FORMATION OF TRYPSIN FROM TRYPSINOGEN BY AN ENZYME PRODUCED BY A MOLD OF THE GENUS PENICILLIUM

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An aqueous solution of crystalline trypsinogen on standing changes autocatalytically into active trypsin without the addition of any activator (kinase). Addition of ammonium sulfate or magnesium sulfate to a solution of trypsinogen greatly accelerates the rate of formation of trypsin as does also the addition of enterokinase or active trypsin (1). The "spontaneous" autocatalytic formation of trypsin from trypsinogen, as well as the accelerating action of salts, enterokinase, or active trypsin takes place best in the range of pH 7.0–9.0. Very little if any trypsin forms in an acid medium at a pH less than 4.0 even with the addition of large amounts of enterokinase or active trypsin. Pure trypsin gradually digests itself in a slightly alkaline medium (2); hence the formation of trypsin at pH 7.0–9.0 is generally accompanied by a considerable loss of the trypsin formed. This paper describes the formation of trypsin from crystalline trypsinogen by means of a new trypsinogen kinase produced, as we accidentally discovered, by a mold of the genus *Penicillium*.[1] It differs markedly from enterokinase in that it brings about the transformation of trypsinogen into trypsin most rapidly at pH 2.5–4.0. In this range of pH trypsin is very stable. Hence the formation of trypsin at pH 2.5–4.0 by the new kinase is not accompanied by any measurable autolysis of the trypsin formed.

The action of the mold kinase in the process of transformation of trypsinogen into trypsin is that of a typical enzyme. The process

[1] The presence of a trypsinogen kinase in bacteria and in a few poisonous mushrooms has been reported as early as 1902; see Delezenne, C., *Compt. rend. Soc. biol.*, **54**, 998, 1902; **55**, 27, 1903.

follows the course of a catalytic unimolecular reaction, the rate of formation of trypsin being proportional to the concentration of kinase used. The ultimate amount of trypsin formed, however, is independent of the concentration of kinase used.

Active trypsin produced by means of mold kinase has been crystallized. It appears to be identical in crystalline form with the crystalline trypsin produced autocatalytically from crystalline trypsinogen at pH 8.0. The two products have, within the experimental error, the same solubility and specific activity.

Mold kinase is most conveniently obtained by cultivating the *Penicillium* mold in a synthetic liquid medium of pH about 4.0. The kinase appears in the medium and its concentration increases during the growth of the mold and continues to increase for some time even after the mold has ceased growing, so that there is no constant relation between the concentration of the kinase in the medium and the weight of the mold.

Mold kinase is rapidly destroyed at pH 6.5 or higher and any culture medium of the *Penicillium* organism of pH above 6.5 fails to show the presence of any kinase activity.

Mold kinase is inactivated when heated at temperatures above 50°C. even at the pH of its maximum stability. The critical thermal increment for the process of heat inactivation at 50°C. and 60°C. is $\mu = 53{,}500$ calories which is comparable with that of denaturation of proteins (3) and of inactivation of trypsin (4) and enterokinase (5). When heated at 70°C. mold kinase is completely inactivated within 5 minutes.

The molecular weight of the mold kinase as determined by diffusion is about 40,000. It does not diffuse through an ordinary collodion membrane.

The high temperature coefficient of inactivation of mold kinase as well as its high molecular weight suggest a possible protein nature for the molecule of mold kinase.

EXPERIMENTAL

I. Some Properties of Mold Kinase

1. Cultivation of the Penicillium Mold.—The mold was originally isolated from an old non-sterile stock of M/400 hydrochloric acid which

showed slight activating power when mixed with a solution of crystalline trypsinogen and which contained a slight growth of mold on the bottom of the container. The mold was cultivated first on potato agar medium; single colonies of the organism on the plate were repeatedly transferred to fresh medium. Single spore preparations of the organism were finally obtained.[2] The pure culture[3] of the organism was henceforth propagated on a liquid medium consisting of:

Sucrose	7.20 gm.	
Dextrose	3.60 "	
$MgSO_4$ (crystals)	1.23 "	
KH_2PO_4	13.62 "	(0.1 mol)
KNO_3	2.00 "	
H_2O	1000 ml.	

The medium was distributed in 250 ml. Pyrex Erlenmeyer flasks, 100 ml. in each flask, and autoclaved for 1/2 hour at 15 pounds steam pressure. 1.0 ml. of concentrated lactic acid was then added to each flask. The flasks were inoculated with 1.0 ml. of a uniform suspension of spores and the mold was allowed to grow at a room temperature of about 20°C.

2. Estimation of Mold Kinase. The Mold Kinase Unit [M.K.U.].— The quantity of mold kinase in any sample is expressed in terms of the velocity with which it transforms crystalline trypsinogen into trypsin under standard conditions.

One mold kinase unit, 1 [M.K.U.], is defined as the amount of kinase that brings about the activation of 0.065 mg. of crystalline trypsinogen at the rate of unity (100 per cent) per unit of time (1 hour) at pH 3.4 and at 35°C.

[2] The isolation and cultivation of single spores was kindly done for us by Dr. Arnold J. Ullstrup of the Department of Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey.

