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The growth and death of wound bacteria in serum, exudate and slough

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INTRODUCTION

When pathogenic bacteria have been implanted in the tissues—for example, by a penetrating wound at an autopsy or some other accident—it can hardly be a matter of indifference to the host whether these organisms are able to multiply rapidly in the extracellular fluid at the site of injury or in his circulating plasma. The subject, however, receives little attention in text-books of pathology or surgery.

During the First World War, Wright, Fleming & Colebrook (1918) reported experiments which suggested that bacteria from wounds could be classified in two groups, those which grow freely from small inocula in fresh human serum and those which will not grow in this environment. The former, which they called 'serophytes', included certain strains of streptococci (in all probability *Streptococcus pyogenes*) and *Staphylococcus aureus*; the latter, described as 'sero-saprophytes', included strains of coliform bacilli. Both groups of bacteria were found to grow well in fresh serum after it had been treated with trypsin; that is, in conditions not very different from those in the exudate of an infected wound in which proteolytic enzymes were being set free from leucocytes. To explain these phenomena Wright and his colleagues suggested that sero-saprophytic organisms required for their growth some of the breakdown products derived from proteolytic digestion of serum protein when enough trypsin was added to overcome its normal antitryptic power.

An alternative hypothesis to account for the difference between serophytes and sero-saprophytes is that the latter fail to grow in fresh serum because they are killed by bactericidal substances, and in particular by the properdin system which is known to be active against many coliform bacilli but inactive against streptococci and staphylococci (Pillemer, Blum, Lepow, Ross, Todd & Wardlaw, 1954; Wardlaw & Pillemer, 1956). On this hypothesis tryptic digestion might, by the destruction of such bactericidal components, convert fresh serum into a suitable culture medium for the organisms which are killed by them.

To throw light on this problem we have repeated some of the experiments of Wright *et al.* (1918) using more conventional techniques, and examined the effect of tryptic digestion of serum on its ability to kill or to support the growth of certain

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Gram-negative bacilli. We have also examined the growth of various wound organisms in fresh and heated serum, in blister fluid and in suspensions of burn slough.

GROWTH OF BACTERIA IN FRESH SERUM

Methods

Strains of bacteria

Six strains of *Str. pyogenes* (8355, 8075, 3814, 3819, 6398, 6549), and four strains each of *Staph. aureus* (1, 8223, 3230, 6587), *Pseudomonas pyocyanea* (1, 8223, 3402, 6626), Proteus (1, 8213, 3406, 6600), *Escherichia coli* (1, 2, 3, 5) and *Str. viridans* (1, 2, 3435, NT 172) were selected for the tests. The strain numbers in parentheses refer to identified cultures maintained in this Unit and not to strains maintained in the National Collection of Type Cultures. The strains of *E. coli* were isolated from faeces, and the other bacteria were isolated from burns and examined by methods described elsewhere (Jackson, Lowbury & Topley, 1951*a* and *b*).

Tests of bacterial growth

Overnight cultures of the test bacteria in nutrient broth were washed three times and resuspended in sterile Ringer's solution. Dilutions of these suspensions were made with Ringer's solution; in some experiments two dilutions were used (10^{-2} and 10^{-5}), and in others a single dilution (10^{-3} or 10^{-4}). Fresh serum was obtained from clotted blood (of L.C., E.J.L. or L.H.). To 0·8 ml. amounts of serum in small tubes, 0·04 ml. amounts of diluted bacterial suspensions were added with standard dropping pipettes. The tubes were sealed by plugging them with cotton-wool impregnated with wax, allowing an air space no greater than about twice the volume of the serum to prevent the loss of carbon dioxide and the development of a highly alkaline reaction during agitation (Fuller, Colebrook & Maxted, 1939). The tubes were incubated at 37° C. in a water bath with constant mechanical agitation. In some of the experiments tubes were incubated in a water bath without agitation; these tubes were closed with rubber stoppers.

