STUDIES ON LYSOSOMES

III. The Effects of Streptolysins O and S on the Release of Acid Hydrolases from a Granular Fraction of Rabbit Liver*

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Several agents which cause injury to erythrocytes are also capable of releasing hydrolytic enzymes from lysosomes. The red cell membrane can be disrupted *in vitro* by exposure to saponin or detergents (1), lecithinase (2), excess vitamin A (3), ultraviolet irradiation (4), or repeated freezing and thawing. These agents and procedures are known to release degradative enzymes, *e.g.* cathepsins, acid DNAase, RNAase, acid phosphatase, and beta-glucuronidase from lysosomes (5–7). It appeared likely, therefore, that the extracellular hemolysins elaborated by streptococci, streptolysin O(SLO), and streptolysin S (SLS) could affect lysosomes *in vitro*, and perhaps *in vivo*, as well.

SLO is a hemolytic protein whose activity is markedly enhanced by cysteine or reduced glutathione; it is readily inactivated by cholesterol (1). The presence of specific antibody in convalescent sera after acute streptococcal infection in man is evidence for the production of the toxin *in vivo* (8). In contrast, SLS appears not to be antigenic, is not inhibited by cholesterol, and is maximally active without reducing agents (9). SLS is the agent responsible for the beta hemolysis of aerobically incubated blood agar plates (10). Both SLO and SLS are lethal in several species (9); injected *in vivo* they induce massive intravascular hemolysis, and this makes difficult the interpretation of specific organ pathology (11, 12). Two applications of SLO induce systolic contracture of amphibian hearts. However after the heart has been pretreated with saponin, only one application is necessary (13). Damage to leucocytes (14) and lymphocytes (15) *in vitro* can also be produced by SLO, and recently both SLO and SLS have been shown to cause degranulation of rabbit polymorphonuclear leucocytes and macrophages (16).

During streptococcal infections, therefore, membranous organelles such as ly-

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sosomes might be targets for the action of the streptolysins. Evidence has been presented which suggests that lysosomal enzymes can alter extracellular materials and can induce inflammation. Protease(s) released from lysosomes by vitamin A for example, caused dissolution of chondromucoprotein of chick and rabbit cartilage *in vivo* and *in vitro* (17, 18); similar mechanisms were held responsible for connective tissue breakdown due to excess vitamin A in amphibian larvae (19). Degradation of collagen can also be effected by enzymes concentrated in the lysosomal fractions of rat liver (20). Finally, extracts prepared from lysosome-rich fractions of rabbit liver have been found to evoke inflammation when injected intradermally (21).

In the experiments to be described, it has been found that SLO and SLS solubilize two hydrolytic enzymes, beta glucuronidase and acid phosphatase, from large granule fractions of rabbit liver, heart, spleen or lymph nodes into the suspending medium. At equivalent levels of hemolytic activity, SLS was at least ten times more active in this respect than SLO; no other streptococcal exotoxins affected lysosomes as significantly. One mitochondrial enzyme, malic dehydrogenase, was released partially by the action of the streptolysins. Although cortisol, cholesterol, and specific antibody inhibited SLO activity, these agents failed to inhibit SLS.

Materials and Methods

Animals.—Young hybrid male albino rabbits were obtained from commercial sources and fed a stock laboratory diet. They weighed approximately 1000 gm.

Streptolysin S.—This was a lyophilized product containing 7500 hemolytic units per mg prepared as described elsewhere (22). The hemolytic assay used for this and other streptococcal products has also been described (22).

Product from a Mutant Lacking SLS.—This was prepared by the method used for the preparation of SLS but was obtained from a mutant that does not synthesize SLS (22); it contained less than 100 hemolytic units per mg dry weight.

RNA Core.—This was a preparation of ribonuclease-resistant RNA used in the production of SLS (22). It contained less than 100 hemolytic units per mg.

Streptolysin O.—Preparation A was obtained from the C 203S strain of Group A streptococcus as described by Kellner *et al.* (23) and further fractionated by continuous-flow electrophoresis. It was then dialyzed and lyophilized. After activation with cysteine, it assayed 16,000 hemolytic units/mg dry weight; non-activated material had 1300 hemolytic units per mg. Preparation C′ was derived from the H46 A strain of Group C streptococcus. It was a lyophilized fraction obtained by continuous-flow electrophoresis of a crude concentrate of extracellular streptococcal proteins. After activation with cysteine, it assayed 7000 hemolytic units/mg; unactivated material had 2800 hemolytic units/mg. Preparation C″ was a partially purified streptolysin O derived from the H46 strain of Group C streptococcus. It was prepared by salting out proteins from culture supernatants with ammonium sulfate, adsorbing the streptolysin to $Ca_3(PO_4)_2$ and eluting the activity with 30 per cent saturated ammonium sulfate. The streptolysin was stored in 80 per cent saturated ammonium sulfate and portions were dialyzed against tap water before use. This preparation had 10,000 hemolytic units/mg in the absence of cysteine, and 20,000 hemolytic units/mg when fully activated. The protein concentration of this and other soluble preparations was estimated from the absorbance of the material at 280 mμ relative to that of a serum albumin standard.

Antibody to SLO.—This was a preparation of "antistreptolysin globulins" 20,000 units

per ml, batch No. 7, 19–9–47, Serum Institute, Carshalton, England. 0.06 ml was added to 1.0 ml of SLO(C′) containing 0.5 mg of SLO, and allowed to stand at room temperature for 10 minutes before addition of the entire mixture to the large granule fraction.

Streptococcal Deoxyribonuclease.—(DNAase A) was a product containing 51 DNAase units and 50 hemolytic units/ml and 0.75 mg per ml.

Streptokinase of high purity was obtained through the generosity of Dr. Alan Johnson; it contained 25,000 streptokinase units per ml, 0.45 mg protein per ml and less than 100 hemolytic units/ml.

Streptococcal proteinase precursor was a crystalline product obtained from cultures of a Group A streptococcus (strain AD 487). It contained 0.25 mg protein per ml and less than 10 hemolytic units/mg.

