ACTIVITIES OF BACTERIAL LEVANS AND OF LIPOPOLYSACCHARIDES IN THE PROCESSES OF INFLAMMATION AND INFECTION

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LEVAN and related high molecular bacterial polysaccharides given systemically to laboratory animals have been shown to modify the inflammatory response (Shilo, Wolman and Hestrin, 1954; Davies, Shilo and Hestrin, 1955; Shilo, Wolman and Wolman, 1956; Hestrin, 1956) and to lower resistance towards localised bacterial infections (Shilo, Feingold and Hestrin, 1953; Hestrin, Shilo and Feingold, 1954*a*; Hestrin, Shilo, Feingold and Wolman 1954*b*; Shilo *et al.*, 1954; Joo and Csizmas, 1957).

Likewise bacterial lipopolysaccharides have been shown to cause fluctuation of resistance of hosts towards intraperitoneal bacterial infections (Tal and Olitzki, 1948; Landy and Pillemer, 1956; Dubos and Schaedler, 1956; Boehme and Dubos, 1958) and to suppress local manifestations of acute inflammation in the dermis (Miles and Niven, 1950; Delauney, Delauney and Lebrun, 1947). It has further been demonstrated that some native levans and dextrans, in common with a number of bacterial lipopolysaccharides, interact with the properdin system *in vitro* (Pillemer, Schoenberg, Blum and Wurz, 1955) and cause a characteristic rise in the plasma properdin level *in vivo* associated with markedly increased resistance of hosts to infection (Pillemer *et al.*, 1955; Landy, 1956; Kiser, Lindh and de Mello, 1956).

Since trace amounts of lipopolysaccharides are sufficient to induce the above-mentioned biological activities and since their widespread occurrence in bacterial cells and in animal and plant tissue has been demonstrated (Landy and Shear, 1957) the question has arisen whether lipopolysaccharide contamination can account for some or all of the previously reported biological activites of neutral polysaccharides.

The purpose of the present work was to clarify which biological activities are inherent to the levan molecule and which are due to contaminating substances. The biological activites investigated were infection-promoting activity (IPA), endothelium-sealing activity (ESA), the suppressor activity on acute inflammation, necrosis-promoting activity (NPA) and the resistance-increasing activity (RIA).

METHODS

Native levans.—For the production of native levans the following strains were used: *Aerobacter levanicum*, isolated by Aschner, Avineri-Shapiro and Hestrin (1942), *Pseudomonas denitrolevaniformans* kindly given to us by Dr. A. Fuchs, Delft, and a *Corynebacterium* sp. isolated by Henis and Aschner (1954). *Aero. levanicum* levan and *Ps. denitrolevaniformans* levan were obtained from sucrose cultures and were purified by ethanol precipitation at

neutral pH as described by Hestrin *et al.* (1954*a*). *Corynebacterium* sp. levan was obtained by the action of resting cells on buffered sucrose solution as described by Avigad and Feingold (1957).

Treatment of levans with alkali. Alkaline ethanol treatment.—Native levans were precipitated from a ~ 3 per cent aqueous solution by addition of 2 volumes of alkaline ethanol, brought to pH 12 by addition of 10 N NaOH. The precipitate was resuspended in distilled water and reprecipitated in 2 volumes of alkaline ethanol. The white flocculate precipitate was dissolved in distilled water and dialysed against distilled water in the cold (4°) until pH 7 was reached (24–48 hr.). The levan was then precipitated by addition of 3 volumes of 96 per cent ethanol to 1 vol. of the solution and the gummy precipitate was converted into a white powder by persistent trituration under absolute ethanol. The powder was washed with petroleum-ether on a Buchner funnel and dried over calcium chloride.

Precipitation of levan by cetyltrimethylammonium bromide (CETAB).—A convenient method of purifying levan was afforded by the use of CETAB as a precipitating reagent in the presence of alkaline aqueous borate solution according to the principle proposed by Palmstierna, Scott and Gardell (1957) and worked out for levan in this laboratory by G. Avigad (1958). The precipitated levan was freed of salts by washing successively in aqueous methanol and petroleum ether.

Estimation of levan concentration.—The concentration of levan solutions was estimated by determining total fructose using the method of Roe, Epstein and Goldstein (1949).