[3] A pure culture of the organism was submitted for identification to Dr. Charles Thom of the U. S. Department of Agriculture at Washington, D. C. Dr. Thom stated that the organism is a member of the genus *Penicillium* belonging to the "very questionable group designated as *lanata-divaricata* with characteristics running all the way from *simplicissimum* through *janthinellum* to *soppi*, the exact strain being very difficult to determine."

Dr. Selman A. Waksman of the State of New Jersey Agricultural Experiment Station kindly tested the organism for production of citric acid, which he found to be negative.

The rational derivation of the definition of the unit is discussed in the section of this paper which deals with the kinetics of formation of trypsin, where a standard method is also described for the determination of the number of [M.K.U.] in a sample of mold kinase. The standard method involves a large number of measurements. For practical purposes the following simplified procedure was developed:

Activation mixture
1.0 ml. sample of kinase, plus
3.5 ml. M/10 citrate buffer pH 3.4, plus
0.5 ml. of stock crystalline trypsinogen in M/200 HCl containing 0.65 mg. protein per ml. (0.1 mg. protein nitrogen per ml.)

The activation mixture is placed for 30 minutes in a water bath at 35°C. 1.0 ml. of the mixture is then added to 5.0 ml. urea-hemoglobin and its active trypsin content, $[T.U.]^{Hb}$, is determined as described by Anson and Mirsky (6). The number of mold kinase units [M.K.U.] per ml. activation mixture corresponding to the number of $[T.U.]^{Hb}$ measured is then read off a standard curve. The standard curve is obtained by plotting the data of $[T.U.]^{Hb}$ *vs.* [M.K.U.] for a series of activation mixtures containing various dilutions of a stock of mold kinase of known [M.K.U.] content, as determined by the standard method.

3. The Rate of Accumulation of Mold Kinase in the Penicillium Culture Medium. —A series of 250 ml. Pyrex Erlenmeyer flasks, each containing 100 ml. of sterilized liquid medium, was inoculated with 1.0 ml. of a uniform suspension of *Penicillium* spores and kept at 20°C. At definite intervals of time, the contents of individual flasks were filtered through dried and weighed Whatman No. 42–9 cm. filter paper. The filtrate in each case was used for analysis for its kinase and phosphate content, while the mold residue on the paper was washed several times with distilled water and then dried on the filter paper for 24 hours at 100°C. and weighed. The phosphate values were used as a basis to correct the data for the concentration of the kinase per milliliter of medium for losses in volume due to evaporation. The pH of the medium rises gradually during the later stage of the growth of the mold even in the presence of M/10 KH_2PO_4. Hence concentrated lactic acid in doses of 1.0 ml. per flask was added whenever indicator tests showed that the pH had risen above 5.6.

Fig. 1 shows that the curve for the growth of the *Penicillium* organism as well as the curve for the accumulation of kinase are of the form typical for growth of microorganisms in a limited amount of nutritive medium. The accumulation of kinase in the medium proceeds initially at a very slow rate as compared with the rate of growth of the mold, but it becomes very rapid at the time when the mold reaches its final growth. The maximum rate of increase of the concentration

of the kinase in the medium coincides approximately with the time of spore formation by the mold. The concentration of kinase in the medium continues to increase for some time even after the mold has stopped growing. Thus there is no constant relation between the rate of growth of the organism and the rate of accumulation of kinase in the medium. A similar observation was made recently by Scribner and Krueger (7) for the rate of growth of phage and bacteria, namely that under certain conditions there is a continuous increase in the concentration of phage in the medium for some time after the bacteria have ceased to multiply.

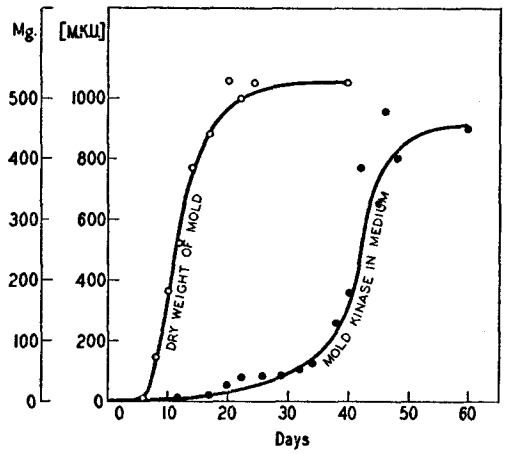

FIG. 1. Rate of formation of trypsinogen kinase in the culture medium of *Penicillium*.

The rate of enzyme formation in cultures of microorganisms depends on experimental conditions and may be either proportional to or independent of the increase in the number of organisms.[4]

4. Stability of Mold Kinase at Various pH.—Mixtures were made of:
1.0 ml. kinase containing 500 [M.K.U.] plus
1.0 ml. of M/10 buffer solutions of various pH, plus
3.0 ml. H_2O. Left at 25°C.
Samples were measured for pH and kinase content immediately after mixing and again after 1.5 hours.

[4] *Cf.* Yudkin, J., *Biol. Rev. Cambridge Phil. Soc.*, 1938, **13**, 93.