Samples of inoculated serum were taken for bacterial counts before incubation began, and again (in some experiments) after 2 hr. or 6 hr., and always after 24 hr. incubation. Counts of viable bacteria by the method of Miles & Misra (1938) were made on horse blood agar (with 1·5% New Zealand agar except in experiments with Proteus and with *Ps. pyocyanea*, for which the agar concentration was increased to 4%). Colonies were counted after 18 hr. incubation at 37° C. and occasionally also after 48 hr. incubation.

Results

Fig. 1 shows the logarithms of viable counts before and after 24 hr. incubation, and in some tests after 2 hr. or 6 hr. incubation. Each graph shows the findings in experiments with all the strains of one species, including both dilutions of inoculum when two dilutions were tested.

The six strains of *Str. pyogenes* and the four strains of *Staph. aureus* showed vigorous growth in serum from large and from small inocula. By contrast, small inocula of *E. coli* were killed and large inocula were either killed or tended to

remain at the same concentration. Results with other organisms were less consistent. In two experiments Proteus was killed, while in other experiments the organisms either grew, or showed little change, or declined in numbers. *Ps. pyocyanea* showed a tendency to multiply, especially from the larger inocula. Two strains of *Str. viridans* were killed, and two others multiplied, one of them from

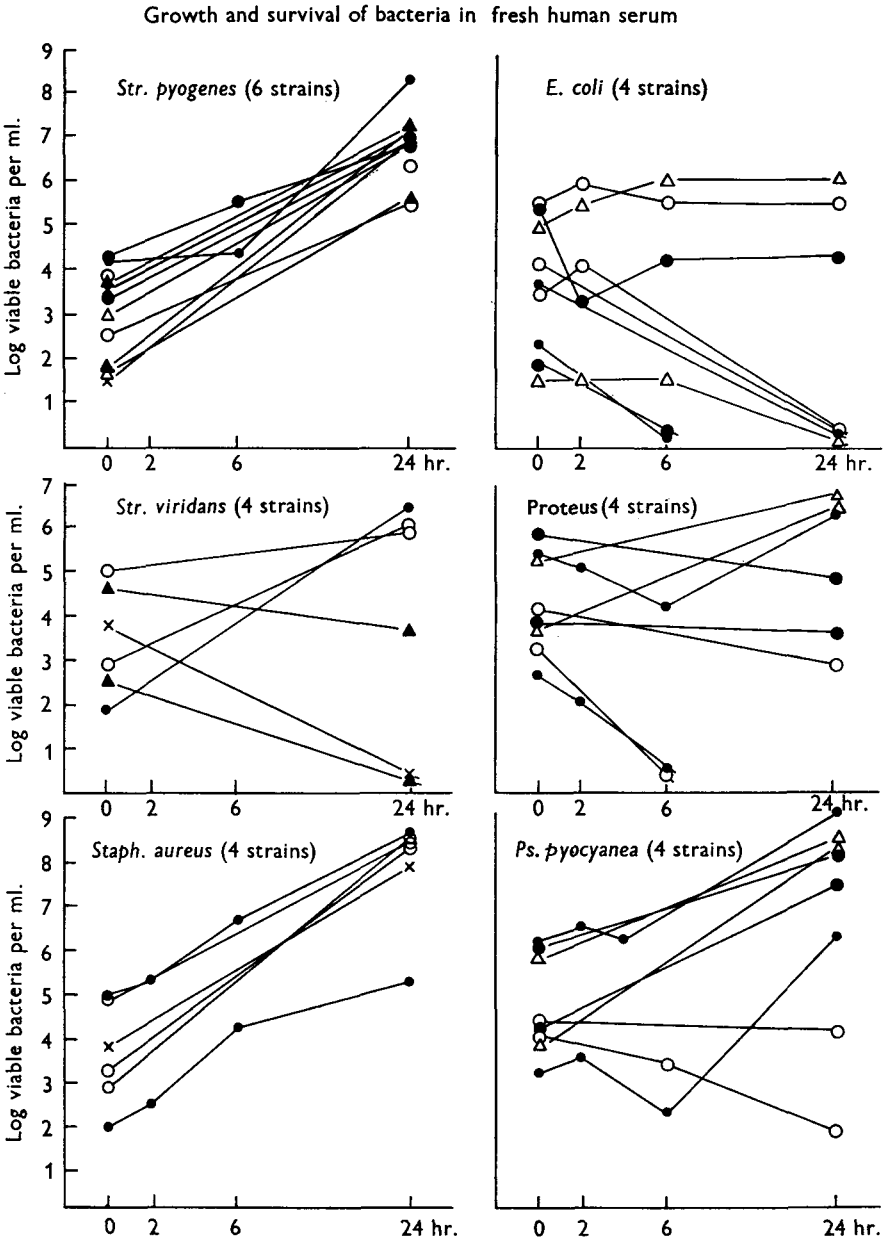

Fig. 1. The growth of six strains of *Str. pyogenes* and four strains each of *Staph. aureus*, *Str. viridans*, *E. coli*, *Ps. pyocyanea* and Proteus in fresh serum. The data are expressed as logarithms of viable counts before and after (and sometimes at intervals during) 24 hr. incubation at 37° C.

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a small inoculum. Unlike the other species tested, *Str. viridans* tended to produce minute colonies at the centre, but not at the periphery of the inocula of serum, suggesting inhibition of growth by serum on the plate. This effect was also found when the test was made with serum heated at 56° C. for 60 min.

EFFECTS OF HEATING AND OF PROTEOLYTIC ENZYMES ON THE SURVIVAL AND GROWTH OF BACTERIA IN SERUM