Native Leuconostoc mesenteroides dextran.—Lot N-378, kindly supplied by Dr. H. Staveley, Commercial Solvents Corporation, Terre Haute, U.S.A.

Lipopolysaccharides.—AE 1688, S_4 derived from *Salmonella abortus equi* and Co08, 1684, S_4 derived from *Bacterium coli* were highly purified samples (Westphal and Luederitz, 1954) and kindly sent to us by Dr. O Westphal, Wander Forschungsinstitut, Freiburg, Germany.

In certain experiments, lipopolysaccharide was subjected to alkaline ethanol treatment for the purification of levans.

Polyvinylpyrrolidone.—Type NP (K90) kindly supplied by Mr. R. D. Goghill, Director of Research of General Aniline & Film Corporation, New York, U.S.A.

Mild acid hydrolysis of polymers.—Mild acid hydrolysis of polymers was carried out in aqueous solution at pH 3·2 (by addition of 1·0 N HCl) and heating for 60 min. at 60°. The pH of the hydrolysed solution was brought to 7 and sodium chloride added to make an 0·85 per cent solution.

Preparation of solutions and their injection.—Solutions of polymers were prepared for injection as previously described (Shilo *et al.*, 1956). Special care was taken to avoid lipopolysaccharide contamination of glassware and syringes.

Bacterial strains used in experimental infections.—*Salmonella typhi* 0901, *Salmonella paratyphi* B, strain 29 KA, *Salmonella paratyphi* C, strain 13 KA were grown in nutrient broth (Difco) for 24 hr. at 37°. *Pseudomonas aeruginosa*, strain S 64 and *Proteus* X 19 were grown on slants of nutrient agar (Difco) for 24 hr. at 37°. *Corynebacterium diphtheriae gravis* was grown on slants of brain heart infusion agar (Difco) for 24 hr. at 37°.

The above listed strains were obtained from the culture collection of the Department of Bacteriology of the Hebrew University-Hadassah Medical School.

Staphylococcus aureus, strains 9 and 33 were grown on slants of nutrient agar (Difco) for 24 hr. at 37°. These strains were isolated from cases of mastitis in sheep by Dr. T. Dishon and kindly put at our disposal.

Animals.—Albino mice 16–20 g. and adult rabbits 1½–2 kg. were used. The skin of the abdomen of the rabbits was depilated with barium sulphide before the intradermal injection of the bacteria.

Test of toxicity.—Toxicity of a given polymer was tested by intravenous injections into rabbits and mice. Diarrhoea in rabbits and mortality in mice served as an indicator of toxicity. Groups of 10 mice were used for the determination of the minimal lethal dose (MLD) of each polymer dose.

Measurement of infection-promoting activity (IPA).—IPA of a polysaccharide was tested by injecting different amounts of the polymer intravenously into mice which were infected intraperitoneally promptly thereafter with a standard sub-

lethal dose of *Salm. typhi* 0901 (1×10^7). The technique was similar to that used in our earlier work (Hestrin *et al.*, 1954*a*). The minimal amount of polymer which caused at least 80 per cent mortality served as a measure of the activity of the polymer. Groups of 10 mice were used at each test level.

Measurement of resistance-increasing activity (RIA).—RIA of a given polymer was tested in mice exposed to levan-enhanced *Salm. typhi* 0901 infection. The polymer to be tested was given intravenously and 24 hr. later the mice were given 10 mg. of alkaline ethanol-treated *Aero. levanicum* levan intravenously and a standard dose of *Salm. typhi* 0901 intraperitoneally. RIA was measured as the minimal amount of polymer given 24 hr. prior to the lethal challenge which saved at least 80 per cent of the infected mice. Groups of 10 mice were used at each tested polymer dose.

Test of endothelium-sealing activity (ESA).—Rabbits received polymer intravenously 15 min. prior to an intravenous injection of a 1 per cent solution of trypan blue (C.I. No. 437, National Aniline Division, New York) in saline. The delay in the appearance of a blue coloration at sites of irritation in the skin served as a measure of ESA. A dose of 0·002 ml. turpentine (commercial preparation) given intradermally into the depilated abdomen with the aid of an Agla microsyringe 3 min. before the administration of polymer served for producing the standard irritation of the skin. Non-levanised animals given irritant and trypan blue served as controls.

