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ACTION OF STREPTOCOCCAL HAEMOLYSINS AND PROTEOLYTIC ENZYMES ON EHRLICH ASCITES TUMOUR CELLS*

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It has been recently shown that various strains of *Streptococcus pyogenes* group A possess a cell-bound haemolysin (CBH) which can be released from the streptococcal cells into the surrounding medium by some surface active materials (Tween 40, Tween 80, Triton) by crystalline albumin, but not by sonic energy (Ginsburg and Grossowicz, 1957; Ginsburg, 1958; Ginsburg and Grossowicz, 1959). This haemolytic factor was designated as streptolysin "D" (SLD) D signifying detergent.

SLD is distinguished from streptolysin O (SLO) streptolysin S (SLS) (Todd, 1938) and from the intracellular haemolysin (IH) described by Schwab (Schwab, 1956) on the bases of different substrate requirements for formation, sensitivity to U.V. irradiation and to sonic energy (Ginsburg, 1958; Ginsburg and Grossowicz, unpublished).

Besides haemolysing RBC of various animal species both cell-free and cell-bound SLD, are also capable of injuring and killing various mammalian cells *in vitro* (Ehrlich ascites tumour cells, fibroblasts, amnion cells, leucocytes) (Ginsburg, 1958; Ginsburg and Grossowicz, 1959).

The purpose of the present study is to show that Ehrlich ascites tumour cells damaged by different streptococcal haemolysins may be disintegrated by various proteolytic enzymes which by themselves are not lethal.

MATERIALS AND METHODS

Streptococcal strain.—A Lancefield group A *Strep. pyogenes* (strain S84 type 3) was obtained from the State Serum Institute, Copenhagen. The streptococcus was cultivated in Brain Heart Infusion (BHI) broth (Difco).

Preparation of washed streptococci.—Streptococci grown in BHI medium for various periods of time at 37° were washed 3 times with 20 ml. (final volume 60 ml.) of buffered saline

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(equal volumes of phosphate buffer 0.067 M, pH-7.4 and saline). The washed streptococci were diluted to contain approximately 5×10^8 cells/ml. and were put in ice until used.

Haemolysins

Cell-bound haemolysin (CBH) was obtained by incubating washed streptococci (5×10^8 cells/ml. obtained from a 6 hr culture) for 5 min. in the presence of glucose, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and cysteine free-base (0.5 mg./ml. of each). Under such conditions a potent haemolytic activity appeared in close association with the streptococcal cells, however no extra-cellular haemolysin was obtained. When however 25 mg./ml. of salt-free crystalline blood albumin (Cutter laboratories) were added to the streptococci the CBH was released into the surrounding medium. This haemolysin was designated as streptolysin D (SLD). CBH and SLD were assayed according to the method of Slade and Knox (1950). Haemolytic activity is expressed as haemolytic units/ml.—HU/ml.

Streptolysin O (SLO).—Supernatants obtained from streptococcal cultures grown for 18 hr. in BHI were used as a source of haemolysin. These supernatants were incubated for 10 min. at 37° with 1 mg./ml. of cysteine in order to secure maximal activation of SLO. SLO was titrated according to the method of Slade and Knox (1950).

Streptolysin S (SLS).—Was prepared and assayed according to the method of Bernheimer (1949).

Proteolytic enzymes

Crystalline streptococcal proteinase—was kindly supplied by Dr. S. D. Elliot, Department of Animal Pathology, School of Veterinary Medicine, Madingley Road, Cambridge, England.

The enzyme was activated with 0.1 per cent cysteine before conducting the experiments. The enzyme solution always contained insoluble material which was removed by centrifugation.

Crystalline trypsin—(Tryptar) was purchased from the Armour Laboratories, Illinois.

