for 6 hr. each with acetone, ethanol and ether.

Sulphur determinations. The HNO_3-$KClO_3$-$Cu(NO_3)_2$ oxidation generally employed for the determination of S in *H*-treated proteins (e.g. Boursnell *et al.* 1946) is lengthy and sometimes violent. The alternative method of alkali fusion gives a very complete oxidation of the S-amino-acids of proteins but with *H*-treated products, it is open to the objection that combined *H* groups may be split off and lost in the form of volatile sulphides or their oxidation products. This difficulty has been overcome by mixing the finely-ground protein (0·3 g.) with a large excess of fusion mixture (8 g. of 1 : 1 Na_2CO_3 and Na_2O_2) and heating in a nickel crucible on a hot-plate at 140–160°. After 0·5–1 hr. the mixture gently fuses, each protein particle burning at red heat in the oxidizing medium. The melt is then heated to redness for 3–4 min., cooled, and sulphate estimated gravimetrically using glass-distilled water. The reagent blank amounted to 1 mg. $BaSO_4$. The S content of crystalline *H* sulphoxide determined by this method was 18·4% (theory, 18·3%).

The combination of H with ovalbumin and fibrinogen. The number of *H* groups in combination with 10^5 g. protein (chosen as a basis for comparison with hexokinase, which has a molecular weight of nearly 10^5) was calculated from the expression

$$\frac{1000\,N^0}{32}\left(\frac{S^H}{N^H}-\frac{S'}{N'}\right),$$

where S' and N' are the S and N contents of the untreated protein (not necessarily dry); S^H and N^H are the values for the *H*-treated sample, and N^0 the true N percentage of the original ash-free protein dried at 105°. The results are summarized in Table 7. Despite the fact that the analytical methods used cannot determine the extent of substitution with any great precision when only small differences of S content are measured, it is nevertheless clear that the number of *H* groups combining under the 'mild' treatment are comparable with those in hexokinase; such differences as exist can largely be explained on



Crystalline yeast hexokinase (× 135).

Table 7. *Treatment of ovalbumin and fibrinogen with H*

Protein	Conditions of treatment	Untreated (dried at 105°) N (%)	Untreated (dried at 105°) S (%)	*H*-treated (air-dried) N (%)	*H*-treated (air-dried) S (%)	Mol. $H/10^5$ g. protein
Fibrinogen*	0·025 M-$NaHCO_3$, 0·06 M-phosphate, 0·15 M-NaCl (*H* in solution)	16·7	1·16	(*a*) 15·85	1·32	7·2
				(*b*) 15·9	1·34	7·8
					1·28	5·8
					Mean	7·0
Ovalbumin	0·1 M-phosphate (*H* in solution)	15·5	1·56	14·0	1·55	4·9
					1·59	6·3
					Mean	5·6
Ovalbumin†	$NaHCO_3$ (liquid *H*)	15·5	1·56	12·95	2·19	33
					2·25	35
					Mean	34
Hexokinase (see Table 6)	(*H* in solution)	—	—	—	—	4·3

(*a*) and (*b*) represent separate experiments, not duplicate analyses.

* After *H*-treatment, the fibrinogen sol in presence of purified thrombin still clots; the clotting time is only slightly delayed, but the clot is at first of loose texture and opalescent, and becomes more rigid on standing.

† *H*-treated ovalbumin after 24 hr. at pH 11 and room temperature contains 14·8% N and 1·95% S (air-dried), corresponding to 15 mol. $H/10^5$ g. protein.

the basis of differences in buffer competition. From these experiments, therefore, it appears that the sensitivity of hexokinase to *H* is not due to any special reactivity of the molecule as a whole, but rather to the dependence of enzyme activity upon groups which are relatively easily substituted by *H*.

As against the six *H* groups combined with 10^5 g. ovalbumin after mild treatment, some 34 are taken up when treated with liquid *H*. These figures run parallel with those already discussed for hexokinase under the two comparable sets of conditions. When allowed to stand at room temperature at pH 11 for 24 hr., 19 of the 34 groups were found to be alkali-labile, indicating that 56% of the groups substituted are probably in ester linkage with carboxyl groups. The residual (alkali-stable) groups may involve amino groups, though according to Herriott *et al.* (1946) these are not substituted in the case of ovalbumin. None of the groups was hydrolyzed by a partially purified liver esterase preparation, although this enzyme is relatively non-specific, hydrolyzing almost all esters.

It may be noted that the extent of *H* substitution in the case of ovalbumin treated with liquid *H* corresponds to one *H* group/26 amino-acid residues. It is remarkable, therefore, that the protein evinces no sign of denaturation and remains soluble both at pH 7 and 5, but, like the native form, is readily heat-coagulated at the latter pH. This is in contrast to the behaviour of nucleoproteins which become insoluble after reaction with *H* (Berenblum, 1940). As recent work (Auerbach & Robson, 1946) suggests that nuclear changes are manifest in *H*-damaged tissue, this difference of behaviour may be of great importance to a complete understanding of the toxicology of mustard gas.

SUMMARY

1. The purification and crystallization of hexokinase from baker's yeast are described. The method was developed independently of those of the Cori and Northrop schools, but the procedure of the latter for the final step of crystallization has been followed. The crystals are of the same form and activity as those of Northrop.

