SPERMINE OXIDASE: AN AMINE OXIDASE WITH SPECIFICITY FOR SPERMINE AND SPERMIDINE

By JAMES G. HIRSCH,* M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, September 25, 1952)

Spermine, a polyamine widely distributed in animal tissues, exhibits potent antimycobacterial activity under certain conditions *in vitro* (1). Of numerous other biological and synthetic amines, only the closely related compound spermidine affects the growth of tubercle bacilli like spermine (2). Recent studies have demonstrated that spermine and spermidine exert a toxic action on acid-fast bacteria only when a certain protein substance is also present in the culture medium (3). This protein, which is found in the alpha globulin fraction of bovine and sheep serum, alters spermine enzymatically; apparently some product of the enzymatic reaction is responsible for the antimycobacterial activity.

The present communication presents data concerning the characteristics of the enzymatic attack on spermine as reflected by the oxygen consumption and the ammonia liberation resulting from this reaction under a variety of conditions. These data establish the presence in bovine and sheep sera of an enzyme which brings about a rapid oxidative deamination of spermine and spermidine. The enzyme differs from previously described amine oxidases in several regards and especially in its substrate specificity. Studies of the influence of the chemical environment on the course of the enzymatic reaction have provided information relating to the characteristics of the enzyme and to the kinetics of its oxidation of spermine.

Methods

The sera used as a source of enzyme were obtained as follows. Fresh blood was allowed to clot firmly at room temperature. The clotted blood was incubated at 38°C. for 1 hour at the end of which time the clot was separated from the walls of the container. After standing overnight at 4°C., the serum was collected by centrifugation and was adjusted to pH 6.8 by the addition of 0.1 N HCl. The serum was then sterilized by filtration through porcelain and stored at 4°C.

Chemicals were obtained from commercial sources except as otherwise noted in the text. Amines or their salts were dissolved in distilled water and adjusted approximately to neutrality with NaOH or HCl. Appropriate dilutions of these stock solutions in distilled water were made to yield the desired concentrations.

* Fellow (1951–52) of the National Tuberculosis Association, administered by the National Research Council

Measurements of oxygen uptake were obtained in the Warburg respirometer using techniques outlined by previous workers (4). Reaction vessels of approximately 16 cc. capacity were employed. The final fluid volume in each flask was 4.2 cc. and the gas phase was room air. Carbon dioxide was removed from the system by placing 0.2 cc. of 10 per cent KOH distributed on a strip of fluted filter paper in the center well of each vessel. The substrate or the enzyme preparation was added from a side arm after an appropriate period of equilibration. Each series of tests included control flasks in which the substrate or the enzyme was omitted or inactivated. Except as otherwise noted in the text, the flasks were incubated at 38°C. and agitated in a 3 cm. linear path 100 times per minute.

Ammonia liberated during the enzymatic reaction was determined by a method devised by Hotchkiss (5). Accurately measured 2 cc. aliquots of the residual reaction mixtures in the Warburg vessels were transferred to 10 cc. volumetric flasks. After the addition of one drop of a 1 per cent alcoholic solution of phenolphthalein, a solution containing 16 mg. of sodium carbonate per cc. was added dropwise until a persistent pink color appeared. To this mixture was added 1 cc. of carbonate buffer (2.12 gm. dry sodium carbonate and 8.4 gm. sodium bicarbonate in 240 cc. water). Glass tubes approximately 0.5 cm. in diameter and 7 cm. in length were fitted with a bulb positioned about 1 cm. from one end. This bulb was of a size sufficient to be supported by the rim of the 10 cc. volumetric flask. Both ends of the glass tubing were left open. Holding the tube by the bulb, the long end was dipped into a 1 N solution of sulfuric acid. On withdrawal a drop of the acid was retained inside the tip of the tube. The outside was wiped clean and the tube was then placed in the volumetric flask so that the lower end containing the acid rested slightly above the mixture in the flask. Distillation of the ammonia was complete after standing 24 hours in a container which excluded room air. The tubes were then carefully removed and their contents washed with distilled water into clean 10 cc. volumetric flasks. 0.2 cc. of 1 N sulfuric acid was added and the flasks were filled nearly to the mark with distilled water. After the addition of 0.5 cc. of Nessler's reagent, the flasks were filled to the mark with distilled water, the contents were then mixed, and the color developed for 15 minutes. The optical densities were determined at 450 mμ in a Coleman Jr. spectrophotometer and compared with the optical densities of blanks and of standard solutions of ammonium sulfate.