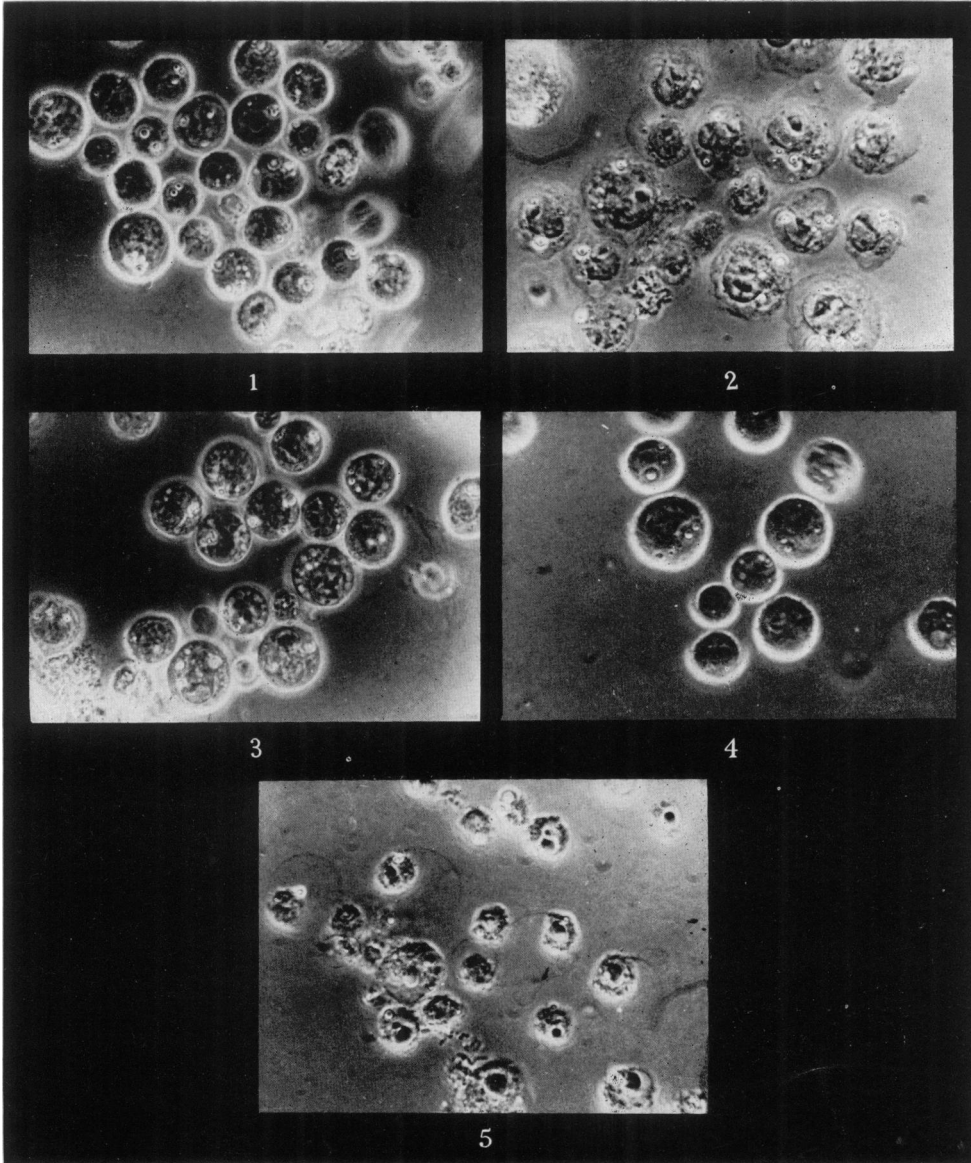
Papain (Nutrition Biochemical Corporation Cleveland Ohio).—The enzyme was activated with 0.1 per cent cysteine before the experiment was conducted. Insoluble material was removed by centrifugation.

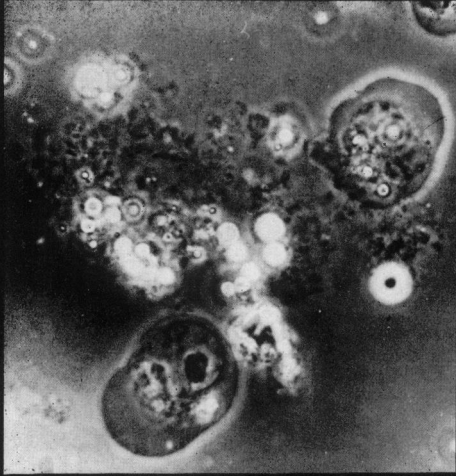
All enzymes were dissolved in buffered saline and were assayed by the method of Clifton and Cannamela (1953).

EXPLANATION OF PLATES

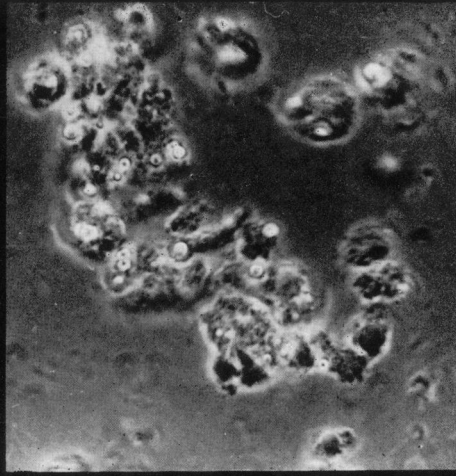
All photographs were taken with a Spencer Phase contrast microscope.