Streptococcal Diphosphopyridine Nucleotidase.—(DPNase) was prepared from cultures of Group C streptococci. It contained 400,000 DPNase units per mg, and 3000 hemolytic units (streptolysin O) when activated (15).

Erythrogenic toxin was obtained through the generosity of Dr. Aaron Stock, and contained 1072 Lf per mg, and less than 20 hemolytic units/ml.

Preparation of a Large Granule Fraction from Rabbit Liver.—The preparation of this fraction has been described in detail previously (24, 25). Briefly, this was a twice-washed fraction of tissue homogenates in 0.25 M sucrose, sedimenting between 800 *g* (10 minutes) and 15,000 *g* (20 minutes). It contained the bulk of mitochondria and lysosomes, as judged by enzyme analyses of the several fractions obtained, and was similar to preparations of rat liver studied by de Duve *et al.* (26) and by Dingle (6). The final suspension was 1:5 (w/v) in 0.25 M sucrose. While in most experiments liver was used, in some experiments preparations were made in identical manner from heart, spleen, and popliteal lymph nodes.

Incubation and Addition of Reagents.—In most experiments, 5.0 ml samples of the large granule fraction were incubated for 60 minutes at 37°C and appropriate amounts of the test materials were added in volumes not exceeding 0.5 ml. Following incubation, the samples were centrifuged at 20,000 *g* (20 minutes) and activity of enzymes released into the clear supernatant was assayed. "Controls" were samples to which had been added 0.5 ml aliquots of solvent: 0.9 per cent NaCl, 0.1 M phosphate buffer (pH 7.0) with or without 3.5 mM cysteine, or sucrose alone. The "total" activity of each enzyme which could be solubilized was determined by mechanically disintegrating samples of the same large granule fractions in a Waring blendor for 4 minutes, centrifuging at 20,000 *g* for 20 minutes, and measuring the activity of enzymes released into the supernatant.

Buffers for pH Studies.—When the effects of varying pH were to be determined, large granule fractions in 0.25 M sucrose were incubated in the presence of 0.05 M acetate buffers from pH 4.5 to 6.5, and in 0.05 M phosphate buffers from pH 7.0 to 7.8. In each case, the pH of the final suspension, as indicated in the appropriate figure, was directly determined.

Enzyme Assays.—Beta glucuronidase, acid phosphatase, and protein were determined as previously described (24, 25). Malic dehydrogenase was determined by the method of Mehler *et al.* (27).

Tabulation of Results.—To compare directly the action of various agents on release of three enzymes, all data have been expressed as "per cent of enzyme activity released from control suspensions." Figures for actual amounts of substrates which were hydrolysed are given; these have also been expressed as "per cent of total activity of the homogenate."

Cholesterol was prepared as a suspension according to the method of Cohen *et al.* (28) and was allowed to react with streptolysin O or S for 20 minutes at 4°C before the streptolysins were added to the granules. The final concentration of cholesterol was 1.5×10^{-4} M.

Saponin (Merck), a commercial grade product, was dissolved in 0.25 M sucrose.

Hydrocortisone 21–sodium hemisuccinate (solu-cortef, The Upjohn Co., Kalamazoo, Michi-

gan) was allowed to stand for 30 minutes with the large granule fraction in the cold before addition of the streptolysins. The final concentration was 1.5×10^{-4} M.

Cortisone acetate in aqueous suspension (Philadelphia Ampoule Labs.) was injected intramuscularly for 3 days (50 mg/kg) before the animals were killed and the large granule fraction prepared from their livers. Vitamin A alcohol (Nutritional Biochemicals Corporation, Cleveland) was dissolved in ethanol. An equal volume (0.5 ml) of ethanol was added to control suspensions.

RESULTS

The Effects of Streptolysin S on Release of Enzymes from the Large Granule Fraction of Rabbit Liver.—In Table I can be seen the effects of SLS on release of two lysosomal enzymes, beta glucuronidase and acid phosphatase, and one mitochondrial enzyme, malic dehydrogenase. In amounts of SLS ranging from 200 to 1000 hemolytic units per ml of suspension (26 to 130 μg/ml), release of the two lysosomal enzymes was accompanied by release of mitochondrial malic dehydrogenase. However, when SLS was added in concentrations of 5 to 100 hemolytic units/ml (0.65 μg/ml to 13.0 μg/ml) significant release of both lysosomal enzymes was observed without a comparable increase in the liberation of malic dehydrogenase. Neither the product obtained from a mutant lacking the ability to produce SLS, nor the RNA core used to induce the formation of SLS by streptococci, was active in releasing enzymes from the large granule fraction. Absence of SLS activity against lysosomes was observed when SLS was heated for 15 minutes at 100°C, or when fully active SLS was permitted to react with the granules for 60 minutes at 4°C. In contrast, exposure of the toxin to cholesterol, before the addition of SLS to the granules, diminished neither its activity against the granules nor against erythrocytes (Table I). Incubation of the granules with cortisol (1.5×10^{-4} M) did not protect them against SLS.

In Fig. 1 can be seen the effects of increasing the time of incubation on release of enzymes by SLS. Samples were incubated in unbuffered sucrose with 500 hemolytic units/ml of SLS and aliquots removed at the times indicated. Control values were obtained from tubes incubated 60 minutes in the absence of SLS. As early as 5 minutes after the addition of SLS, the two lysosomal enzymes appeared in the supernatant; malic dehydrogenase was not solubilized until 30 minutes had elapsed.

The effect of varying pH on release of enzymes by SLS is shown in Fig. 2. Below pH 5 the lysosomes were unstable (5), as demonstrated by release of acid phosphatase and beta glucuronidase from tubes containing buffer alone. Optimal release of these enzymes was at pH 7.5, and at this pH significant malic dehydrogenase activity was also liberated. However, at pH 6.5 (where the majority of experiments were performed), SLS acted chiefly to release lysosomal enzymes. Control values for pH experiments were taken from samples in unbuffered sucrose (pH 6.5) in 0.05 M NaCl.

