PURIFICATION OF BACTERIAL ARGINASE

EUGENIA SORU*
WITH THE TECHNICAL ASSISTANCE OF ELENA GHYKA
Department of General Biochemistry and Immunochemistry, "Dr. I. Cantacuzino" Institute, Bucharest (Rumania)
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Arginase (L-arginine-ureohydrolase EC 3.5.3.1), terminal enzyme of the urea ornithine cycle[1], exists in few bacterial species. In an investigation[2] of 53 species this enzyme was only found in the group of pathogenic staphylococcus[3] and in the *Bacillus anthracis* group.

For the investigation of bacterial arginases from a chemical as well as from an immunological point of view, the purity of these enzymes is essential. For the purification of staphylococcus arginase continuous electrophoresis on filter paper, as described by GRASSMANN and coworkers[4] for the purification of liver arginase, has been used previously[5] as a final stage. The present communication describes a more efficient procedure for obtaining both the purified staphylococcus and *B. anthracis* arginase preparations. The procedure includes gel filtration on Sephadex G-25, followed by horizontal zone electrophoresis in Sephadex G-200.

MATERIALS AND METHODS

Strains

For the extraction of staphylococcus arginase the strains *Staphylococcus aureus* Wood and *Staphylococcus aureus* 33-R were used, and for *B. anthracis* arginase the pathogenic strains $C_{12}R$ and $C_{14}R$ were used.

Growth of cultures

Preparation of acetone dried cells. Cultures were incubated for 24 h at 37° on nutrient agar (2%) pH 7.2. All the following manipulation stages were performed in the cold. The bacteria, suspended in saline solution, were harvested by centrifugation, washed twice with the same solution and once with distilled water. The cells were dehydrated by washing many times, successively, with cold acetone, acetone–ether mixture and ether and then filtered on a Büchner funnel under suction and finally stored under reduced pressure in a desiccator containing concentrated sulphuric acid.

Preparation of cell extracts. 10 g of the acetone dried cells were suspended in 100 cc distilled water or 0.001 *M* $MnCl_2$ solution and allowed to autolyse under a toluene layer for 24–48 h at 4°. The cell debris collected by centrifugation could be utilized for a new extraction. The supernatant containing arginase was filtered

* Present address: Strada B. P. Haşdeu 37, Bucharest 5, Rumania.

through a thick filter paper to remove the traces of toluene. Four volumes of acetone were added to the clear extract and the precipitate formed under these conditions containing the crude enzyme was collected by centrifugation and dried under reduced pressure.

Enzyme assay. Arginase activity was determined according to ENGEL AND ENGEL[6]. A unit of arginase is defined as the amount of enzyme that catalyzes the formation of 1 μmole of urea under the experimental conditions indicated by the method concerned[7]. Specific activity is defined as enzyme units per mg N.

Proteins were determined by Lowry's modified method[8], *nitrogen* by micro-kjeldahl.