- FIG. 1.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate-saline buffer containing glucose (500 $\mu\text{g./ml.}$) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (500 $\mu\text{g./ml.}$) and cysteine (500 $\mu\text{g./ml.}$). $\times 500$.
- FIG. 2.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate saline buffer containing glucose, Mg^{++} and cysteine and 5×10^8 cells/ml. of streptococci (strain S84) possessing 1000 H.U./ml. of SLD. At the left lower corner of the picture streptococcal chains are seen attached to tumour cells. $\times 500$.
- FIG. 3.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate saline buffer containing glucose Mg^{++} cysteine, 5×10^8 cells/ml. of streptococci and 25 $\mu\text{g./ml.}$ of aureomycin. The streptococci were preincubated for 10 min. at 37° with aureomycin before the mixture was added to tumour cells. Aureomycin was found to abolish the haemolytic activity of the streptococci. $\times 500$.
- FIG. 4.—Ehrlich tumour cells incubated for 90 min. at 37° in phosphate saline buffer containing glucose, Mg^{++} , cysteine and 100 $\mu\text{g./ml.}$ of streptococcal proteinase. $\times 500$.
- FIGS. 5, 6, 7.—Ehrlich tumour cells incubated for 30, 60 and 90 minutes respectively, at 37° in the presence of phosphate saline buffer containing glucose Mg^{++} , cysteine, streptococci (5×10^8 cells/ml.—possessing 1000 H.U./ml. SLD), and 100 $\mu\text{g./ml.}$ of streptococcal proteinase. Note the various stages of cell disintegration and the accumulation of cell debris. (Fig. 5. $\times 500$. Figs. 6 and 7 $\times 710$).
- FIG. 8.—Ehrlich tumour cells incubated for 90 min. at 37° with SLD (1000 H.U./ml.). Similar results were obtained with SLO, and SLS. $\times 710$.
- FIG. 9.—Ehrlich tumour cells incubated for 90 min. at 37° with SLD (1000 H.U./ml.), trypan blue 25 $\mu\text{g./ml.}$ and proteinase (100 $\mu\text{g./ml.}$). Trypan blue was found to abolish the haemolytic activity of cell-free SLD. (Ginsburg, 1958). $\times 710$.

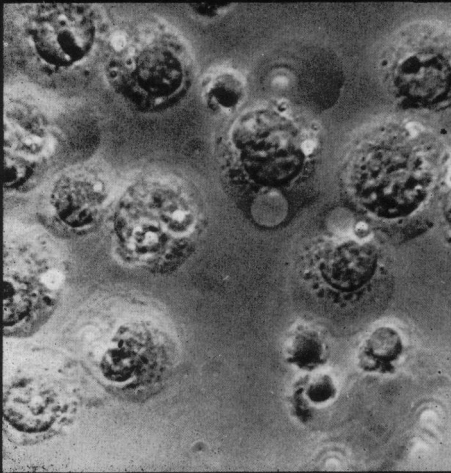




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Ehrlich ascites tumour cells

Ehrlich tumour cells were obtained from mice infected with the Landchutz strain. The tumour cells were maintained by weekly passages in white mice. The ascites fluid was removed from mice killed by decapitation. The cells were washed with buffered saline and diluted to contain approximately 10^7 cells/ml.

RESULTS

Action of streptococci possessing CBH and streptococcal proteinase on Ehrlich ascites tumour cells

All experiments on the action of streptococcal haemolysins and proteolytic enzymes on tumour cells were performed at 37° and were recorded by direct observations with a phase microscope.

Untreated tumour cells.—Untreated cells (7 days old) (Fig. 1) appeared round, the edge of the cells smooth, the nucleus when visible was surrounded by a narrow rim of cytoplasm and only few granules could be seen scattered in the cytoplasm. Such cell suspensions always contained 2–3 per cent of dead cells as demonstrated by vital staining with trypan blue (2 per cent). A minimum of 500 cells were counted in each measurement.