The conversion of serum by tryptic digestion into a favourable culture medium for coliform bacilli and other sero-saprophytes was reported by Wright *et al.* (1918). If this change is due to the inactivation of bactericidal proteins of the properdin system, many bacteria which fail to grow in fresh serum should grow as well in serum which has been heated at 56° C. for 60 min. as in serum digested with trypsin. The following experiments were made in order to throw light on this problem.

Growth of Gram-negative bacilli in heated and in trypsinized serum

Methods

Fresh serum from one subject (L.H.) was divided in two portions, one of which was heated in a water bath at 56° C. for 60 min. Half of the heated and half of the unheated portions were treated with trypsin in the manner described by Pillemer, Ratnoff, Blum & Lepow (1953). 0·1 ml. amounts of a solution containing 10 mg. crystalline trypsin (Armour) per ml. physiological saline were added to 0·9 ml. amounts of unheated and of heated serum; these mixtures were incubated in a water bath at 37° C. for 1 hr. The untrypsinized portions of the heated and of the unheated serum were diluted with 1 part in 10 of sterile physiological saline and incubated for 1 hr. at 37° C. Tests of growth in each of these portions were made by the method described above with three strains of bacteria previously shown to be killed on incubation with unheated serum; the strains selected were *E. coli* 1, Proteus

Table 1. *Growth of Gram-negative bacilli in fresh serum and in serum modified by heating and by tryptic digestion*

Medium	Organism	Viable counts per 0·02 ml. after incubation for 0 hr.	6 hr.	24 hr.
Fresh serum	*E. coli* 1	20	< 1	< 1
	Proteus 8213	34	< 1	< 1
	Ps. pyocyanea 8223	170	80	11
Heated serum (56° C. for 60 min.)	*E. coli* 1	80	900	37,000
	Proteus 8213	60	6,000	11,000
	Ps. pyocyanea 8223	170	1,600	8×10^5
Trypsinized serum	*E. coli* 1	80	430	21,000
	Proteus 8213	110	4,100	9×10^7
	Ps. pyocyanea 8223	160	1,200	9×10^5
Trypsinized heated serum	*E. coli* 1	30	37,000	8×10^7
	Proteus 8213	60	28,000	8×10^7
	Ps. pyocyanea 8223	120	8,000	14×10^7

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8213 and *Ps. pyocyanea* 8223. The inoculum was 0·02 ml. of a 10^{-4} dilution in Ringer's solution of thrice-washed nutrient broth culture.

Results

Table 1 shows the results of these tests. Both heating and tryptic digestion rendered the serum capable of supporting the growth of each of the strains. The strain of Proteus grew better in trypsinized than in heated serum, and the strains of *Ps. pyocyanea* and of *E. coli* grew better in trypsinized heated serum than in serum which had only been trypsinized or heated.

