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STAPHYLOCOCCAL LEUCOCIDINS

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THE staphylococcus is known to produce at least two leucocidins: (*a*) The α-haemolysin, which attacks white as well as red cells. Its leucocidal action, however, is confined to cells of the rabbit. (*b*) The so-called Panton-Valentine (P-V) leucocidin, which attacks human as well as rabbit leucocytes, and is not haemolytic. This leucocidin is almost certainly the same as that originally described by van der Velde (1894; Denys and van der Velde, 1895). It has not always been clear that these two leucocidins are distinct (*e.g.*, Neisser and Wechsberg, 1901), but there is now little doubt that they are (*e.g.*, Panton and Valentine, 1932; Valentine, 1936; Wright, 1936; Proom, 1937; Jensen and Maaløe, 1950).

The present work forms part of an investigation into the nature of the P-V leucocidin and its relation to haemolysins and other extracellular substances produced by staphylococci. We have shown also that there is a third leucocidin.

METHODS

Strains of Staphylococci

A number of strains of staphylococci, both coagulase-positive and negative, were classified into 5 groups, as follows: (1) 60 coagulase-positive strains from human pyogenic infections, (2) 10 coagulase-positive strains from nasal carriers, (3) 5 coagulase-positive strains from cases of food poisoning, (4) 12 coagulase-positive strains from animal sources, and (5) 23 coagulase-negative saprophytic strains. Most of the work was done with strain V8 from group 1, received from Dr. F. C. O. Valentine of the London Hospital. It was isolated from a case of chronic furunculosis of 5 years' standing, was deficient in pigment, and produced high titres of P-V leucocidin.

Production of Leucocidin

For production of culture filtrate on the scale of litres the aerated column device of van Heyningen and Gladstone (1953*a*) was used. This supplied 4 l. of culture every 24 hr. For small-scale experiments (10 ml.) a modification of Monod's rocking T-culture tube was used (van Heyningen and Gladstone, 1953*b*).

The medium used was a modification of Gladstone and Fildes' (1940) CCY medium, as follows: Bacto Casamino acids, 160 g.; yeast diffusate, 1600 ml.; Na lactate 70 per cent

syrup, 113·6 ml.; Na glycerophosphate, 160 g.; $MgSO_4.7H_2O$, 0·16 g.; $MnSO_4.4H_2O$, 0·084 g.; 0·32 per cent (w/v) $FeSO_4.7H_2O$, 0·32 per cent (w/v) citric acid, 16 ml.; KH_2PO_4, 3·28 g.; $Na_2HPO_4.12H_2O$, 50·0 g.; distilled water to 8000 ml. The yeast diffusate was obtained by dialysing a suspension of 200 g. of Bacto yeast extract in 200 ml. distilled water against 1800 ml. distilled water in the cold overnight with constant agitation. For small-scale experiments the medium was distributed in 100 ml. quantities in flat 8-oz. (227-ml.) bottles and sterilized by autoclaving. For larger scale preparation the medium was sterilized in 8 l. volumes in 10-l. bottles by autoclaving at 15 lb. per sq. in. (1·1 kg. per sq. cm.) for 30 min.

The organisms for the inoculum were preserved in the dry state *in vacuo*. From this they were grown on tryptic meat agar slopes overnight at 37°. The slopes were then kept at room temperature and used for inoculation for a period of about 4 weeks, when they were discarded and fresh dry samples used. For the larger cultures the initial inoculum was 2 ml. of a visible suspension made from these slopes into 200 ml. medium in the column. This was grown overnight and the column then filled to the 2·2-l. mark. The first harvest (2 l.) was made after 10-hr. growth and the apparatus refilled with fresh medium. Thereafter 2 l. were removed every morning and evening, leaving 200 ml. to inoculate the fresh medium. Growth was measured in a photo-electric densitometer and expressed as mg. dry wt. per ml.

Cultures greater than 100 ml. were Seitz-filtered after centrifuging. Loss due to adsorption was negligible when 100 ml. or more of crude culture supernatant was passed through a 16-cm. pad. Smaller quantitites were tested without filtering. The leucocidin in the culture filtrate was concentrated by precipitation with ammonium sulphate at full saturation. Studies on the purifications of the leucocidin are in progress.

Assay of Leucocidin

We assayed leucocidin roughly by determining the minimal leucocidal dose and more accurately by titrating it against anti-leucocidin. In both methods we used a direct microscopical method of assessing death of the leucocyte. In addition it was also possible, though not convenient, to use a Ramon flocculation method of assay. Valentine's (1936) original anti-leucocidal K serum (B8760, Wellcome Research Laboratories) was no longer available, but we obtained from the Wellcome Research Laboratories 6 samples of antitoxin, 4 of which contained high titres of anti-leucocidin. One of these (KCP2029) was shown by Valentine (personal communication) to have a content of anti-leucocidin four times that of the K serum, and this was used in most of our assays. We gave this serum an arbitrary value of 100 anti-leucocidal units per ml. In addition it contained 340 units α-antitoxin and 12 units β-antitoxin per ml.

Microscopical "wet" method

Most of the work was carried out by observing the effect of leucocidin on living leucocytes in wet preparations. All glassware was scrupulously cleaned, since grease (probably the free fatty acid) is toxic to leucocytes. Slides, cover glasses and test tubes were immersed in concentrated sulphuric acid containing a few crystals of $NaNO_3$ at 60° and thoroughly rinsed. Slides and cover glasses were kept in alcohol. Tubes were dried at 37°, and not in the hot air oven, to avoid deposition of volatile grease. Pipettes were kept in sulphuric-bichromic acid mixture and rinsed with running tap water and dried with acetone as required.

Unless otherwise stated, assays were made with *human* leucocytes, generally from a single subject. Comparison between different subjects, however, showed no individual variation. When a drop of blood is allowed to clot on a grease-free cover glass, the polymorphonuclear leucocytes migrate from the clot and adhere to the glass. Harris (1953) showed that the clot and adherent red cells can be washed off without disturbing the leucocytes and that these remain viable for some hours. When the cover glass preparation is inverted over a drop of serum, or other suitable medium, on a slide and incubated, microscopical examination shows active amoeboid movements of the cells as they detach themselves from the glass surface.

A drop of blood was taken from a finger prick and allowed to clot on a cover glass and incubated at 37° for 30 min. in a moist chamber. Unless otherwise stated the clot and any adherent red cells were washed off with 0·5 per cent gelatine in normal saline (pH 7·6) and the preparation stored in this solution until required. Leucocytes remain viable and active for 2 hr. or more.