TABLE I

Release of Enzymes from a Granular Fraction of Rabbit Liver in 0.25 M Sucrose by Streptolysin S and Related Products

Streptolysin preparation	µg/ml of granule suspension	Hemolytic units/ml of granule suspension	No. of experiments	Per cent of enzyme activity released by controls*		
				Beta glucuronidase	Acid phosphatase	Malic dehydrogenase
Controls	—	—	24	100‡	100§	100‖
Streptolysin S (SLS)	130.0	1000	6	975 ± 92¶	951 ± 102¶	301 ± 103¶
	65.0	500	6	874 ± 108¶	812 ± 96¶	297 ± 68¶
	32.5	250	4	730	660	241
	26.0	200	4	710	400	192
	13.0	100	4	550	327	108
	6.5	50	4	331	199	112
	0.65	5	2	225	140	103
	0.13	1	2	102	98	107
Streptolysin S lacking mutant	130.0	<10	2	110	97	98
	65.0	<5	2	106	116	141
	32.5	<2.5	1	108	106	87
RNA core, used in SLS preparation	130.0	<10	2	71	106	90
	65.0	<5	1	95	105	96
	32.5	<2.5	1	92	122	98
SLS heated at 100°	32.5	—	1	111	126	35
SLS, granules at 4°	32.5	250	1	104	85	115
SLS + cholesterol	130.0	1000	2	929	—	320
SLS + cortisol (1.5 × 10″ M)	130.0	1000	3	963	942	293

* All experiments in the presence of 0.09 per cent NaCl. Values are means.

‡ Controls released 10.4 ± 1.7 per cent of the "total" beta glucuronidase activity of the granules (17.5 ± 2.5 µg phenolphthalein/100 µg protein/hr.).

§ Controls released 11.3 ± 1.2 per cent of the "total" acid phosphatase activity of the granules (5.91 ± 1.7 µg phenolphthalein/100 µg protein/hr.).

‖ Controls released 24.2 ± 6.8 per cent of the "total" malic dehydrogenase activity of the granules (250 ± 86 malic dehydrogenase units/µg protein).

¶ $p = <0.01$.

The Effects of Streptolysin O on Release of Enzymes from the Large Granule Fraction of Rabbit Liver.—Streptolysin O added to large granule fractions in the absence of cysteine caused no appreciable release of enzymes (Table II) and these preparations were only slightly hemolytic. In the presence of 3.5 mM

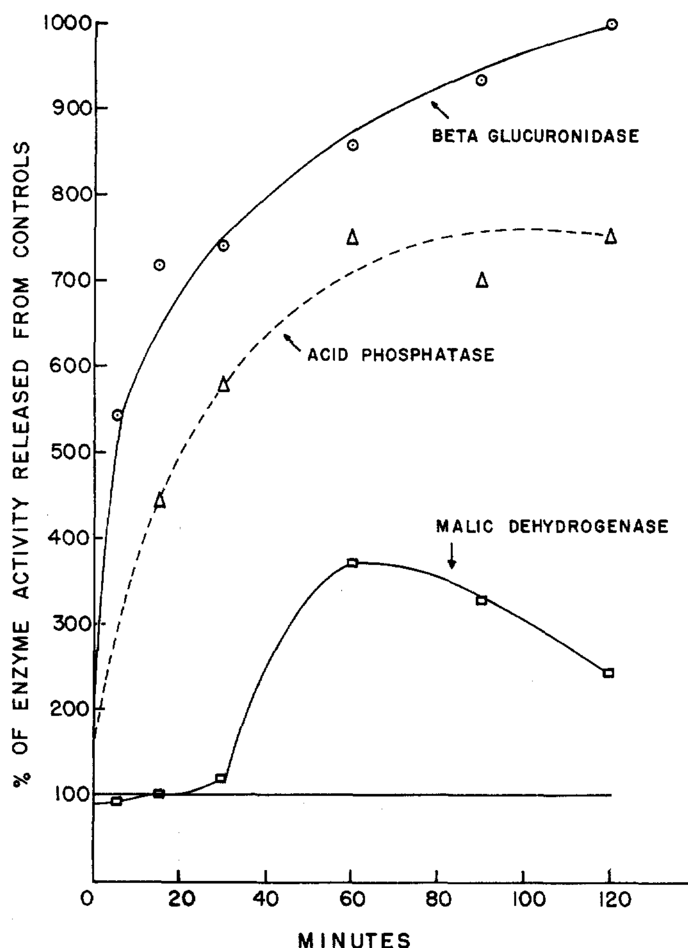

FIG. 1. Release of three enzymes from the large granule fraction of rabbit liver, in 0.25 M sucrose, by streptolysin S (500 hemolytic units/ml). Granules were incubated for periods indicated above, centrifuged for 20 minutes at 20,000 *g*, and enzyme activities appearing in the supernatant were determined. Results are expressed as per cent of activity released from control suspensions to which 0.09 per cent NaCl was added.

cysteine, however, three preparations of SLO, ranging in hemolytic activity from 160 to 2000 hemolytic units/ml of suspension (10 to 100 μg/ml) released enzymes from the granules. With rare exceptions, release of the two lysosomal enzymes exceeded liberation of malic dehydrogenase. However, since cysteine alone caused a twofold increase in malic dehydrogenase activity released from "controls" this finding cannot be interpreted as evidence against a significant effect of SLO on mitochondria.

