STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA

VIII. The Effect of Calcium and Strontium Ions on the Activation of the Plasma Proteolytic Enzyme*

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The proteolytic enzyme in human plasma which is active at neutrality appears to exist in the circulating blood in the form of an inactive precursor. *In vitro*, the enzyme in clotted blood may become active "spontaneously" so that fibrinolysis occurs (1). This spontaneous activation is readily detectable in crude preparations of the enzyme (2), but is not observed when the enzyme is more highly purified (3).

The conditions under which the proteolytic enzyme of the plasma becomes active have not been clarified. Scant attention has been paid to the effect of various ions on the process of activation. In the experiments to be described, the influence of various metallic ions was studied. It was not possible to demonstrate that calcium or strontium altered the properties of activated plasma proteolytic enzyme. However, the activation of the enzyme from its precursor proceeded more rapidly in the presence of appreciable amounts of these cations. In the concentrations tested, other monovalent and bivalent cations did not accelerate activation. The effect of calcium ions was observed in crude preparations of the proteolytic enzyme, but was not apparent in solutions of more purified enzyme. Since the concentrations of calcium which were effective were comparable to those existing in the circulating blood, it seems possible that the phenomena observed may be significant in relation to the physiologic activation of the enzyme.

Methods

Nomenclature.—The term *plasma proteolytic enzyme* designates the substance or substances in plasma capable of digesting fibrinogen, fibrin, and certain other proteins *in vitro* at or near neutrality. This enzyme has also been called plasmin (4) and fibrinolysin (5). It is ordinarily present in plasma as an inactive *precursor*. The filterable principle of cultures of hemolytic streptococci which activates the precursor will be called *streptokinase* in accordance

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with current usage (4). The term *enzyme* designates the crude enzyme preparations used and does not imply either a purified product or even a single substance.

Preparation of Enzyme.—The general methods used in the preparation of precursor have been described elsewhere (6, 7). In most experiments, a globulin fraction was separated from oxalated human plasma in the following manner. One volume of plasma was diluted with 19 volumes of water and acidified with 1 per cent acetic acid to pH 5.2–5.4. The precipitate which formed was separated by centrifugation and dissolved in buffer in a volume usually equal to that of the original sample of plasma. This solution of globulin contained the precursor of the plasma proteolytic enzyme.

In certain experiments, the precursor was partially purified by a modification of the method described by Christensen and Smith (3). A solution of globulin, prepared in the manner just described, was mixed with 5 volumes of acetone. After 10 minutes, the precipitate which formed was separated by centrifugation, resuspended in acetone, and recentrifuged. The precipitate was then dried with a current of air, and suspended in a volume of 0.02 N hydrochloric acid one and a half times that of the original sample of plasma. The precipitate was mixed vigorously with the acid, and the suspension was then filtered through Whatman No. 2 filter paper. The acid extract was mixed with 10 volumes of acetone and centrifuged. After drying, the precipitate was dissolved in buffer in a volume equal to that of the original specimen of plasma. All these procedures were performed at 4°C.

Chloroform-activated enzyme was prepared by dissolving the crude precipitate of globulin in a volume of buffer equal to one-fourth that of the original specimen of plasma. This solution of precursor was mixed with an equal volume of chloroform, and the mixture was incubated at 25°C. for 16 hours. The chloroform was removed by centrifugation and aeration of the supernatant solution. The enzyme was then diluted to the original plasma volume.

Streptokinase-activated enzyme was prepared by mixing a solution of crude globulin with streptokinase, and incubating the mixture at 25°C. for 10 minutes. The mixture was then diluted with 19 volumes of water and acidified to pH 5.2 with 0.1 per cent acetic acid. The reprecipitated globulin was separated by centrifugation, dissolved in buffer, and diluted suitably. The amount of streptokinase which was needed to activate the precursor maximally was determined on a small aliquot of the crude globulin solution. The streptokinase used in all experiments was a purified preparation which contained 53 units of streptococcal desoxyribonuclease for every 169 units of streptokinase.[1] The streptokinase was dissolved in buffer.