Test of suppressor activity of polymer on acute inflammation.—Polymer was injected intravenously into rabbits together with an intradermal standard dose of *Staph. aureus*, strain 9 (3×10^6 organisms) as described by Shilo *et al.* (1956). The suppressor effect of polymer on the acute inflammatory response was judged both on the basis of the induced delay of the appearance of macroscopic signs of acute inflammation (oedema and erythema) and of histologically-detectable diapedesis. Diapedesis was examined in histological sections fixed in Carnoy's fluid and stained with haematoxylin and eosin and Gram's stain. Non-levanised rabbits injected with a standard dose of staphylococci served as controls.

Measurement of necrosis promoting activity (NPA) of polymer in staphylococcal lesions in the skin.—Polymer was injected intravenously into rabbits simultaneously with an intradermal standard dose of *Staph. aureus*, strain 9 (3×10^6 organisms) as described by Shilo *et al.* (1956). The time of appearance and the size of the area of necrosis were regarded as measures of activity. Lesions were traced on cellophane paper and their areas were measured with the aid of a planimeter.

RESULTS

All tested preparations of native levan irrespective of source manifested IPA (Table II). On the other hand, only those levan preparations which had been obtained from sucrose cultures of Gram-negative organisms (*Aero. levanicum* and *Ps. denitrolevaniformans*) and which had been treated only by ethanol precipitation at a neutral pH manifested RIA (Tables I and II). The latter activity is one which such levan preparations share with a pure lipopolysaccharide preparation (Tables I and II).

The effect of mild acid treatment and of alkali on the RIA and IPA of preparations of levan and lipopolysaccharide are shown in Tables I and II. The RIA of these levan preparations was not destroyed by partial degradation of the macro-

TABLE I.—*Resistance-increasing Activity of Polymer Preparations in Respect to Levan-enhanced* Salm. typhi *Infection in Mice*

Polymer		Treatment		Resistance-increasing activity observed at different polymer doses (mg.)						
Kind	Source	A*	H†	0·001	0·010	0·05	1·0	5·0	10·0	30·0
Levan	*Aero. levanicum*	−	−	..	9/20	18/20	19/20	..	..	..
		−	+	..	..	9/10	8/10	..	..	..
		+	−	..	..	..	..	0/20	2/20	1/10
		+	+	..	..	..	..	..	..	0/20
	Ps. denitro-levaniformans	−	−	..	5/10	8/10	9/10	..	..	..
		+	−	..	..	..	..	..	0/10	..
		−	+	..	..	7/10	9/10	..	..	..
		+‡	+	..	..	..	..	..	..	0/8
	Corynebacter sp.	−	−	..	..	..	..	..	..	0/10
		−	+	..	..	..	..	..	..	0/10
Dextran	*Ln. mesenteroides*	−	−	..	..	..	..	..	..	0/20
Lipopoly-saccharide	*Salm. abortus equi* (AE 1688 S_4)	−	−	7/10	20/20	19/20	..	..	..	..
		−	+	7/9	8/10	9/10	..	..	..	..
		+	−	..	..	2/10	..	..	..	..
	Bact. coli (Co08 S_5)	−	−	6/10	19/20	10/10	..	..	..	..
Polyvinylpyrrolidone (K 90)		−	−	..	..	..	..	..	..	0/20

Resistance-increasing activity is expressed as number of mice (numerator of fraction) among those tested (denominator of fraction) which survived challenge (levan-enhanced *Salm. typhi* infection) delivered 24 hr. after injection of the specified dose of the polymer.

* Alkaline ethanol treatment.

† Mild acid hydrolysis.

‡ Alkaline CETAB treatment.