The results are given in Fig. 2 which shows that mold kinase is quite stable between pH 2.0 and 6.0 and is very rapidly destroyed at pH 7.0. Mold kinase thus differs from enterokinase which has an optimum stability in a region extending from pH 6.0–8.0 (8).

5. Inactivation of Mold Kinase by Heat.—Mold kinase is completely inactivated within 5 minutes when heated at 70°C. At lower temperatures the inactivation proceeds at a measurable rate. Fig. 3 shows the curves for inactivation at 50°C. and 60°C. of dialyzed mold kinase made up in M/50 KH_2PO_4 and containing about 6 [M.K.U.] per

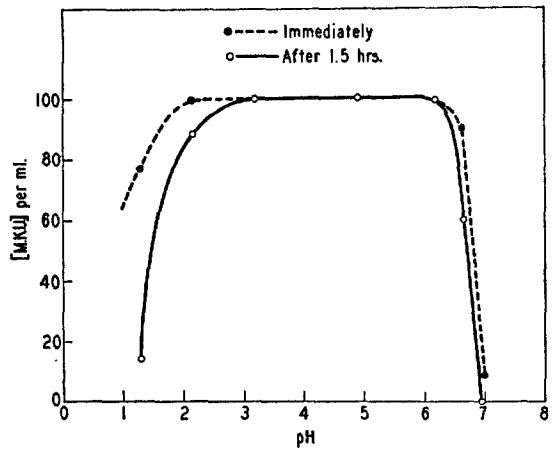

FIG. 2. Stability of mold kinase at various pH.

ml. The inactivation at both temperatures evidently follows a unimolecular course in accordance with the equation

$$-\frac{dM}{dt} = KM \tag{1}$$

which when integrated gives

$$\ln \frac{M_0}{M} = Kt \tag{2}$$

M_0 = initial concentration of kinase
M = concentration of kinase at any time t
K = velocity constant of inactivation

K is the slope of the straight line obtained when values for $\ln \frac{M_0}{M}$ are plotted against corresponding t values. The curves show that the velocity constants for inactivation of mold kinase pH 4.0 at 50°C. and 60°C. are:

$K_{50} = 0.0073$ per minute
$K_{60} = 0.088$ per minute

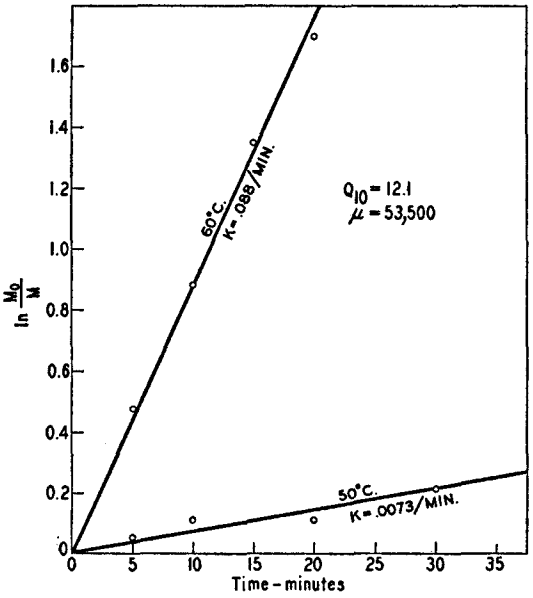

FIG. 3. Rate of inactivation of mold kinase at 50°C. and 60°C. $Q_{10} = 12.1$. $\mu = 53{,}500$ calories per mol.

with a temperature coefficient of $Q_{10} = 12.1$.

Substituting $\ln \frac{K_{60}}{K_{50}}$ in Arrhenius equation

$$\ln \frac{K_2}{K_1} = \frac{\mu(T_2 - T_1)}{2T_1T_2} \tag{3}$$

we get a value for "the critical thermal increment" of inactivation of mold kinase

$\mu_{50-60} = 53{,}500$ calories per mol

which is of the same high order as that obtained for denaturation of proteins and of inactivation of enzymes.

6. Chemical Nature of Mold Kinase. Molecular Volume.—We have not made as yet any serious attempt to isolate the mold kinase in pure form. Preliminary studies showed that the kinase can be purified to some degree by dialysis and fractional precipitation with ammonium sulfate. It is non-volatile and can be concentrated by vacuum distillation at about 35°C.

The rapid inactivation of mold kinase when heated to 70°C. as well as the high temperature coefficient of inactivation suggest a possible protein nature for the molecule of mold kinase which is in agreement with its high molecular volume of 31,000 cm.^{3} and molecular weight of 41,000 gm. (assuming a density of 1.3) as determined by the diffusion method of Northrop and Anson (9). The value of the diffusion coefficient is

$$D = 0.05 \text{ cm.}^2\text{/day at } 10°\text{C.}$$

II. Formation of Trypsin from Crystalline Trypsinogen by Mold Kinase

1. Effect of pH on Rate of Formation.—Activation mixtures were made consisting of:

1.0 ml. mold kinase solution containing 50 [M.K.U.] per ml., plus
3.5 ml. M/10 citrate buffer of various pH, plus
0.5 ml. of 0.065 per cent solution of crystalline trypsinogen in M/200 HCl.

Mixtures were allowed to stand for 30 minutes at 35°C. The amount of formed trypsin in each mixture was then determined by the hemoglobin method.