The same technique was used for *mouse* leucocytes, but is unsatisfactory with *guinea-pig* and *rabbit* leucocytes, since the clot fails to retract and migration of the leucocytes is scanty. Leucocytes were obtained from these animals after injecting saline intraperitoneally (about 200 ml. per kg.). After 18 hr. the animal was killed and the peritoneal cavity washed out with saline containing 0·3 per cent sodium citrate. The cells, of which about 90 per cent were polymorphs, were concentrated by light centrifugation and suspended in gelatine saline. A loopful of the suspension was placed on a cover glass at the time of assay.

For *sheep* leucocytes blood was obtained from a slaughter-house within 2 hr. of killing and lightly centrifuged. The buffy coat was removed and washed with 20 volumes of 0·5 per cent gelatine saline and finally suspended in an equal volume. One loopful of the suspension was placed on a cover glass at the time of assay. In addition to leucocytes it contained many red blood corpuscles.

Minimum leucocidal dose (*MLeD*).—A preliminary approximate estimation of the content of leucocidin in a culture filtrate was made by determining the minimum leucocidal dose. Serial 2-fold (or 1·41-fold) dilutions of the culture filtrate were made in gelatine saline. One drop of each dilution was placed on a slide (two dilutions on one slide) and the cover glass preparation of leucocytes inverted over it. The excess fluid was blotted off with filter paper and the preparation sealed with paraffin wax. Salts deposited on the surface of the cover glass were removed with damp filter paper. The slides were placed in an air incubator at 35° for 30 min. In order that they should reach temperature rapidly they were placed on a metal bar kept in the incubator. After incubation they were observed on a warm microscope stage with 1/12 in. (0·21 cm.) oil immersion lens under light phase contrast. The MLeD was the smallest amount of leucocidin to bring about the changes described under " Experimental ", and shown in Fig. 1–7, as evidence of death of the leucocyte. At this low level of leucocidin the end point was difficult to determine and the assay could only be approximate.

The test dose (*L*+).—The L+ dose is defined in the usual way as the smallest amount of leucocidin which when mixed with 1 unit of anti-leucocidin in a total volume of 1 ml. brings about the death of the leucocyte. Appropriate mixtures containing constant amounts of anti-leucocidin and logarithmically falling (20 per cent steps) amounts of leucocidin were made in tubes and 1 drop from each tube transferred to a slide, on which the coverslip with adhering leucocytes was inverted. The preparations were then sealed, incubated at 35° for 30 min. and examined as described above. A well-defined end point was obtained in the first mixture to contain an excess of leucocidin.

Normally the test was done at the 0·3 unit level, but with crude filtrates it was necessary to work at lower levels, *viz.*, 0·1 to 0·005 unit, since it was advisable to dilute these at least 1/5 in order to dilute out non-specific factors toxic to leucocytes. The L+ dose contains roughly 500 MLeD and consequently the end points of the titrations are still sharp at these low antitoxic levels.

P-V method

Valentine's (1936) method of assay was also used. Two ml. of saline containing 2 per cent sodium citrate were mixed with 2 ml. of fresh human blood and lightly spun for 10 min. The supernatant was removed and the red and white cells washed once with 10 ml. saline and re-suspended in 2 ml. saline. The usual leucocidin-antileucocidin mixtures were made and 0·1 ml. of the washed blood cells added to 1 ml. of each mixture and incubated at 37° for 1 hr. Films were made by streaking each mixture on a slide with a platinum wire, staining with Loeffler's methylene blue and examining with a 1/7 in. (0·36 cm.) oil immersion lens in a light microscope. According to Valentine, dead, but not live, leucocytes are mechanically destroyed in making the film, and the end-point is taken as that preparation showing no recognizable polymorphs.

Flocculation assay

The Lf dose was also determined by a modification of the semi-micro method of van Heyningen and Gladstone (1953*c*).

Assay of Haemolysins

α- and β-Haemolysins.—The haemolytic test doses (Lh) of these haemolysins were determined in the usual way, with 20 per cent differences in toxin in successive mixtures. For α-haemolysin, rabbit red blood cells were used and for β-haemolysin, sheep cells. Since the β-haemolysin

is a " hot-cold " haemolysin the toxin-antitoxin red cell mixtures were left at 4° overnight after incubation at 37°. When the haemolysin content was small the M.H.D. rather than the Lh was determined.

δ-Haemolysin.—Since this toxin is apparently not antigenic, only the M.H.D. could be determined. Human or horse red blood cells were used for these estimations.

Other Estimations

Hyaluronidase was estimated by the mucin clot prevention test of Tolksdorf, McCready, McCullagh and Schwenk (1949) on hyaluronic acid substrate (British Drug Houses), and the test controlled with testicular hyaluronidase (" Rondase ", Evans Medical Supplies, Ltd.).

Coagulase was estimated by the method of Gillespie, Devenish and Cowan (1939). Culture filtrates were mixed with an equal volume of human plasma diluted 1/10 and incubated at 37° in the water bath for 3 hr. and then for 18 hr. at room temperature.

Lipase was estimated by an antibody-combining test. Most commercial samples of α-antitoxic horse serum contain antilipase (Davies, 1954). To one of these, the anti-leucocidal standard serum KCP2029, an arbitrary unitage of 100 antilipase units was assigned. The lipase-antilipase mixtures were placed in capillary tubes, and on these were layered equal volumes of 10 per cent egg yolk extract in saline. Excess lipase was demonstrated by an opaque zone of highly refractile fatty acid particles at the junction of the fluids.

It was necessary to carry out the lipase antibody-combining assay at the 1-unit level in order to achieve a sufficiently high concentration of free enzyme at the end point.

For rapid surveys of lipase production by growing organisms, the diameter of the zone of clearing and blue coloration surrounding colonies plated on Nile blue fat plates was measured. These plates were similar to those used by Davies (1954) except that olive oil, emulsified in the agar by shaking on a Mickle homogenizer, was used in place of horse fat. Later, egg yolk plates (Gillespie and Alder, 1952) were used and the diameter of the zone of precipitated fatty acid surrounding the colonies measured.

Phosphatase production by growing organisms on phenolphthalein plates was detected by the method of Barber and Kuper (1951).