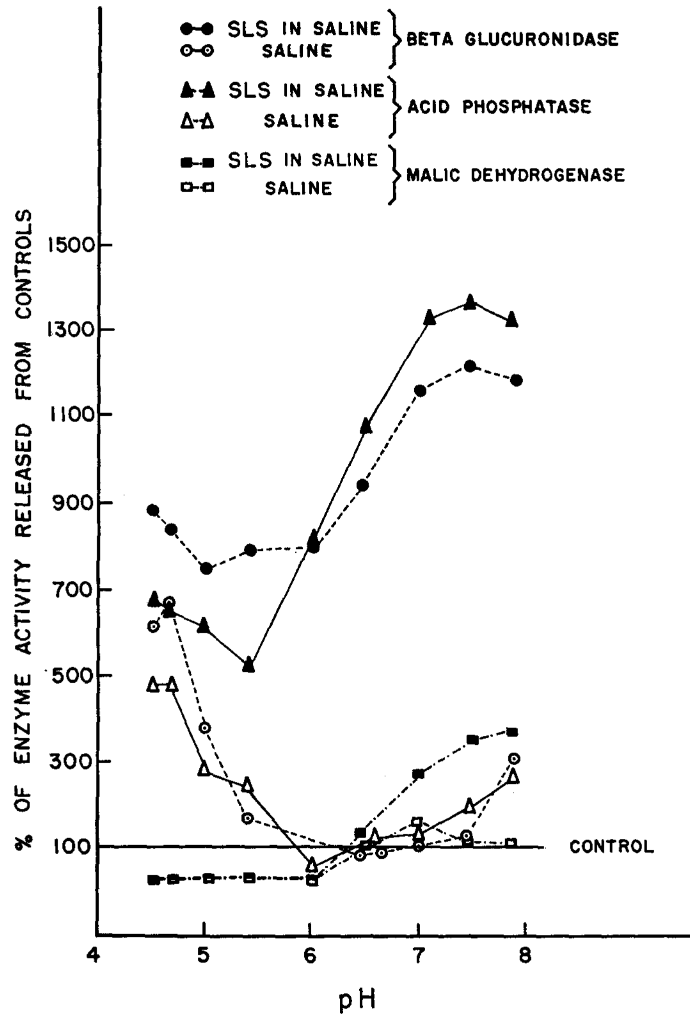

FIG. 2. Effect of varying pH on release of three enzymes from the large granule fraction of rabbit liver in 0.25 M sucrose. Granules were incubated with streptolysin S (500 hemolytic units/ml) at 37° for 60 minutes, in various buffers (see text), centrifuged for 20 minutes at 20,000 *g*, and enzyme activities appearing in the supernatant were determined. Results are expressed as per cent of enzyme activity released from control suspensions in unbuffered sucrose containing 0.05 M NaCl.

The effects of varying the time of incubation on release of enzymes by SLO are shown in Fig. 3. Both acid phosphatase and beta glucuronidase were solubilized in samples containing SLO and cysteine to a greater extent than in samples incubated with cysteine alone. Release of beta glucuronidase appeared to

TABLE II

Release of Enzymes from a Granular Fraction of Rabbit Liver in 0.25 M Sucrose by Streptolysin O and Related Products

Streptolysin preparation	Cysteine 3.5 mM	μg/ml granule suspension	Hemolytic units/ml granule suspension	No. of experiments	Per cent of enzyme activity released by controls*		
					Beta glucuronidase	Acid phosphatase	Malic dehydrogenase
Controls	+ −	—	—	36	100‡	100‡	100§
Streptolysin O (prep. C′)	−	25.0	70	2	177	95	100
	+	25.0	175	2	205	142	61
Streptolysin O (prep. A)	−	10.0	13	2	76	132	92
	−	25.0	33	2	108	165	87
	−	50.0	65	2	119	144	79
	+	10.0	160	5	174‖ ± 13	121 ± 18	151 ± 28
	+	25.0	400	5	241¶ ± 18	162¶ ± 19	180¶ ± 24
	+	50.0	800	7	311¶ + 23	194¶ + 21	140¶ + 32
Streptolysin O (prep. C″)	+	20.0	400	2	220	242	163
	+	50.0	1000	2	385	412	157
	+	100.0	2000	3	450	490	188
SLO (C″) + antibody	+	25.0	<10	2	96	103	97
Antibody alone	+	25.0	<10	2	112	113	142
SLO (A) + cholesterol	+	25.0	8	1	93	97	68
	+	50.0	16	1	118	113	139
Cholesterol alone	+	7.5 $\times 10^{-5}$ M	—	1	89	87	96
	+	1.5 $\times 10^{-4}$ M	—	1	79	83	69
SLO (A) heated 100°	+	50.0	<40	3	108	103	82
SLO (A), granules at 4°C	+	50.0	800	2	102	96	62
SLO (C″) + cortisol (1.5 $\times 10^{-4}$ M)	+	100	2000	4	220	118	142
Cortisol	+	1.5 $\times 10^{-4}$ M	—	3	86	82	102
SLO (A) and cortisone pretreatment	+	50.0	800	3	161	112	124

* All experiments in presence of 10 mM $PO_4^{=}$. Values are means.

‡ See footnotes to Table I.

§ In the presence of 3.5 mM cysteine, 53.5 per cent of the total malic dehydrogenase activity of the granules was released.

‖ $p < 0.05$

¶ $p < 0.01$

precede that of acid phosphatase; this was also observed in experiments with SLS. Control values for these experiments were taken from suspensions containing 0.01 M $PO_4^=$ with 3.5 mM cysteine, incubated for 60 minutes.

