133. ZYMOHEXASE

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With an Addendum by E. C. Bate-Smith

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Meyerhof & Lohmann [1934, 1, 2] discovered zymohexase, an enzyme of wide distribution in animal and plant tissues which catalyses the scission of 1:6-diphosphofructose into two molecules of triosephosphate. Although they made a careful and thorough study of the properties of the enzyme and the reversibility of the reaction catalysed they made no attempt to obtain the enzyme in a purified state.

We have isolated zymohexase from rabbit skeletal muscle *ca.* 150 times more active per mg. dry weight than the original muscle. At the highest purity level 1 mg. of the enzyme catalyses the formation of 915 mg. triosephosphate per hour at 38°. The enzyme is a colourless protein accounting for some 4% of the total water-soluble protein of rabbit skeletal muscle. As yet we have no clue as to the nature of its prosthetic group.

I. *Estimation of enzyme activity*

Hexosediphosphate is converted in presence of the enzyme into two molecules of triosephosphate:

$$\text{hexosediphosphate} \rightleftharpoons 2 \text{ triosephosphate}.$$

The reaction is reversible with the equilibrium point in favour of hexosediphosphate formation under physiological conditions. It follows that the rate of triosephosphate formation cannot be used as a strict measure of enzyme concentration, since the rate will fall off as the equilibrium point is approached. To obviate this difficulty triosephosphate can be "fixed" with cyanide and the reaction then proceeds quantitatively from left to right.

Triosephosphate cyanohydrin can be estimated as inorganic phosphate after exposure to *N* soda for 20 min. at room temperature [Meyerhof & Lohmann, 1934, 1, 2]. Hexosediphosphate is not estimated under these conditions.

We have arbitrarily defined as the unit of zymohexase activity an amount which catalyses the formation of 1 mg. P equivalent of triosephosphate per 3 min. at 38° and *p*H 7·3. The test mixture was as follows: 1 ml. enzyme, 1 ml. *M*/4 HCN (neutralized to *p*H 7·3) and 1 ml. *M*/5 borate buffer *p*H 7·3. After the mixture had been equilibrated in a water bath maintained at 38°, 1 ml. *M*/20 hexosediphosphate (previously warmed to the same temperature) was rapidly introduced. At the end of 3 min. the reaction was stopped by rapid addition of 5 ml. 5% trichloroacetic acid. The inorganic P formed after alkaline hydrolysis was estimated by the method of Fiske & Subbarow [1925]. The quantity of enzyme used in the test should be between the limits of 0·5 and 1·5 units. It is important to prepare the neutralized cyanide solution fresh for each experiment.

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The sample of hexosediphosphate used in our experiments was isolated from fermenting dried yeast in the form of the acid barium salt following an unpublished method communicated to us by Prof. Robison. The barium salt was dissolved in dilute HCl and decomposed with slightly more than the theoretical amount of Na_2SO_4. The $BaSO_4$ precipitate was washed several times with water and the combined solutions were neutralized to *p*H 7·3. The final strength of the hexosediphosphate solution was determined by estimation of inorganic P after enzymic conversion into triosephosphate followed by alkaline hydrolysis.

We have used the ratio $\frac{\log I_0/I \text{ at } 280\ m\mu}{\text{enzyme units/ml.}}$ as a measure of the purity level. The density measurements were made in a 2 cm. cell. The factor for converting density values in terms of dry weight per mg. is remarkably constant throughout the entire purification process, and for practical purposes a solution containing 1 mg. dry weight of protein per ml. can be assumed to have a density of 1·8 in a 2 cm. cell.

II. *Method of isolation*

The skeletal muscle of one large rabbit (*ca.* 4 kg.) provides sufficient starting material for attaining the highest purity level. The following are the details of a typical preparation.

(1) 827 g. of fresh rabbit skeletal muscle were finely minced and mixed with 1240 ml. water. After 30 min. the suspension was filtered through muslin. The extract (1192 ml.) was treated with 357 g. $(NH_4)_2SO_4$, i.e. 30 g. for each 100 ml. The precipitate was centrifuged off and discarded. The supernatant fluid (1310 ml.) was further treated with 131 g. $(NH_4)_2SO_4$, i.e. 10 g. for each 100 ml. The precipitate was centrifuged hard for *ca.* 1 hr. and dissolved in 3·1 vol. water (final volume 500 ml.). The enzyme solution was then heated to 58° in a water bath at 70° and maintained at 58° for 2 min. The heating was so arranged that the maximum temperature was reached in <1 min. Vigorous shaking was necessary to prevent local overheating. After 2 min. exposure the solution was rapidly cooled to room temperature (time *ca.* ½ min.). The protein coagulum was filtered off with suction. The filtrate (486 ml.) was pale red though water clear. The yields of enzyme obtained in these procedures were as follows:

Stage	Volume ml.	Total units	$\frac{\log I_0/I \text{ } 280\ m\mu}{\text{Enzyme units/ml.}}$
First extract	1192	15,620	5·6
After addition of 30 g. $(NH_4)_2SO_4$ per 100 ml.	1315	14,990	—
Precipitate of 2nd $(NH_4)_2SO_4$ addition	500	12,500	2·3
After heating to 58° for 2 min.	486	12,200	1·3

(2) The enzyme solution was then submitted to the first fractionation with ammoniacal $(NH_4)_2SO_4$ (6 ml. of 0·88 NH_3 to 94 ml. saturated $(NH_4)_2SO_4$). Four fractions were obtained by addition of 0·66, 0·70, 0·83 and 1·0 final volumes respectively of the $(NH_4)_2SO_4$ reagent and the precipitates were dissolved in water:

Fraction	Volume ml.	Total units	$\frac{\log I_0/I \text{ } 280\ m\mu}{\text{Enzyme units/ml.}}$
I	39	3370	0·9
II	29	2540	0·6
III	23	1630	0·9
IV	33	1130	1·6

Fraction IV was discarded whereas fractions I and II were each resolved with the $(NH_4)_2SO_4$ reagent into three successive fractions, *a*, *b* and *c*. Fraction I*a* was discarded; I*b* and I*c* were retained. Fraction III*a* was retained; III*b* and III*c* were discarded. Finally all the best fractions, viz. I*b*, I*c*, II and III*a*, were combined. The final volume after the complete first fractionation series was 100 ml. containing 6750 units at the ratio 0·58 stage.