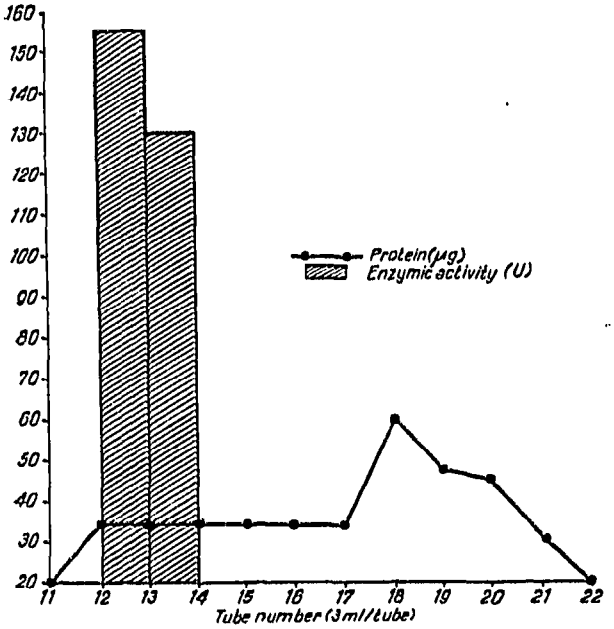
Gel filtration

Sephadex-G-25 fine* (200–400 mesh), water regain 2.5 $\pm$ 0.2, was used. The Sephadex beads were washed three times with a $MnCl_2$–NaCl (0.005 *M*–0.153 *M*) solution[9], the "fines" being removed by decantation after each wash. The gel suspension was stored 24 h in the cold in the same solution to allow the swelling of the dextran gel to proceed to completion. A column 2 × 25 cm, fitted with a sintered glass disc, was filled with the gel suspension and allowed to stand for 48 h so that the gel particles could pack down and complete equilibration with the eluent solution could be obtained. The crude enzyme preparation (30 mg), dissolved in 2 ml eluent, was introduced on the top of the column and eluted for 50 min. Fractions of 3 ml effluent were collected, the flow rate being 0.2 ml/min. Each fraction was examined with regard to protein content and arginase activity. Figs. 1 and 2 show the elution patterns of *B. anthracis* and staphylococcus arginase preparations respectively. The effluents showing arginase activity were pooled and mixed with four volumes of cold acetone. The enzymically active precipitate was dried under reduced pressure and stored in the cold.

Electrophoresis in Sephadex G-200

The arginase preparation obtained by gel filtration as described above is submitted to further purification by horizontal zone electrophoresis on Sephadex G-200 gel[10]. 3 g of Sephadex G-200 (100–400 mesh)* powder was suspended in 60 ml of veronal–acetate buffer, pH 8.5, μ = 0.046. The gel, after having been left a few hours to eliminate air bubbles, is poured into a plastic tray 10 × 30 × 0.2 cm and this gel-covered plate is placed in a common electrophoresis box, the connection with the electrode vessels being made by means of thick filter paper sheets. The gel plate is left overnight for complete equilibration with the electrode vessels containing the same buffer as the gel suspension, 20 mg of a solution in the same buffer of the arginine preparation obtained by gel filtration is mixed with dry Sephadex G-200 and the paste introduced in a groove cut in the middle of the gel plate. Electrophoresis is carried out for 3 h at 400 V, 30 mA. To extract the electrophoretically separated enzyme the gel plate was cut into 1 cm sections, each of which was placed in a test tube together with 2 ml of $MnCl_2$–NaCl (0.005 *M*–0.153 *M*) solution. The gel suspensions were left overnight in the cold for complete extraction of the proteins, after which the gel bed was removed by vacuum filtration on sintered glass funnels. The enzyme activity and

* Pharmacia, Uppsala.



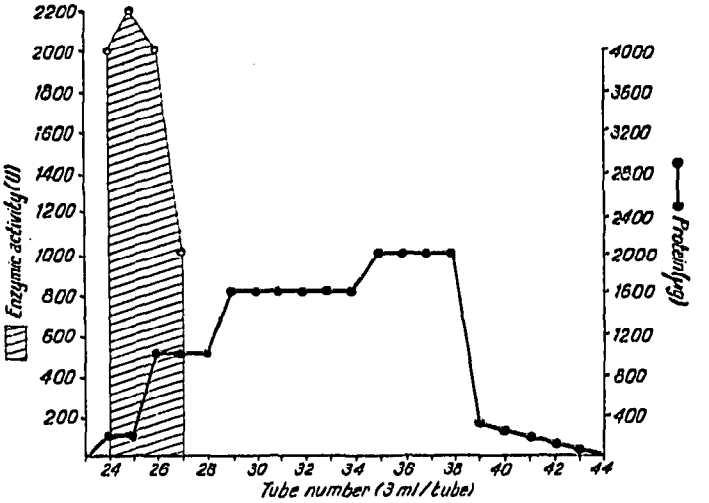
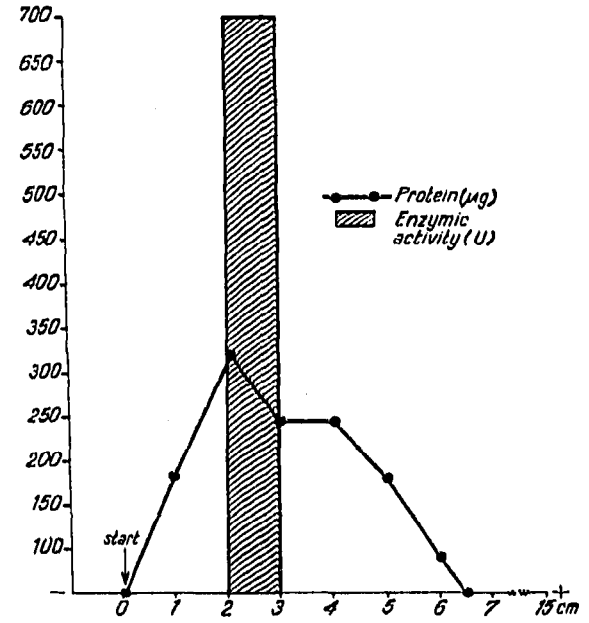


Fig. 1. Fractionation of a *B. anthracis* arginase preparation on a Sephadex G-25 (fine) (2 × 25 cm) column (eluent $MnCl_2$–NaCl (0.005 *M*–0.153 *M*)). The shaded section corresponds to the enzymically active protein.