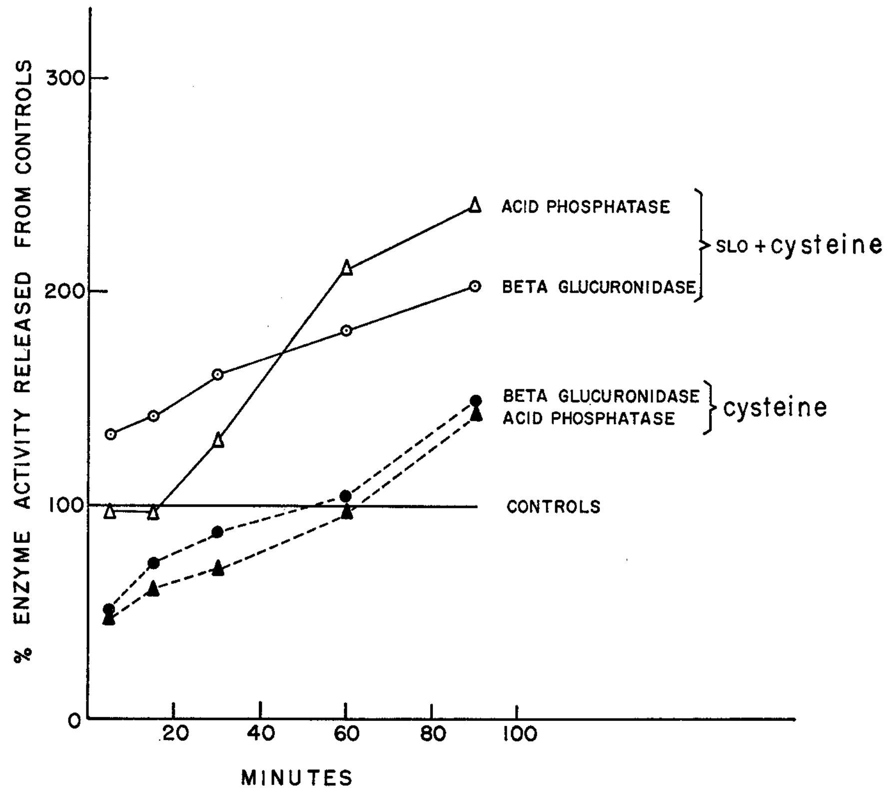

FIG. 3. Release of two acid hydrolases from the large granule fraction of rabbit liver in 0.25 M sucrose. Granules were incubated either with streptolysin O (preparation A, 400 hemolytic units/ml) and 0.01 M phosphate with 3.5 mM cysteine, or with phosphate and cysteine alone. After periods indicated above, they were centrifuged at 20,000 *g* for 20 minutes, and enzyme activities appearing in the supernatant were determined. Results are expressed as per cent of enzyme activity released from control suspensions containing 0.09 per cent NaCl.

In Fig. 4, the effect of varying pH on release of granular enzymes may be seen. As in the experiments with SLS, release of the two lysosomal enzymes below pH 5 was as marked in tubes containing buffer and cysteine alone, as in tubes containing SLO as well. The pH optimum of SLO action on the granules appeared to be 6.5, a finding in accord with the pH optimum of this agent on erythrocytes (29).

The Inhibition of SLO Action on Granules by Specific Antibody, Cholesterol, and Glucocorticoids.—Release of enzymes was largely abolished when SLO was incubated with specific antibody before addition to the granules (Table II). Antibody alone had no significant effect on the granules. Similar inhibition

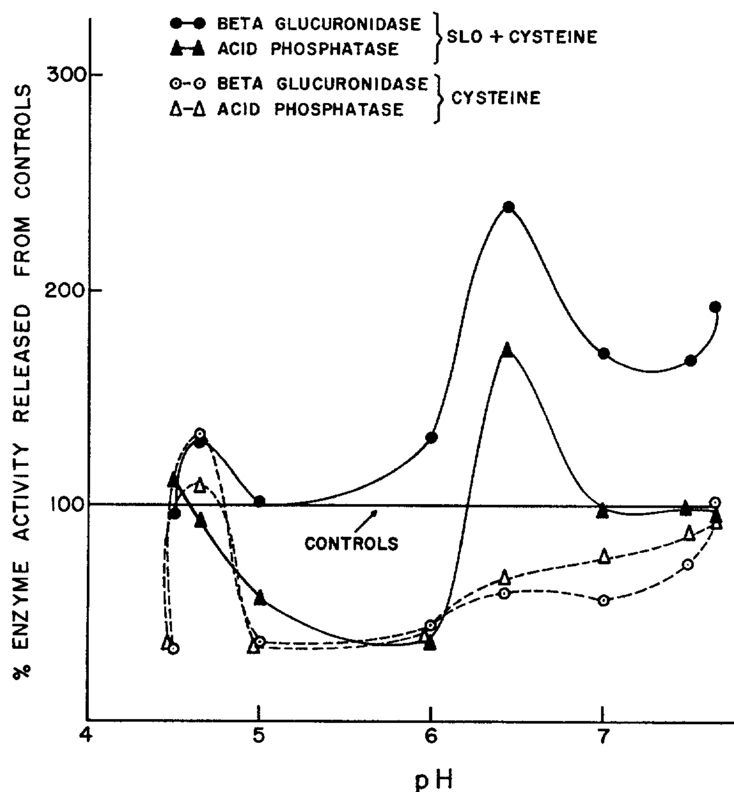

FIG. 4. Effect of varying pH on release of two acid hydrolases from the large granule fraction of rabbit liver in 0.25 M sucrose. Granules were incubated either with streptolysin O (preparation A, 400 hemolytic units/ml) and 0.01 M phosphate with 3.5 mM cysteine, or with phosphate and cysteine alone. After incubation with various buffers (see text) for 60 minutes at 37°, they were centrifuged at 20,000 *g* for 20 minutes, and enzyme activities appearing in the supernatant were determined. Results are expressed as per cent of enzyme activity released from control suspensions containing 0.09 per cent NaCl.

was noted if SLO had been reacted with cholesterol. At a final concentration of 1.5×10^{-4} M, cholesterol had a minimal inhibitory effect on release of enzymes, and this is in agreement with de Duve's findings (26).

When rabbits were pretreated with cortisone, large granule fractions prepared from their livers proved resistant to the action of SLO. This also is shown

in Table II. In these experiments, control values were obtained from samples prepared from the livers of cortisone-treated animals. Such treatment decreased the "total" content of beta glucuronidase by 22 per cent of control values and of malic dehydrogenase by 16 per cent of control values, but did not alter the acid phosphatase content significantly (25).

TABLE III

*Release of Hydrolytic Enzymes from a Granular Fraction of Rabbit Tissues in 0.25 M Sucrose by Streptolysin O**

Organ	Hemolytic units of SLO added/ml of granule suspension	Per cent of "total" activity of large granule fraction released into 20,000 g supernatant	
		Beta glucuronidase	Acid phosphatase
Liver	800	22.4	23.5
	400	16.7	19.8
	200	14.7	16.9
	0	11.0	14.4
Heart	800	56.0	83.7
	400	53.1	88.0
	200	61.0	54.0
	0	25.7	57.3
Spleen	800	80.5	61.0
	400	80.0	55.8
	0	65.0	45.0
Lymph nodes	800	55.0	94.0
	400	46.2	86.4
	200	44.6	76.3
	0	33.0	64.0

* Preparation "A," mean of two experiments.

‡ All experiments in the presence of 3.5 mM cysteine, 0.01 M $PO_4^{=}$.