EXPERIMENTAL

Morphological Appearances of Human Leucocytes exposed to Leucocidin

Fig. 1–7 show the effect of adding a preparation of leucocidin (0·2 L+ per ml.) to human leucocytes. Changes are observed within minutes, the first being a loss of motility by the cell and a withdrawal of its pseudopodia. The granules lose

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EXPLANATION OF PLATES

Photographs taken under light phase contrast.

FIG. 1–7.—Changes in human polymorphonuclear leucocytes brought about by P–V leucocidin. Magnification × 600.

FIG. 1.—Normal leucocytes.

FIG. 2.—The same field as Fig. 1, 2 min. after the addition of 1 L+ P–V leucocidin per ml., showing withdrawal of pseudopodia.

FIG. 3.—The same field as Fig. 1, 4 min. after the addition of leucocidin, showing cytoplasmic protrusions.

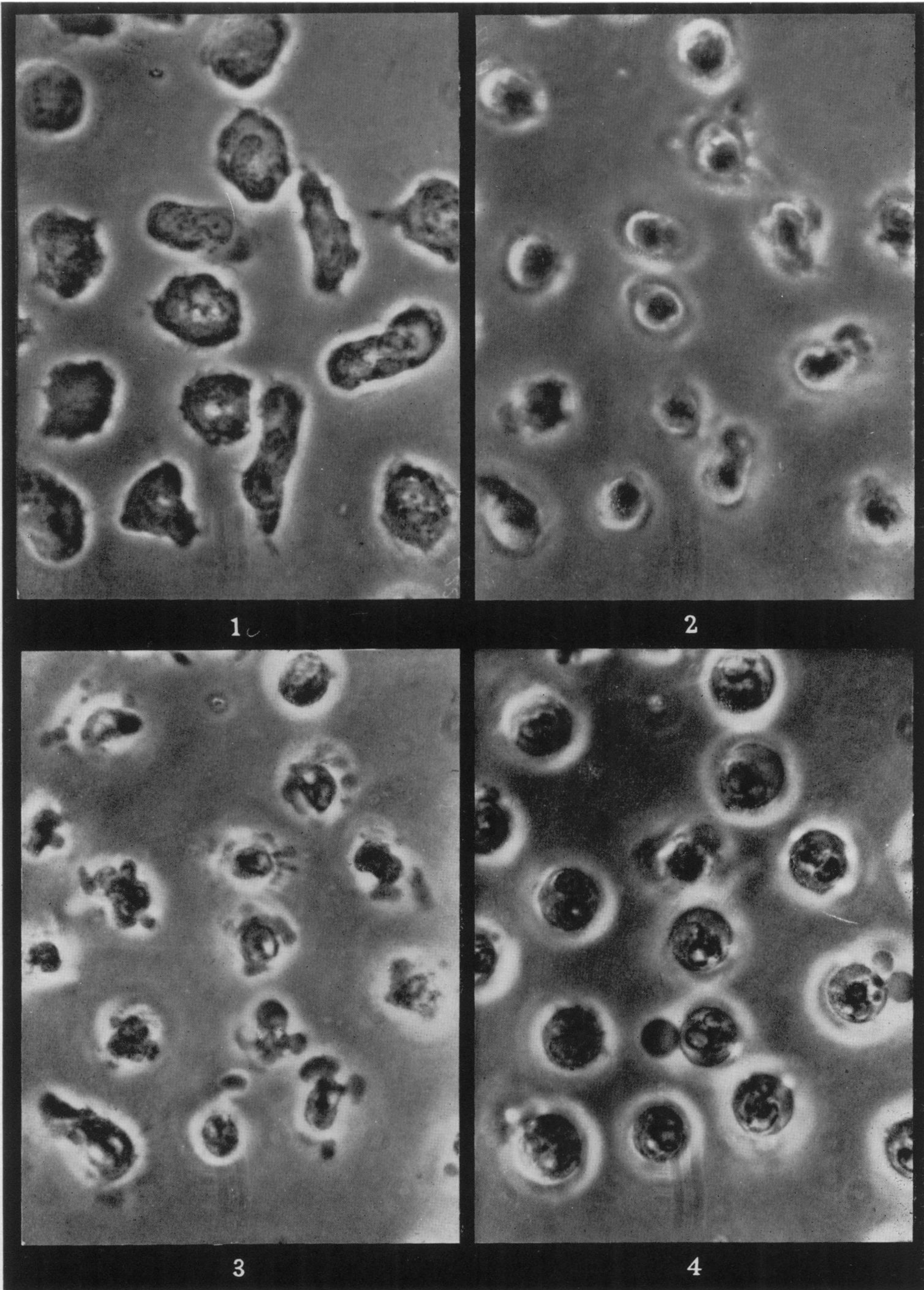
FIG.—4. The same field 6 min. after the addition of leucocidin. The nuclei are becoming prominent but still retain their polymorphic appearance. The cells are spherical and some cytoplasmic extrusions have been nipped off and show as spherical bodies.

FIG. 5.—The same field 8 min. after the addition of leucocidin. Many of the granules have disappeared. The nuclei are losing their polymorphic appearance and becoming spherical.

FIG. 6.—Fifteen min. after the addition of leucocidin. The granules have disappeared except for a few at the periphery of the cell. Nuclei are all spherical.

FIG. 7.—Eighteen hr. after the addition of leucocidin. The nuclei have disappeared and the cells are degenerate with foamy cytoplasm. No lysis has taken place.

FIG. 8.—The effect of δ-haemolysin on human polymorphs showing greatly swollen nucleus with the granules pushed to one side of the cell. " Signet ring " form. Magnification × 700.