Fig. 2. Fractionation of a staphylococcus arginase preparation on a Sephadex G-25 (2 × 25 cm) column. Eluant: $MnCl_2$–NaCl (0.005 *M*–0.153 *M*). The shaded section corresponds to the enzymically active protein.



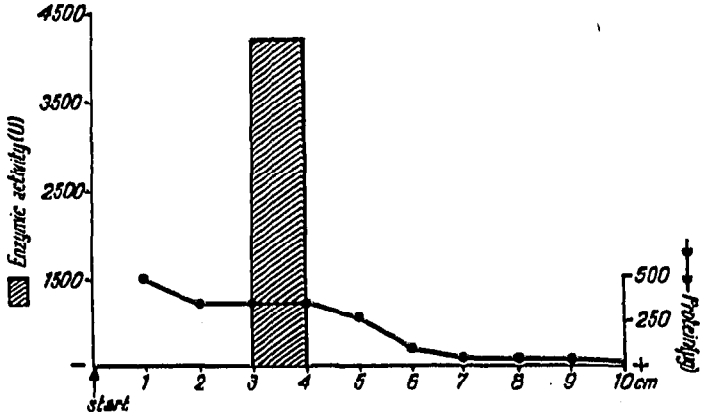


Fig. 3. Patterns of staphylococcus arginase obtained by horizontal zone electrophoresis in Sephadex G-200 (10 × 30 × 0.2 cm); veronal–acetate buffer pH 8.6, μ = 0.045; 400 V, 30 mA for 3 h. The shaded section corresponds to the enzymically active protein.

Fig. 4. Patterns of *B. anthracis* arginase obtained by horizontal zone electrophoresis in Sephadex G-200 (10 × 30 × 0.2 cm); veronal–acetate buffer, pH 8.6, μ = 0.045; 400 V, 30 mA for 3 h. The shaded section corresponds to the enzymically active protein.