(3) The second fractionation series also involves the use of the ammoniacal $(NH_4)_2SO_4$ reagent. The additions of the reagent were so regulated as to distribute the precipitated protein into three more or less equal fractions which we shall refer to as fractions 1, 2 and 3 respectively:

Fractions	Volume ml.	Total units	$\frac{\log I_0/I \text{ 280 m}\mu}{\text{Enzyme units/ml.}}$
1	41	3200	0·60
2	50	2660	0·45
3	13	580	0·72

Fraction 3 was discarded. Fractions 1 and 2 were each resolved into two successive fractions which we shall refer to by the subscripts *a* and *b*. Fractions 1*b* and 2*a* were combined, final volume 60 ml. containing 4220 units at the ratio 0·42 stage. The yield of enzyme at this stage was 27 %.

(4) The above solution was dialysed against running water for 12 hr. and then mixed with 1/10 volume *M*/5 citrate buffer *p*H 6. Saturated $(NH_4)_2SO_4$ solution (not ammoniacal) was added in three instalments sufficient to bring the saturation to 51·5, 54 and 62 % respectively, the corresponding fractions for which we shall refer to as A, B and C:

Fraction	Volume ml.	Total units	$\frac{\log I_0/I \text{ 280 m}\mu}{\text{Enzyme units/ml.}}$
A	25	624	0·85
B	50	1764	0·36
C	40	1008	0·33

Fraction A was discarded whereas fractions B and C were combined and refractionated again into three successive fractions A^1, B^1 and C^1. The latter two together contained 800 units at the ratio 0·28 stage—the limit we have reached by our methods. The final enzyme solution was dialysed exhaustively against water, frozen at −10° and dried in high vacuum over H_2SO_4. The dried enzyme which retains enzymic activity unimpaired weighed 127 mg. The yield of enzyme at the ratio 0·28 purity level was about 5 %. The degree of concentration relative to the first extract of minced muscle was $\frac{7·2}{0·28}=26$. Assuming that the enzyme is homogeneous at the ratio 0·28 stage it follows that zymohexase accounts for *ca.* 4 % of the dry weight of the soluble protein of rabbit skeletal muscle and for *ca.* 0·7 % of the dry weight of whole muscle.

The first three stages in the method of purification (i.e. as far as ratio 0·4) are easily reproducible and there is no need to carry out estimations of activity apart from the initial one. But in proceeding from ratio 0·4 to 0·28 the purity of each fraction must be determined. The successful prosecution of the final $(NH_4)_2SO_4$ fractionations depends on a variety of factors such as the rate of stirring, the temperature of the room, the rate of addition of the reagent, etc. It is in fact a matter for experimentation in each preparation, and the element of practice is important.

III. *Properties of the homogeneous enzyme*

Elementary composition. The enzyme powder at the ratio 0·28 stage was dried at 100° *in vacuo* and found to have the following elementary composition: C, 50·6%; H, 7·04%; N, 15·8%; S, 1·25%. There was no detectable amount of ash. Phosphorus was estimated as phosphate according to Fiske & Subbarow [1925] after incineration with H_2SO_4 and H_2O_2. No trace of P was found in 15 mg. of protein, the limit of detection being 0·01 mg. The P content could not be greater than 0·05%. Since the molecular weight of the enzyme is *ca.* 100,000 the minimum P content corresponding to 1 atom P per molecule would be 0·03%. It is only possible to say that there cannot be more than 1 atom of P per molecule of enzyme, if that. Total carbohydrate was estimated by the method of Pirie [1936]. We are grateful to Mr R. Markham for carrying out the analysis. In 20 mg. protein, less than 0·01 mg. carbohydrate (expressed as ribose) were found, which approaches the limit of detection of the method. In a unit of molecular weight 100,000 one molecule of ribose would correspond to a carbohydrate content of 0·14%. The value found was less than 0·05%. No significant amount of iodine was detectable.

The analytical data rule out the possibility of zymohexase being a metalloprotein or a combination of protein and purine nucleotides. The absence of carbohydrate and P is perhaps a little surprising in an enzyme whose substrate is a phosphorylated hexose. Thus far we have been unable to detect any substance of non-amino acid nature as a part of the molecule. This does not necessarily mean or imply that the enzyme has no special prosthetic group.

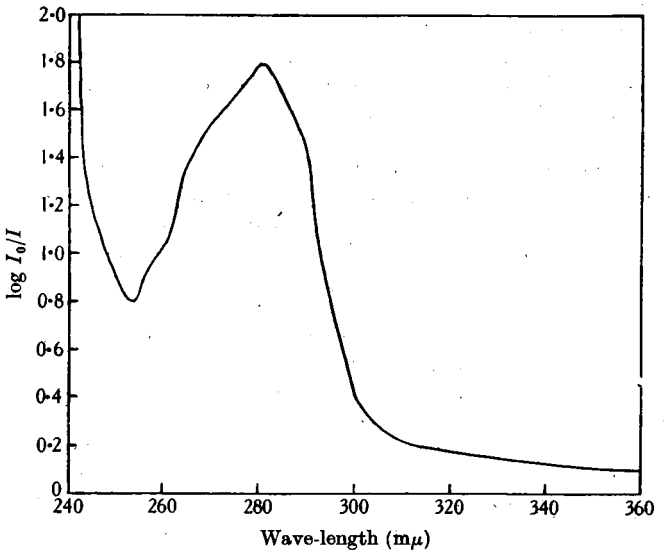

Fig. 1. Ultraviolet absorption spectrum of zymohexase concentration 0·1%. Tube-length 2 cm.