The addition of cortisol, (1.5×10^{-4} M) to the large granule fractions, 30 minutes before addition of SLO, significantly prevented the solubilization of enzymes. Concentrations below this were not inhibitory; no significant *in vitro* inhibition of any of the three enzymes was observed when 10^{-4} M hydrocortisone was added to supernatants of blendorised preparations. Control values for these experiments were obtained from samples incubated in the presence of cortisol without SLO.

Effect of Activated Streptolysin O on Release of Lysosomal Enzymes from Organs other than Liver.—In Table III can be seen the consequences of addition of SLO, activated with 3.5 mM cysteine, to large granule fractions of rabbit

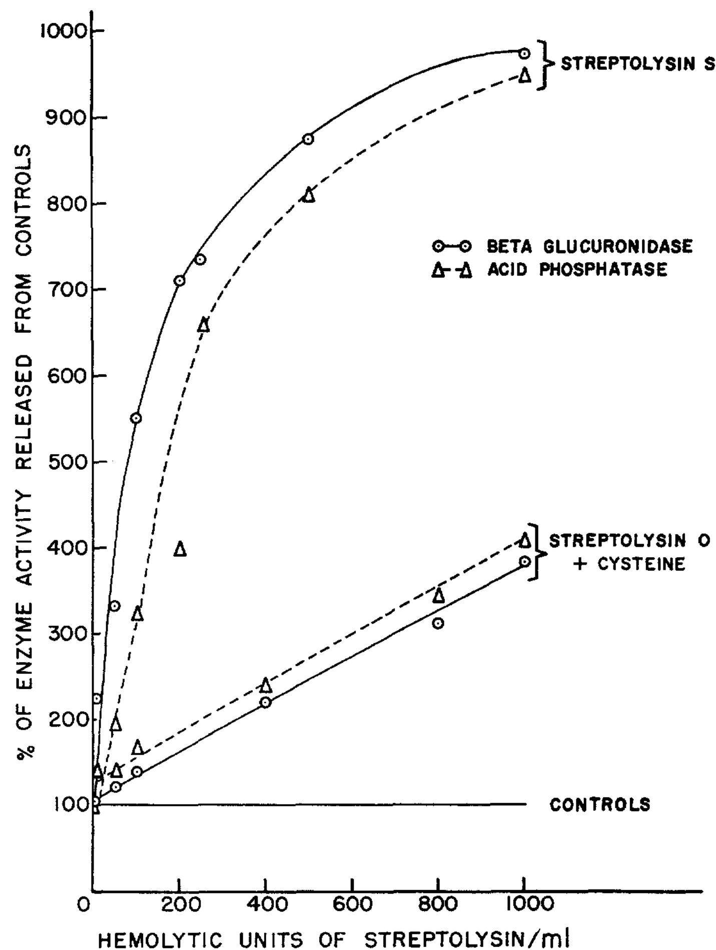

FIG. 5. Comparison of the effects of streptolysin S and streptolysin O on lysosomes and erythrocytes. The "lysosomal activity," expressed as per cent of enzyme released from control suspensions, of the two streptolysins is plotted on the ordinate as a function of the hemolytic activity, expressed as units, plotted on the abcissa.

liver, heart, spleen, and lymph nodes. While the activity of enzymes released from granules prepared from the various organs varied considerably, significant release of lysosomal enzymes by SLO was observed in all organs. Because of the difficulty with which such tissues as heart or lymph nodes were homogenized, it was neither possible to compare the distribution of enzyme activity of various organs directly, nor to judge whether SLO affected the granules of one organ more than others.

Comparison of the Effects of SLS and SLO on Lysosomes and Erythrocytes.—The "lysosomal activity" of SLO and SLS (expressed as per cent of enzyme released from control suspensions) was plotted as a function of hemolytic activity. From the data in Fig. 5 it is apparent that, for any given level of hemolytic action, considerably more lysosomal enzyme activity was released by

TABLE IV

Release of Enzymes from a Granular Fraction of Rabbit Liver in 0.25 M Sucrose

Material added	μg/ml of granule suspension	Hemolytic units/ml of granule suspension	No. of experiments	Per cent of enzyme activity released by controls*		
				Beta glucuronidase	Acid phosphatase	Malic dehydrogenase
Controls	—	—	36	100‡	100‡	100‡
Erythrogenic toxin	200	1	1	80	112	216
Streptokinase	50	10	1	100	129	102
DNAase A	75	5	2	91	67	84
DPNase	25	<40	2	97	69	176
Streptococcal proteinase, no cysteine	50	<1	2	98	118	87
Proteinase, + 3.5 mM cysteine	50	<1	2	178	141	175
	25	<1	1	145	116	112
Saponin	1250	—	2	254	—	—
Vitamin A alcohol	28.6	—	2	403	384	135

* All experiments in the presence of 0.09 per cent NaCl. Values are means.
‡ See footnotes to Table I.

streptolysin S than by streptolysin O. This was also the case when the two agents were compared on a *weight* basis (Tables I and II); 50 μg/ml of SLO (800 hemolytic units/ml) had approximately the same activity on release of lysosomal enzymes as 6.5 μg/ml of SLS (50 hemolytic units).

Effects of other Agents on Release of Enzymes from the Large Granule Fraction of Rabbit Liver.—In Table IV are listed the effects of some other streptococcal products on release of enzymes from lysosomes and mitochondria. While both erythrogenic toxin and DPNase appeared to release malic dehydrogenase from the granules, only the activated proteinase precursor liberated hydrolytic

enzymes from lysosomes. At concentrations of 50 μg/ml, however, the proteinase released enzyme activities no greater than those released by less than 1 μg/ml of streptolysin S, or 10 μg/ml of streptolysin O (Tables I and II). At equivalent w/v concentrations, SLS released 4 to 5 times and SLO approximately twice the enzyme activities solubilized by the activated proteinase. To compare the effects of two previously studied agents on lysosomes and mitochondria, saponin and vitamin A alcohol were added to the suspensions. On a weight/volume basis 28.6 μg/ml of the vitamin had the same activity as approximately 8 μg/ml of SLS or approximately 100 μg/ml of SLO. Much greater amounts of saponin were needed.