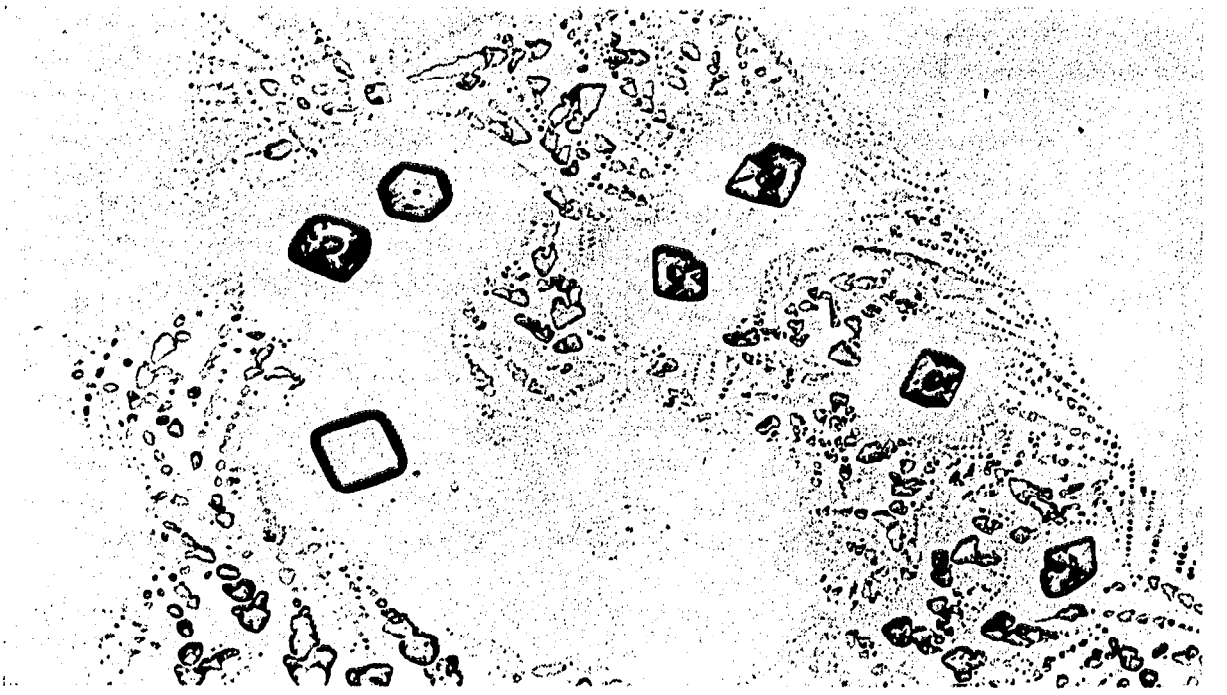


Fig. 5. Crystals of staphylococcus arginase.

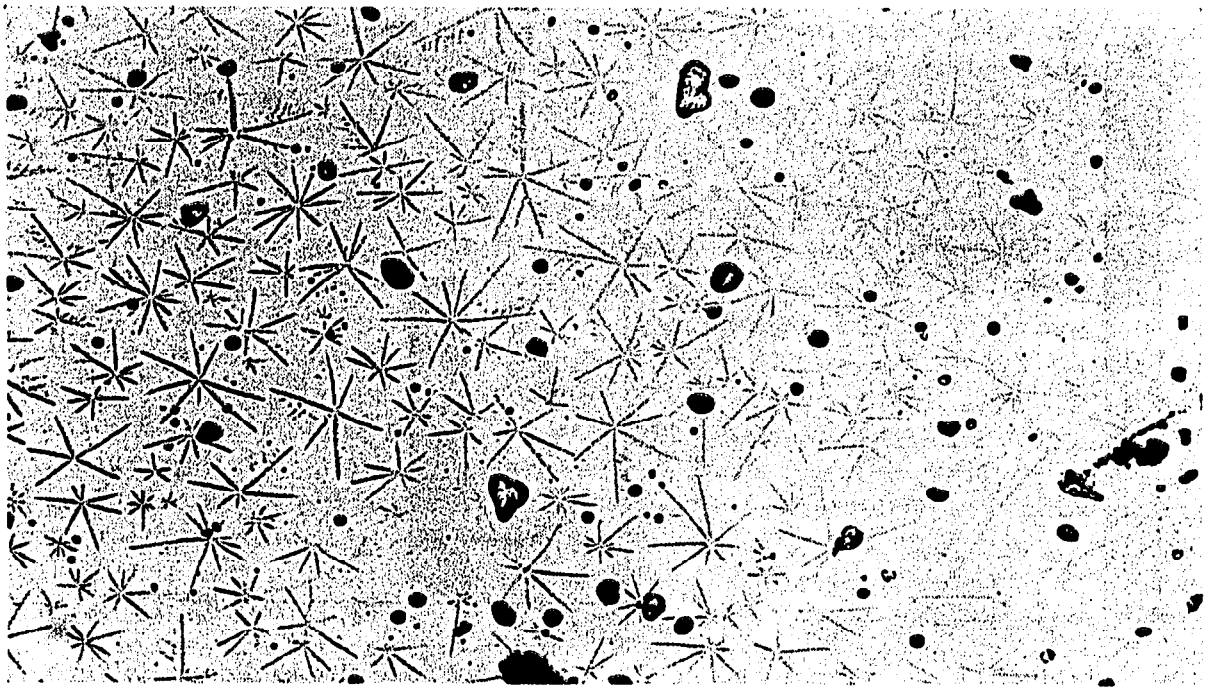


Fig. 6. Crystals of *B. anthracis* arginase.

protein content of the extraction liquid were then determined. Nitrogen was determined after dialysis. Figs. 3 and 4 show the electrophoresis patterns of the staphylococcus and *B. anthracis* arginase respectively.

Crystallization

This was carried out on the purified enzymes according to the procedure described by BACH *et al.* for liver arginase[11]. Figs. 5 and 6 show different aspects of staphylococcus and *B. anthracis* arginase crystals.

Antiarginase serums

These were prepared on rabbits by five intradermal injections, each consisting of 2 ml incomplete Freund's adjuvant containing 10 mg of the enzyme and administered over a 5-week's period. Antibody titre was determined by the ring precipitation test and gel diffusion test.