The results are given in Fig. 4 which shows that mold kinase, unlike enterokinase, transforms trypsinogen into trypsin in an acid medium only, the range of pH favorable for the reaction extending approximately from 2.0–4.5 with a region of optimum rate at about pH 3.4.

2. Effect of Varying the Concentration of Mold Kinase on the Extent of Formation of Trypsin from Trypsinogen.—A series of activation mixtures was made each one containing:

1.0 ml. of stock of 0.065 per cent of crystalline trypsinogen, plus
8.0 ml. of M/10 citrate buffer pH 3.4, plus
1.0 ml. of various concentrations of mold kinase made up in M/10 citrate buffer pH 3.4, the concentrations varying from 0.75 – 7.5 [M.K.U.] per ml.

The mixtures were left at 4°C. Samples of 1.0 ml. of each mixture were tested at various times for tryptic activity.

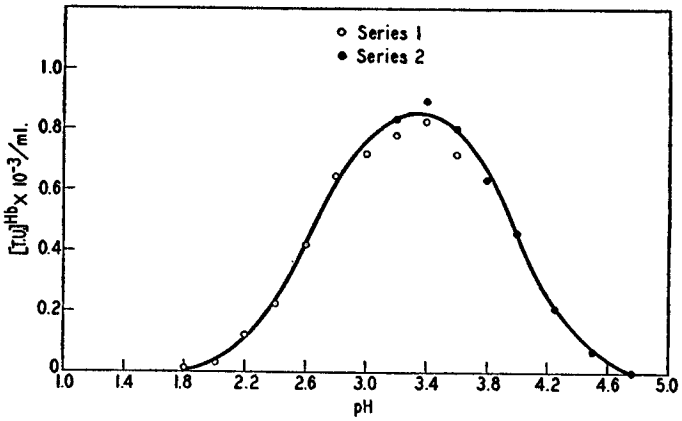

FIG. 4. Effect of pH on rate of activation of crystalline trypsinogen by mold kinase.

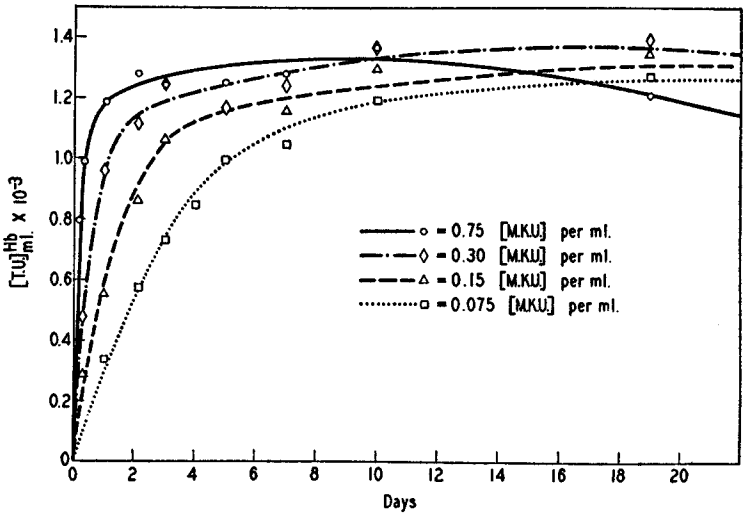

FIG. 5. Effect of kinase concentration on extent of formation of trypsin from crystalline trypsinogen at pH 3.4 and 5°C.

Fig. 5 gives the results of the measurements. It is evident that the rate of formation of trypsin increases with the increase in concentra-

tion of kinase used, but the ultimate amount of trypsin formed is independent of the concentration of kinase added. The same series was repeated with a concentration of trypsinogen 20 times as high as that used in the first series. The result appeared to be the same, thus proving that the kinase in its action resembles a typical catalyst.

3. Is Formation of Trypsin Accompanied by Loss of Protein?—It had been observed that during the process of autocatalytic formation of trypsin from crystalline trypsinogen at pH 7.6 there is a gradual loss of protein as measured by the amount of precipitate formed on addition of trichloracetic acid. This loss may be due either to autolysis of the trypsin formed, which is known to take place readily at pH 7.0 or higher, or to a possible cleavage of the trypsinogen molecule during the reaction. The change of trypsinogen into trypsin by mold kinase at pH 3.4 is accomplished under conditions where no autolysis of the trypsin formed can take place,[5] and any possible loss of protein in the activation mixture consequently could be attributed to a partial hydrolysis of the trypsinogen molecule during the process of activation.

A preliminary experiment showed, however, that there is no measurable loss of protein when crystalline trypsinogen is activated by mold kinase at pH 3.4 and 5°C.