TABLE II.—*Comparison of Toxicity, Resistance-increasing Activity and Infection-promoting Activity of Polymer Preparations in Mice*

Polymer		Treatment		Minimum effective dosage[1] (mg.)		
Kind	Source	A*	H†	Toxicity	Resistance increase	Infection promotion
Levan	*Aero. levanicum*	−	−	10	0·1	5
		−	+	10	0·1	×
		+	−	×	×	5
		+	+	×	×	×
	Ps. denitrolevaniformans	−	−	5	0·1	1
		−	+	..	0·1	..
		+	−	×	10	5
		+‡	−	×	×	5
	Corynebacter sp.	−	−	×	×	2
		−	+	×	×	×
		+	−	×	×	2
Lipopolysaccharide	*Salm. abortus equi* (AE 1688 S_4)	−	−	0·1	0·001	××
		−	+	0·1	0·001	××

[1] See methods.

*, †, ‡ See Table I.

× No effect with max. dose tested (30 mg.).

×× No effect up to toxic dose (0·1 mg.).

molecule on mild acid hydrolysis. IPA, on the other hand, is known to be destroyed by such treatment (Hestrin *et al.*, 1954*a*), a feature which has been confirmed by the present experiments and extended to all the examined levans. Comparison of IPA of the different alkali-treated levans showed them to be of a similar order, *Corynebacterium* levan having highest activity. Preparations of levan which showed RIA and exerted toxic effect in animals were rendered non-toxic towards mice and rabbits and lost their RIA (Table II) by repeated precipitations with alkaline ethanol. Similar results were obtained with *Pseudomonas* levan treated with alkaline CETAB (Table II). RIA of lipopolysaccharide preparations was similarly abolished by contact with alkali in aqueous solution but was not destroyed by acid treatment which sufficed to degrade levan partially (Table I). Thus it is evident that RIA and IPA in a levan preparation were separable functions.

The IPA of alkali-treated levan in respect to intraperitoneal infections produced by several unrelated species of bacteria was evaluated in experiments summarised in Table III. It can be seen that IPA is not limited to *Salm. typhi* infections but extends also to infections produced by several other Gram-negative and Gram-positive organisms.

TABLE III.—*Infection-promoting Activity of Alkali Treated Levan Towards Intraperitoneal Infections in Mice with Different Bacterial Species*

Bacteria[1]	Mortality[2] within 48 hr.	
	With levan[3]	Without levan
Salm. typhi 0901	10/10	0/10
Salm. paratyphi B	9/10	0/10
Salm. paratyphi C.	6/10	0/10
Ps. aeruginosa	9/10	0/10
Pr. X 19	10/10	1/10
Bact. coli, 104	4/10	1/10
Staph. aureus, 9	10/10	0/10
Staph. aureus, 33	10/10	0/10

[1] Highest number of organisms which caused no mortality in absence of levan.
[2] Number of mice dying (numerator) out of those challenged (denominator).
[3] 10 mg. per mouse i/v at time of infection.

Endothelial-sealing-activity (ESA) of all alkali treated levans was clearly demonstrated by the trypan blue test. In every case the escape of trypan blue into sites of skin irritation was retarded for 30 min. or more as compared with the nearly immediate (5 min.) blueing in the non-levanised control animals, thus confirming and extending the effect described by Davies *et al.* (1955) for *Aerobacter* levans purified by precipitation at neutral pH. The suppressor activity on acute inflammation, necrosis-promoting-activity (NPA) and ESA of different levan preparations as well as dextran, PVP and lipopolysaccharides were compared using methods described by us previously (Shilo *et al.*, 1956 ; Davies *et al.*, 1955) (Table IV and Fig. 1). Macroscopically, suppressor activity on acute inflammation and NPA were exhibited by all the native levans tested and by the acid hydrolysates of *Aerobacter* and *Pseudomonas* levans not treated by alkali. Hydrolysates of *Aerobacter* and *Pseudomonas* levans treated by alkali and of untreated *Corynebacterium* levan were devoid of these activites.