Discussion

The data presented above indicate that streptolysins O and S release beta glucuronidase and acid phosphatase from the lysosomes, and to a lesser degree, malic dehydrogenase from the mitochondria, of granular fractions from rabbit liver. At equivalent levels of hemolytic activity, SLS was far more active in releasing enzymes from the granules than SLO. This observation could reflect a true difference in the susceptibility of the granules to the two agents, or reflect differences in uptake and distribution of the two streptolysins among the mitochondria, which constitute the bulk of particles in the suspensions used. In the experiments described it has been assumed that increases in enzyme activity appearing in the granule supernatants represented the solubilization of enzymes after lysosomal or mitochondrial injury.

The methods employed, based on those of Dingle (6) rely on measurements of enzymes freed from their parent granules, rather than on measurements of the availability of enzymes to their substrate. The latter parameter, perhaps a more sensitive one, is measured more appropriately by the methods of de Duve *et al.*, who have also studied the effects of various enzymes and fat-soluble agents on lysosomes *in vitro* (26). The two phenomena, access to substrate and release of soluble enzymes, conceivably may be independent. However, the good agreement between the findings of Dingle (6) and de Duve *et al.* (26) on the "labilising" effect of vitamin A on lysosomes *in vitro*, and the agreement between de Duve's observation of the "stabilising" action of cortisol on lysosomes *in vivo* (30) and *in vitro* (26) and our own later findings (7, 24, 25), suggest that the phenomena are related, and that both methods may be used to measure them reliably. Indeed moderate injury may alter the availability of enzyme to substrate, while drastic insult to the particles may solubilize the enzymes (5); under these circumstances *in vivo*, lysosomal enzymes can escape into the circulation (31, 32).

It is unlikely that trace contaminants in the preparations were responsible for granule injury. Although very little is known at present of the intimate chemistry of the two streptolysins, the preparations used were highly purified (22, 23) and are among the most potent described (9). Since a product derived from a mutant lacking only the ability to produce SLS, and prepared in the same fashion as SLS (22), failed

to affect subcellular particles, and since the hemolytic action of the SLS preparations was directly proportional to the "lysosomal" activity, it is reasonable to conclude that SLS itself was the agent responsible for lysosomal rupture. Similarly, evidence that the hemolytic activity of SLO preparations paralleled "lysosomal" activity, that both actions were markedly enhanced by cysteine, that cholesterol and specific antibody inhibited both hemolysis and effects on lysosomes, and that the pH optimum of both the hemolytic action and the effect on lysosomes was 6.5, also make it unnecessary to postulate the presence of any agent other than SLO.

No direct evidence is available concerning the mechanisms of streptolysin action on subcellular particles. The streptolysins may however act enzymatically, for enzymes such as proteases and lecithinase have been shown to release acid hydrolases from lysosomes (5, 33) and are also hemolytic (1). However, judging from the greater concentrations of proteolytic enzymes (compared to streptolysins) necessary to damage, lysosomes (33) it may be inferred that the two lysins are unlike trypsin, chymotrypsin or pancreatin in their actions. The biological actions of SLO and SLS are by no means restricted to lysosomes or erythrocytes. Recently streptolysin S has been shown to cause "swelling" of mitochondria in concentrations of 33 hemolytic units/ml; SLO caused swelling at 333 hemolytic units/ml (34). It was apparent that, at the higher concentrations of SLS used in the present experiments, malic dehydrogenase *was* released from mitochondria, but to a lesser extent than the acid hydrolases. Although streptolysins share the property of affecting several membrane systems, their actions are by no means identical. Agents such as antibody, cortisol, or cholesterol, which inhibit, and cysteine, which activates SLO, have no effects on SLS action. Similarly, the pH optima of the two agents are quite distinct. On morphologic grounds, too, their actions are dissimilar, because differences in the sequence of granule lysis (16) and subsequent cell damage (14, 16) were seen when the streptolysins were added to leucocytes *in vitro*.

The role that membrane damage by streptolysins might play during acute streptococcal infections, or during such late sequelae as rheumatic fever, is obscure. Although the present report demonstrates that minute amounts of streptolysin S are active in causing release, from lysosomes, of hydrolytic enzymes which could act upon the tissues of the host, it remains to be determined whether such a mechanism is operative in streptococcal disease.

Summary

Streptolysins O and S from hemolytic streptococci have been added to granular fractions of tissue homogenates in 0.25 M sucrose prepared from rabbit liver, heart, spleen and lymph nodes. At concentrations ranging from 0.65 μg/ml to 2.0 mg/ml of streptolysin S, and from 10 μg/ml to 1.0 mg/ml of streptolysin O, two lysosomal enzymes (beta glucuronidase and acid phosphatase) and, to a lesser degree, one mitochondrial enzyme (malic dehydrogenase) were released into the supernatants of the reaction mixture. Although the hemolytic action of each lysin paralleled the effect on lysosomes, at equiva-

lent levels of hemolytic activity, SLS was approximately 10 times more active on lysosomes than SLO. SLO was inhibited by cholesterol, cortisol, and specific antibody *in vitro*; pretreatment of animals with cortisone decreased the susceptibility of their isolated lysosomes to SLO. These agents failed to prevent SLS action on lysosomes. SLO had a pH optimum of 6.5 against lysosomes while SLS was maximally active at 7.5. No other streptococcal extracellular products were as active on lysosomes as the streptolysins, although activated streptococcal proteinase precursor released some hydrolases from the granules. Similarities between the actions of SLO and SLS on red cells and lysosomes suggested that the membranes bounding lysosomes and erythrocytes have common properties.