Absorption spectrum. A solution of the enzyme showed no absorption in the visible region; however, the ultraviolet absorption spectrum was that of a typical protein (cf. Fig. 1). From the absorption spectrum in $N/10$ NaOH the tyrosine and tryptophan contents of the enzyme were calculated by the method of Holiday [1936]. There was 10·6% tyrosine but no tryptophan.

Catalytic constants. At the extinction ratio 0·286 stage 1 mg. of the enzyme is equivalent to 6·3 catalytic units. Thus 1 mg. zymohexase under the conditions of our standard test at 38° and *p*H 7·33 forms 6·3 mg. triosephosphate-P in 3 min. or 126 mg. triosephosphate-P per hour. 1 mg. triosephosphate-P is equivalent to 5·48 mg. triosephosphate (expressed as free acid). Therefore under standard conditions, i.e. *p*H 7·3 and 38°, 1 mg. zymohexase forms 691 mg. triosephosphate per hour. At *p*H 9 and 38° the rate is 915 mg. per hour.

Assuming a molecular weight of 100,000 calculation shows that one molecule of zymohexase would form 6600 molecules of triosephosphate per min. at 38° and *p*H 7·3. At 38° and *p*H 9 the "turnover number" would be 8800.

General properties. On drying the frozen solution the enzyme is obtained in the form of a pure white feathery powder. There is no loss of activity on drying. The dried enzyme dissolves readily in distilled water, yielding a colourless, crystal clear solution. The enzyme is relatively stable either in solution or in the dried form. Aqueous neutral solutions may be kept some weeks at 0° with negligible loss of activity. The enzyme is less stable at *p*H 6 and very unstable at *p*H 4 or less. Dilute ammoniacal $(NH_4)_2SO_4$ is an especially favourable medium for preserving activity.

Temperatures higher than 50° destroy the enzyme:

Temperature	50°	60°	70°
Time of exposure (min.)	5	5	5
% inactivation	8	35	91

In presence of 10 % $(NH_4)_2SO_4$ the resistance to high temperatures is greater, e.g. heating to 58° for 3 min. in this medium produces no appreciable inactivation. This is made use of in the method of preparation.

Organic solvents must be avoided in the preparation of the enzyme. Strong aqueous solutions of alcohol and acetone inactivate the enzyme rapidly at room temperature and even at 0° or below.

Homogeneity of the purified enzyme. We have consistently failed to advance the purity of the enzyme beyond the ratio 0·28 stage. Fractionation of the enzyme at this stage merely yielded a series of identical fractions.

In the addendum, Dr E. C. Bate-Smith discusses the results obtained by cataphoresis of the enzyme in the Tiselius apparatus. At the lower stages of purity several components were observed; at the ratio 0·28 stage, there was only one component.

Mr J. St J. Philpot has examined the purified enzyme in the ultracentrifuge. Two different preparations were found to contain only a single, sharply defined component, though the sedimentation constants were not identical in the two cases. Unfortunately, the investigation could not be continued, and therefore no definite statements of molecular weight can be made on the strength of the available evidence.

The purity of our enzyme preparation was also checked by tests for other enzymes known to be present in the original muscle extract. No trace was found of the following eight enzymes; lactic, malic, α-glycerophosphoric and triosephosphoric dehydrogenases, phosphorylase, enolase, isomerase and adenylic acid deaminase. Some 10–15 mg. of the enzyme were employed in each of these tests. The triosephosphoric dehydrogenase is present in small amount at the ratio 0·6 stage; with further ammonium sulphate fractionation the impurity is easily eliminated. Thus at the ratio 0·45 stage the test for the triosephosphoric enzyme was completely negative.

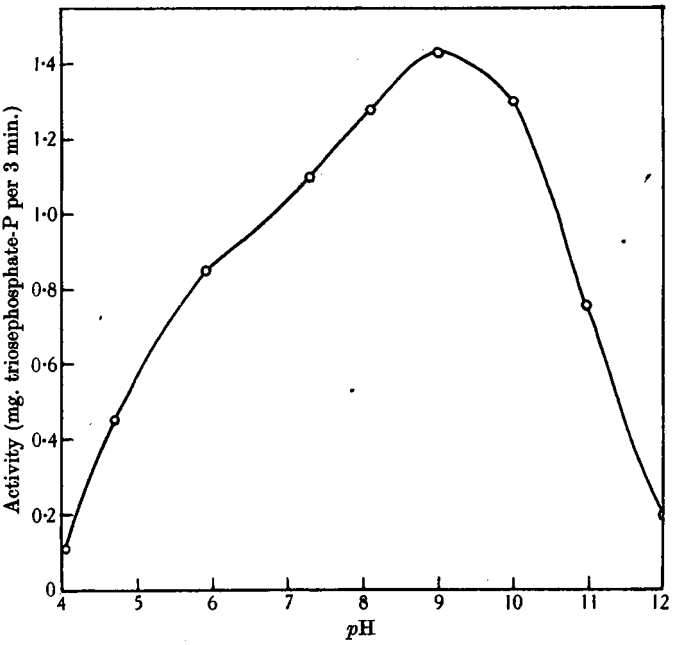

Fig. 2. *p*H-activity curve.