RESULTS

Oxygen Consumption and Ammonia Liberation during the Incubation of Spermine and Certain Animal Sera

In the preceding report (3) it was shown that sheep and bovine serum contained a protein substance which rendered spermine inhibitory for the growth of tubercle bacilli *in vitro*, and that spermine was altered chemically during incubation with these sera. Serum from man, rabbit, and guinea pig did not manifest similar activity.

Using techniques described above, experiments were performed to measure oxygen uptake and ammonia liberation resulting from the incubation of spermine and sera from various animals. As is seen in Table I, the reaction between spermine and sheep serum or bovine serum was associated with oxidation and deamination. Under similar conditions, mixtures of human, rabbit, or guinea pig sera and spermine did not consume oxygen or release ammonia. Chemical tests for residual spermine at the conclusion of the reaction period established that the oxidation and deamination, when they occurred, were correlated with chemical alteration in the spermine molecule.

The Substrate Specificity of the Amine Oxidase Present in Sheep Serum

Crude preparations of diamine oxidase are known to be capable of attacking spermine (6). Experiments were therefore performed to determine the relationship of the amine oxidase present in sheep serum to the recognized monoamine and diamine oxidases.

TABLE I

The Oxidative Deamination of Spermine in the Presence of Various Serum Preparations

5×10^{-4} M spermine in 0.025 M phosphate buffer pH 6.8 containing	Oxygen consumed*	Ammonia produced*	Residual spermine‡*
10 per cent sheep serum	+	+	0
" " " bovine "	+	+	0
" " " human "	0	0	+
" " " rabbit "	0	0	+
" " " guinea pig serum	0	0	+
No serum	0	0	+

* After incubation at 38°C. for 1 hour.

‡ Estimated by picrate precipitation.

TABLE II

The Substrate Specificity of the Amine Oxidase Present in Sheep Serum

12–25 μl. O_2 consumed per μM during 1st hr.*		
Spermine		
Spermidine		
0–2.5 μl. O_2 consumed per μM during 1st hr.*		
Tyramine	Putrescine	Diethylenetriamine
β-Phenylethylamine	Cadaverine	Triethylenetetramine
n-Propylamine	Histamine	Tetraethylenepentamine
n-Butylamine	1,2-Propanediamine	γ-Aminopropyl-*N*-pyrroline
Di-*n*-butylamine	1,3-Propanediamine	Piperazine
Thiamine	*N*,*N*-Dimethyl-1,3-propanediamine	Piperidine
L-Proline	*o*-Phenylenediamine	2-Aminopyridine

* In 0.025 M mixed phosphate buffer pH 6.8 containing a final concentration of 10 per cent sheep serum and 5×10^{-4} M amines as listed.

The results presented in Table II indicate that the amine oxidase in sheep serum reacted with various substrates in a fashion unlike that of previously described enzymes in this classification. The rate of oxidative attack on spermine and spermidine was at least ten times higher than on any of the other amines tested. The absence of significant oxidation of typical monoamines (tyramine, *n*-propylamine) or typical diamines (putrescine, histamine) under

these conditions clearly distinguished the enzyme present in sheep serum (spermine oxidase) from monoamine oxidase and diamine oxidase.