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BIBLIOGRAPHY

1. Ponder, E., Hemolysis and Related Phenomena, New York, Grune and Stratton, Inc., 1948, 258.
2. Wintrobe, M. M., Clinical Hematology, Philadelphia, Lea and Febiger, 3rd edition, 1951, 576.
3. Dingle, J. T., and Lucy, J. A., Studies on the mode of action of vitamin A. 5. The effect of vitamin A on the erythrocyte membrane, *Biochem. J.*, 1962, **84,** 611.
4. Cook, J. S., Hemolysis by ultraviolet irradiation, *in* Progress in Photobiology, (B. Ch. Cristensen and B. Buchman, editors), Amsterdam, Elsevier Publishing Company, 1961, 453.
5. de Duve, C., Lysosomes, a new group of cytoplasmic particles, *in* Subcellular Particles, (T. Hayashi, editor), New York, Ronald Press Co., 1959, 128.
6. Dingle, J. T., Studies on the mode of action of vitamin A. 3. The release of a bound protease by the action of vitamin A., *Biochem. J.*, 1961, **79,** 500.
7. Weissmann, G., and Dingle, J. T., Release of lysosomal protease by ultraviolet irradiation and inhibition by hydrocortisone, *Exp. Cell Research*, 1961, **25,** 207.
8. Todd, E. W., The differentiation of two distinct serological varieties of streptolysin, streptolysin O and streptolysin S, *J. Path. and Bact.*, 1938, **47,** 423.
9. Bernheimer, A. W., Streptolysins and their inhibitors, *in* Streptococcal Infections, (M. McCarty, editor), New York, Columbia University Press, 1954, 19.
10. Herbert, D., and Todd, E. W., The oxygen stable haemolysin of group A haemolytic streptococci (streptolysin S), *Brit. J. Exp. Path.*, 1944, **25,** 242.
11. Barnard, W. G., and Todd, E. W., Lesions in the mouse produced by streptolysins O and S, *J. Path. and Bact.*, 1940, **51,** 43.
12. Halbert, S. P., Bircher, R., and Dahle, E., The analysis of streptococcal infections. V. Cardiotoxicity of streptolysin O for rabbits *in vivo*, *J. Exp. Med.*, 1961, **113,** 785.

13. Cantoni, G. L., and Bernheimer, A. W., The toxic action of preparations containing the oxygen-labile hemolysin of streptococcus pyogenes. IV. Comparison of the cardiotoxic action with that of saponin, *J. Pharm. and Exp. Therap.*, 1947, **91,** 31.

14. Bernheimer, A. W., and Schwartz, L. L., Leucocidal agents of haemolytic streptococci, *J. Path. and Bact.*, 1960, **79,** 37.

15. Bernheimer, A. W., Recent studies on streptolysin O and streptococcal diphosphopyridine nucleotidase, *Rev. Pediat.*, 1960, **15,** 237.

16. Hirsch, J. G., Bernheimer, A. W., and Weissmann, G., Motion picture study of the toxic action of streptolysins on leucocytes, *J. Exp. Med.*, 1963, **118,** 223.

17. Dingle, J. T., Lucy, J. A., and Fell, H. B., Studies on the mode of action of excess of vitamin A. I. The effect of excess of vitamin A on the metabolism and composition of embryonic chick limb-cartilage grown in organ culture, *Biochem. J.*, 1961, **79,** 497.

18. Thomas, L., McCluskey, R. T., Potter, J. L., and Weissmann, G., Comparison of the effects of papain and vitamin A on cartilage. I. The effects in rabbits, *J. Exp. Med.*, 1960, **111,** 705.

19. Weissmann, G., Alterations in connective tissue and intestine produced by hypervitaminosis A in *Xenopus laevis*, *Nature*, 1961, **192,** 235.

20. Frankland, D. M., and Wynn, C. H., The degradation of acid-soluble collagen by rat-liver preparations, *Biochem. J.*, 1962, **85,** 276.

21. Janoff, A., and Kaley, G., data to be published.

22. Bernheimer, A. W., Formation of a bacterial toxin (streptolysin S) by resting cells, *J. Exp. Med.*, 1949, **90,** 373.

23. Kellner, A., Bernheimer, A. W., Carlson, A. S., and Freeman, E. B., Loss of myocardial contractility induced in isolated mammalian hearts by streptolysin O, *J. Exp. Med.*, 1956, **104,** 361.

24. Weissmann, G., and Thomas, L., Studies on lysosomes. I. The effects of endotoxin, endotoxin tolerance, and cortisone on release of acid hydrolases from a granular fraction of rabbit liver, *J. Exp. Med.*, 1962, **116,** 433.

25. Weissmann, G., and Thomas, L., Studies on lysosomes. II. The effect of cortisone on the release of acid hydrolases from a large granule fraction of rabbit liver induced by an excess of vitamin A., *J. Clin. Inv.*, 1963, **42,** 661.

26. de Duve, C., Wattiaux, R., and Wibo, M., The effects of fat-soluble compounds on lysosomes *in vitro*, *Biochem. Pharmacol.*, 1962, **9,** 97.

27. Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate, *J. Biol. Chem.*, 1948, **174,** 961.

28. Cohen, B., Schwachman, H., and Perkins, M. E., Inactivation of pneumococcal hemolysin by certain sterols, *Proc. Soc. Exp. Biol. and Med.*, 1937, **35,** 586.

29. Herbert, D., and Todd, E. W., Purification and properties of a hemolysin produced by group A hemolytic streptococci (streptolysin O), *Biochem. J.*, 1941, **35,** 1124.

30. Beaufay, H., van Campenhout, E., and de Duve, C., Tissue fractionation studies. II. Influence of various hepatotoxic treatments on the state of some bound enzymes in rat liver, *Biochem. J.*, 1959, **73,** 617.

31. Janoff, A., Weissmann, G., Zweifach, B., and Thomas, L., Pathogenesis of experimental shock. IV. Studies on lysosomes in normal and tolerant animals subjected to lethal trauma and endotoxemia, *J. Exp. Med.*, 1962, **116,** 451.
32. Weissmann, G., Uhr, J. W., and Thomas, L., Acute hypervitaminosis A in guinea pigs. I. Effects on acid hydrolases, *Proc. Soc. Exp. Biol. and Med.*, 1963, **112,** 284.
33. Beaufay, H., and de Duve, C., Tissue fractionation studies IX. Enzymic release of bound hydrolases, *Biochem. J.*, 1959, **73,** 604.
34. Keiser, H., Bernheimer, A. W., and Weissmann, G., Studies on lysosomes. IV. Solubilization of enzymes during mitochondrial swelling induced by streptolysin S and vitamin A, submitted for publication.