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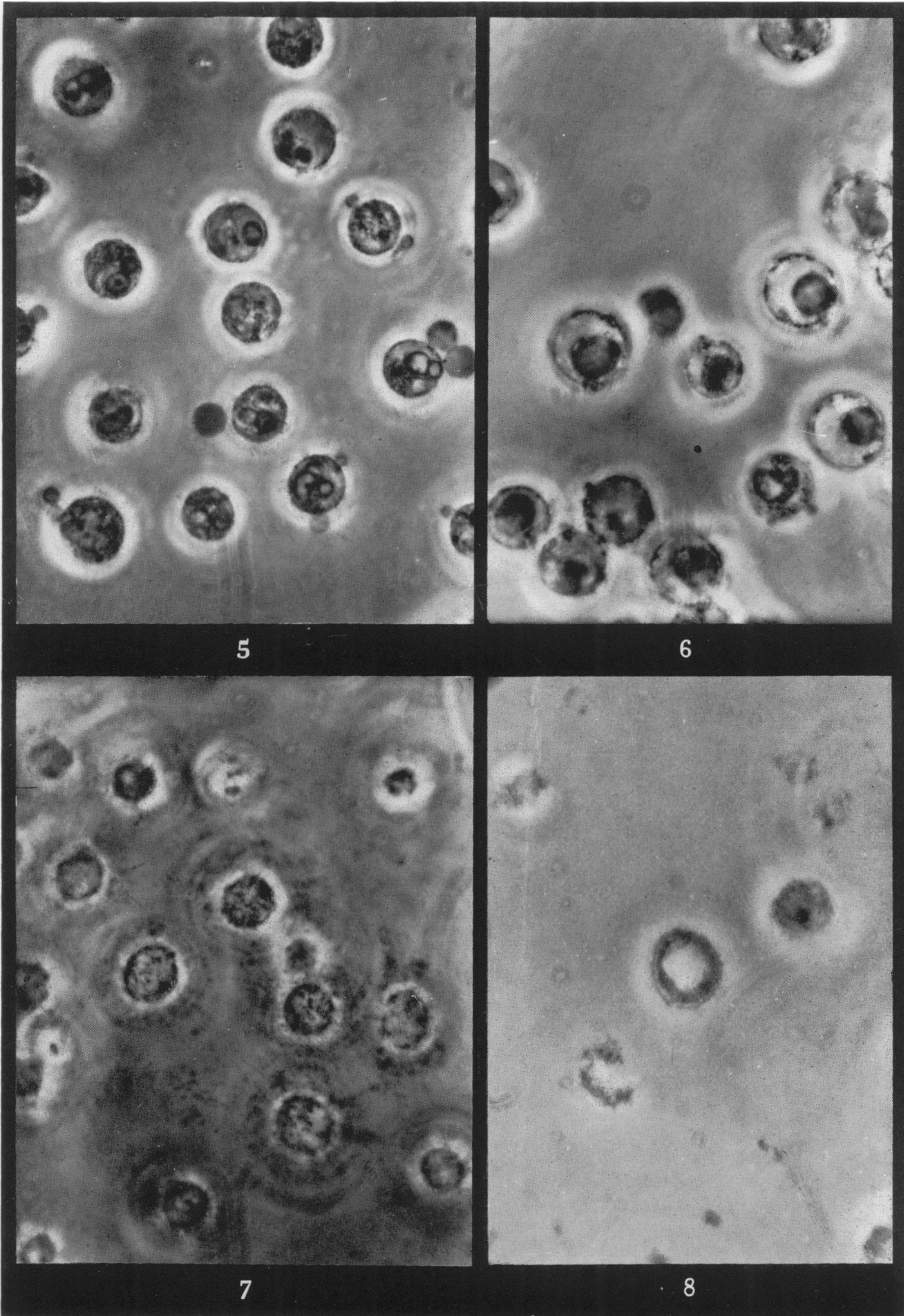

Gladstone and van Heyningen.

their orderly streaming and undergo Brownian movement. Rounded protoplasmic extrusions, quite unlike the " snail horn " pseudopodia, are violently put out and as violently withdrawn without any movement of translation of the cell as a whole (Fig. 3). Sometimes these protoplasmic extrusions are nipped off in the form of spheres which separate from the cell, taking granules with them (Fig. 4), but the cell as a whole does not disintegrate. After a minute or two these rounded extrusions cease to appear, the cell becomes spherical but not greatly swollen, and most of the granules disappear (Fig. 5). The remaining granules lose their Brownian movement and become closely applied to the wall of the cell. At this stage the nucleus becomes very prominent and heavily outlined. The next stage is the loss of the lobulated appearance of the nucleus which becomes spherical (Fig. 6). Cells at this stage stained by Sheehan and Storey's (1947) Sudan black method are entirely devoid of fat-staining granules. Eosinophiles show the same changes as polymorphs. The final stage is a gradual loss of the outline of the nucleus, the cell eventually becoming a practically empty vesicle containing a few granules (Fig. 7). Apart from the extrusion of parts of the cytoplasm as described above, no disruption of the cell ever occurs. We tested the lethal action of 0·1 N-HCl, 0·1 N-NaOH, 0·0033 M sodium oleate and distilled water and, although they are all lethal, in no case did they bring about these changes. With distilled water there was the same rounding of the cell, loss of streaming, and active Brownian movement of the granules, but there was swelling of the cell without disappearance of the granules and the nucleus became less obvious and showed no prominent outline.

Comparison between " Wet " and P-V Methods of Assay

A preparation of leucocidin concentrated by precipitation with $(NH_4)_2SO_4$ from a culture filtrate of strain V8 was assayed by both the " wet " and the P-V methods of estimation, the leucocidin being diluted in both ordinary and gelatine saline since Valentine (1936) used the former and we the latter. The usual clear end point was obtained with the " wet " method, distinguishing between 20 per cent steps in toxin concentration, irrespective of the dilution fluid used. With the P-V method the end point was much more difficult to determine ; in under-neutralized mixtures the polymorphs were still recognizable, although markedly degenerate. Moreover, mixtures giving a clear end point by the " wet " method contained many apparently normally staining cells in addition to those that were degenerate. The end point therefore appeared to be based on a subjective assessment as to what were normally staining and what were degenerate cells. Consequently we were unable to fix the end point within less than a 2-fold range and in subsequent work the " wet " method was used throughout.

Comparison between L+ and Lf Methods of Assay

Table I shows that when constant amounts of crude leucocidin were mixed with amounts of horse antiserum varying by 20 per cent steps over a 10-fold range, only a single zone of flocculation was obtained. The Table also shows that the first flocculating mixture contains about 40 per cent less toxin per unit of antitoxin than the first mixture to show excess free leucocidin, which suggests that the flocculation zone is due to the leucocidin, since the Lf dose is usually smaller than the L+ dose. This was confirmed by assays by both the Lf and the

L+ methods of 7 toxins against 4 antisera. The results of these are shown in Tables II and III.

TABLE I.—*Flocculation in Mixtures of Crude Leucocidin and Antiserum*

Each tube contained constant amounts of crude leucocidin solution, varying amounts of antiserum dilution and saline to constant volume. One drop of each mixture tested for leucocidal action by the "wet" method, the rest incubated at 37°.