Measurement of Proteolytic Activity.—Proteolytic activity was determined by measuring the rate of fibrinolysis. In some experiments, the fibrinogen present in the solution of globulin was clotted by the addition of bovine thrombin,[2] and the clot lysis time was then measured at 37°C. In other experiments, the precursor or enzyme was prepared in such a manner that no clottable fibrinogen was present. In these instances, a solution of bovine or human fibrinogen was mixed with the globulin and clotted by the addition of bovine thrombin. In all experiments, fibrinolysis was measured in pyrex tubes with an internal diameter of 8 mm. If the period of observation was longer than 1 hour, a drop of toluene was added to each tube as a bacteriostatic agent. The *clot lysis time* was the interval between the time that thrombin was added to the globulin and the time that the clot which formed completely reliquefied. Care was taken to avoid disturbing the clots during the period of observation. If the clots were agitated, retraction appeared to occur and it became difficult to estimate the clot lysis time. The data presented in the tables are representative of repeated experiments. In most instances, duplicate determinations were performed and the difference be-

[1] Purified streptokinase was obtained through the courtesy of the Lederle Laboratories, Pearl River.

[2] Bovine thrombin was obtained through the courtesy of Parke, Davis, and Company, Detroit.

tween the clot lysis times in each pair usually did not vary by more than 10 per cent of the mean time.

Preparation of Fibrinogen.—Human fibrinogen was prepared by a modification of the method described by Holmberg (8). Pooled oxalated plasma was filtered through a Seitz EK filter to reduce the concentration of prothrombin. The filtered plasma was suspended in one-tenth its volume of powdered barium sulfate and incubated at 37°C. for 10 minutes. The barium sulfate was separated by centrifugation. The supernatant plasma was then mixed at 4°C. with one-fourth its volume of ammonium sulfate solution, saturated at 4°C. After 10 minutes, the precipitate which formed was separated by centrifugation at 4°C., dissolved in water, and reprecipitated with one-fourth its volume of saturated ammonium sulfate. The precipitate was again dissolved in water, mixed with an equal volume of saturated sodium chloride solution, and, after 10 minutes, recentrifuged. This precipitate was redissolved and reprecipitated by half saturation with sodium chloride three or four times, and the final precipitate was diluted with water to reduce the concentration of sodium chloride to 0.85 per cent. Human fibrinogen prepared in this manner contained 80 to 95 per cent clottable protein. In the experiments to be described, the solutions of fibrinogen were used on the day that the preparation was completed, but such fibrinogen could be dried, with some loss of coagulability. It was necessary to remove prothrombin both by Seitz filtration and adsorption on barium sulfate to prevent the formation of thrombin during the process of preparation.

Bovine fibrinogen[3] was dissolved in buffer and the undissolved residue was separated by filtration through Whatman No. 1 filter paper. In all experiments, 250 mg. of purified fibrinogen was dissolved in 100 ml. of buffer. Assay of the filtered solution demonstrated approximately 150 mg. of clottable protein per 100 ml. of solution.

Preparation of Buffer.—In all experiments, the buffer used was 0.025 M barbital and 0.125 M sodium chloride, at pH 7.5.

Preparation of Solutions of Metallic Ions.—A 0.15 N aqueous solution of the chloride salt of each of the elements tested was diluted as required with buffer. There was no difference in the behavior of calcium chloride distributed by Merck & Co. Inc., Mallinkrodt Chemical Works, and The Baker Laboratories, Inc.

Determination of Fibrinogen.—The concentration of fibrinogen was determined as fibrin by a method previously described (9).

EXPERIMENTAL

1. The Effect of Various Cations on Fibrinolysis in Crude Preparations of Globulin.—The chloride salts of various metallic elements were tested for their effect on fibrinolysis in clots formed from solutions of human globulin.

A globulin fraction was prepared from fresh, oxalated human plasma by the method described. The precipitate was dissolved in a volume of buffer equal to that of the plasma from which it had been prepared. A volume of 0.3 ml. of globulin was added to each of 11 tubes containing 0.3 ml. of the chloride salt of the ion to be tested, serially diluted with buffer. The mixtures were clotted by the addition of 0.1 ml. of a solution of bovine thrombin containing 10 National Institutes of Health (N.I.H.) units per ml. The tubes were stoppered, incubated at 37°C. in a water bath, and observed at frequent intervals for evidences of fibrinolysis.