Control of the purity of arginase preparations

The homogeneity of the arginase preparations purified by the above methods was checked by the agar gel double diffusion method as described by OUCHTERLONY[12], by the immunoelectrophoresis method of GRABAR AND WILLIAMS[13], by electrophoresis on cellulose acetate strips[14] and by thin-layer gel filtration on Sephadex G-200 (superfine < 400)[15].

RESULTS

The purification stages of bacterial arginases as described above and outlined in schematic form in Fig. 7 and Table I consist in the extraction of the enzyme by autolysis of acetone dehydrated bacteria, under a toluene layer; precipitation with acetone (1:4) of the crude enzyme from the aqueous extract; gel filtration of the crude

TABLE I

PURIFICATION OF *Staphylococcus aureus* AND *B. anthracis* ARGINASES

Fraction	*Protein (mg/ml)*		*N (mg/ml)*		*Total arginase units* (μmol. urea/ml)*		*Specific activity (units/mg N)*		*Purification (x fold)*	
	Staph. aureus	*B. anthracis*	*Staph. aureus*	*B. anthracis*	*Staph. aureus*	*B. anthracis*	*Staph. aureus*	*B. anthracis*	*Staph. aureus*	*B. anthracis*
A	8	27.00	1.79	5.18	186.66	90.33	103	17.47	0	0
B	5.3	2.70	0.58	1.05	113.33	58.16	195	55.38	1.83	3.11
C	1.25	15.00	0.56	3.15	171.33	570	308	171.42	2.99	9.81
D	0.25	0.44	0.03	0.05	123.33	185	4110	3700	39.81	217.54
E	0.06	0.07	0.008	0.008	43.33	39.10	5416	4885	52.58	273.32
F	0.06	0.06	0.006	0.006	46.66	28.10	7773	5600	75.45	320.35

A = Autolyzed bacterial suspension (48 h at 4° under toluene layer); B = Supernatant of centrifuged A fractions; C = Acetone precipitate of B. D = Effluents of gel filtration of C on Sephadex G-25 (fine); E = Eluates of electrophoresis of separated fractions of D in Sephadex G-200 gel. F = Crystallized arginase preparations.

* One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmole of urea under the experimental conditions indicated.