4. Kinetics of Formation of Trypsin. (a) Effect of Varying Concentration of Mold Kinase on the Rate of Reaction.—The formation of trypsin from crystalline trypsinogen by mold kinase at pH 3.4 is not accompanied by any autocatalytic formation of trypsin. This is due to the fact that trypsin is inactive at this pH. The time rate of formation of trypsin by the mold kinase follows the course of a catalytic unimolecular reaction, and is expressed by the differential equation

$$\frac{dA}{dt} = KM(A_e - A) \tag{4}$$

where K equals velocity constant, M is the initial concentration of kinase in the activation mixture which, like any other catalyst, re-

[5] A slight hydrolysis of the trypsin formed takes place if activation proceeds at 35°C. or higher due to the action of a very weak proteolytic enzyme which was found to be associated with the mold kinase; like kinase the optimum pH range of the proteolytic activity is between 3.0–4.0. Its effect on the formed trypsin becomes negligible if activation is accomplished at a temperature of 5–10°C.

mains unchanged during activation, A is the concentration of trypsin in the activation mixture at any time t, and A_e is the final concentration of trypsin when the activation is complete. ($A_e - A$ = concentration of trypsinogen at any time t.) When integrated equation 4 becomes

$$\ln \frac{A_e}{A_e - A} = (KM)t \tag{5}$$

(KM) being the slope of the straight line obtained when the values of $\ln \frac{A_e}{A_e - A}$ are plotted against the corresponding values of t.

Fig. 6 shows the experimental time rate curves for the activation at 35°C. of a definite amount of crystalline trypsinogen by two concentrations of mold kinase in the ratio of 1.0 to 2.5. The experimental points in each case are on a straight line except for the slight deviation of the last few points due to the action of the proteolytic enzyme associated with the kinase (see footnote 5). The slopes of the straight lines are proportional to the concentrations of kinase used, namely

$KM_1 = 0.013$ per minute

$KM_2 = 0.0325$ " "

and

$$\frac{KM_1}{KM_2} = 2.5$$

(*b*) *Effect of Varying the Concentration of Trypsinogen on the Velocity Constant of Formation of Trypsin.*—Equation 5 predicts that for any given concentration of kinase the velocity constant K should be independent of the magnitude of the initial concentration of trypsinogen used. Actually, this prediction does not hold true as has been found to be the case with a large number of other enzymes such as urease (10), trypsin (11), invertase (12), etc.

Fig. 7 shows the experimental curves for the rate of activation of two different amounts of crystalline trypsinogen by mold kinase at pH 3.4 and 6°C., the concentration of mold kinase used being identical in both cases. There is a decrease in the velocity constant as the

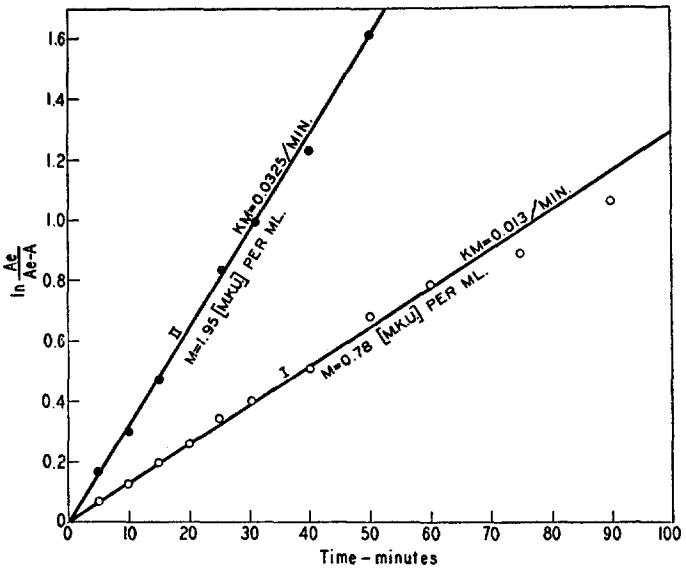

FIG. 6. Rate of formation of trypsin from crystalline trypsinogen by mold kinase. Concentration of trypsinogen = 0.01 mg. protein nitrogen per ml.

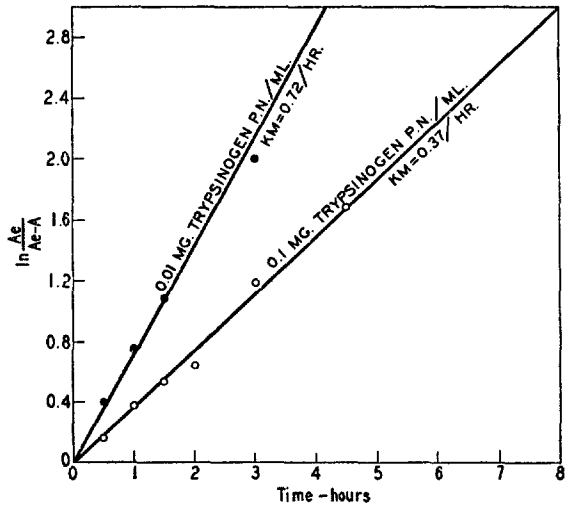

FIG. 7. Effect of concentration of trypsinogen on the velocity constant of the formation of trypsin by mold kinase.

concentration of trypsinogen used is increased. The velocity constant is reduced 50 per cent for a tenfold increase in initial concentration of trypsinogen.

Activation mixtures used:
14.0 ml. M/10 citrate buffer pH 3.4, plus
4.0 ml. solution of mold kinase containing 15 (M.K.U.) per ml., plus
2.0 ml. solution of crystalline trypsinogen in M/200 HCl containing either 1.0 or 0.1 mg. protein nitrogen per ml.
Mixed cold and left at 6°C. Samples tested for tryptic activity at various intervals of time.