In repeated experiments, the clots formed by the addition of thrombin to globulin usually lysed within 18 to 30 hours. Repeatedly, when small amounts

[3] Bovine fibrinogen was obtained through the courtesy of Dr. Walter Seegers.

of calcium or strontium chloride were added to this mixture, lysis was accelerated (Table I). Calcium chloride appeared to be somewhat more effective than strontium chloride. Similar concentrations of the chloride salts of potassium, lithium, barium, magnesium, zinc, gold, copper, mercury, and cadmium did not accelerate fibrinolysis. In the higher concentrations tested, a white flocculent precipitate appeared when the chloride salts of zinc, gold, copper, mercury, and cadmium were added to the globulin solution, but in no case was fibrinolysis accelerated.

In the experiments which have been described, the fibrinogen in the globulin solution was clotted by the addition of thrombin. The lysis-accelerating

TABLE I

The Effect of Calcium and Strontium Salts on the Rate of Fibrinolysis

Clot lysis time at 37°C. of mixtures of 0.3 ml. globulin solution, 0.3 ml. of solutions of calcium chloride or strontium chloride, serially diluted with buffer, and 0.1 ml. of a solution of bovine thrombin containing 10 N.I.H. units per ml.

Final concentration of salt	Clot lysis time	
	Calcium chloride	Strontium chloride
m.eq./l.	*hrs.*	*hrs.*
32	8	10
16	8	10
8	8	10
4	10	11½
2	10	22 *
1	10½	25
0.5	11½	25
0.25	22 *	26
0.125	24	26½
0.062	25	26½
0	25	25

* The tubes were not observed in the interval between 12 and 22 hours from the start of the experiment.

effect of calcium ions was also readily demonstrable without the addition of thrombin. However, under these conditions, the clotting time varied with the concentration of calcium in the mixture. In order to eliminate this variable, the fibrinogen was clotted by the addition of thrombin in all experiments.

The method by which the calcium and strontium ions accelerated the lytic process in crude globulin preparations remained unexplained. The effect was not related to any appreciable change in the concentration of hydrogen ions in the reaction mixture. The pH of mixtures of either salt, globulin, and thrombin was measured after lysis had occurred. In different experiments, the pH varied from approximately 7.0–7.7, but within this range there appeared to be no correlation between pH and the rate of fibrinolysis.

There was no evidence that calcium or strontium acted by combining with

some diffusible substance. Fresh oxalated human plasma was dialyzed against barbital buffer for 24 hours at 0 to 4°C., using Visking no-jax cellophane sausage casings. The globulin was then prepared in the usual manner. Again calcium ions accelerated fibrinolysis in clots prepared by the addition of thrombin to this globulin solution. This observation suggests that the effect of calcium was not due to its combination with a dialyzable substance.

The lysis-accelerating effect of calcium and strontium ions did not seem to be the result of simple solution of fibrin in the presence of these substances. Bovine fibrin, prepared by the mixture of bovine fibrinogen and bovine thrombin, did not dissolve within 5 days in the presence of 0.065 to 32 m.eq. per liter of calcium or strontium chloride. Human fibrin, prepared from human fibrinogen and bovine thrombin, lysed within 60 hours, presumably because it was contaminated with small amounts of plasma proteolytic enzyme. The rate of lysis was not influenced by the presence of calcium chloride over the same range of concentrations. The effect of calcium and strontium ions, therefore, did not seem to be due to a direct lytic action of these ions on fibrin.

Another possibility was that calcium and strontium interfered with the clotting action of thrombin. It was possible that progressively less fibrin was formed as the concentration of these ions increased, and the time needed for lysis might thus be shortened.

The globulin fraction was precipitated from fresh oxalated human plasma and dissolved in a volume of buffer equal to that of the original sample. In duplicate, a volume of 0.3 ml. of globulin was pipetted into each of 11 pyrex tubes containing 0.3 ml. of a solution of calcium chloride, serially diluted with buffer. About 0.3 ml. of crushed pyrex glass was added to each tube to facilitate the separation of fibrin by centrifugation (9). The contents of each tube was then clotted by the addition of 0.1 ml. of a solution of bovine thrombin containing 50 N.I.H. units per ml. The amount of fibrin formed in the presence of each concentration of calcium was then determined in one set of tubes, and the other set was observed for lysis at 37°C.

In repeated experiments, the amount of fibrin formed was not influenced by the addition of calcium ions (Table II), nor did the presence of crushed glass have significant effect on the rate of fibrinolysis.

2. The Effect of Calcium Ions on Active Plasma Proteolytic Enzyme.—The data thus far presented demonstrate that the clots formed by the addition of thrombin to crude preparations of human plasma globulin lysed more rapidly when calcium ions were added to the mixture. A number of experiments were performed to determine whether the effect of calcium ions was upon the active plasma proteolytic enzyme or upon its precursor.