Of special interest in connection with the specificity of spermine oxidase was the failure of sheep serum to attack diethylenetriamine and triethylenetetramine, compounds similar in structure to spermidine and spermine except for the length of the carbon chain between amine groups. Also insusceptible to

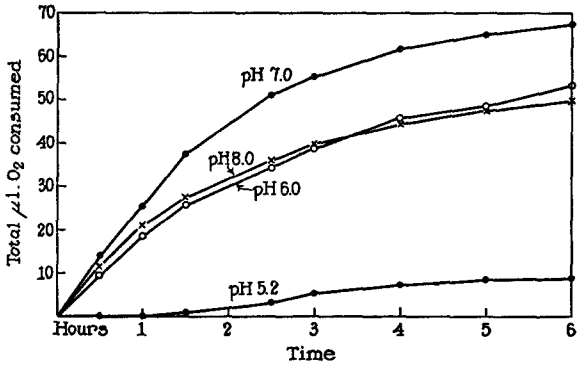

FIG. 1. The influence of the pH of the medium on the course of the oxidation of spermine by an enzyme present in sheep serum. Each vessel contained 2 μM spermine and 5 per cent sheep serum in 0.05 M mixed phosphate buffer, with a final volume of 4 cc. in the reaction chamber.

TABLE III

The Oxygen Consumed during the Reaction between Spermine and Sheep Serum in Various Media

5×10^{-4} M spermine—25 per cent sheep serum in	Oxygen uptake μl. O_2 taken up per μM spermine in 30 min.
Distilled water	23.4
0.85 per cent NaCl	22.5
0.025 M mixed phosphate buffer pH 6.8	24.0
0.05 M sodium citrate — HCl buffer pH 6.6	23.7
0.05 M tris(hydroxymethyl)aminomethane buffer pH 6.6	24.9

oxidation by this enzyme were isolated components of the spermine molecule (*n*-propylamine, 1,3-diaminopropane, *n*-butylamine, 1,4-diaminobutane) and products of its chemical degradation (1,3-diaminopropane, γ-aminopropyl-*N*-pyrroline) (7, 8).

The Influence of the Physicochemical Environment on the Activity of the Spermine Oxidase Present in Sheep Serum

Fig. 1 illustrates the effect of the pH of the reaction medium on the rate of oxidation of spermine by the enzyme present in sheep serum. The reaction

proceeded in a pH range of at least 6.0 to 8.0, with an optimal rate in the vicinity of neutrality.

Because of the limited solubility of spermine phosphate, concentrations of spermine higher than 5×10^{-4} M could not be used in a phosphate buffer system. The activity of spermine oxidase was therefore studied in various media free of phosphate ion. The results outlined in Table III show that the oxygen consumption resulting from the incubation of spermine and sheep serum was essentially the same whether these substances were dissolved in distilled water, physiological saline, phosphate buffer, citrate buffer, or tris(hydroxymethyl)aminomethane buffer.

Extensive studies of the relationship between the temperature and the rate of the reaction were not made. In one experiment in which flasks containing spermine and sheep serum in phosphate buffer were shaken at 18.5°C., the rate of oxygen consumption was approximately 60 per cent of that observed in similar tests at 38°C.

TABLE IV

The Relationship between the Concentration of Sheep Serum (Enzyme) and the Rate of the Reaction

5×10^{-4} M spermine in 0.025 M mixed phosphate buffer containing	Rate of reaction μl. O_2 consumed per μM spermine per min. during 1st 20 min. of incubation
20 per cent sheep serum	0.80
10 " " " "	0.57
5 " " " "	0.30
2.5 " " " "	0.18

The Kinetics of the Oxidative Deamination of Spermine by an Enzyme Present in Sheep Serum

As is shown in Table IV, the oxidative attack on spermine proceeded the more rapidly the higher the concentration of sheep serum. The rate of the reaction was, however, not precisely proportional to the concentration of serum.

Fig. 2 illustrates the rate of oxygen consumption when varying concentrations of spermine were incubated with the same concentration of sheep serum. The rate of the reaction during the early phase was independent of the concentration of the substrate, and the oxygen uptake plotted against time produced a straight line. However, in the latter phase of the reaction, the rate gradually diminished with a rapidity which was determined, at least in part, by the relative original concentrations of enzyme and substrate.