2. The addition of cysteine or NaSH to autolyzed baker's yeast accelerates the liberation of hexokinase from the particulate components of the cell and retards inactivation.

3. As found by van Heyningen (1942) for impure hexokinase, the pure enzyme is stabilized by glucose and is activated by Mg ions. This activation is not antagonized by Ca ions.

4. The enzyme is inhibited by SH poisons (including lachrymators), suggesting that hexokinase is an SH enzyme. These results confirm those of van Heyningen (1942) using impure hexokinase.

5. Since vesicant action is accompanied by *in vivo* inactivation of hexokinase (Dixon *et al.* 1942), the uptake of vesicant by yeast hexokinase has been studied, using $\beta\beta'$-dichlorodiethyl sulphide containing radioactive S. By comparison with other proteins treated with vesicant under similar conditions, it is concluded that the sensitivity of hexokinase to mustard gas is not due to any special reactivity, but to the dependence of enzyme activity on groups which are relatively easily substituted.

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REFERENCES

Auerbach, C. & Robson, J. M. (1946). *Nature, Lond.*, **158**, 878.
Bach, S. J., Dixon, M. & Zerfas, L. G. (1942). *Nature, Lond.*, **149**, 48.
Bach, S. J., Dixon, M. & Zerfas, L. G. (1946). *Biochem. J.* **40**, 229.
Bailey, K. & Webb, E. C. (1944*a*). Report to Ministry of Supply by Dixon, no. 30.*
Bailey, K. & Webb, E. C. (1944*b*). *Biochem. J.* **38**, 394.
Banks, T. E. (1946). *Brit. J. Radiol.* **19**, 333.
Berenblum, I. (1940). Report to Ministry of Supply by Peters, no. 16.*
Berger, L., Slein, M., Colowick, S. P. & Cori, C. F. (1945–6). *J. gen. Physiol.* **29**, 379.
Boursnell, J. C., Francis, G. E. & Wormall, A. (1946). *Biochem. J.* **40**, 743.
Cori, C. F., Colowick, S. P., Berger, L. & Slein, M. (1942). By communication.*
Cori, C. F., Colowick, S. P., Berger, L. & Slein, M. (1943). By communication.*
Dixon, M. (1943). Report to Ministry of Supply by Dixon, no. 19.*
Dixon, M. & Needham, D. M. (1946). *Nature, Lond.*, **158**, 432.
Dixon, M., Needham, D. M. & van Heyningen, R. (1942). Report to Ministry of Supply by Dixon, no. 11.*
Herriott, R. M., Anson, M. L. & Northrop, J. H. (1946). *J. gen. Physiol.* **30**, 185.
Kunitz, M. & McDonald, M. R. (1945–6). *J. gen. Physiol.* **29**, 393.
Mackworth, J. F. (1941). Report to Ministry of Supply, by Dixon, no. 4.*
Mackworth, J. F. (1948). *Biochem. J.* **42**, 82.
Meyerhof, O. (1927). *Biochem. Z.* **183**, 176.
Northrop, J. H., Kunitz, M. & McDonald, M. R. (1942). By communication.*
van Heyningen, R. (1942). Report to Ministry of Supply by Dixon, no. 10.*

* Information available on application to Ministry of Supply, London.

The Metabolism of the Lungs from Normal Mice and from Mice Exposed to Phosgene

BY E. BOYLAND AND F. F. MCDONALD, *Experimental Station, Porton, Wilts.*

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The respiration and glycolysis of the lungs of normal mice, and of mice previously exposed to phosgene, have been measured in order to determine if the oedema following exposure is preceded by inhibition of the carbohydrate metabolism. This was done in the hope that knowledge of the nature of the changes might lead to a rational therapy of phosgene poisoning.

EXPERIMENTAL

The measurements were made with the Warburg apparatus at 38°. The lungs were suspended in Ringer-phosphate in the presence of O_2 for respiration measurements and in Ringer-bicarbonate in 95% N_2/5% CO_2 for measurement of the glycolysis. To minimize injury to the lung, the whole lobes from mice of about 20 g. wt. were used for the measurements. Lungs of larger animals would have necessitated the use of minced or sliced tissue. Manometric readings were taken at intervals over a 2 hr. period. The tissue in each experiment was weighed (*a*) before the manometric measurements were made, (*b*) in the wet state at the end of the manometric measurements and (*c*) after drying to constant wt. at 100°. The metabolic quotients (Q) are expressed in μl./mg. dry wt./hr.

RESULTS

Comparison of metabolism of whole lobes and cut lung tissue from normal mice

The lungs of freshly killed normal mice were removed and washed with Ringer solution. The lobes of one lung were weighed, and placed whole in a Warburg vessel, while the lobes of the other lung were minced into fine pieces with scissors before being placed in a second vessel. Some values for metabolism of whole and minced tissue are given in Table 1. The mean values for Q_{O_2} with glucose are 7·3 for whole tissue, 7·4 for minced tissue (eight determinations of each type). Similar experiments have shown that the respiration in the absence of glucose, and the glycolysis in the presence of glucose, are not seriously changed by mincing of the tissue. These results indicate that measurements made on whole lobes of small mice are not affected by the rates of diffusion of O_2 or CO_2 in the tissue and give a true picture of the activity of the enzymes within the tissue. The