A solution of calcium chloride was serially diluted with buffer and 0.3 ml. of each dilution was pipetted into a pyrex tube. To each tube were added in succession 0.3 ml. of a solution of active bovine plasma proteolytic enzyme[4] containing 1 unit per ml., 0.1 ml. of a solution

[4] Bovine plasma proteolytic enzyme (fibrinolysin) was obtained through the courtesy of Parke, Davis and Company, Detroit.

of bovine thrombin containing 10 units per ml., and 0.3 ml. of a solution of bovine fibrinogen. The tubes were shaken, incubated at 37°C., and the clots which formed were observed for evidences of fibrinolysis.

Calcium chloride did not augment the lytic action of bovine plasma proteolytic enzyme on bovine fibrin, and in fact, in higher concentrations, was slightly inhibitory (Table III). The same result was obtained when the substrate was human fibrin formed by the addition of bovine thrombin to human fibrinogen. Furthermore, calcium ions did not appear to accelerate fibrinolysis by human plasma proteolytic enzyme which had been activated by chloro-

TABLE II

The Effect of Calcium Ions on the Amount of Fibrin Formed by the Addition of Thrombin to a Solution of Globulin

Clot lysis time at 37°C. and the amount of fibrin formed in a mixture of 0.3 ml. of human globulin, 0.3 ml. calcium chloride solution, serially diluted with buffer, and 0.1 ml. of a solution of bovine thrombin containing 50 N.I.H. units per ml.

Final concentration of calcium chloride	Amount of fibrin formed	Clot lysis time
m.eq./l.	*mg.*	*hrs.*
32	0.54	6
16	0.56	6
8	0.53	6
4	0.55	7 ½
2	0.54	9 ½
1	0.55	17 ½*
0.5	0.42	17 ½
0.25	0.53	17 ½
0.125	0.51	20 ½
0.063	0.52	20 ½
0	0.57	20 ½

* The tubes were not observed in the interval between 9 ½ and 17 ½ hours from the start of the experiment.

form, by streptokinase, or by a combination of both. These experiments confirmed the view that the observed effect of calcium ions was not upon the reactivity of the substrate.

3. The Effect of Calcium Ions upon the Activation of the Precursor of the Plasma Proteolytic Enzyme by Chloroform and by Streptokinase.—Calcium ions did not appear to exert their lysis-accelerating effect either by an action upon the fibrin substrate or upon the active form of the plasma proteolytic enzyme. These observations suggested that calcium accelerated fibrinolysis by increasing the rate at which the proteolytic enzyme in the globulin fraction was activated from its precursor. *In vitro*, this precursor may be activated by treatment of the crude globulin fraction either with chloroform or with strep-

tokinase. The effect of calcium ions on the rate of activation of the enzyme by chloroform was tested.

The globulin fraction of plasma was prepared in the usual manner, but dissolved in a volume of buffer one-eighth that of the original plasma. A volume of 0.25 ml. of calcium chloride solution, serially diluted with buffer, was pipetted into each of eleven 40 ml. round bottomed pyrex centrifuge tubes, followed by 0.25 ml. of the globulin solution and 0.50 ml. of chloroform. The tubes were stoppered, shaken, and incubated for 18 hours at 25°C. At the end of that time, 0.25 ml. of 0.3 N sodium oxalate solution and 1.25 ml. of buffer were added to each tube. The mixtures were allowed to stand for 10 minutes and were then centrifuged. 1 ml. of the supernatant fluid of each mixture was then diluted with 19 ml. of water and acidified with 0.1 per cent acetic acid to pH 5.2–5.4. The precipitates which formed were separated by centrifugation and each was dissolved in 1 ml. of buffer. The fibrinolytic activity of the enzyme preparations was then measured by mixing 0.3 ml. of globulin, 0.3 ml. of bovine fibrinogen solution, and 0.1 ml. of bovine thrombin solution.

TABLE III

The Effect of Calcium Ions on the Rate of Lysis of Clots by Bovine Plasma Proteolytic Enzyme.

Clot lysis time at 37°C. of a mixture of 0.3 ml. of a solution of bovine plasma proteolytic enzyme containing 1 unit per ml., 0.3 ml. of a solution of calcium chloride, serially diluted with buffer, 0.1 ml. of a solution of bovine thrombin containing 10 N.I.H. units per ml., and 0.3 ml. of a solution of bovine fibrinogen.