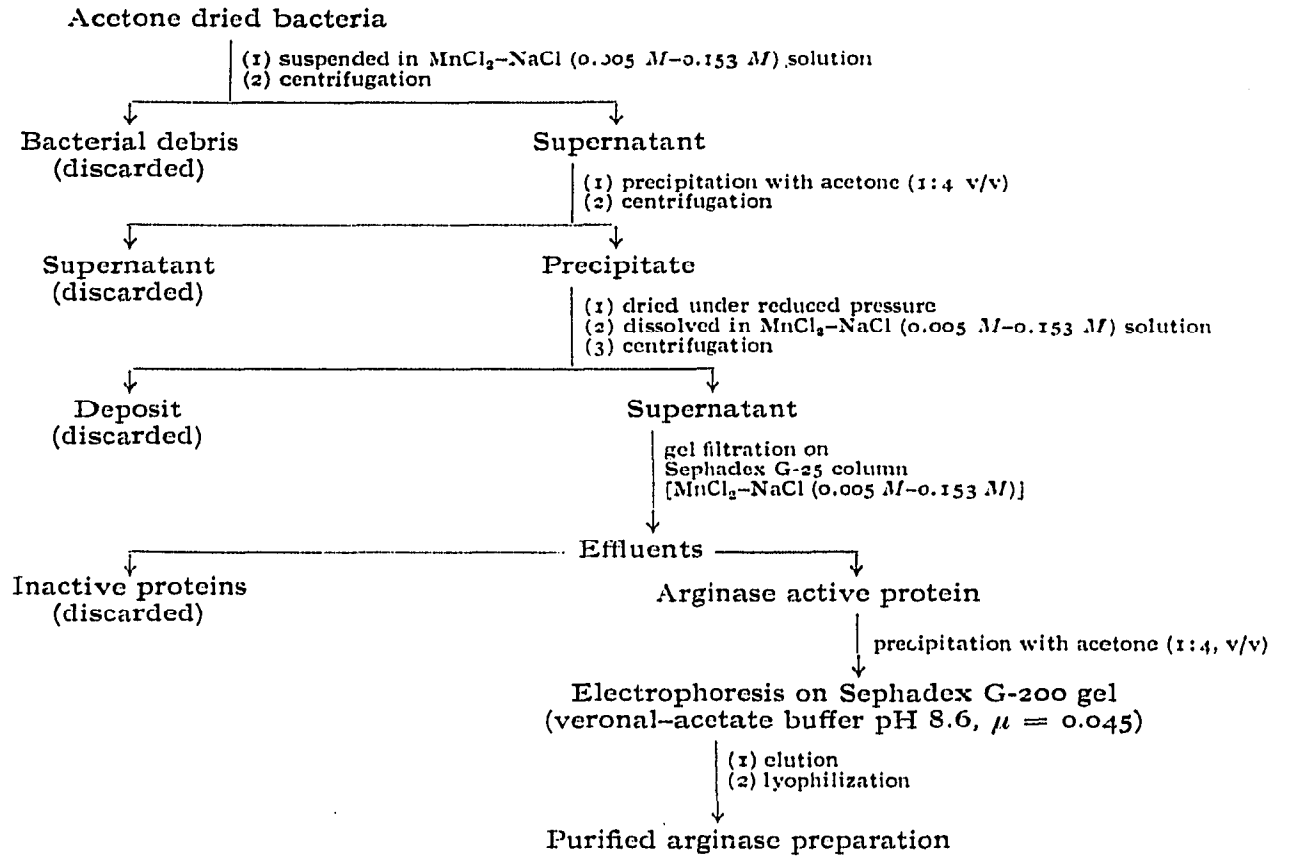
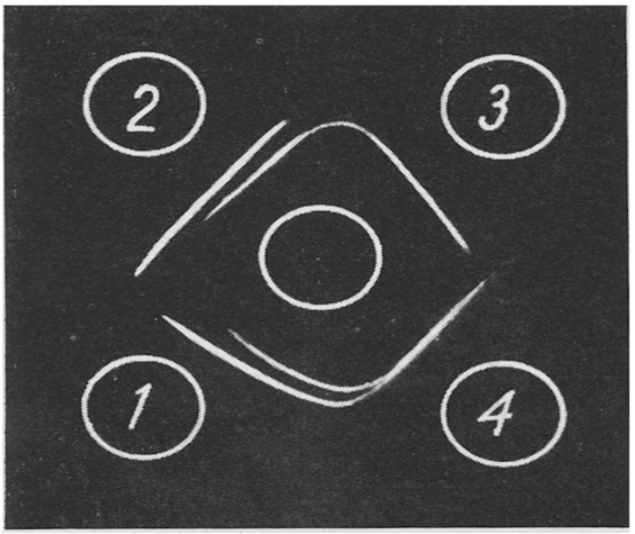


Fig. 7. Outline of procedure used for purification of bacterial arginase.

preparation on Sephadex G-25 (fine) equilibrated with MnCl–NaCl (0.005 M–0.153 M) solution; and further purification by horizontal electrophoresis on Sephadex G-200 gel (veronal–acetate buffer pH 8.6, μ = 0.045). The arginase preparations so obtained were found to be homogeneous in the double diffusion in gel test (Figs. 8 and 9), and on immunoelectrophoresis in agar gel (Fig. 10). Thin layer gel filtration on Sephadex G-200 superfine < 400) and electrophoresis on cellulose acetate strips (Figs. 11 and 12) also showed homogeneity. An activity of 5416 AU/mg N and a purification of 52.58 times was obtained for staphylococcus arginase, while for *B. anthracis* arginase the activity was 4885 AU/mg N and a purification of 273.33 times was obtained. The crystallized preparations obtained by applying the procedure of BACH *et al.*[11] show an increased specific activity: 7773 AU/mg N for staphylococcus arginase and 5600 AU/mg N for *B. anthracis* arginase. The crystal form of the two bacterial arginases differed from one another as is shown in Figs. 5 and 6. The antigenic specificity of the bacterial arginases was shown by the double diffusion and immunoelectrophoresis in agar gel test. The antiserums were prepared by immunization of rabbits by subcutaneous injections of the enzyme preparations in incomplete Freund's adjuvant at weekly intervals over a period of 5 weeks. The precipitation lines appear only when using the arginase preparation and its corresponding antiserum. No cross reactions were detected, either between staphylococcus arginase and *B. anthracis* antiserum, or between *B. anthracis* arginase and staphylococcus antiserum (Figs. 13, 14 and 15).