Tumour cells incubated with streptococci.—The tumour cells (10^7 /ml) began to swell 10 min. after the addition of streptococci (5×10^8 /ml.) possessing CBH activity (1000 H.U./ml.). The nucleus became more obvious and many glistening cytoplasmic granules appeared in the swollen cells. The average diameter of most of the cells was twice that of controls 90 min. after the addition of streptococci (Fig. 2). The nucleus was swollen and some streptococcal chains were attached to the swollen tumour cells. Only 20 per cent viable cells were present as shown by staining with trypan blue. No cell disintegration or cell debris could be demonstrated and no reduction in the total number of cells could be observed even after 90 min. of incubation (Table I).

The swelling and the death of the cells could not be prevented by ascitic fluid, rabbit sera, or by penicillin (1000 u/ml.) but could be prevented to a large extent by aureomycin (25 μ g./ml.). Aureomycin was found to inhibit CBH production (Ginsburg and Grossowicz, 1957). Tumour cells treated with streptococci-aureomycin mixtures appeared somewhat swollen and contained no more than 5 per cent of dead cells.

Tumour cells incubated with streptococcal proteinase.—The concentrations of proteinase used in these experiments (10–100 μ g./ml., 0.2–2 u/ml.) had no visible effect on the tumour cells for the first 60 min. of the experiment. After further incubation (90 min.) some cells appeared somewhat swollen (Fig. 4). However, no change in the total number of the cells could be demonstrated (Table I). Staining with trypan blue revealed that only 2–4 per cent of dead cells were present.

The combined action of streptococci and of proteinase on tumour cells.—The first signs of cell disintegration could be observed 30 min. after the addition of streptococcal proteinase to streptococci-tumour mixtures (Fig. 5). Most of the swollen cells still contained eccentric nuclei which appeared distorted. The cytoplasm which appeared empty and swollen was surrounded by a cell membrane, and many free nuclei were seen floating in the medium. After 60 min. of incubation many free globules appeared in the medium together with large accumula-

TABLE.—*The Combined Action of Haemolytic Streptococci Possessing CBH and Proteinase on Ehrlich Ascites Tumour Cells*

Reaction mixture Tumour cells†	Number of cells		Percentage of cells disappearing*
	Before treatment	After treatment	
streptococci‡	1.4×10^7	1.4×10^7	—
streptococci + aureomycin§ (25 $\mu\text{g./ml.}$)	1.4×10^7	1.4×10^7	—
proteinase (25 $\mu\text{g./ml.}$)	1.4×10^7	1.4×10^7	—
proteinase (50 $\mu\text{g./ml.}$)	1.4×10^7	1.4×10^7	—
proteinase (100 $\mu\text{g./ml.}$)	1.4×10^7	1.4×10^7	—
proteinase (100 $\mu\text{g./ml.}$) + ascitic fluid ¶	1.4×10^7	1.4×10^7	—
streptococci + proteinase (25 $\mu\text{g./ml.}$)	1.4×10^7	2.5×10^6	82
streptococci + proteinase (50 $\mu\text{g./ml.}$)	1.4×10^7	1.7×10^6	88
streptococci + proteinase (100 $\mu\text{g./ml.}$)	1.4×10^7	6.8×10^5	95
streptococci + proteinase (25 $\mu\text{g./ml.}$) + ascitic fluid	1.4×10^7	7.3×10^6	48
streptococci + proteinase (50 $\mu\text{g./ml.}$) + ascitic fluid	1.4×10^7	5.4×10^6	62
streptococci + proteinase (100 $\mu\text{g./ml.}$) + ascitic fluid	1.4×10^7	4.4×10^6	68
streptococci + proteinase (100 $\mu\text{g./ml.}$) + aureomycin (25 $\mu\text{g./ml.}$) + ascitic fluid	1.4×10^7	1.4×10^7	—

* The figures are the average of 3 experiments. All cells that still possessed a cell membrane, no matter how damaged they appeared, were counted. Cell counts were performed after 90 minutes of incubation at 37°.

† 1.4×10^7 cells/ml. suspended in phosphate saline buffer.

‡ Strain S84 (type 3)— 5×10^8 cells/ml. obtained from a 6 hr culture grown in BHI medium. The cells were washed with phosphate saline buffer and were resuspended in buffered saline containing glucose $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and cysteine (0.5 mg./ml.) of each and incubated for 5 min. at 37° before they were added to tumour cells. The cells possessed 1000 H.U./ml. of CBH.

§ Aureomycin was found to abolish the haemolytic activity of the streptococci. Streptococci were incubated for 5 min. at 37° with aureomycin before they were added to tumour cells.