Tube	Antitoxin units per ml.	Time of flocculation (min.)	Leucocidal action
1	72·5	78	0
2	58·0	61	0
3	46·4	35	0
4	37·1	24	0
5	29·7	14	0
6	24·9	11	0
7	20·3	9	0
8	16·2	11	0
9	13·0	22	+
10	10·2	28	+
11	8·7	>120	+
12	7·3	>120	+

TABLE II.—*L+ and Lf Values of Various Toxins against Standard Antitoxin KCP2029*

Toxin	L+ per ml.	Lf per ml.	Lf/L+
U_1	500	555	0·9
U_2	250	380	0·65
V8C	312	641	0·48
	312	781	0·39
ZC	8	13	0·62
69B	40	62	0·64
A	20	25	0·8
IIA	111	178	0·62

TABLE III.—*Antileucocidin Contents of Antisera Estimated by L+ and Lf Methods*

	Units per ml.					
	Test toxin V8C			Test toxin ZC		
Antitoxin	L + method	Lf method	Difference	L + method	Lf method	Difference
CPP 68/36	144	128	+16	160	130	+30
CPP 80/63	128	100	+28	128	144	−16
CPP 81/B5	125	161	−36	160	156	+4
RK 5749/36	149	161	−12	160	130	+30

Mean difference between unitage by L + and unitage by Lf = 5·5.
Standard error mean difference = 232·12.
$t = 0{\cdot}023$, 7 d.f.
Differences therefore are not significant.

The flocculation reaction requires a concentration of leucocidin of at least 5 Lf units per ml. and a corresponding amount of antiserum to give flocculation in a reasonable time, and since this was too wasteful of reagents the method was not routinely used.

The Production of Leucocidin

Little attempt has been made to determine the optimal conditions for leucocidin production since the medium did not appear to be critical provided it supported good growth of the organism. Except in the semi-solid agar medium used by Valentine (1936), relatively poor growth was obtained in stagnant cultures in tryptic heart broth or CCY. However, when these cultures were aerated by continuous shaking in air good yields of organisms and of leucocidin were obtained. Carbon dioxide was not only unnecessary, but in concentrations greater than 10 per cent was inhibitory to growth and to the production of leucocidin. Medium L described under " Methods " was found to be satisfactory and used throughout.

One litre of L medium inoculated with V8 strain was grown overnight in the large-scale aeration apparatus. Nine hundred ml. were then removed and the apparatus filled up to 1000-ml. mark with fresh medium. Samples were taken immediately and at hourly intervals, and the growth and leucocidin content estimated (Table IV). The concentration of leucocidin per unit weight of organism is at its maximum at the very beginning of growth and remains roughly constant for the next 18 hr. or more.

TABLE IV.—*Rate of Production of Leucocidin in Medium L* (10 *per cent Inoculum*)

Growth			
Hours	mg. per ml.	L+ per ml. culture	L+ per mg. organism
0	1·4	0·16	—
1	1·4	0·16	—
2	1·75	0·32	0·18
3	2·6	0·35	0·13
4	3·2	0·64	0·2
5	4·4	0·8	0·18
6	4·8	0·8	0·17
7	5·1	1·0	0·2
9	5·2	1·0	0·19
10	5·3	1·0	0·19
18	7·5	1·0	0·16
42	8·8	1·2	0·14

Since the production of many bacterial toxins is improved when the iron content of the medium is reduced we thought it worth while to test whether this was the case with the leucocidin. The culture medium was de-ferrated by calcium phosphate precipitation in the usual way, but leucocidin production was unaffected.

The Effect of P-V Leucocidin on Rabbit, Mouse, Sheep and Guinea-pig Leucocytes

The work so far reported is concerned with human leucocytes. It was found that a leucocidin preparation had the same MLeD titre when tested against human and rabbit leucocytes, and that the cells underwent exactly the same changes. On the other hand, mouse, sheep and guinea-pig leucocytes appeared to be completely refractory to solutions containing 500 MLeD per ml. for human or rabbit leucocytes.

Relationship of P-V Leucocidin to Other Active Products

α-Haemolysin.—That the α-haemolysin is distinct from the P-V leucocidin is evident from the following:

(1) Strain V8 produced a poor zone of haemolysis on rabbit blood agar in 20 per cent CO_2 and no haemolysin was found in culture filtrates when grown under conditions giving 0·8 L+ leucocidin per ml. On the other hand, an old laboratory strain (N.C.T.C. 804) gave large zones of α-haemolysin on rabbit blood agar in 20 per cent CO_2 and culture filtrates contained 16 M.H.D. per ml., but less than 0·05 L+ leucocidin per ml. (2) A dried preparation of α-haemolysin (S883, Wellcome Research Laboratories) in a concentration of 2·3 Lh per ml. was found to have no action on human leucocytes. When tested on rabbit leucocytes, however, the cells were killed, but without the changes found with P-V leucocidin—beyond cessation of movement and withdrawal of pseudopodia they underwent little change. We have confirmed therefore the observation of Wright (1936) that α-haemolysin is lethal to rabbit leucocytes. A proviso must be made: since neither the production of γ-toxin by the strains mentioned above nor the content of γ-toxin in the α-toxin preparation was known, the conclusions relating to α-toxin may also apply to γ-toxin.

β-Haemolysin.—Culture supernatants of V8 in medium L containing 1 L+ leucocidin per ml. produced no hot-cold haemolysis of sheep red cells. A solution of β-haemolysin (S879, Wellcome Research Laboratories) containing 32 M.H.D. per ml. appeared to have no effect on human leucocytes. Thus the β-haemolysin and P-V leucocidin are separate entities. This observation is contrary to that of Flaum (1938), but confirms that of Wright (1936).

δ-Haemolysin.—The δ-haemolysin is the only staphylococcal toxin known to attack horse and human red blood cells (Marks and Vaughan, 1950). The V8 strain was tested for the production of this haemolysin by growing it on tryptic meat agar plates layered with cellophane in an atmosphere of 20 per cent CO_2 (Williams and Harper, 1947; Marks and Vaughan, 1950). The growth was washed off with 1·5 ml. saline per plate and the organism removed by centrifuging. The supernatant was tested on washed horse red cells, but no haemolysis was detected. It appeared, therefore, to contain no δ-haemolysin. When assayed for leucocidin it was found to contain 8 L+ per ml.; conversely, V8 strain grown in medium L and producing 1 L+ P-V leucocidin per ml. failed to lyse horse red blood cells. Furthermore, δ-haemolysin and leucocidin have quite different properties. δ-Haemolysin is not specifically neutralized by staphylococcal antitoxic horse serum (Marks, 1951), whereas all of 6 samples of antitoxin obtained by us from the Wellcome Research Laboratories contained high titres of antileucocidin. δ-Haemolysin is stable to autoclaving at 115° for 20 min., whereas leucocidin is destroyed at 56° for 30 min.