There were several possible explanations for the diminution in the rate of the reaction late in its course: (*a*) The enzyme might be slowly denatured or inactivated by the physicochemical elements in the medium or by a product formed during the enzymatic reaction. (*b*) The rate of attack might become dependent on the concentration of substrate when this concentration fell below a certain level. (*c*) One of the products of the reaction might compete with the

unchanged spermine for a position on the enzyme, or might complex with unchanged spermine and prevent combination with the enzyme. (*d*) The oxygen uptake might reflect a series of enzymatic processes. As to the last of these possibilities, no evidence pointing to the participation of more than one enzyme was found. Also, the final values for oxygen uptake and ammonia liberation, which will be presented later, did not correspond to whole values in terms of oxygen atoms added and ammonium radicals removed from the spermine

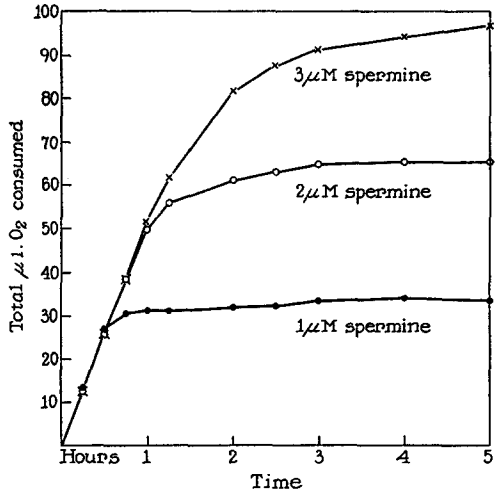

FIG. 2. The relationship between the concentration of spermine and the course of its oxidation by an enzyme present in sheep serum. Each flask contained spermine as indicated and 20 per cent sheep serum in 0.05 M mixed phosphate buffer pH 7.0, with a final volume of 4 cc. in the reaction chamber.

molecule, thus suggesting that the reaction was not proceeding to completion. To gather further information regarding which of these mechanisms was at work, the following experiment was performed.

In the reaction chamber of one flask were placed 2 cc. of 0.1 M mixed phosphate buffer at pH 7.0, 0.4 cc. sheep serum, and 1.2 cc. of water. In each of two side arms of this flask was placed 0.2 cc. of 0.01 M spermine. Another vessel contained 0.2 cc. of 0.01 M spermine, 2 cc. of 0.1 M mixed phosphate buffer pH 7.0, and 1 cc. of water in the reaction chamber and 0.4 cc. of sheep serum in each of two side arms. Other flasks were similar except for the use of spermidine in place of spermine. The general procedures used were the same as those described in an earlier section. After an appropriate period of equilibration, the contents of one of the side arms of each flask was added and the oxygen uptake recorded until no further oxygen was being consumed. At this point the contents of the second side arm of each flask was mixed with the fluid in the reaction chamber, thus bringing about in fashion suitable for accurate measurement the introduction of additional enzyme or additional substrate at a time when the original reaction had essentially come to a stage of equilibrium.

In the experiment illustrated in Fig. 3, the reaction between sheep serum and spermine or spermidine was allowed to proceed to equilibrium and then addi-

tional enzyme or substrate was introduced from a side arm. It is seen that the consumption of oxygen promptly resumed following the introduction of spermine or spermidine, while the addition of sheep serum to the system was without effect. Thus, the slowing of the rate of the original reaction could hardly have been due to inactivation of the enzyme. The observations were compatible with an alteration in the kinetics of the reaction at low concentrations of substrate, or with the formation of a product which competitively inhibited the oxidation of residual spermine.