Final concentration of calcium	Clot lysis time
m.eq./l.	*min.*
22.5	28
11.25	29
5.62	29
2.81	29
1.40	26
0.70	31
0.35	22
0.175	24
0.087	24
0.044	19
0	19

In order to determine whether the reprecipitated globulin solutions still contained the same amount of potential proteolytic activity, every other globulin solution was assayed for precursor by the addition of streptokinase. 0.3 ml. of serial dilutions of the various globulin solutions was mixed with 0.3 ml. of bovine fibrinogen solution, 0.3 ml. of streptokinase solution (containing 63 units per ml.) and 0.1 ml. of bovine thrombin solution. The lysis time of the clots which formed was measured at 37°C.

In repeated experiments, the proteolytic activity produced in the plasma globulin fraction by activation of the enzyme from its precursor was somewhat variable from tube to tube. However, there was no correlation between

the concentration of calcium during the period of activation and the degree of proteolytic activity which developed. The data obtained suggested that the activation of the plasma proteolytic enzyme by chloroform was not increased by the presence of calcium ions in the concentrations tested (Table IV). In these experiments, the amount of precursor in the final preparation of globulin was the same regardless of the concentration of calcium during the process of activation.

TABLE IV

The Effect of Calcium Chloride on the Rate of Activation of Plasma Proteolytic Enzyme by Chloroform

Clot lysis time of mixtures of 0.3 ml. of chloroform-activated plasma proteolytic enzyme, 0.3 ml. of a solution of bovine thrombin containing 10 N.I.H. units per ml., and 0.3 ml. of a solution of human fibrinogen (approximately 250 mg. per 100 ml. clottable protein). The plasma proteolytic enzyme was prepared by incubating 0.25 ml. of concentrated globulin, 0.25 ml. of calcium chloride solution, serially diluted with buffer, and 0.5 ml. of chloroform, for 16 hours at 25°C.

Concentration of calcium during period of activation	Clot lysis time
m.eq./l.	*min.*
37.5	25
18.7	13*
9.4	27
4.7	24
2.34	22
1.17	18
0.58	15
0.29	13
0.15	16
0.07	16
0	17

* Checked.

The effect of calcium on the rate of activation of the precursor by streptokinase was also investigated.

In one such experiment, globulin, partially purified in the manner described, was dissolved in a volume of buffer equal to that of the plasma from which it had been prepared. A mixture of 0.3 ml. of the solution of globulin was mixed with equal parts of a solution of streptokinase, serially diluted with buffer, and bovine fibrinogen solution, and the mixture was clotted by the addition of 0.1 ml. of a solution of bovine thrombin containing 10 units per ml. The clot lysis time at 37°C. was measured. In this experiment, clot lysis was most rapid when the final concentration of streptokinase in the mixture was approximately 20 units per ml.

A volume of 0.3 ml. of the globulin solution was mixed with 0.3 ml. of calcium chloride solution, serially diluted with buffer, 0.3 ml. of streptokinase solution, 0.4 ml. of bovine fibrinogen solution, and 0.1 ml. of bovine thrombin solution. The final concentration of

streptokinase in the mixture was 0.8 units per ml., or approximately 1/25th that concentration which had been found to result in the most rapid fibrinolysis. The mixtures were incubated at 37°C. and the clots which formed were observed for fibrinolysis.

In the concentrations tested, the presence of calcium chloride did not affect the activation of plasma proteolytic enzyme by suboptimal concentrations of streptokinase (Table V). The same result was obtained when mixtures of calcium chloride, globulin, and streptokinase were allowed to stand at room temperature for 20 to 40 minutes before the addition of fibrinogen and thrombin.

TABLE V

The Effect of Calcium Ions on the Rate of Activation of Plasma Proteolytic Enzyme by Streptokinase

Clot lysis time at 37°C. of a mixture of 0.3 ml. purified plasma proteolytic enzyme, 0.3 ml. calcium chloride solution, serially diluted with buffer, 0.3 ml. of a solution of streptokinase, containing 4 units per ml., 0.4 ml. solution of bovine fibrinogen, and 0.1 ml. solution of thrombin containing 10 N.I.H. units per ml.