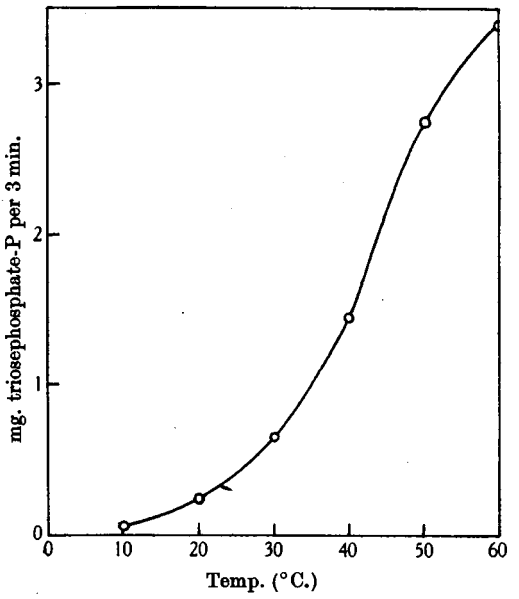

Fig. 3. Effect of temperature on velocity.

IV. *Kinetics*

Apart from the studies of Meyerhof & Lohmann [1934, 1, 2; 1935] on the reversibility of the reaction catalysed by zymohexase there is little known of the kinetics of the enzyme. This gap is in part due to the lack of a suitable method for measuring the activity of the enzyme. The cyanide fixation method described in Section I obviates the principal difficulty and has enabled us to determine the effect of various conditions on the activity of the enzyme.

The *p*H-activity curve is shown in Fig. 2. The enzyme is active over a wide range of *p*H with a maximum at *ca.* *p*H 9. The activity at *p*H 11 is *ca.* 50% of the activity at *p*H 9. Even at *p*H 12 the enzyme shows some activity. Activity falls off rapidly on the acid side of neutrality. These facts may be correlated with the marked stability of the enzyme to alkali, and its rapid denaturation at *p*H 4 and below.

Between 30° and 50° the Q_{10} is very nearly 2 (cf. Fig. 3). Below 30° the Q_{10} is greater than 2 and above 50° it is less than 2. In the higher range of temperature increased velocity is of course compensated by increased rate of destruction during the course of the experiment.

The rate of formation of triosephosphate is practically independent of the initial concentration of hexosediphosphate (cf. Table 1). The Michaelis constant, i.e. the half-speed concentration, is less than 0·001 *M*.

Table 1. *Effect of substrate concentration*

Hexosediphosphate concentration (*M*)	Velocity, in mg. P per 3 min.
0·005	1·25
0·0083	1·34
0·0166	1·36
0·033	1·43
0·05	1·47

The additions in each case were 1 ml. enzyme, 1 ml. borate buffer *p*H 7·3, 1 ml. *M*/4 KCN and varying amounts of hexosediphosphate; final volume 5 ml. Temp. 38°.

Table 2. *Effect of enzyme concentration*

Enzyme ml.	Time min.	Triosephosphate-P found (mg.)
0·2	15	0·66
0·5	6	0·92
1·0	3	1·04
2·0	1·5	1·08

The additions in each case were, 1 ml. *M*/20 hexosediphosphate, 1 ml. *M*/4 KCN, 1 ml. borate buffer *p*H 7·3, and varying amounts of enzyme; total volume 5 ml. Temp. 38°.

The effect of cyanide concentration on the velocity of triosephosphate formation has an important bearing on our standard test of enzyme activity. The final cyanide concentration must be 0·05 *M* for the reaction velocity to be maximal:

HCN concentration (*M*)	0	0·01	0·025	0·05	0·1
mg. triosephosphate-P formed in 3 min.	0·4	0·72	0·97	1·11	1·12

The velocity of triosephosphate formation is proportional to the concentration of enzyme only within restricted limits under the conditions of our standard test (cf. Fig. 4). In the range 0·5 to 1·5 enzyme units a linear relation obtains. The lack of proportionality at higher enzyme concentrations is referable

to the exhaustion of substrate before the end of the experimental period. When employing low enzyme concentrations the experimental period has to be extended in order to obtain sufficient P for accurate estimation. There is clearly destruction of the enzyme in the course of prolonged contact with the substrate (Table 2).

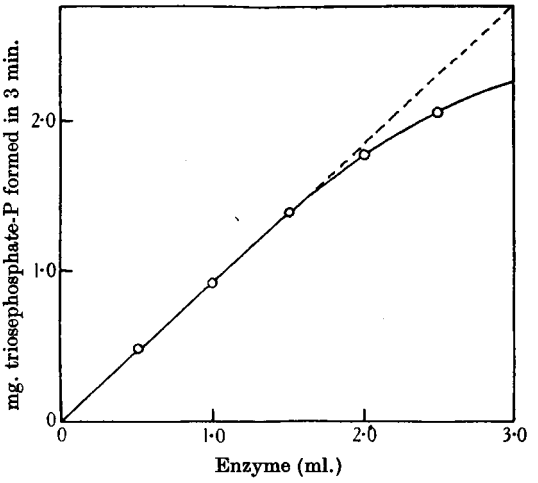

Fig. 4. Effect of enzyme concentration.

V. *Specificity of substrate*

Zymohexase does not attack glucose, fructose or fructose-6-phosphate. Solutions of glucose and fructose in presence of the enzyme show no change in optical relation over a period of 24 hr. Under the conditions of the standard test fructose-6-phosphate does not yield alkali-labile P.

