STUDIES ON A SARCOSINE OXIDASE OF BACTERIAL ORIGIN

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Few studies have been carried out on amino acid oxidases of bacterial origin. Stumpf and Green (1944) described an *l*-amino acid oxidase of *Proteus vulgaris*, the specificity of which could not be sharply defined due to the difficulties involved in its separation and purification. No data on the action of this enzyme on glycine and sarcosine are reported. Ratner, Nocito, and Green (1944) discovered in mammalian livers an oxidase attacking both these substrates by oxidative deamination. From the same source, Handler, Bernheim, and Klein (1941) extracted a specific sarcosine oxidase, capable of breaking down sarcosine to formaldehyde and glycine.

It was found by Kopper and Robin (1950) that a sarcosine oxidase, identical in mode of action, occurred in an atypical strain of *Pseudomonas aeruginosa* characterized by its ability to decompose creatinine (Kopper, 1947). The present study was undertaken to obtain information about the general properties and behavior pattern of this enzyme.

EXPERIMENTAL

1. Preparation of the "Enzyme Solution"

The organisms were grown in *coli*-flasks on meat extract agar in which 0.5 per cent creatinine had been incorporated. After 72 hours' incubation at room temperature they were washed off with distilled water and centrifuged. Following two subsequent washings and centrifugations, they were suspended in 2 volumes of 50 per cent toluene and allowed to autolyze for 1 hour. The autolysate was centrifuged and the supernatant discarded.

Attempts were made to purify the enzyme present in the crude bacterial autolysate. Drying over H_2SO_4 and lyophilization inactivated the enzyme completely. Its activity was also lost upon dialysis against distilled water for 48 hours and could not be restored by adding to the dialysate undialyzed bacterial protein heated at 55°C. for 10 minutes. Dialysis against dilute phosphate buffer of pH 7 for 96 hours did not result in loss of enzyme activity.

Autolyzed *Pseudomonas* cells were suspended in 4 volumes of distilled water, allowed to stand at 5°C. overnight, and centrifuged. This procedure was repeated on 3 successive days. It was found that most of the activity was retained in the super-

natant taken off on the 2nd day and that the residue of the 3rd day was completely inactive. The supernatant, an opalescent particle suspension, seemed to resemble Stumpf and Green's cell-free *l*-amino acid oxidase preparation, which had been obtained by fractional centrifugation of bacteria disintegrated by exposure to ultrasonic vibrations, but, unlike the *Proteus* enzyme, it tended to become rapidly inactivated.

In view of this experience it was decided to use the unpurified bacterial autolysate. When kept at 5°C. it remained active for about 2 weeks; however, because of a gradual decrease in activity, it was used only for 1 week following its preparation. Always immediately prior to use a 1/10 dilution in phosphate buffer was prepared. This will be referred to as the "enzyme solution" or "sarcosine oxidase."

2. *Materials and Methods*

Eastman-Kodak's sarcosine hydrochloride was used. An amount of 141 mg., equivalent to 100 mg. of sarcosine, was dissolved in enough NaOH to neutralize it, and then made up to a volume of 10 ml. with distilled water. Dilutions were prepared from this stock solution.

Enzyme activity was measured by the amount of formaldehyde produced rather than by the quantity of oxygen consumed. Eastman-Kodak's reagent, 1,8-dihydroxynaphthalene-3,6-disulfonic acid (chromotropic acid), was selected as most suitable for determining small quantities of HCHO, in accordance with the method outlined by MacFadyen (1945). This test allows the accurate determination of as little as 0.1 μg. of HCHO. Color intensities were read in a Leitz photoelectric colorimeter (C filter) and compared with standards obtained with known amounts of HCHO.