Final concentration calcium	Clot lysis time
m.eq./l.	*min.*
16	28
8	29
4	29
2	41
1	28
0.5	26
0.25	43
0.125	28
0.031	28
0	29

Human serum contains substances which appear to inhibit the activation of the plasma proteolytic enzyme in the presence of streptokinase (4). The effect of these substances is reduced by heating the serum to 56°C. for 30 minutes (7). Calcium salts were without effect on the activation of plasma proteolytic enzyme by streptokinase in crude globulin solutions prepared from heated as well as unheated serum.

4. The Effect of Calcium on the "Spontaneous" Activation of Purified Plasma Proteolytic Enzyme.—Plasma proteolytic enzyme, partially purified by a modification of the method described by Christensen and Smith (3), exhibited varying degrees of spontaneous proteolytic activity. 0.3 ml. of a solution of "purified" enzyme was mixed with equal parts of calcium chloride solution, serially diluted with buffer, and bovine fibrinogen solution. This mixture was then clotted by the addition of 0.1 ml. of bovine thrombin solution containing

10 units per ml. In different preparations, the clots which formed lysed within 6 to 72 hours. The presence of calcium chloride in concentrations varying from 0.045 to 22.5 m.eq. per liter did not influence the rate of fibrinolysis under these conditions.

DISCUSSION

In the experiments which have been described, the rate of lysis of fibrin clots prepared from a crude globulin fraction of human plasma was accelerated by small amounts of calcium chloride. Over the range of concentrations tested, calcium did not accelerate fibrinolysis by active bovine or human plasma proteolytic enzyme, an observation previously reported by Tagnon and his associates (10). Neither did calcium appear to influence the rate of activation of plasma proteolytic enzyme by chloroform or by steptokinase. The calcium ions appeared to exert their effect by accelerating the rate at which the plasma proteolytic enzyme in the globulin fraction became "spontaneously" active.

The mechanism by which calcium exerts its accelerating action on fibrinolysis was not explained. When the precursor of the enzyme was partially purified by acid extraction, calcium was no longer effective. This experiment suggested that the action of calcium was indirect. Perhaps calcium potentiated a kinase in plasma capable of activating the proteolytic enzyme. Such a kinase has been described by Schmitz (11), and others. Another possibility is that calcium might have combined with some substance which interfered with the process of activation. If this were the case, the substance with which calcium combined was probably not diffusible, since calcium increased the rate of fibrinolysis in clots prepared from dialyzed plasma.

The biological significance of the presence of a potent proteolytic enzyme in plasma is as yet unexplained. The concentration of diffusible calcium ions in human plasma is about 2.5 m.eq. per liter. Calcium ions in this concentration were sufficient to accelerate fibrinolysis significantly. For this reason, it is tempting to assume that calcium may play a role *in vivo* in the activation of the plasma proteolytic enzyme from its precursor. Other positive ions tested were not acceleratory except for strontium, which is the element corresponding to calcium in the next higher period of the periodic table. The present observations support the view that the proteolytic enzyme in plasma may become active *in vivo* and may provide a means by which fibrin deposits can be removed.

SUMMARY

Calcium ions accelerated the activation of the proteolytic enzyme of plasma from its precursor in the globulin fraction. Calcium did not appear to potentiate fibrinolysis by active bovine or human plasma proteolytic enzyme, nor

did it accelerate the activation of the precursor of this enzyme by chloroform or by streptokinase. Experiments with partially purified plasma proteolytic enzyme suggested that the acceleratory effect of calcium was mediated indirectly. Since the concentration of calcium which was effective was comparable to that present in plasma, it is possible that the phenomena reported are of biological significance.

BIBLIOGRAPHY

1. Goodpasture, E. W., *Bull. Johns Hopkins Hosp.*, 1914, **25,** 330.
2. Hedin, S. G., *J. Physiol.*, 1904, **30,** 195.
3. Christensen, L. R., and Smith, D. H., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74,** 840.
4. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28,** 559.
5. Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12,** 1.
6. Ratnoff, O. D., *J. Exp. Med.*, 1948, **87,** 199.
7. Ratnoff, O. D., *J. Exp. Med.*, 1948, **87,** 211.
8. Holmberg, C. G., *Ark. Kemi, Mineral. och Geol.*, 1944, **17A,** No. 28.
9. Ratnoff, O. D., and Menzie, C., *J. Lab. and Clin. Med.*, 1951, **37,** 316.
10. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Inv.*, 1942, **21,** 525.
11. Schmitz, A., *Z. Physiol. Chem.*, 1936, **244,** 89.