Lohmann [1935] discovered in muscle extract "aldolase", an enzyme which catalyses the aldol condensation of dihydroxyacetonephosphate with various aliphatic or aromatic aldehydes, e.g.

dihydroxyacetonephosphate + *d*-glyceraldehyde → fructose-1-phosphate
dihydroxyacetonephosphate + acetaldehyde → 5-desoxyxyloketose-1-phosphate.

The analogy between the catalytic actions of zymohexase and aldolase respectively was sufficiently close as to lead the discoverer to the surmise that they were the same enzyme. This point we have been able to clinch by the demonstration that preparations of zymohexase at the highest purity level still manifest strong aldolase activity.

The method of testing for aldolase activity is a little complicated and requires a few explanatory remarks. Hexosediphosphate in presence of zymohexase is partially and reversibly resolved into one molecule each of dihydroxyacetonephosphate and glyceraldehydephosphate. In absence of a ketone fixative the reaction does not go to completion. If aldolase is present addition of acetaldehyde should result in the formation of methyltriosephosphate by combination with dihydroxyacetonephosphate. The new compound formed contains alkali-labile P and can be estimated as triosephosphate. The removal of dihydroxyacetonephosphate by aldol condensation will disturb the zymohexase equilibrium and more will be formed to satisfy the equilibrium requirements. The net result should be therefore an increase in alkali-labile P after addition of

acetaldehyde. Table 3 summarizes a typical experiment demonstrating increased production of alkali-labile P in presence of various concentrations of acetaldehyde.

Table 3. *Aldolase reaction*

In all experiments, 2 ml. enzyme, 1 ml. buffer *p*H 7·3, 2 ml. *M*/20 hexosediphosphate. Temp. = 38°.

Exp.

1 Incubated 10 min.
2 Incubated 20 min.
3 Incubated 10 min., then 4·4 mg. acetaldehyde added, incubated a further 10 min.
4 Incubated 10 min., then 17·6 mg. acetaldehyde added, incubated a further 10 min.
5 Incubated 10 min., then 88 mg. acetaldehyde added, incubated a further 10 min.

Triosephosphate-P found (mg.)

Exp. 1. 0·50. Exp. 2. 0·50. Exp. 3. 0·74. Exp. 4. 1·15. Exp. 5. 1·94

VI. *Products of reaction*

Meyerhof & Lohmann [1935] originally formulated the catalytic scission of fructosediphosphate as follows:

fructosediphosphate $\rightleftharpoons$ 2 dihydroxyacetonephosphate.

The basis of this formulation was the observation that the triosephosphate formed in crude muscle extracts was more than 95% dihydroxyacetonephosphate. Later Meyerhof & Kiessling [1935] discovered in muscle extracts, isomerase, an enzyme which catalyses the following reactions:

d-3-glyceraldehydephosphate $\rightleftharpoons$ dihydroxyacetonephosphate.

It thus became clear that the presence only of the ketotriosephosphate in muscle extracts did not necessarily exclude the formation of the aldotriosephosphate. Since the isomerase equilibrium is vastly in favour of the former, no appreciable quantity of aldotriosephosphate should be present in a solution containing both zymohexase and isomerase.

It has now become possible to test this hypothesis directly. Purified zymohexase preparations which are free from isomerase should catalyse the formation of equal quantities of the two triosephosphates. Both the aldo- and keto-triosephosphates contain alkali-labile P. But whereas the aldotriosephosphate is oxidized by iodine to phosphoglycerate whose P is with difficulty hydrolysable, the ketotriosephosphate is unaffected by iodine. We should expect therefore that half the total triosephosphate formed from fructosediphosphate should disappear after iodine treatment. Some typical results are shown in Table 4. It is clear that within the limits of experimental error iodine treatment halves the total alkali-labile P.

Table 4. *Ratio of glyceraldehydephosphate to dihydroxyacetonephosphate*

Enzyme and hexosediphosphate were incubated for 10 min. at 37° without addition of cyanide. Aliquots were then estimated for alkali-labile P before and after oxidation with iodine.

Exp.	Glyceraldehyde-phosphate-P mg.	Dioxyacetone-phosphate-P mg.	Ratio
1	0·187	0·131	59/41
2	0·22	0·13	63/37
3	0·49	0·67	42/58
4	0·25	0·25	50/50
5	0·21	0·30	41/59
		Mean	51/49

VII. *The equilibrium constant*

The equilibrium constant K for the reactions catalysed by zymohexase is given by the equation

$$(1)\quad K=\frac{[\text{dihydroxyacetonephosphate}]\,[\text{glyceraldehydephosphate}]}{[\text{hexosediphosphate}]}.$$

Since the two triosephosphates are formed in equal quantity,

$$(2)\quad K=\frac{[\frac{1}{2}\text{ total triosephosphate}]^2}{[\text{hexosediphosphate}]}.$$

K has the dimensions of a concentration and will be expressed in terms of g. mol. per litre. As in all dissociating systems, dilution of the system at equilibrium will bring about an increased breakdown of hexosediphosphate.

Solutions of enzyme and hexosediphosphate were incubated at various temperatures until equilibrium had been reached. The total triosephosphate was then estimated as alkali-labile P. The amount of hexosediphosphate remaining was obtained by subtraction of the triosephosphate-P from the original total P. In these experiments the quantity of enzyme used was sufficient to permit the attainment of final equilibrium within 5–10 min. The instability of triosephosphate would otherwise prove an interfering factor. Table 5 shows that K is independent of the concentration of enzyme (within the limits of experimental error), and that the enzyme obeys the essential criterion of a true catalyst. K is also independent of the initial substrate concentration within the limits of concentration tested. The small variations in K are well within the limits of experimental error. The amount of triosephosphate present at equilibrium is small, and this not too easily estimated quantity must be halved and then squared in the calculation of K. Although K remains the same for all substrate-concentrations, the percentage of hexosediphosphate converted into

Table 5. *Effect of enzyme concentration on the equilibrium constant*

ml. enzyme	mg. triosephosphate-P at equilibrium	mg. hexosediphosphate-P at equilibrium	K
0·3	0·311	2·51	$1{\cdot}24\times10^{-4}$
1·0	0·293	2·53	$1{\cdot}21\times10^{-4}$
3·0	0·292	2·53	$1{\cdot}21\times10^{-4}$

The reaction mixture contained in each case 1 ml. $M/20$ hexosediphosphate, 1 ml. $M/4$ borate buffer pH 7·33, and varying amounts of enzyme; final volume 5 ml. Temp. 38°. Values of K in g. mol./litre. All values are the means of duplicate observations.