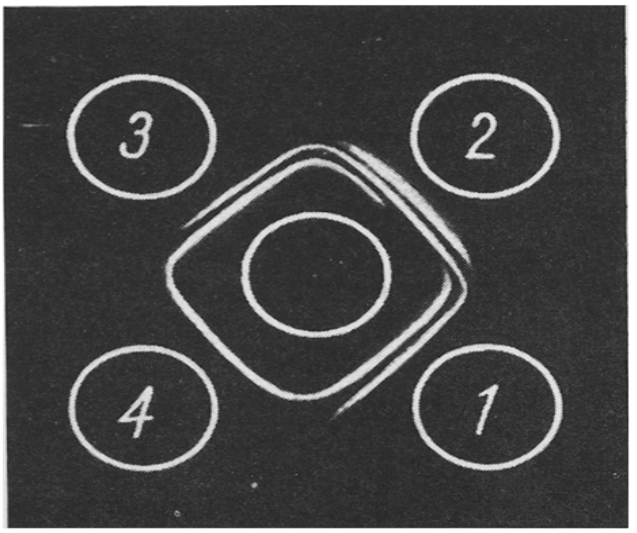


Fig. 8. Agar gel double diffusion experiment. Central well: antistaphylococcal crude arginase serum; well 1 and 2: partially purified arginase (Sephadex G-25 column effluents); well 3 and 4: purified arginase (by electrophoresis in Sephadex G-200).

Fig. 9. Agar gel double diffusion experiment. Central well: anti-*B. anthracis* crude arginase serum; well 2: crude arginase preparation; well 1 and 3: arginase active effluents from Sephadex G-25 column; well 4: *B. anthracis* arginase purified by electrophoresis in Sephadex G-200.

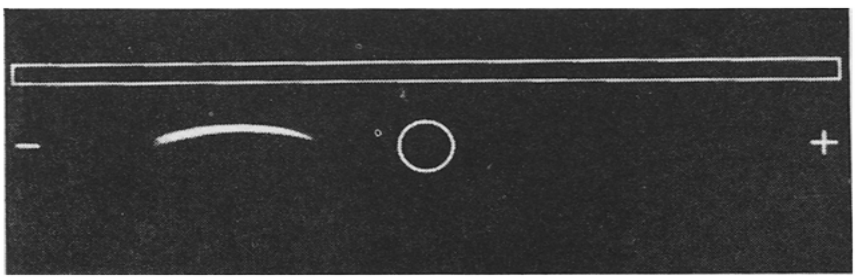


Fig. 10. Immunoelectrophoretical homogeneity of staphylococcus arginase.

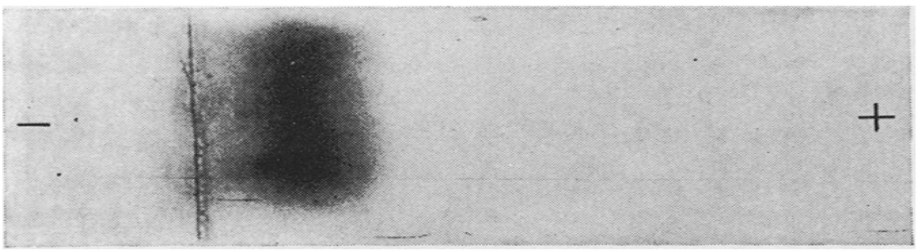


Fig. 11. Electrophoresis on cellulose acetate sheets of purified staphylococcus arginase.

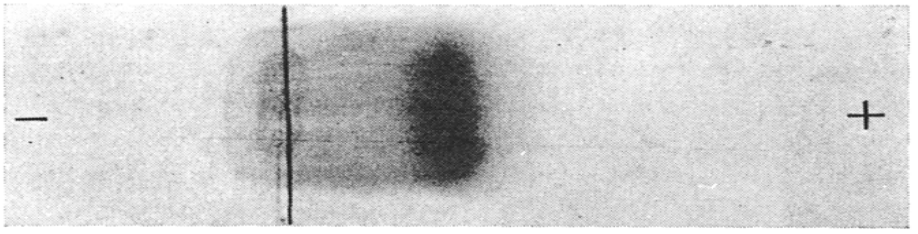


Fig. 12. Electrophoresis on cellulose acetate sheets of purified *B. anthracis* arginase.