Lipase.—Culture filtrates of V8 strain contain lipase, and all 6 samples of α-antitoxic horse serum tested contained antilipase. When lipase and leucocidin antibody-combining assays were carried out simultaneously on the same samples, no correlation was found between these two activities. For example, a crude culture filtrate containing 0·8 test dose of P-V leucocidin per ml. contained 0·3 test dose of lipase per ml., whereas an $(NH_4)_2SO_4$ concentrate containing 15 L+ per ml. contained no lipase. Moreover there appeared to be no correlation between lipase and P-V leucocidin production with other strains of staphylococci.

Colonies of some strains which produced no P-V leucocidin were surrounded by wide zones of opacity on egg yolk plates or clear blue areas on Nile blue-olive oil plates, whereas other strains producing no zones on these plates gave good yields of P-V leucocidin.

Phosphatase.—Another enzyme produced by coagulase-positive staphylococci is phosphatase (Barber and Kuper, 1951), which appears to be bound to the bacterial cell. It was found to have no relationship to the P-V leucocidin. Although all of 83 coagulase-positive strains produced phosphatase on phenolphthalein phosphate nutrient agar, no phosphatase could be demonstrated in liquid culture supernatants containing high titres of leucocidin. Moreover, some strains which failed to produce P-V leucocidin were phosphatase-positive on plates.

Protease.—Culture filtrates of strain V8 in medium L contain protease, active on gelatine and casein, and this is precipitated together with P-V leucocidin by $(NH_4)_2SO_4$. However, the protease and the leucocidin are evidently not identical since they can be separated by differential elution after adsorption on a column of calcium phosphate. This work is being continued.

Coagulase.—Although V8 strain is coagulase-positive it does not always produce coagulase, and failed to do so in cultures grown under optimal conditions for production of leucocidin; *e.g.*, 18 hr.-culture filtrates from medium L, containing 1 L+ leucocidin per ml. were devoid of coagulase. Conversely, strain "Newman" (N.C.T.C. 8178) produced high titres of coagulase in medium L, but no leucocidin. Although it is clear from these observations that P-V leucocidin is not coagulase, and that it is not produced by all coagulase-positive strains, none of 23 coagulase-negative strains produced any P-V leucocidin.

Hyaluronidase.—Hyaluronidase is produced by the staphylococcus, but it is clearly a separate entity from leucocidin since it may be absent from culture supernatants of V8 containing 1 L+ of leucocidin per ml.

Appearance of a New Leucocidin

So far all the work had been done with strain V8. Other strains of staphylococci from each of the 5 groups listed under "Methods" were now tested. The organisms were grown in T-tubes in medium L overnight and the supernatants of the cultures tested at a dilution of 1/5 on human leucocyte preparations. We were at once confronted with a new phenomenon. Whereas many of the coagulase-positive strains produced P-V leucocidin, certain of these, and a few of the coagulase-negative strains, seemed to produce an additional factor, for they not only destroyed the leucocyte but lysed it as well. The changes observed in the cell before lysis are quite different from those observed with P-V leucocidin. The effect seems to be mainly on the nucleus; it becomes swollen, loses its lobulated appearance and bulges the cell, pushing the granules to one side and giving the appearance of a signet ring (Fig. 8). The cell does not present the regular spherical appearance observed with P-V leucocidin, and the granules do not disappear. At this point the cell may or may not lyse. Indeed, a characteristic phenomenon is the lack of uniform reaction of individual cells. Some lyse rapidly, others remain like signet rings, and others show little change beyond withdrawal of pseudopodia and loss of movement. In this the reaction differs markedly from that obtained with P-V leucocidin, where every cell is uniformly affected. When lysis takes place Brownian movement of the granules

becomes more and more marked, the cell ruptures and the granules stream out. The enlarged nucleus is extruded either as a spherical or, more usually, as an egg-shaped body divided by a fine septum representing the demarcation of its original two lobes. The nucleus becomes less and less evident and finally disappears. A small mass of granular débris is all that remains of the cell.

This new lytic agent, or " leucolysin ", cannot usually be demonstrated in culture supernatants diluted more than 1/40. It does not diffuse through cellophane membranes ; is precipitated by saturated ammonium sulphate ; is stable to heating at 100° for 30 min. ; and is inhibited by relatively high concentrations of normal human and horse serum or by 1 per cent serum albumin. We have not found specific antibody to this lysin in any sample of staphylococcus antiserum, and are inclined to doubt whether it is antigenic.

It is clear that the " leucolysin " is distinct from the P-V leucocidin. At this point Dr. Elek and Dr. Lack directed our attention to a note by Jackson and Little (1956) to the effect that δ-haemolysin is lethal to human leucocytes. The properties of our " leucolysin " suggested that it might be δ-haemolysin : it is produced by most, if not all, coagulase-positive and by some coagulase-negative staphylococci ; it acts on red blood cells of all species of animals tested, including horse cells which are resistant to α- and β-haemolysins ; it is stable to autoclaving at 115° for 20 min. ; it is readily adsorbed on a Seitz filter ; it does not diffuse through dialysing membranes ; it is not specifically neutralized by antitoxin, but is inhibited by normal serum and serum albumin.

Culture supernatants rich in " leucolysin " were tested for δ-haemolysin. Serial 2-fold dilutions of the culture supernatants were made in gelatine saline and an excess of the standard antiserum added to neutralize any P-V leucocidin that might be present. Drops of each mixture were tested for their action on human leucocytes in the usual way, and then 0·5 ml. of a 3 per cent washed human or horse red blood cell suspension added to the remaining mixture and incubated at 37° for 1 hr. and then overnight at room temperature, and a rough visual estimation made of the degree of haemolysis.

Table V shows : (1) A correlation between " leucolytic " and δ-haemolytic activities ; (2) that the sensitivity of human leucocytes to " leucolysin " is of the same order as that of human or horse red blood cells to δ-haemolysin ; (3) that about 8 times as much δ-haemolysin was produced per unit volume of medium in medium L than on cellophane layered on tryptic meat agar, and that CO_2 was unnecessary ; (4) that " leucolysin " and δ-haemolysin are equally stable to heating to 100° for 30 min.