Table 6. *Effect of substrate concentration on the equilibrium constant*

Initial hexosediphosphate concentration M	mg. triosephosphate-P at equilibrium	mg. hexosediphosphate-P at equilibrium	$\frac{\text{Triosephosphate-P}}{\text{Initial hexosediphosphate-P}}\times100$	K
0·005	0·231	1·15	16·3 %	$1{\cdot}30\times10^{-4}$
0·01	0·305	2·52	11·5 %	$1{\cdot}19\times10^{-4}$
0·02	0·435	5·18	8·3 %	$1{\cdot}21\times10^{-4}$
0·03	0·534	7·93	6·3 %	$1{\cdot}16\times10^{-4}$

All reaction mixtures contained 1 ml. enzyme, 1 ml. borate buffer pH 7·33, and varying amounts of hexosediphosphate, in a total volume of 5 ml. Temp. 38°. All values are the means of duplicate estimations, except those for 0·01 M hexosediphosphate, which are the means of 7 different estimations. K is expressed in g. mol./litre.

Table 7. *Effect of temperature on the equilibrium constant*

Temp.	mg. triose-phosphate-P at equilibrium	mg. hexose-diphosphate-P at equilibrium	$\frac{\text{Triosephosphate-P}}{\text{Initial hexosediphosphate-P}} \times 100$	K
28°	0·220	2·60	7·8	$6{\cdot}0 \times 10^{-5}$
38°	0·305	2·52	11·5	$1{\cdot}19 \times 10^{-4}$
48°	0·417	2·40	14·8	$2{\cdot}34 \times 10^{-4}$

All reaction mixtures contained 1 ml. enzyme, 1 ml. borate buffer *p*H 7·2, and 1 ml. *M*/20 hexosediphosphate, in a total volume of 5 ml. All values are the means of duplicate estimations except those for 38°, which are the means of 7 different estimations. *K* is expressed in g. mol./litre.

triosephosphate decreases with increased substrate concentration. As can be deduced from equation (3) below, the percentage of triosephosphate formed is proportional to the square root of the dilution (Fig. 5).

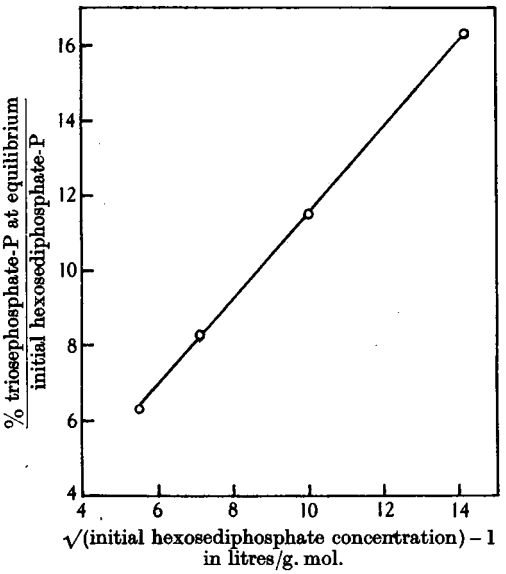

Fig. 5. Effect of hexosediphosphate concentration on equilibrium. Details as in Table 5.

The effect of temperature on the equilibrium constant is shown in Table 7. According to the van't Hoff isochore, the plot of log *K* against *I*/*T* should yield a straight line. The linear relation found is shown in Fig. 6.

The heat of reaction can be calculated from the equation:

$$\ln K_1 - \ln K_2 = -\frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right).$$

The values found are:

Temp. range	ΔH in g. cal.
28°–38°	12,720
38°–48°	13,580
28°–48°	13,130

Meyerhof & Lohmann [1935], by direct calorimetric measurements on muscle extracts, found $\Delta H = 14{,}000$ g. cal. The presence of isomerase in muscle extracts

does not appear to affect the value of ΔH; the conversion of glyceraldehydephosphate into dihydroxyacetonephosphate must therefore involve little heat change.

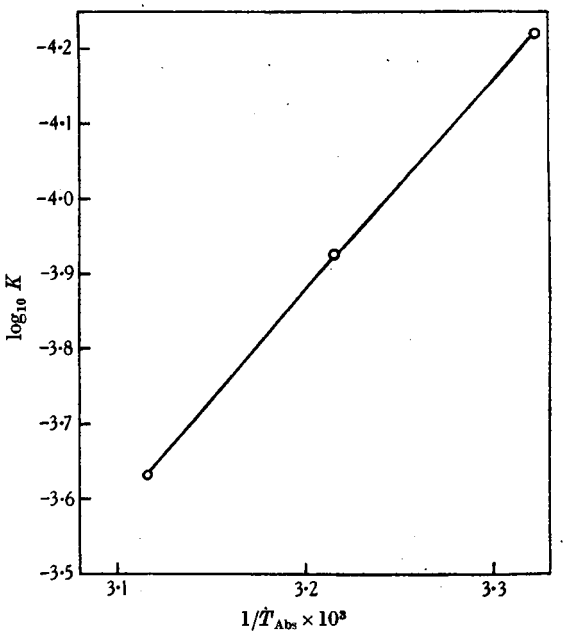

Fig. 6. Variation of equilibrium constant with temperature.