The effect of heating and of proteolytic enzymes on the bactericidal power of serum

Methods

For these tests we employed a culture of *Shigella sonnei*, strain 377, which the late Professor Pillemer had found peculiarly sensitive to the bactericidal action of the properdin system (Wardlaw & Pillemer, 1956; Lowbury & Ricketts, 1957).

Table 2. *Bactericidal action and complement of fresh serum and of serum altered by heat or by proteolytic enzymes*

Medium	% Shigella 377 surviving after 2 hr. in serum at 37° C.	Complement titre, 100 % haemolysis (units per ml.)
Fresh serum (E.L.)	0·003	42
Trypsinized serum (E.L.)	100	Nil
'Varidase'-treated serum (E.L.)	114	Nil
Serum (E.L.) heated at 56° C. for 1 hr.	86	Nil
Fresh serum from healthy subjects		
(*a*) 3 subjects	Range 0·003–0·045	< 20
(*b*) 11 subjects	Mean: 0·05 Range 0·0001–0·33	Range 20–33

Fresh serum (E.L.) was divided into four portions. One portion was trypsinized as described above; another was treated for 1 hr. at 37° C. with one-tenth of its volume of a solution of streptokinase and streptodornase ('Varidase')—the final concentration of streptokinase being 1200 units in 3 ml. of serum; a third portion was heated for 1 hr. at 56° C. and the fourth portion was mixed with one-tenth of its volume of physiological saline and incubated for 1 hr. at 37° C. to serve as a control. For the bactericidal tests an overnight broth culture was washed three times and a series of tenfold dilutions in Ringer's solution was prepared. Then 0·04 ml. volumes of these dilutions were inoculated into 0·2 ml. volumes of each of the samples of serum prepared as above, and incubated for 2 hr. at 37° C. Before and after incubation replicate drops of each of these inoculated samples were tested by the viable-count method of Miles & Misra (1938).

The several samples of serum were also tested for complement activity by a standard method (Pillemer, Blum, Lepow, Wurz & Todd, 1956) and the 100 % haemolysis titre recorded. Similar tests were made on sera from fourteen healthy subjects.

Results

These are shown in Table 2. All the normal sera showed a high but variable degree of bactericidal activity against Shigella 377, and a fairly consistent level of complement activity. Serum which had been treated with trypsin or with streptokinase showed neither bactericidal activity nor any detectable complement. Inactivation of complement by 'varidase' was previously reported by Pillemer *et al.* (1953). They attributed the whole of this activity to activation of serum plasminogen by the streptokinase component.

Antitryptic activity of fresh serum

Wright and his colleagues (1918) considered the antitryptic activity which they detected in fresh serum to have some protective function, for it resists the changes by which serum is converted into a hospitable medium for coliform bacilli and other sero-saprophytes. We made the following experiment to determine the amount of trypsin required to overcome this antitryptic power.

Method

To 0·9 ml. amounts of fresh serum (L.H.) were added 0·1 ml. amounts of doubling dilutions of crystalline trypsin in saline, from 20 mg. to 1·25 mg./ml., and 0·1 ml. of saline without trypsin as a control. After incubation for 1 hr. in stoppered tubes in a water bath at 37° C. the contents of each tube were tested (1) for bactericidal action against Shigella 377; (2) for complement; (3) for residual trypsin, by spotting on to photographic film, incubating at 37° C. for 1 hr. in a Petri dish containing moist filter-paper, and rinsing with cold water; a sharply defined transparency appeared in the presence of active trypsin.

Table 3. *Effect of different concentrations of trypsin on bactericidal and complement activity of serum*

Trypsin added to serum (mg./ml. serum)	% Shigella 377 surviving in serum after			Complement after 2 hr. (100 % haemolysis units)	Residue of trypsin in serum after 2 hr.
	30 min.	1 hr.	2 hr.		
2·0	119	112	119	Nil	+
1·0	105	100	111	Nil	+
0·5	81	91	91	Trace	±
0·25	1·5	0·02	0·0006	25	Trace
0·125	0·9	0·008	0·001	42	Nil
Nil	0·8	0·007	0·002	42	Nil

Results

These are shown in Table 3. Serum treated with trypsin at concentrations less than 0·5 mg./ml. retained its bactericidal activity and complement, and trypsin could not be detected in it (except for a trace after digestion with 0·25 mg. trypsin/ml.). After treatment with trypsin at and above 0·5 mg. per ml. bactericidal and complement activity were destroyed, and residues of trypsin were detected.