The basic type of experiment, before the introduction of variables, was conducted as follows: In test tubes of 1/2 inch diameter, 200 μg. of sarcosine and 0.1 ml. of enzyme solution were mixed and made up to 1 ml. with M/15 phosphate buffer of pH 7. The tubes were placed in a 37°C. water bath and shaken at 275 to 285 oscillations per minute for 1 hour. To each tube, 0.2 ml. of a 6 N H_2SO_4 solution was then added, which caused precipitation of the bacterial protein. This was removed by centrifugation. To the supernatants, 9 volumes of chromotropic acid solution were added, the mixtures placed in a boiling water bath for 30 minutes, and the resulting colors read. The amount of HCHO obtained from 200 μg. of sarcosine fluctuated between 21 and 35 μg., which corresponds to 31 to 52 per cent substrate decomposition.

An alternative method frequently employed in estimating the activity of oxidizing enzymes is the determination of the reduction time of methylene blue in evacuated Thunberg tubes. Decolorization of methylene blue by sarcosine oxidase in the presence of the substrate was observed, but no trace of HCHO could be detected when the tubes were opened and aliquots tested with chromotropic acid. It was believed at first that HCHO, under anaerobic conditions, might be oxidized in turn by an enzyme present in the unpurified bacterial autolysate; but the latter, in the presence of HCHO, failed to reduce methylene blue to its leuco base even after 1 hour's incubation. Upon addition of reducing agents, such as sodium cyanide and cysteine, however, HCHO would disappear in evacuated Thunberg tubes, regardless of the presence or absence of enzyme solution and methylene blue. Sarcosine, when acted upon by sarcosine oxidase, also becomes a reducing agent; this might explain the absence of HCHO as a breakdown product under anaerobic conditions.

The results reported in this study were all obtained by means of the aerobic technique.

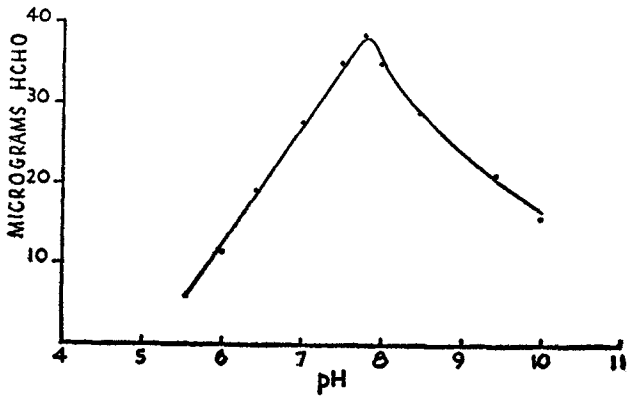

FIG. 1. Effect of pH on the activity of sarcosine oxidase.

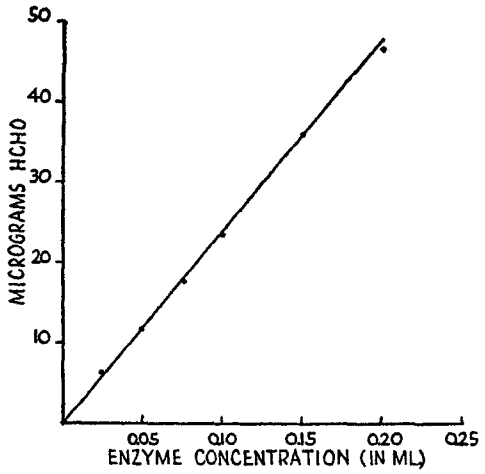

FIG. 2. Relation of enzyme concentration to the activity of sarcosine oxidase.

RESULTS

The pH activity curve of sarcosine oxidase is shown in Fig. 1. Sørensen's M/15 phosphate buffer and Clark and Lub's M/5 borate buffer solutions were prepared and the experiments set up in the usual way. The pH optimum is reached at 7.8. The curve resembles that obtained by Handler et al. (1941) for animal sarcosine oxidase.

The effect of enzyme concentration on substrate decomposition is presented in Fig. 2. As with many enzymes, a direct relationship exists.

Fig. 3 shows the relation of substrate concentration to enzyme activity.

As with *Proteus* *l*-amino acid oxidase, a point is soon reached beyond which no further increase in activity occurs. The reason for this must be sought in the saturation of enzyme by substrate molecules.