(*c*) *The Equation for the Kinetics of Formation of Trypsin As a Basis for the Definition of the Mold Kinase Unit* [*M.K.U.*].—The numerical value of the velocity constant K for the rate of formation of trypsin from crystalline trypsinogen by mold kinase as given in equation 4 depends on the value of the unit chosen to express the magnitude of the kinase concentration M. K can be made equal to unity by choosing the appropriate unit for M. Writing equation 4 in the form of

$$-\frac{dG}{G\,dt} = KM$$

where G is the concentration of trypsinogen at any time t and putting $K = 1$, we get

$$-\frac{dG}{G\,dt} = M$$

M equals unity when

$$-\frac{dG}{G\,dt} = 1$$

i.e., when the activation proceeds at the rate of 100 per cent per unit of time (under specified conditions of temperature, pH, and initial concentration of trypsinogen). The standard activation mixture used in our studies consisted of the following:

1.0 ml. of a solution of crystalline trypsinogen in M/200 HCl containing 0.1 mg. protein nitrogen per ml., plus
2.0 ml. of solution of kinase pH 3.4, plus
7.0 ml. of M/10 citrate buffer pH 3.4.

Activation was allowed to proceed at 35°C. The hour was taken as the unit of time. The definition of the mold kinase unit under these conditions is as follows: One mold kinase unit, 1 [M.K.U.], is the amount of kinase that brings about activation of 0.065 mg. crystalline trypsinogen (0.01 mg. protein nitrogen) at pH 3.4 and 35°C. at the rate of 100 per cent per hour.

The standard method of determining the concentration of kinase in the activation mixture in [M.K.U.] per ml. consists in measuring the tryptic activity of the mixture at various intervals and then plotting the values of $\ln \frac{A_e}{A_e - A}$ against time. The slope of the straight line drawn through the plotted points gives the concentration of kinase in [M.K.U.] per ml. of activation mixture. Thus in Fig. 6, where the data plotted have been obtained under the standard conditions mentioned before, the slope of line I is 0.013 per minute or 0.78 per hour, and the slope of II is 0.0325 per minute or 1.95 per hour; the concentrations of mold kinase in the activation mixtures are then 0.78 [M.K.U.] per ml. and 1.95 [M.K.U.] per ml. correspondingly. It is evident that this method of measuring kinase activity involves the determination of tryptic activity of several samples. As a general routine, only the 30 minute sample is measured and the concentration of kinase corresponding to the found value of A is read off a standard curve described before. It can also be calculated by substituting A in the equation

$$\ln \frac{A_e}{A_e - A} = 0.5\,M$$

The value of A_e is readily determinable for any stock of trypsinogen by using an excess of kinase in the activation mixture.

(*d*) *Effect of Temperature on the Rate of Formation of Trypsin.*— Fig. 8 shows the curves for the rate of formation of trypsin from crystalline trypsinogen by mold kinase at various temperatures. The activation mixtures used were of the standard type containing 0.01 mg. trypsinogen protein nitrogen and 0.75 [M.K.U.] per ml. The plotted curves for $\ln \frac{A_e}{A_e - A}$ are all straight lines. The velocity constants were obtained by dividing the values for the slopes of the lines by the value of $M = 0.75$. When plotted against the reciprocals of the corresponding absolute temperatures the values of $\ln K$ do *not*

fall in a straight line, as shown in Fig. 9. The calculated temperature coefficients Q_{10} as well as the values for μ are given in Table I. The temperature coefficient Q_{10} for the range of 25–35°C. is much lower

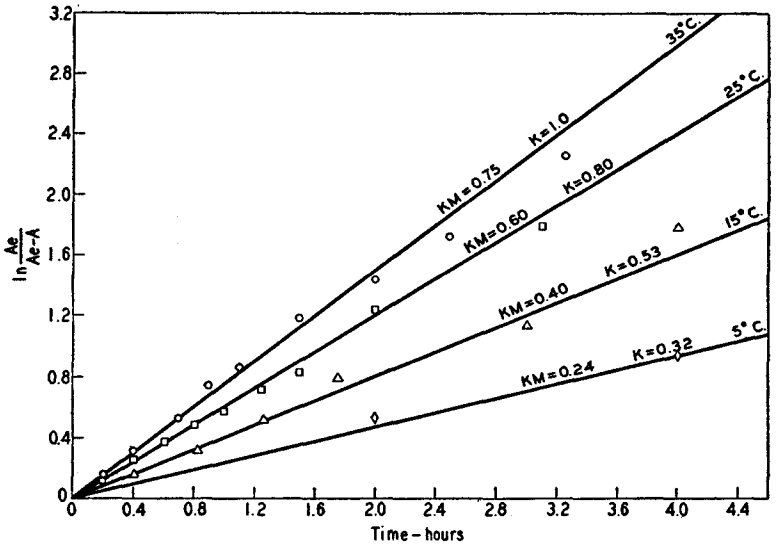

FIG. 8. Effect of temperature on the rate of formation of trypsin by mold kinase.