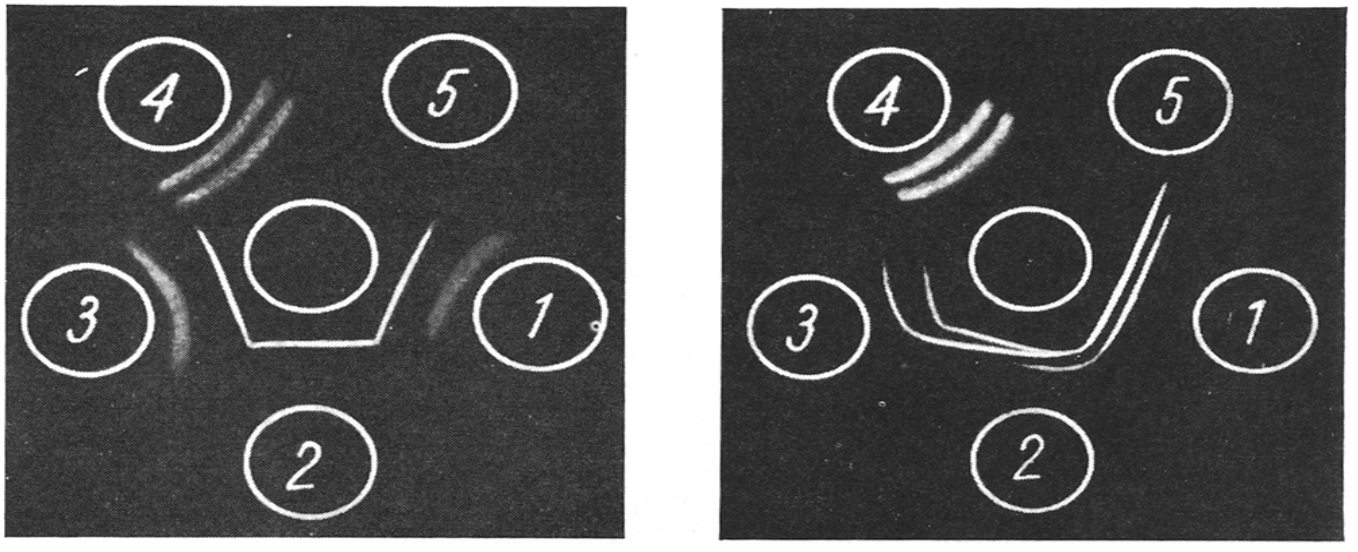


Fig. 13. Immunological specificity of staphylococcal arginase. Gel diffusion test. Central well: antistaphylococcal crude arginase serum; well 4: crude staphylococcal arginase; well 3 and 1: arginase active effluents from Sephadex G-25 column; well 2: purified staphylococcus arginase (by electrophoresis in Sephadex G-200 gel); well 5: *B. anthracis* arginase.

Fig. 14. Immunological specificity of *B. anthracis* arginase. Gel diffusion test. Central well: anti-*B. anthracis* crude arginase serum; well 2 and 4: crude *B. anthracis* arginase; well 1 and 3: partially purified *B. anthracis* arginase; well 5: staphylococcus arginase.

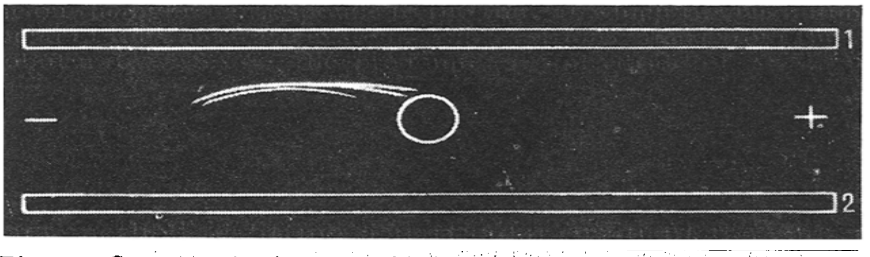


Fig. 15. Immunological specificity revealed by immunoelectrophoretic analysis of staphylococcus (1) and *B. anthracis* arginase (2); central well: antistaphylococcal arginase serum.

SUMMARY

A method is described for purification of staphylococcus and *B. anthracis* arginases. It consists in the extraction of the crude enzyme by autolysis of the acetone dehydrated bacteria, gel filtration on a Sephadex G-25 column and horizontal zone electrophoresis on Sephadex G-200. Crystallized preparations were obtained by acetone treatment in the cold. The crystal forms of the two bacterial arginases are different. The arginase preparations so obtained are homogeneous in the double diffusion and immunoelectrophoresis in agar gel tests, and also on thin layer gel filtration on Sephadex G-200 superfine and electrophoresis on cellulose acetate strips. The immunological specificity of the two bacterial arginases was shown by double diffusion and immunoelectrophoresis in agar gel.

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