TABLE IV.—*Effect of Alkali Treatment and Mild Acid Hydrolysis on ESA and NPA of Polymers in* Staphylococcus *Infection*

Polymer			Treatment		Quantity	Suppression of acute inflammation			
Kind	Source		A*	H†	mg./kg.	Erythema[1]	Diapedesis[2]	NPA[3]	ESA[4]
Levan	*Aero. levanicum*		−	−	100	−	−−	+	+
			−	+	100	−	−−	+	−
			+	−	100	−	−	+	+
			+	+	100	+	+	−	−
	Corynebacterium sp.		−	−	100	−	..	+	+
			−	+	100	+	..	−	−
			+	−	100	−	..	+	+
Lipopolysaccharide	*Salm. abortus equi* AE 1688 S_4		−	−	0·02	−	−−	+	..
			−	+	0·02	−	..	+	..
			+	−	0·02	+	..	−	..
Polyvinylpyrrolidone (K 90)			−	−	250	+	..	−	−
None			−	−	−	+	+	−	−

[1] + : Visible erythema and oedema < 3 hr. ; − : absent ⩾8 hr.
[2] + : Marked leucocyte diapedesis < 1 hr. ; − : absent 5 hr. ; −− : absent > 8 hr.
[3] + : Dermonecrosis 7 hr. (spread to 14 − 20 sq. cm. 24 hr.) ; − : No dermonecrosis.
[4] + : No blueing ⩾ 30 min. ; − : Blueing 5–10 min.
*, † See Table I.

Histological examinations of animals given alkali treated *Aerobacter* levan showed no diapedesis even after 5 hr. although marked leucostasis was evident already 1 hr. after infection. In rabbits injected intravenously with hydrolysates of alkali treated *Aerobacter* levan significant diapedesis of leucocytes was found already 1 hr. after infection, phlegmonous infiltration after 3 hr., and formation of minute abscesses was evident after 5 hr.

Intravenous injection into rabbits of minute amounts (5–20 μg.) of the purified lipopolysaccharide resulted in suppression of oedema, erythema and diapedesis for 8 hr. or more. These results confirmed and extend the findings of Delauney *et al.* (1947) and Miles and Niven (1950) obtained with cruder endotoxin preparations.

The strong suppressor activity on acute inflammation and NPA serve as a delicate test for lipopolysaccharide contamination in levan preparations, provided the levan is first depolymerised by mild acid hydrolysis. In our test system clear effects were obtained by the injection of 5 μg. of purified lipopolysaccharide/kg. body weight. The presence of 5 μg. of lipopolysaccharide in a 100 mg. dose of levan, *i.e.* 0·005 per cent, could easily be detected.

In the levanised rabbit, suppression of the inflammatory response and NPA have also been shown to occur in dermal infections with *Ps. aeruginosa*, *Pr.* X 19 and *C. diphtheriae gravis* (Table V).

DISCUSSION

Treatment of levan from Gram-negative organisms with alkaline ethanol or CETAB at alkaline pH removes resistance-increasing activity (RIA) and toxicity but not infection-promoting activity (IPA), endothelium-sealing activity (ESA),

TABLE V.—*Effect of Levan on the Induction of Necrosis in Dermal Lesions Caused by Different Bacteria*

Bacteria	Levan[1]	Area of dermonecrosis and of erythema in sq. cm. with different doses of bacteria[2]		
		0·1 ml.	0·01 ml.	0·001 ml.
Staph. aureus, strain 9	+	15·0	9·5	4·0
	−	0 (3·0)	0 (1·5)	0
Ps. aeruginosa	+	20·0	9·5	2·5
	−	1·5 (3·5)	0 (1·0)	0 (0·25)
Pr. X 19	+	4·2	0	0
	−	0 (2·0)	0	0
C. diphtheriae gravis	+	34·5	0	0
	−	0 (3·0)	..	..

[1] 100 mg./kg. alkali treated *Aero. levanicum* levan 5 min. before infection.
[2] Numbers given represent the average area of dermonecrosis in two rabbits. Numbers in brackets represent the total area of lesion including erythema. Where bracketed values are not given no eythema was visible.

suppressor activity on acute inflammation and necrosis-promoting activity (NPA) from the original preparation of levan. This suggests that during the treatment some contaminating substance of the levan might have been inactivated or removed. Alkaline treatment of lipopolysaccharide is known to remove many of their biological activities (Landy and Johnson, 1955) indicating that the contaminant might be a lipopolysaccharide. The destruction of the neutral polysaccharide by mild acid hydrolysis showed that RIA and toxicity could not have been due to high molecular levan *per se* and further indicated the presence of a contaminating substance in some of the levan preparations which has both RIA and toxicity.