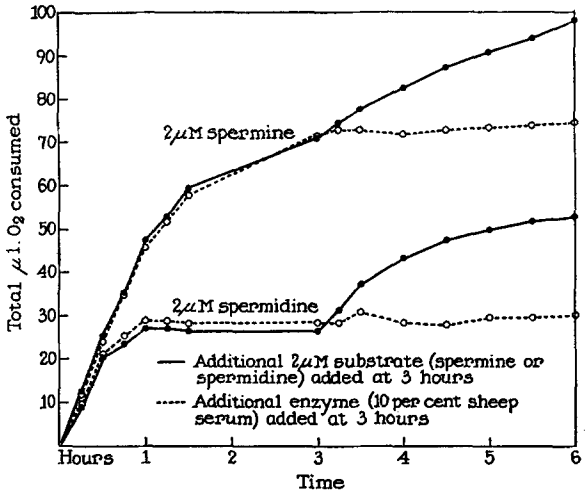

FIG. 3. An experiment dealing with the kinetics of the oxidation of spermine and spermidine by an enzyme in sheep serum. The reaction chamber of each flask contained 2 μM substrate and 10 per cent sheep serum in phosphate buffer (final volume 4 cc.). After oxygen uptake had essentially stopped, additional enzyme or substrate was introduced from a side arm. See text for experimental details.

The quantitative values obtained for oxygen uptake and ammonia liberation on incubation of spermidine or spermine in the presence of high concentrations of sheep serum are presented in Table V. As was previously pointed out, these are indeterminate. It is, however, apparent that the values approach those corresponding to the uptake of 4 atoms of oxygen and the liberation of 3 ammonia radicals for each molecule of spermine; for spermidine the values approach 2 atoms of oxygen consumed and 2 molecules of ammonia formed.

The Effect of Various Amines and Enzyme Inhibitors on the Oxidation of Spermine by Sheep Serum

In order to gather further information pertaining to the characteristics of spermine oxidase, and in order to determine in another manner its relation to the recognized amine oxidases, studies were made of the influence of various chemicals on the oxidation of spermine by sheep serum.

A summary of the results of experiments in which amines were added to the spermine–sheep serum system is presented in Table VI. The addition of *n*-propylamine, guanidine, putrescine, or histamine in amounts equimolar to that of spermine had no effect on the oxygen uptake. On the other hand, partial inhibition of oxygen consumption resulted from the addition of equi-

TABLE V

Quantitation of Oxygen Uptake and Ammonia Formation Resulting from the Action of Sheep Serum on Spermine and Spermidine

Substrate*	Oxygen uptake μl. O_2 taken up per μM substrate in 6 hrs.	Ammonia formation μg. NH_3 nitrogen liberated per μM substrate in 6 hrs.
Spermine	36.6	41.6
Spermidine	18.6	25.1

* Flasks containing 50 per cent sheep serum pH 7.2 in distilled water and a final concentration of 5×10^{-4} M substrates. No buffer was added. The pH of the reaction mixtures was unchanged at the end of the incubation period.

TABLE VI

The Oxidation of Spermine by Sheep Serum in the Presence of Various Amines and Enzyme Inhibitors

12 per cent sheep serum in 0.05 M mixed phosphate buffer pH 6.8 containing 5×10^{-4} M spermine and	Oxygen uptake μl. O_2 consumed per μM spermine in 90 min.	12 per cent sheep serum in 0.05 M mixed phosphate buffer pH 6.8 containing 5×10^{-4} M spermine and	Oxygen uptake μl. O_2 consumed per μM spermine in 90 min.
No added amine	25.8	5×10^{-4} M semicarbazide	1.9
5×10^{-4} M *n*-propylamine	26.4	" " hydroxylamine	0
" " guanidine	26.7		
" " putrescine	28.2	5×10^{-4} M iodoacetamide	25.8
" " histamine	24.3		
" " diethylenetriamine	17.1	5×10^{-4} M NaF	27.6
5×10^{-4} M triethylenetetramine	8.7	" " ethylenediamine tetraacetic acid	26.4
		10^{-4} M KCN	18.0
		10^{-3} M "	3.3

molar amounts of diethylenetriamine or triethylenetetramine, compounds similar in structure to spermidine and spermine except for the length of the carbon chain between nitrogen atoms. Presumably these two amines inhibited the oxidation of spermine by competing with spermine for position on the enzyme.