TABLE I

Temperature Coefficient for the Rate of Formation of Trypsin

Temperature	K	Q_{10}	μ
°C.	*per hour*		*calories per mol*
5	0.32		
5–15		1.66	8,100
15	0.53		
15–25		1.51	7,000
25	0.80		
25–35		1.25	4,250
35	1.00		

than the temperature coefficient of about 1.8 found for the rate of digestion of hemoglobin by pepsin (13) or trypsin (14).

The values of μ obtained here for the formation of trypsin compare

in magnitude with those obtained for hydrolysis of ethyl butyrate by pancreatic lipase (15) as well as for the decomposition of hydrogen peroxide by liver catalase (16).

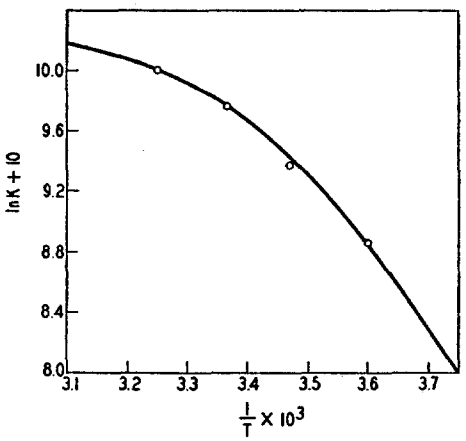

FIG. 9. Velocity constant of activation *vs.* reciprocal of absolute temperature.

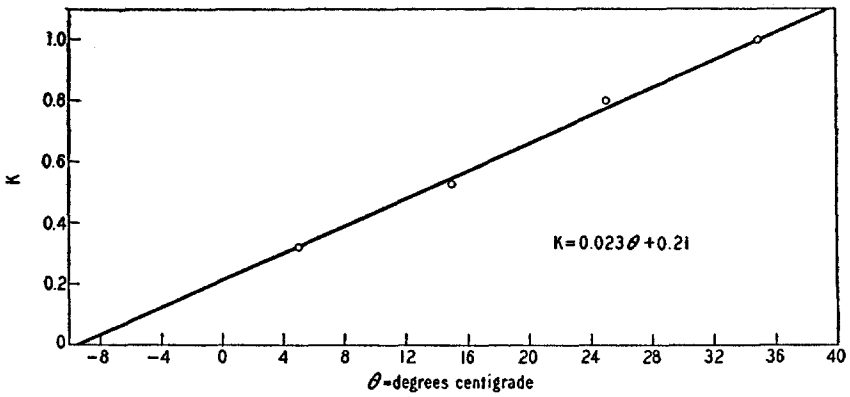

FIG. 10. Linear relation between temperature and velocity constant of activation.

It is to be observed that when the velocity constants for the rate of activation of crystalline trypsinogen by mold kinase are plotted against temperature directly they fall in a straight line (Fig. 10) the equation of which is

$$K = 0.023\theta + 0.21 \qquad (6)$$

where θ = temperature in degrees centigrade. The velocity constant thus appears to increase by a definite amount of 0.023 for a one degree rise in temperature. Equation 6 can be readily changed into the convenient formula

$$K = K_0(1 + 0.11\,\theta)$$

where K_0 is the value of K at 0°C. = 0.21.

5. Preparation of Crystalline Trypsin from Trypsinogen by Mold Kinase.—30 ml. of dialyzed crystalline trypsinogen containing 10 mg. protein nitrogen per ml. were mixed with 120 ml. M/10 citrate buffer pH 3.4 and 6 ml. of solution of mold kinase containing 2,000 [M.K.U.] per ml. The mixture was left at 5°C. for 18 hours. Solid ammonium sulfate was then added so as to bring the solution to 0.7 saturation. The precipitate formed was filtered with suction and washed with saturated magnesium sulfate in N/50 sulfuric acid at room temperature. Filter cake (7.5 gm.) was dissolved in 7.5 ml. ice cold 0.4 M borate buffer pH 9.0 and 7.5 ml. saturated magnesium sulfate added. Solution was left at 5°C. Crystals of typical trypsin needles began to appear rapidly, and the crystallization was complete in a few hours. The crystals were filtered after 24 hours and the yield was 3 gm. of crystal cake of a specific activity of 0.19 $[T.U.]^{Hb}_{mg.P.N.}$ which is the same as found for the purest trypsin crystals formed from crystalline trypsinogen by autocatalysis (17).

The solubility of the new crystalline trypsin in saturated magnesium sulfate at 10°C. is the same as that of the trypsin formed from trypsinogen by autocatalysis and a solution saturated with the crystals of either one of the trypsin preparations does not dissolve any crystals of the other kind, thus indicating the identity of the two preparations. The new preparation of the crystalline trypsin gives a solubility curve with saturated magnesium sulfate typical of a pure substance, as shown in Fig. 11. Since the trypsin formed from trypsinogen by the mold kinase is the same as that formed by trypsin the mold kinase must cause the same change in the molecule of trypsinogen as does trypsin.