Michaelis and Menten (1913) worked out general rate laws for the action of invertase on sucrose by assuming a chemical combination of the enzyme with its substrate as the governing step in the hydrolysis of the sugar. The enzyme-substrate equilibrium can be represented by the equation:

$$k_s = \frac{(E)(S)}{(ES)}$$

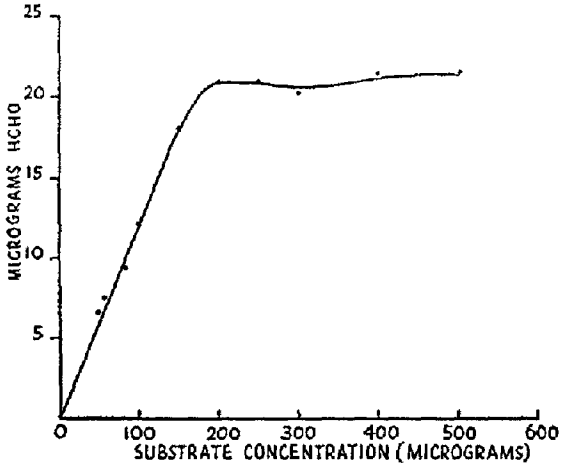

FIG. 3. Relation of substrate concentration to the activity of sarcosine oxidase.

The constant k_s could be determined by simple mathematical calculation leading to the equation:

$$\frac{v}{V_m} = \frac{(S)}{k_s + (S)} \quad \text{or} \quad k_s = (S)\left(\frac{V_m}{v} - 1\right) \tag{1}$$

in which v represents the initial velocity at the substrate concentration (S) V_m the maximum velocity, k_s, therefore, being equivalent to the substrate concentration at which half the limiting velocity is reached. Lineweaver and Burk (1934) developed graphic methods for determining dissociation constants of enzyme-substrate compounds. Equation (1) can be written:

$$\frac{1}{v} = \frac{k_s}{V_m(S)} + \frac{1}{V_m} \tag{2}$$

V_m and k_s are constants. A plot of $1/v$ against $1/S$ must, therefore, give a straight line, if one molecule of enzyme combines with one molecule of sub-

strate. The intercept of this line on the $1/v$ axis is $1/V_m$ and its slope k_s/V_m. Thus the constants are easily determined. Fig. 4 gives the results for sarcosine oxidase. From this graph V_m is calculated as 1.75 μg. of HCHO and k_s as 146.7 μg. of sarcosine.

When equation (2) is multiplied by (S), it assumes the form:

$$\frac{(S)}{v} = \frac{k_s}{V_m} + \frac{(S)}{V_m} \tag{3}$$

By plotting S/v against S a straight line is again obtained (Fig. 5). The inter-

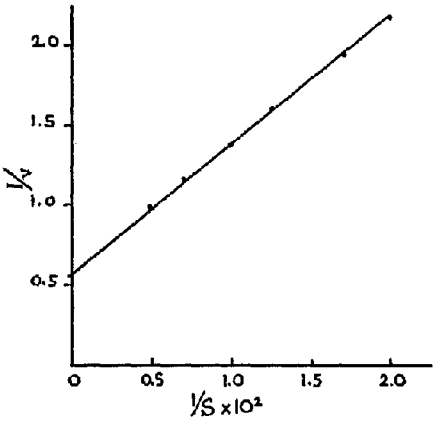

FIG. 4

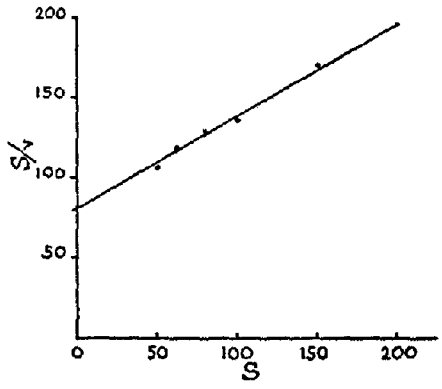

FIG. 5

FIGS. 4 and 5. Tests of the sarcosine oxidase–sarcosine intermediate. S = substrate concentration; v = the initial velocity, that is the amount of HCHO produced per minute, as determined by averaging values obtained from experiments run for 30, 40, and 50 minutes.