¶ Supernatant obtained from Ehrlich tumour cells. 0.5 ml. of undiluted fluid were added to 0.5 ml. of reaction mixture.

tions of cell debris (Fig. 6). A few swollen cells showing pseudopod-like structures could still be seen. The process of disintegration advanced rapidly, and 90 min. after the addition of streptococcal proteinase (100 $\mu\text{g./ml.}$) over 95 per cent of the cells disappeared (Table I). The rest of the cells were in various stages of disintegration (Fig. 7). When however the production of CBH was blocked by aureomycin, no cell disintegration or reduction in the total number of cells occurred upon addition of proteinase, and only few cells appeared somewhat swollen (Table I).

Changes in cells were obtained even in the presence of 50 per cent ascitic fluid or rabbit's serum, however the percent of cells remaining was somewhat higher than that obtained in the absence of body fluid and was roughly proportional to the amount of proteinase present (Table I).

Dilute streptococcal suspensions ($< 10^4$ cells/ml.) which after 60 min. of incubation had no effect on the cells, in the presence of proteinase caused marked swelling and some disintegration. These experiments suggested that CBH and proteinase act synergistically.

The action of SLD, SLO, SLS and proteinase on Ehrlich tumour cells.—The above experiments were repeated using SLD, SLO, SLS (1000 H.U./ml., 500 H.U./ml. and 1000 H.U./ml. respectively). Similar results were obtained when tested in washed system. The cells that were in contact with the various haemolysins swelled (Fig. 8) and upon addition of proteinase (100 $\mu\text{g./ml.}$) marked

disintegration of the cells occurred. This effect was completely inhibited by anti-SLD (Fig. 9), SLO and SLS agents (trypan blue, in the case of SLD and SLS (Ginsburg, 1958; Bernheimer, 1954) cholesterol and rabbit serum (Van Heyningen, 1950) and ascitic fluid in the case of SLO).

The effect of SLD, SLS and various other proteolytic enzymes on Ehrlich tumour cells.—Crystalline trypsin (10 $\mu\text{g./ml.}$ –1 u/ml.) and crude papain (50 $\mu\text{g./ml.}$ –1 u/ml.) were capable of disintegrating tumour cells previously injured by streptococcal haemolysins (SLD, SLS) when tested in washed systems. On the addition of ascitic fluid or rabbit serum the cells did not disintegrate but did show considerable swelling. The possibility that this effect was due to the inhibition of the proteolytic enzymes by the body fluids (Shulman, 1952) was then tested. It was found that 1 ml. of ascitic fluid or rabbit serum could inhibit the fibrinolytic activity of 1000 $\mu\text{g.}$ of trypsin, 500 $\mu\text{g.}$ of papain but only about 20 $\mu\text{g.}$ of streptococcal proteinase. These results explain why low concentrations of trypsin, papain capable of acting on washed injured cells are inactive in the presence of body fluids. If however the concentrations of trypsin and papain were sufficiently high (1500 $\mu\text{g./ml.}$) cell disintegration occurred.

The mechanism of the cytotoxic-pathological effects observed was further investigated by exposing tumour cells to hypotonic solutions (0.5–0.6 per cent NaCl). This treatment caused swelling of the cells similar to that obtained by haemolysin treatment, on the further addition of small amounts of trypsin or papain (50 $\mu\text{g./ml.}$) marked disintegration of the cells occurred. (Quantitation of the remaining cells is hampered by the fact that trypsin and papain cause the agglutination of the tumour cells into a sticky mass which traps both intact cells and cell debris.) These concentrations of proteolytic enzymes had no effect on cells in isotonic medium. This would suggest that the primary damaging action of the haemolysin is on the cell membrane.

DISCUSSION

The results show that living Ehrlich tumour cells injured by various streptococcal haemolysins can be disintegrated by various proteolytic enzymes of animal, plant or streptococcal origin. The mechanisms of the action of these 2 types of agents either singly or in combination is little known. The fact that the haemolytic agents induced changes in cells expressed by marked swelling of the cells, by pseudopod-like formation and by the death of the cells suggests that as in the case of red blood cells the permeability and integrity of the cell membrane has been impaired.

As to action of proteolytic enzymes on living cells, it is interesting to note that relatively high concentrations of these enzymes (*e.g.* trypsin, papain) are routinely used for tissue culture purposes without causing any apparent permanent damage to the cells, since they can be easily propagated further (Chang, 1954; Younger, 1954).