III. Activation of Chymo-Trypsinogen by Penicillium Kinase

In addition to its powerful activating effect on trypsinogen mold kinase appears to have a slight activating effect on chymo-trypsinogen. The rate of activation of chymo-trypsinogen is only about 2 per cent of that of trypsinogen. Preliminary experiments indicate that the

optimum pH range for activation of chymo-trypsinogen by the mold kinase is the same as for activation of trypsinogen. The pH range of stability of the kinase is also the same with respect to activation of either chymo-trypsinogen or trypsinogen. It thus appears that both kinase activities are associated with one and the same substance.

Methods

1. Preparation of Crystalline Trypsinogen.—Method of Kunitz and Northrop (18).

2. Protein Nitrogen by Turbidity.—5 ml. of sample are mixed in 150 × 15 mm. test tube with 5 ml. 5 per cent trichloracetic acid made up in 0.25 saturated ammonium sulfate. The mixture is allowed to stand at least 5 minutes at 20°C. and then placed for 10 minutes in a water bath at 85°C. The test tube is immersed

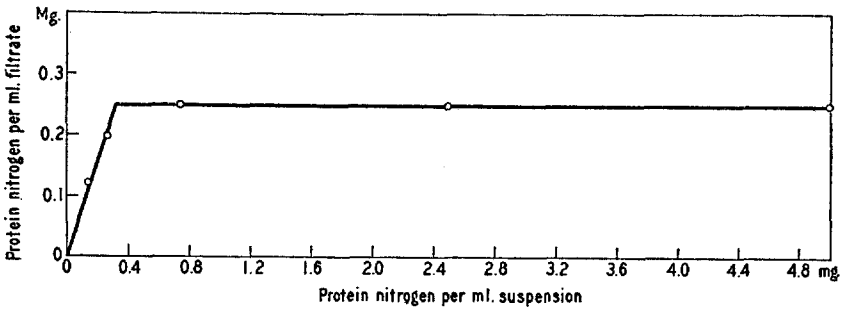

FIG. 11. Solubility of crystalline trypsin in saturated magnesium sulfate pH 4.0 at 10°C. in the presence of increasing quantities of solid phase.

in the hot water to a depth about 2.0–5.00 mm. above the level of the liquid inside the tube and is loosely stoppered during the heating. The tube is stoppered tightly immediately after removal from the bath and left to cool to room temperature. The turbidity of the well mixed suspension is determined in a Klett photoelectric colorimeter (19) against a suspension of the same protein material of a known protein nitrogen concentration as determined by Kjeldahl analysis. A more convenient way is to draw a calibration curve for the turbidity of several concentrations of the standard solution as read against a glass disk or a solution of M/25 copper sulfate in M/10 sulfuric acid. This avoids the necessity of preparing fresh standards. The range is from 0.001–0.005 mg. protein nitrogen per ml. suspension.

3. Tryptic Activity Determinations.—The tryptic activity was measured by the rate of digestion of hemoglobin as described by Anson and Mirsky (6) except that the suspension after the addition of trichloracetic acid was allowed to stand 1/2 hour before filtering. 2.0 ml. of 1 per cent of merthiolate in 1.4 per cent borax

were added to each liter of stock of hemoglobin solution, as a preservative, instead of toluene. The latter has been found to interfere with the measurement of tryptic activity.

4. Phosphorus Determination.—The colorimetric method of Fiske and Subbarow (20).

SUMMARY

1. A powerful kinase which changes trypsinogen to trypsin was found to be present in the synthetic liquid culture medium of a mold of the genus *Penicillium*.

2. The concentration of kinase in the medium is increased gradually during the growth of the mold organism and continues to increase for some time even after the mold has ceased growing.

3. Mold kinase transforms trypsinogen to trypsin only in an acid medium. It differs thus from enterokinase and trypsin which activate trypsinogen best in a slightly alkaline medium.

4. The action of the mold kinase in the process of transformation of trypsinogen is that of a typical enzyme. The process follows the course of a catalytic unimolecular reaction, the rate of formation of a definite amount of trypsin being proportional to the concentration of kinase added. The ultimate amount of trypsin formed, however, is independent of the concentration of kinase used.

5. The formation of trypsin from trypsinogen by mold kinase is not accompanied by any measurable loss of protein.

6. The temperature coefficient of formation of trypsin from trypsinogen by mold kinase varies from $Q_{5-15} = 1.70$ to $Q_{25-30} = 1.25$ with a corresponding variation in the value of μ from 8100 to 4250.

7. Trypsin formed from trypsinogen by means of mold kinase is identical in crystalline form with the crystalline trypsin obtained by spontaneous autocatalytic activation of trypsinogen at pH 8.0. The two products have within the experimental error the same solubility and specific activity. A solution saturated with the crystals of either one of the trypsin preparations does not show any increase in protein concentration or activity when crystals of the other trypsin preparation are added.

8. The *Penicillium* mold kinase has a slight activating effect on chymo-trypsinogen the rate being only 1–2 per cent of that of trypsinogen. The activation, as in the case of trypsinogen, takes place only in an acid medium.

9. Mold kinase is rapidly destroyed when brought to pH 6.5 or higher, and also when heated to 70°C. In the temperature range of 50–60°C. the inactivation of kinase follows a unimolecular course with a temperature coefficient of $Q_{10} = 12.1$ and $\mu = 53{,}500$. The molecular weight of mold kinase, as determined by diffusion, is 40,000.

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