cept on the S/v axis is k_s/V_m and the slope $1/V_m$. The values derived from this graph are: $V_m = 1.81$, $k_s = 152.5$. The importance of this plot is not only to check the values obtained from Fig. 4, but also to discover any departure from a straight line, which would be due to substrate inhibition. The results indicate that such inhibition does not occur and that enzyme and substrate combine in equimolecular proportion.

The effect of temperature on enzyme activity is shown in Fig. 6. These experiments were run for only 15 minutes. Over a longer period of time inactivation at the higher temperatures becomes appreciable.

To determine the rate of enzyme destruction at about 50°C., the following experiments were set up. Test tubes containing 0.1 ml. of enzyme solution were immersed in a water bath at the desired temperature for varying lengths of time. They were then immediately placed in ice water to check further

destruction of the enzyme. The residual activity of the enzyme was determined. The rates of heat inactivation of sarcosine oxidase at 48° and 52°C. are presented in Fig. 7.

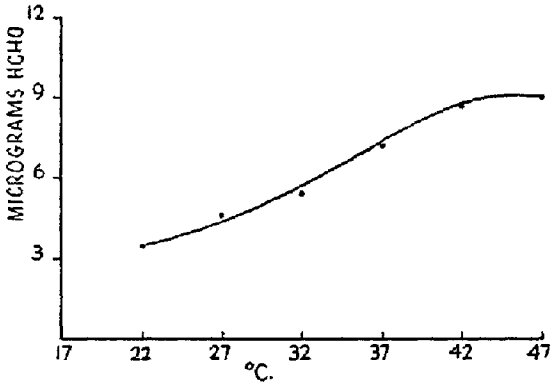

FIG. 6. Effect of temperature on the activity of sarcosine oxidase.

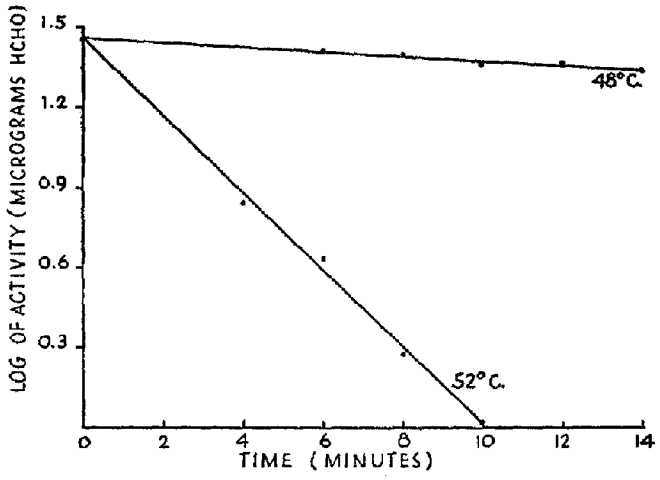

FIG. 7. Rate of heat inactivation of sarcosine oxidase.

The thermal inactivation of the enzyme follows the equation of a first order reaction:

$$2.3 \log A_0/A = kt$$

where A_0 is the activity of the unheated enzyme solution, A the activity of the enzyme heated for time t, and k the constant of heat inactivation. The average values for k are 0.0218 at 48°C. and 0.1475 at 52°C. This considerable

TABLE I

Relation of Enzyme Concentration to Rate of Heat Inactivation of Sarcosine Oxidase

Temperature of preheating	Time of preheating	Inactivation with enzyme concentration			
		1/10	1/13	1/20	1/40
°C.	*min.*	*per cent*	*per cent*	*per cent*	*per cent*
48	14	7.2	6.7	11.2	20.0
	16	16.4	18.1	33.4	40.0
	18	25.0	24.8	41.3	54.3
52	4	78.2	77.8	87.3	100.0
	6	85.0	87.6	100.0	100.0
	8	91.1	92.4	100.0	100.0