Further evidence that leucolysin is δ-haemolysin lay in the finding of complete correlation between the leucolytic action on human leucocytes and the haemolytic action on horse red cells in the supernatants of 45 strains. Since many of these strains also produced P-V leucocidin it was necessary to add excess anti-P-V leucocidin, or to heat the preparation at 56° for 30 min.

Leucolytic Synergism between β- and δ-Haemolysins

Marks and Vaughan (1950) noted that δ-haemolysin has a synergic effect in the haemolysis of sheep red cells by β-haemolysin. β-Haemolysin alone has no leucolytic action, but we found that it is capable of enhancing the leucolytic action of δ-haemolysin. A supernatant from a culture in medium L, containing 5 minimum leucolytic doses of δ-haemolysin per ml. was diluted to 1/20, well

TABLE V.—*Correlation between δ-Haemolysin and " Leucolysin " in Culture Supernatants of Various Strains of Staphylococci*

" Leucolysin " tested against human leucocytes ; δ-haemolysin tested against 1 per cent suspension of human or horse red blood cells ; 1 unit anti-P-V leucocidin added to each ml. dilution.

Organisms	Medium	Leucolytic and haemolytic activity* Dilution of culture supernatant 1/5	1/10	1/20	1/40	1/80
Group 1.—*Coagulase-positive pyogenic source*						
V8	Medium L	0, (0)	0, (0)	0, (0)	0, (0)	0, (0)
V8	Agar	0, (0)	0, (0)	0, (0)	0, (0)	0, (0)
F	,,	++, (++)	+, (+)	+, (+)	0, (±)	0, (0)
Foggie	Medium L	++, (++)	++, (++)	+, (++)	+, (±)	0, (0)
Newman	Agar	++, (++)	++, (++)	++, (+)	+, (±)	0, (0)
Nl	Medium L	++, (++)	+, (+)	±, (±)	0, (0)	0, (0)
Group 2.—*Coagulase-positive, nasal carrier*						
56/3411	Medium L	++, (++)	++, (++)	++, (+)	+, (±)	0, (0)
Group 3.—*Coagulase-positive, food poisoning*						
1074	Medium L	++, (++)	+, (++)	±, (+)	0, (0)	0, (0)
Group 4.—*Coagulase-positive, animal sources*						
Z92	Agar	++, (++)	+, (+)	+, (+)	0, (±)	0, (0)
143/54	Medium L	++, (++)	+, (++)	+, (+)	+, (±)	0, (0)
42/54	,,	++, (++)	+, (+)	±, (±)	0, (0)	0, (0)
42/54	Medium L (heated 100°, 30′)	++, (+)	+, (±)	±, (0)	0, (0)	0, (0)
42/54	Agar	++, (++)	+, (+)	0, (0)	0, (0)	0, (0)
Group 5.—*Coagulase-negative*						
N.C.T.C. 6153	Agar	+, (+)	+, (+)	± (0)	0, (0)	0, (0)

All cultures in medium L (10 ml.) shaken in T-tubes in air for 18 hr. ; agar cultures grown under conditions suitable for production of δ-haemolysin (Williams and Harper, 1947), *viz.*, 18-hr. growth on cellophane layered on tryptic meat agar in 20 per cent CO_2 and extracted with 1·5 ml. saline. (Supernatants from agar cultures were thus 8 times as concentrated as those from T-tubes.)

* In each column the signs on the left refer to leucolytic activity (++ = complete lysis, +, ± = changes in cell short of lysis), and those on the right, in brackets, to haemolytic activity (++ = complete haemolysis, + = 20–80 per cent haemolysis, ± = trace).

beyond its limit of leucolytic action. When β-haemolysin in a final concentration of 1·34 Lh per ml. was added to this dilution the preparation lysed human leucocytes strongly. The effect was not due to the enhancing action of proteolytic enzyme on δ-haemolysin (Marks, 1952), since our preparation of β-haemolysin contained no proteolytic enzyme.

Effect of δ-Haemolysin on Leucocytes of Other Species

Leucocytes from rabbits, guinea-pigs, mice and sheep were tested for their susceptibility to δ-haemolysin, and all except sheep cells appeared to be as susceptible as those of man. The experiments with sheep leucocytes were not

quite clear since it was impossible to rid the preparation of red cells, and these may have adsorbed the haemolysin. Marks and Vaughan (1950) have shown that the haemolytic action of δ-haemolysin depends on the concentration of red cells.

Estimation of P-V Leucocidin in the Presence of δ-Haemolysin

The leucolytic action of δ-haemolysin interferes with the assay of P-V leucocidin, unless the concentration of the P-V leucocidin is such that the δ-haemolysin can be diluted out, which is often the case. In other cases P-V leucocidin cannot be ruled out unless a test can be devised to detect it in the presence of δ-haemolysin.

We have confirmed Marks' (1951) observation that serum albumin inhibits the haemolytic action of δ-haemolysin, and have shown further that it inhibits its lytic action on human leucocytes. However, the concentration of human serum albumin used (1 per cent) also inhibited P-V leucocidin to some extent, and therefore we sought another inhibitor.

Cholesterol is known to inhibit certain haemolysins, *e.g.*, the oxygen-labile bacterial haemolysins and saponin (see Cohen, Halbert and Perkins, 1942). A suspension of 24 mg. cholesterol per ml. was made as follows: 25 ml. of a saturated solution of cholesterol in acetone at 37° was poured with continuous vigorous stirring into 500 ml. H_2O at pH 8. Coarse particles of cholesterol were strained off on cotton wool, the acetone was boiled off and the suspension reduced to about 1/5 of its original volume by boiling, to give the desired concentration of cholesterol, and finally re-filtered to remove coarse particles.

Various amounts of this suspension were added to culture filtrates containing δ-haemolysin and the mixtures tested for leucolytic and haemolytic action. Table VI shows that cholesterol, in a final concentration of 1·6–3·2 mg. per ml.,

TABLE VI.—*Effect of Cholesterol on the Leucolytic and Haemolytic Action of δ-Haemolysin*

(Excess anti-P-V leucocidin added in all cases.)