The lipopolysaccharide nature of the contaminant is consistent with the fact that levan produced by Gram-negative organisms rich in lipopolysaccharides contained an active contaminant whereas levan from *Corynebacter* sp. and dextran from *Leuconostoc mesenteroides*, both Gram-positive organisms known to have a low content of lipopolysaccharides, behaved as if they were initially free of this contamination.

The data obtained with purified, or initially lipopolysaccharide-free levans indicate that IPA, ESA, suppression of the inflammatory response and NPA are likely to be inherent properties of the native levan molecule, whereas RIA and toxicity, may probably be attributed to lipopolysaccharide contamination.

The previously described biological properties of *Aero. levanicum* levan and of *Ln. mesenteroides* dextran can now be further extended to other polysaccharides of the levan family of different molecular shape. *Corynebacter* sp. levan which differs from other levans in that the length of its basal chain is 1·6 times as high as that found for *Aero. levanicum* levan (Avigad and Feingold, 1957) or for *Ps. prunicola* levan (Bell and Dedonder, 1954), was shown to be similar in its biological properties to *Aerobacter* and *Pseudomonas* levans.

Although similarities between some of the biological activites of neutral polysaccharides and lipopolysaccharides are apparent, there are also important differences between the 2 groups of substances. Whereas lipopolysaccharides

act in trace amounts suggesting a trigger-like induction of a chain of reactions, often resulting in shock, neutral polysaccharides were shown not be shocking agents (Davies *et al.*, 1955) and are biologically active only when given in comparatively large amounts. It therefore seems likely that different mechanisms underlie the biological activities of the 2 groups of polysaccharides.

The findings that levan preparations are often contaminated with lipopolysaccharide makes it necessary to reconsider if some activites ascribed to levan may not have been due to a contaminant. Such a possibility may have to be considered for the following activities : the properdin-binding activity of levan *in vitro* (Pillemer *et al.*, 1955), its effect on the bactericidal activity of serum (Wardlaw, 1958), change of resistance to experimental infections (Kiser *et al.*, 1956), evocation of local Schwartzmann reaction by levan (Hestrin and Davies, 1956) as well as the effect of levan on formation of granulation tissue (Wolman and Wolman, 1956).

The use of native levan in the study of experimental infections has been hampered by the residual toxicity to animals present in some of the samples (Shilo *et al.*, 1954 ; Davies *et al.*, 1955). The proposed procedures of treatment by alkaline ethanol and alkaline CETAB make it easy to obtain non-toxic levans of high infection-promoting activity, endothelium-sealing activity and suppressor activity of acute inflammation. The levan of *Corynebacterium* sp., initially free of lipopolysaccharide activity, seems a suitable material for the study of the IPA and other biological activities of levans

SUMMARY

Native levans from *Aero. levanicum* and *Ps. denitrolevaniformans* were found to contain a contaminant showing lipopolysaccharide activity, namely resistance-increasing activity and toxicity. Treatment of these levan preparations with alkaline ethanol or alkaline cetyltrimethylammonium bromide destroyed these activities without loss of the other biological properties of the polymer, *e.g.* infection-promoting activity, endothelium-sealing activity, suppressor activity on acute inflammation and necrosis-promoting activity. Partial degradation of the levan by acid destroyed infection-promoting activity, but not resistance-increasing activity and toxicity and thus furnished further proof for a lipopolysaccharide-like contaminant. *Corynebacterium* sp. levan was found to be initially free from lipopolysaccharide activity but active in respect to promotion of infection, sealing of the endothelium, suppression of acute inflammation and promotion of necrosis in staphylococcal lesions.

The infection-promoting activity of levan towards intraperitoneal infections in mice, the inflammation modifying activity and the induction of necrosis in dermal infections in rabbits has been shown to occur with a variety of unrelated micro-organisms.