TABLE II

Inhibition of Sarcosine Oxidase by Chemical Compounds

Chemical compound	Concentration	Inhibition
		per cent
Copper sulfate	M/10,000	100.0
Silver nitrate	M/10,000	100.0
Mercuric chloride	M/10,000	100.0
Sodium cyanide	M/1000	100.0
	M/10,000	66.7
Cysteine	M/1000	100.0
	M/10,000	21.7
Sodium sulfide	M/100	71.0
	M/1000	13.0
Sodium sulfite	M/100	61.2
	M/1000	23.1
Sodium benzoate	M/100	100.0
	M/1000	63.8
Sodium fluoride	M/100	16.0
Sulfanilamide	M/100	35.0

change over a narrow temperature range is characteristic of enzyme inactivation as well as protein denaturation.

Destruction rates are usually considered in their relation to the correspond-

ing heats of enzyme inactivation or critical thermal increments. These can be calculated with the aid of the van't Hoff-Arrhenius equation

$$\frac{d \ln k}{dt} = \frac{\Delta H}{RT^2}$$

Integrated between the limits T_2 and T_1, this equation assumes the form:

$$\ln \frac{k_2}{k_1} = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

Since $k_1 = 0.0218$, $k_2 = 0.1475$, $T_1 = 321°$, $T_2 = 325°$, and $R = 1.99$ calories, the value of ΔH can be calculated as 103,000 calories per mol.

Casey and Laidler (1950) have pointed out that rates of heat inactivation of pepsin are dependent on enzyme activity at low enzyme concentrations. It seemed of interest to determine whether their observations would also be valid for sarcosine oxidase. Amounts of 0.1 ml. of 1/10, 1/13, 1/20, and 1/40 dilutions of the bacterial autolysate were exposed to different temperatures for varying lengths of time and their residual activity was measured. Table I summarizes the results obtained. It is to be noted that, while the values for enzyme concentrations 1/10 and 1/13 are practically identical, further dilution of the enzyme is associated with progressively higher rates of heat inactivation.

Table II presents the results of experiments on inactivation of sarcosine oxidase by various chemical substances. The enzyme is inhibited by heavy metal salts, reducing agents and benzoate, and to a lesser degree by fluoride and sulfanilamide.

DISCUSSION

Pseudomonas sarcosine oxidase displays a behavior pattern similar to that of *Proteus* l-amino acid oxidase. Both these enzymes, unlike animal amino acid oxidases, do not seem to require a dialyzable cofactor for action. The observed loss of activity of the *Pseudomonas* enzyme through continuous perfusion with distilled water could possibly be due to a shift of pH to a level at which a more rapid inactivation would take place.

The observation made in this study that HCHO disappears in the presence of reducing agents under anaerobic conditions may explain a phenomenon reported in a previous communication (Kopper and Robin, 1950), in which it was pointed out that decomposition of sarcosine by resting cells of atypical *P. aeruginosa* failed to yield any HCHO. It may be assumed that live bacteria are capable of disposing of HCHO through reduction, a process, non-enzymic in character, made possible by the high reduction potential characteristic of living cells. Further experimental work to verify this hypothesis is planned.

SUMMARY

A "sarcosine oxidase" was prepared from a creatinine-decomposing strain of *Pseudomonas aeruginosa*.

The enzyme is inactivated by drying, lyophilization, and dialysis against distilled water. No dialyzable cofactor was found.

Optimal activity of the enzyme is reached at pH 7.8. Enzyme activity is directly proportional to enzyme concentration and also to substrate concentration up to the point of saturation of enzyme with substrate molecules. One molecule of enzyme combines with one molecule of substrate.

Data concerning the effect of temperature and of a variety of chemical compounds on the enzyme are presented.

Its inactivation by heat follows the course of a first order reaction, and the critical thermal increment between 48° and 52°C. was calculated to be 103,000 calories per mol. The relationship of enzyme concentration to heat inactivation rates is illustrated.

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