The free energy change for the conversion of hexosediphosphate into triosephosphate can be calculated from the relation:

$$\Delta F = -RT \ln K.$$

The values for different temperatures are:

Temp.	ΔF in g.-cals.
28°	5820
38°	5590
48°	5340

VIII. *Isomerase equilibrium*

The equilibrium between dihydroxyacetonephosphate and glyceraldehydephosphate catalysed by isomerase has hitherto been difficult to evaluate. No technique is available for estimating either of the two triosephosphates with the required degree of accuracy. The only quantity that can be determined with reasonable accuracy is the sum of the two triosephosphates.

From a knowledge of the equilibrium constant $K_{\text{zymohexase}}$ (cf. equation (1)) it is possible to arrive at $K_{\text{isomerase}}$ by the following indirect method. Hexosediphosphate is added to dialysed muscle extract and the equilibrium ratio $\frac{\text{total triosephosphate}}{\text{nitial hexosediphosphate}}$ is then measured.

This will be different from the ratio obtained with purified zymohexase. The presence of isomerase in the crude muscle extract is responsible for the alteration in the ratio. The calculation is made as follows.

If a = g.mol. hexosediphosphate present initially,
$a-x$ = g.mol. hexosediphosphate present at equilibrium,
$x-y$ = g.mol. glyceraldehydephosphate present at equilibrium,
$x+y$ = g.mol. dihydroxyacetonephosphate present at equilibrium,
$2x$ = g.mol. total triosephosphate at equilibrium,
V = volume.

then
$$K_{\text{zymohexase}} = \frac{(x+y)(x-y)}{(a-x)V}, \qquad \ldots(3)$$

$$K_{\text{isomerase}} = \frac{x+y}{x-y}. \qquad \ldots(4)$$

Therefore
$$y = \frac{K_{\text{isomerase}}-1}{K_{\text{isomerase}}+1}x. \qquad \ldots(5)$$

Substituting this value of y in equation (3) and simplifying,

$$K_{\text{zymohexase}} = \frac{\frac{4x^2 K_{\text{isomerase}}}{(K_{\text{isomerase}}+1)^2}}{(a-x)V}. \qquad \ldots(6).$$

Therefore
$$K_{\text{isomerase}}^2 + \left(2 - \frac{4x^2}{(a-x)VK_{\text{zymohexase}}}\right) K_{\text{isomerase}} + 1 = 0. \qquad \ldots(7)$$

To solve this quadratic equation for $K_{\text{isomerase}}$ the only quantity required is x, which is half the sum of the total triosephosphate. $K_{\text{zymohexase}}$ is known.

The following are the protocols of a typical experiment designed to evaluate $K_{\text{isomerase}}$. Dialysed muscle extract (1 ml.) was mixed with 1 ml. of 0·0091 M hexosediphosphate (2·82 mg. P) in a final volume of 5 ml. at 38° and pH 7·3. At equilibrium the triosephosphate-P formed was 0·84 mg., and the hexosediphosphate-P remaining was 1·98 mg. Therefore $x = 2{\cdot}71 \times 10^3$ mol. and

$$(a-x) = 6{\cdot}38 \times 10^3 \text{ mol.}$$

and the ratio
$$\frac{\text{total triosephosphate-P}}{\text{initial hexosediphosphate-P}} 100 = 29{\cdot}8\,\%.$$

These results are in good agreement with those of Meyerhof & Lohmann [1934, 1]. The ratio for purified zymohexase under the same experimental conditions was 11·5%. Substituting the values for x and $a-x$ in equation (7), we find that $K_{\text{isomerase}} = 36{\cdot}3$,

i.e. the ratio
$$\frac{\text{glyceraldehydephosphate}}{\text{total triosephosphate}} 100 = 2{\cdot}7\,\%.$$

From $K_{\text{isomerase}}$ the free energy of the conversion of glyceraldehydephosphate into dihydroxyacetonephosphate can be calculated. At 38°, $\Delta F = -2220$ g. cal.

IX. *Inhibitors*

Table 8 summarizes the effects of various agents on zymohexase. $Na_2S_2O_4$, ascorbic acid, reduced and oxidized glutathione, iodoacetic acid and H_2O_2 have no appreciable effect. There is no suggestion therefore of an oxidizable or reducible group being concerned in the activity of the enzyme.

Iodine in very low concentration inhibits the enzyme completely. No correlation was observed between the degree of inhibition and the number of tyrosine molecules in the enzyme.

The inhibitory effect of heavy metals has an important practical bearing on the purification of the enzyme. There are sufficient amounts of heavy metals

Table 8. *Effect of various substances on zymohexase activity*

Reagent	Final concentration	% inhibition
Sodium hydrosulphite	0·1%	0
Ascorbic acid	0·1%	0
Reduced glutathione	0·1%	0
Iodoacetic acid	*M*/500	0
H_2O_2	*M*/500	0
I_2	*M*/50,000	100
I_2	*M*/150,000	46
I_2	*M*/500,000	18
$AgNO_3$	*M*/50,000	100
$CuSO_4$	*M*/50,000	100
$ZnSO_4$	*M*/50,000	34
$Hg(NO_3)_2$	*M*/50,000	16

In each experiment, 1 ml. enzyme was incubated with 1 ml. of the various substances for 5 min. at 38°. Buffer, cyanide and hexosediphosphate were then added, and the activity tested in the usual way. A control was run simultaneously.

in commercial $(NH_4)_2SO_4$ to cause considerable inactivation of the enzyme, especially at higher purity levels. It is essential to use analytical reagents and glass-distilled water throughout the purification process.