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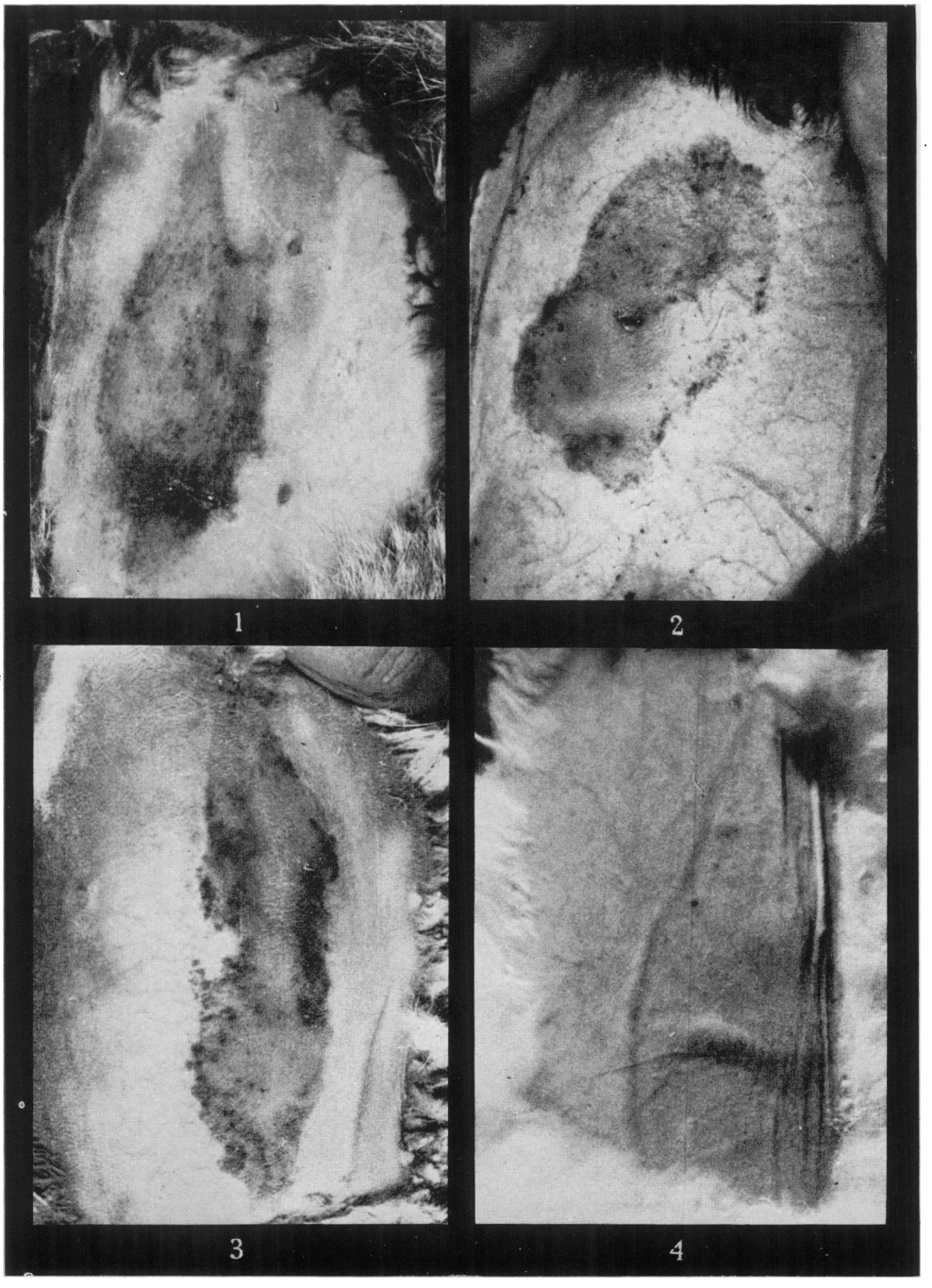
EXPLANATION OF PLATE

Necrosis-promoting activity of *Aero. levanicum* levan preparations in staphylococcal lesions in rabbits

The rabbits received 100 mg./kg. of different *Aero. levanicum* levan preparations i.v. simultaneously with an intracutaneous injection of 3×10^6 *Staph. aureus*, strain 9 organisms.

FIG. 1 : Native *Aero. levanicum* levan precipitated by ethanol at neutral pH.
FIG. 2 : Same as 1 after partial degradation by acid (pH 3·2, 60 min., 60°).
FIG. 3 : Native levan treated with alkaline ethanol (pH 12).
FIG. 4 : Same as 3 after partial degradation by acid (pH 3·2, 60 min., 60°).
Pictures were taken 24 hr. after challenge.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