Table VI also records the essentially complete blocking of the oxidation of spermine by the carbonyl reagents semicarbazide and hydroxylamine, suggesting that spermine oxidase has ketone or aldehyde groupings which are essential

for its enzymatic action. In this regard, spermine oxidase resembles diamine oxidase (6).

The inclusion of a final concentration of 5×10^{-4} M iodoacetamide in the reaction medium did not affect the oxygen consumption of spermine–sheep serum mixtures, thus indicating that the enzyme does not need free sulfhydryl radicals for its action.

In general, metal-complexing agents had no effect on the reaction between spermine and sheep serum. It was pointed out in a previous section that the use of a citrate buffer did not inhibit oxygen consumption. As shown in Table VI, flasks containing added fluoride or ethylenediaminetetraacetic acid took up oxygen in a fashion similar to controls containing no sequestering agents. The addition of cyanide ion, however, did inhibit the reaction between spermine and sheep serum. The degree of inhibition varied with the concentration of cyanide, suggesting that cyanide acted not by means of cation binding, but

TABLE VII

The Effect of Low Concentrations of Mercuric Chloride on the Oxidation of Spermine and Spermidine by Sheep Serum

50 per cent sheep serum pH 7.2 in distilled water containing	Oxygen uptake μl. O_2 taken up per μM substrate in 6 hrs.	Ammonia formation μg. NH_3 nitrogen liberated per μM substrate in 6 hrs.
5×10^{-4} M spermine	36.6	41.6
" " " $+ 10^{-4}$ M $HgCl_2$	63.4	41.0
" " spermidine	18.6	25.1
" " " $+ 10^{-4}$ M $HgCl_2$	36.9	25.1

rather in a competitive fashion, probably by competing with spermine for the receptor positions (? carbonyl groups) on the enzyme molecule.

In the course of experiments dealing with antiseptic agents which might be used in the spermine–sheep serum system, it was unexpectedly observed that the presence of low concentrations of mercuric chloride led to an increase in the quantity of oxygen consumed by this system. Table VII presents data from these experiments and shows that the inclusion of a final concentration of 10^{-4} M mercuric chloride in the flasks containing spermine or spermidine and sheep serum brought about the uptake of approximately twice as much oxygen as in control vessels containing no mercuric ion. The quantity of ammonia formed during the reaction was unaffected by the addition of mercuric chloride. Although the mechanism by which mercuric chloride enhanced oxygen consumption is obscure, presumably the additional oxygen was used for the conversion to carboxyl groups of the alcohol or aldehyde radicals formed by the action of spermine oxidase. In contrast to the action of mercuric chloride,

the addition of low concentrations of magnesium chloride or ferric chloride did not alter the oxygen taken up by the spermine–sheep serum system.

DISCUSSION

Enzymes responsible for the oxidative deamination of amines have been classified as monoamine oxidases or diamine oxidases. Studies have revealed variation in the substrate specificity of these enzymes depending on the tissue used as a source for their preparation (6), and have also indicated that the susceptibility of amines to attack may be determined by the configuration of the substrate molecule rather than by the number of amino groups *per se* (9).

The oxidative deaminase described in the present communication differs from previously described enzymes of this category. The kinetics of its reaction, its resistance to various competitive inhibitors, and its failure to bring about oxidation of histamine or putrescine clearly distinguish the enzyme in sheep serum from histaminase or diamine oxidase. The amine oxidase in sheep serum also differs from monoamine oxidase in solubility, in susceptibility or resistance to various inhibitors, and especially in the lack of ability to attack tyramine or *n*-propylamine. Since sheep serum did not act on diethylenetriamine or triethylenetetramine, the enzyme cannot be classified as a triamine oxidase or a tetramine oxidase. All studies thus far point to specificity for the oxidative deamination of spermine and of the closely related compound spermidine. The name spermine oxidase therefore seems justified, at least for the present.