A. *Constant δ-Haemolysin (8 M.H.D., 8 MLeD per ml.), Varying Cholesterol.*

Cholesterol mg. per ml.	Leucolysis (human leucocytes)	Haemolysis (human red cells)
6·4	0	0
3·2	0	0
1·6	0	±
0·8	+	++
0·4	++	++
0	++	++

B. *Varying δ-Haemolysin, With and Without Cholesterol (Final Concentration 1·25 mg. per ml)*

Dilution of supernatant	No cholesterol		With cholesterol	
	Leucolysis	Haemolysis	Leucolysis	Haemolysis
1/5	++	++	+	++
1/10	++	++	0	+
1/20	+	++	0	0
1/40	+	+	0	0
1/80	0	+	0	0
1/160	0	0	0	0

The signs have the same significance as in Table V.

inhibits both the leucolytic and the haemolytic action of δ-haemolysin in a concentration of 8 M.H.D. or MLeD per ml. On the other hand, further tests showed that 6·4 mg. cholesterol per ml. had no effect on P-V leucocidin in a concentration of 1 L+ per ml. Cholesterol can therefore be used to inhibit δ-haemolysin in the presence of P-V leucocidin.

Survey of 110 *Strains of Staphylococci for Production of δ-Haemolysin and P-V Leucocidin*

Each of 110 strains from all 5 groups was grown in a T-tube in medium L and the 18-hr. culture supernatant tested in a dilution of 1/5 ; (*a*) without further addition, to detect any leucocidal or haemolytic activity ; (*b*) *plus* 1 unit anti-P-V leucocidin per ml., to detect δ-haemolysin ; (*c*) *plus* 2·4 mg. cholesterol per ml., to detect P-V leucocidin. One drop of each mixture was tested for its leucocidal action on human leucocytes, and to the remainder was added 0·5 ml. of 3 per cent washed horse red blood cells. The results of this survey are summarized in Table VII. This shows that P-V leucocidin and δ-haemolysin are randomly distributed amongst coagulase-positive staphylococci, irrespective of the source of the strains, except that only a few strains from animal sources produce P-V leucocidin. Although no P-V leucocidin is produced by coagulase-negative strains, many of these do produce δ-haemolysin.

TABLE VII.—*Distribution of P-V Leucocidin and δ-Haemolysin in* 110 *Strains of Staphylococci*

Group	Coagu-lase	Source	Number tested	Number producing P-V leuco-cidin*	Number producing δ-haemo-lysin*	Number producing both	Number producing neither
1	+	Pyogenic	60	31	39	21	11
2	+	Nasal carrier	10	2	9	2	1
3	+	Food poisoning	5	3	4	2	0
4	+	Animal sources	12	2	8	2	4
5	—	Saprophytic	23	0	7	0	16

* Supernatants from 18-hr. cultures in medium L tested at a dilution of 1/5. Hence numbers refer to strains producing more than 5 minimum leucocidal or haemolytic doses.

DISCUSSION

It is clear that there are at least three quite distinct staphylococcal leucocidins : (1) The α-haemolysin, sometimes known as the Neisser-Wechsberg leucocidin, active only on rabbit leucocytes without gross changes in their morphology ; (2) the Panton-Valentine leucocidin, not related to any of the 4 haemolysins, or to any of the other known active products of the staphylococcus, and active on rabbit and human leucocytes with characteristic changes in morphology, but not active on guinea-pig, mouse or sheep leucocytes ; (3) δ-haemolysin, active on the leucocytes of all species tested except those of sheep, and not only lethal to the leucocyte, but lytic as well. These leucocidins are randomly distributed amongst coagulase-positive staphylococci, irrespective of source, whether animal or human. δ-Haemolysin is also produced by some coagulase-negative saprophytic varieties.

From the differences between the changes in the leucocyte brought about by these leucocidins, it is evident that they act on the cell in different ways. The P-V leucocidin appears to act by altering the permeability of the membrane so that cytoplasm may be extruded through it without rupturing it. The rounded form assumed by the cell could be explained if the membrane (a non-rigid structure) were to become freely permeable to water, salts and protein. A spherical but greatly swollen form is also found when the cell is placed in distilled water, but this is the result of the increased osmotic pressure within the cell. P-V leucocidin does not cause a swelling of the cell, so that the spherical appearance cannot be due to increase in osmotic pressure. The disappearance of the granules due to P-V leucocidin can be demonstrated by their failure to stain for fat. We thought at one time that this might be due to the presence of lipase in the culture supernatants, but all attempts to equate lipase with P-V leucocidin have failed.

Lysis of the cell has never been observed when P-V leucocidin is free from δ-haemolysin. Where lysis has been observed (Proom, 1937) it is likely that δ-haemolysin was also present. Marks and Vaughan (1950) have called attention to the similarity between the lytic action of fatty acids on red blood cells and that of δ-haemolysin, and also to certain chemical properties common to these substances. They suggest that δ-haemolysin might be a lipoid-protein complex. Leucocytes are also lysed by sodium oleate, but only in the relatively high concentrations 0·01 M. At lower concentrations it is lethal to leucocytes, but there are no nuclear changes such as those found with δ-haemolysin. Recently, a lipid extracted from *Corynebacterium ovis* has been shown to be leucocidal (Carne, Wickham and Kater, 1956). It would be of interest to determine whether the changes observed in the leucocytes bore any resemblance to those observed with δ-haemolysin.

SUMMARY

Staphylococci produce three leucocidins: α-haemolysin active on rabbit polymorphs, the Panton-Valentine (P-V) leucocidin active on human and rabbit cells, and δ-haemolysin active on rabbit, guinea-pig, human and mouse cells. The P-V leucocidin was assayed by antitoxin-combining methods involving determinations of the L+ dose by microscopical examination using living human leucocytes, and the Lf dose by a Ramon flocculation method. A close correlation between L+ and Lf doses was obtained. The P-V leucocidin produces characteristic changes in the polymorph but never ruptures the cell. It cannot be equated with any of the four known haemolysins or with any other known active products of the staphylococcus. It is produced by 38 out of 87 coagulase-positive staphylococci tested, but not by any of 23 coagulase-negative strains. Staphylococci from human pyogenic infections and food poisoning more often produced it than strains from nasal carriers or from animal sources. δ-Haemolysin destroys polymorphs by an action on the nucleus, which becomes greatly swollen. In higher concentration the cell is lysed. Its leucolytic action on human leucocytes runs parallel with its haemolytic action on horse or human red cells. It is inhibited by cholesterol, so enabling P-V leucocidin to be assayed in its presence. It is formed by most coagulase-positive staphylococci irrespective of their source and by some coagulase-negative strains. The possible mode of action of these leucocidins is discussed.

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