Glucose, fructose and fructose-6-phosphate, although not attacked by the enzyme, competitively inhibit the scission of hexosediphosphate (cf. Table 9). Fructose-6-phosphate shows this phenomenon best. α-Glycerophosphate at the same concentration has no effect on the enzyme.

Table 9. *Competitive inhibition*

Each experiment contained 1 ml. enzyme, 1 ml. borate buffer, *p*H 7·3, 1 ml. *M*/4 KCN, 1 ml. *M*/10 hexosediphosphate and 1 ml. of the various compounds listed below.

Substance added	None	*M*/10 glucose	*M*/10 fructose	*M*/10 fructose-6-phosphate	*M*/10 β-glycero-phosphate
mg. triosephosphate-P found in 3 min.	1·29	1·22	1·08	0·89	1·28
% inhibition	—	5·4	16·3	31	0

X. *Crystalline form*

In the course of the purification of zymohexase a marked tendency of the $(NH_4)_2SO_4$ precipitates to take on crystalline form was observed. After some hours the precipitates show pronounced anisotropy of flow when examined with polarized light. The crystals which form are very small needles. During the final stages of the purification of the enzyme the $(NH_4)_2SO_4$ precipitates show no tendency to form these crystals and it must be concluded therefore that some protein other than the enzyme is responsible for the anisotropic crystals.

Recently Baranowski [1939] and Bailey [1940] have isolated from rabbit skeletal muscle a protein (the so-called "myogen B") whose general properties resemble closely those of the crystalline material found in impure preparations of zymohexase. Through the kindness of Dr Bailey we had an opportunity of testing a highly purified crystalline preparation of this protein for zymohexase activity. No trace of activity was found.

It is an interesting commentary on the value of the crystalline state as an index of purity of proteins that one of the best tests for the homogeneity of zymohexase preparations is that the $(NH_4)_2SO_4$ precipitates should show no tendency to crystallize. As long as the precipitate shows anisotropy of flow

in polarized light the enzyme is still inhomogeneous. It would appear from our results that this so-called "myogen B" tends to crystallize even when present to the extent of only 10 % of the total protein. At this stage the crystals of course are grossly contaminated with other proteins.

XI. *Zymohexase and muscle proteins*

There is constant reference in the literature to the number of proteins present in skeletal muscle. Myosin, myohaemoglobin, myoalbumin, myogens A and B and globulin X are assumed to represent the principal proteins of skeletal muscle. This view is somewhat naïve in view of our knowledge of the enzymic equipment of skeletal muscle. One could enumerate some fifty enzymes known to be present in skeletal muscle. Making the not unjustifiable assumption that all these enzymes are proteins it follows that at least fifty different proteins should be found in skeletal muscle. The concentration will vary from one enzyme to another. Some like zymohexase are present in relatively high concentration (*ca.* 5 % of the total water-soluble protein) whereas others like catalase are present only in minute concentration. There can be no question therefore that what have hitherto been regarded as the main proteins of skeletal muscle are merely mixtures of enzymes (or other proteins) with superficially similar properties.

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ADDENDUM

CATAPHORETIC BEHAVIOUR OF ZYMOHEXASE

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The water-soluble proteins of rabbit muscle show two distinct boundaries in the Tiselius apparatus. Two preparations made by extracting the minced muscle with 7 % LiCl solution and dialysing against distilled water until no further precipitation of globulins occurred gave the following data. The cataphoresis was carried out in 0·05 *M* phosphate buffer at *p*H 7·0.

	Electrophoretic mobility × 10^5 cm.^{2} volt^{-1} sec.$^{-1}$)	
	Boundary 1	Boundary 2
Preparation 1	−5·3	−1·6
Preparation 2 (*a*)	−5·4	−1·6
(*b*)	−5·2	−1·6

These fractions corresponded in properties to myoalbumin and myogen, respectively [cf. Bate-Smith, 1937]. Myogen, the protein forming boundary 2, was definitely not homogeneous.

Preparations of zymohexase were submitted by Dr Green and his co-workers. Successive preparations were of increasing activity and the cataphoretic results showed them to be increasingly homogeneous. The final preparation appeared to be completely homogeneous. A fast-moving fraction (at *p*H 7) in the first preparation was absent from the later ones. The mobility of the main fraction showed that it formed part of the myogen complex. The cataphoretic data obtained with these preparations were as follows.

Preparation	Activity ratio	*p*H	Electrophoretic mobility × 10^5 main boundary
1	0·6	7·4	−1·1
2	0·4	6·5	−0·6
		6·0*	+0·7
3	0·34	6·0*	+0·85
4	0·28	7·0	−0·85
		6·0*	+0·35

* Prolonged cataphoresis at *p*H 6·0 leads to inactivation of the enzyme.

Judging from preparations 2 and 4, the main constituent of the zymohexase preparation has an isoelectric point in 0·05 *M* phosphate at *p*H ~ 6·3. This was confirmed by observing the cataphoretic behaviour of particles of heat-coagulated enzyme in the apparatus described by Dummett and Bowden. The particles were isoelectric at *p*H 6·3. The isoelectric point of "myogen" has been reported by different observers to lie between 6·3 [Weber, 1925] and 6·7 [Bate-Smith, 1937]. Zymohexase is therefore one member of the complex of substances forming the myogen fraction, its isoelectric point lying at the most acid end of the range covered by the group. In bulk it probably represents about 10 % of the whole myogen complex, which again forms 10 % of the total muscle protein. It is interesting to note that Finn [1932] obtained strong evidence of the inhomogeneity of myogen now confirmed in several quarters.

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