Although spermine has been shown to be widely distributed in animal tissues, occurring in high concentration in certain organs such as the prostate and pancreas (10, 11), little is known of its origin, function, or fate in the body. Since serum containing spermine oxidase brings about the rapid oxidative deamination of spermine *in vitro* under conditions similar to those present physiologically, perhaps this enzyme plays a role in the metabolism of spermine *in vivo*. Further study of the distribution of spermine oxidase in various organs and tissues should shed light on this possibility. The lack of demonstrable spermine oxidase activity in the serum of man, rabbit, and the guinea pig does not necessarily reflect the situation in the organs of these animals, since in a previous investigation spermine activator was demonstrated in the guinea pig kidney, while the serum of this animal was devoid of similar activity (3).

Purification of the spermine oxidase present in sheep and bovine serum would be desirable for more trustworthy studies of the kinetics of its reaction with spermine and the quantitation of the chemical exchanges resulting from this reaction. In previous work, employing the tubercle bacillus in a biological test system, the general properties and stability of the enzyme were established (3), but no program directed towards purification has been undertaken.

In order to evaluate the possible role of the spermine system in influencing host-parasite relationships in tuberculosis, present investigations in our laboratory deal with attempts to isolate and characterize the products of the enzymatic attack on spermine.

SUMMARY

Sheep serum and bovine serum contain an enzyme which brings about a rapid oxidative deamination of certain biological amines. This enzyme differs from previously described amine oxidases in several regards and especially in its substrate specificity. Studies thus far indicate that only spermine and the closely related compound spermidine serve as substrates for the enzyme in sheep serum. For this reason, the enzyme has been named spermine oxidase.

Spermine oxidase is active in a variety of fluids of various ionic strength and buffer composition. The reaction takes place between pH 6.0 and pH 8.0 with an optimal rate in the vicinity of neutrality.

Under certain conditions, the rate of oxygen consumption during the initial phase of the reaction is independent of the concentration of substrate. The diminution in rate observed during the latter phase of the enzymatic attack appears to be due to an alteration in the kinetics at low concentrations of substrate, or to competitive inhibition by a product of the reaction.

Carbonyl reagents almost completely block the action of spermine oxidase, while certain amines and the cyanide ion bring about partial inhibition. Thiol reagents and sequestering compounds do not alter the course of the oxidative process. In the presence of low concentrations of mercuric chloride, the sheep serum–spermine system consumes approximately twice as much oxygen as controls containing no mercuric ion. The mechanism by which the mercuric ion stimulates additional oxygen uptake is obscure.

BIBLIOGRAPHY

1. Hirsch, J. G., and Dubos, R. J., *J. Exp. Med.*, 1952, **95,** 191.
2. Hirsch, J. G., *J. Exp. Med.*, 1953, **97,** 323.
3. Hirsch, J. G., *J. Exp. Med.*, 1953, **97,** 327.
4. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Minneapolis, Burgess Publishing Company, 1945.
5. Hotchkiss, R. D., unpublished observations.
6. Zeller, E. A., *in* The Enzymes, (J. B. Sumner, and K. Myrbäck, editors), New York, Academic Press, Inc., 1951, 544.
7. Wrede, F., Fanselow, H., and Strack, E., *Z. physiol. Chem.*, 1926, **153,** 291.
8. Wrede, F., Fanselow, H., and Strack, E., *Z. physiol. Chem.*, 1926, **161,** 66.
9. Blaschko, H., and Duthie, R., *Biochem. J.*, 1945, **39,** 478.
10. Dudley, H. W., and Rosenheim, O., *Biochem. J.*, 1925, **19,** 1034.
11. Hämäläinen, R., *Acta Soc. med. Duodecim*, 1947, series A, **23,** 97.