The data also indicate that there is a synergistic effect of streptococcal proteinase and various streptococcal haemolysins (SLO, SLS, SLD) on tumour as well as on normal cells (Ginsburg, unpublished), which results in a complete disintegration of cells. The fact that tumour cells exposed to hypotonicity became very sensitive to the action of trypsin whereas cells incubated in normal saline were unaffected suggests the possibility that streptococcal haemolysins

affect the permeability of tumour cells in such a way that proteolytic enzymes may penetrate into the interior of the cell, and that proteolytic enzymes which by themselves cause no apparent changes to the cell membrane attack certain substrates which have been unmasked due to the action of haemolysins.

Moreover, the cytopathological action of streptococcal proteinase on mammalian cells injured by streptococcal haemolysins has been shown to be unspecific. As neither streptococcal proteinase, nor SLS or SLD, can be inhibited by various body fluids suggest, that such synergistic effect may occur *in vivo* in foci of infections with group A haemolytic streptococci. The lack of specificity of streptococcal proteinase in this effect suggest that other proteolytic enzymes of tissue or blood origin (cathepsins, plasmin) may act in a similar manner.

These suggestions on the *in vivo* action of these agents is supported by the findings of Kellner and Robertson (1954*a, b*) who have shown that cardiac lesions bearing some similarity to those seen in human beings or laboratory animals infected with streptococci could be produced in rabbits by the intravenous injection of streptococcal proteinase as well as by various other proteolytic enzymes. Similarly the intravenous injection of SLS to various laboratory animals resulted in histological damage to various internal organs (Barnard and Todd, 1940). It is possible that these factors may play a role in the production of cardiac lesions and other injuries caused by haemolytic streptococci. Studies on this point are in progress.

SUMMARY

The combined action of streptococcal haemolysins (streptolysin D, streptolysin S and streptolysin O) and various proteolytic enzymes (proteinase, trypsin, papain) on Ehrlich tumour cells is described.

Tumour cells incubated with various streptococcal haemolysins swell, send out large pseudopodia and appear dead when vitally stained with trypan blue.

Various proteolytic enzymes (proteinase, trypsin, papain) which cause no apparent injury to tumour cells disintegrate cells which have been previously damaged by streptococcal haemolysins. The action of haemolysins and proteolytic enzymes is synergistic in nature.

The combined action of streptolysin O and the various proteolytic enzymes on tumour cells is completely abolished by ascitic fluid and rabbit's sera. On the other hand these body fluids do not affect the action of streptolysin D, streptolysin S and streptococcal proteinase on these cells.

The possible action of streptococcal haemolysins and proteolytic enzymes *in vivo* is discussed.

The author is indebted to Prof. Jack Gross, head of the Department of Experimental Medicine and Cancer Research for his interest and advice during the performance of this work.

The author also wishes to thank Mrs. E. Salomon for taking the photographs.

REFERENCES

- BARNARD, W. G. AND TODD, E. W.—(1940) *J. Path. Bact.*, **51**, 43.
BERNHEIMER, A. W.—(1949) *J. exp. Med.*, **90**, 973.—(1954) In 'Streptococcal Infections'. Columbia University Press, N.Y.
CHANG, R. S.—(1954) *Proc. Soc. exp. Biol. N.Y.*, **87**, 440.

- CLIFTON, E. E. AND CANNAMELA, D. A.—(1953) *J. appl. Physiol.*, **6**, 42.
- GINSBURG, I. —(1958) Ph.D. thesis submitted to the senate of the Hebrew University, Jerusalem.
- Idem* AND GROSSOWICZ, N.—(1957) *Bull. Res. Council Israel*, **6E**, XXIV.—(1958) *ibid.*, **7E**, 237.—(1959) *Cancer Res.*, in the press.
- KELLNER, A. AND ROBERTSON, T.—(1954a) *J. exp. Med.*, **99**, 387.—(1954b) *ibid.*, **99**, 495.
- SCHWAB, J. H.—(1956) *J. Bact.*, **71**, 94.
- SHULMAN, N. R.—(1952) *J. exp. Med.*, **95**, 593.
- SLADE, A. D. AND KNOX, G. A.—(1950) *J. Bact.*, **60**, 301.
- TODD, E. W.—(1938) *J. Path. Bact.*, **47**, 423.
- VAN HEYNINGEN, A. W.—(1950) 'Bacterial Toxins'. Blackwell, Oxford.
- YOUNGER, J. S.—(1954) *Proc. Soc. exp. Biol. N.Y.*, **85**, 202.
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