Effect of spontaneous release of plasmin on bactericidal activity of serum

As streptokinase inactivates complement and destroys the bactericidal activity of serum against Shigella 377, the question arises (e.g. Pillemer *et al.* 1953), whether the spontaneous fibrinolytic activity found in the serum of severely ill patients (see Astrup, 1956) might have a similar effect. The following experiment was made.

Method

Serum obtained by heart puncture shortly after death from two patients with extensive burns was tested for fibrinolysis and for complement; one of the sera was also tested for bactericidal activity against Shigella 377. Tests for fibrinolysis were made by a modification of the method described by Pillemer *et al.* (1953). Serum (0·2 ml.) was mixed with 0·2 ml. of a solution containing 1 mg. of fibrinogen/ml. physiological saline in a small tube. One drop (0·02 ml.) of thrombin (1 unit/ml. physiological saline) was added. The tubes were closed with rubber bungs, incubated in a water bath at 37° C. and examined at frequent intervals for the appearance of a clot, after which they were examined at intervals of 5 min. for 1 hr., and then at longer intervals. Serum from a healthy subject was tested as a control.

Results

Both sera (see Table 4) were actively fibrinolytic, but complement was present, and bactericidal activity against Shigella 377 was retained in the serum tested for

Table 4. *Fibrinolysis, bactericidal action and complement of cadaver serum*

Serum from	Approximate clot lysis time	% Shigella 377 surviving after 2 hr. incubation	Complement titre (100% haemolysis units/ml.)
J. K. (cadaver)	Complete in < 35 min.	Not tested	25
H. B. (cadaver)	Nearly complete in 40 min. and after 18 hr.	0·35	< 20
E. L. (control)	No lysis after 18 hr.	0·003	42

this property. No chemotherapy which would have been active against the Shigella was being used in the treatment of this patient.

GROWTH OF WOUND FLORA IN BLISTER FLUID AND IN SLOUGH SUSPENSION

Method

Two strains each of *E. coli*, *Ps. pyocyanea*, Proteus and *Staph. aureus*, and three strains of *Str. pyogenes* were tested for growth by viable counts taken before and after 24 hr. incubation in the following media; (1) fresh serum; (2) serum heated at 56° C. for 1 hr.; (3) blister fluid from burns of patients receiving no antibiotic; (4) a suspension in Ringer's solution of a homogenate made with a blender of a freshly excised new full-skin-thickness burn which had not been cleaned with

antiseptic before excision; and (5) nutrient broth. Two and sometimes three sizes of inoculum were used in most of these tests, and the incubation and sampling were carried out in the manner described above.

Results

These are shown in Table 5. In fresh serum the strains of *E. coli* were killed, those of *Str. pyogenes* and *Staph. aureus* multiplied, and the behaviour of

Table 5. *Growth of bacteria in serum, blister fluid and slough suspension*

	Viable bacteria (thousands/ml.) before and after 24 hr. incubation at 37° C. in									
	Fresh serum		Heated serum		Slough suspension		Blister fluid		Nutrient broth	
Organism	0 hr.	24 hr.	0 hr.	24 hr.	0 hr.	24 hr.	0 hr.	24 hr.	0 hr.	24 h
E. coli 1	6·5	0†	13	1500	130	26×10^4	130	0	16	$2\times$
	1·0	0	4	1850	15	4×10^5	1·3	0	—	—
	—	—	—	—	1·25	17×10^4	—	—	—	—
E. coli 2	17·5	0	22	6000	18·5	29×10^4	200	210	15·5	$5\times$
	—	—	—	—	—	—	2·0	0	—	—
Proteus 8213	15	0·8	45	8×10^5	100	25×10^4	210	4000	31	$37\times$
	1·7	0	3	550	33	4×10^5	2·1	0·05	—	—
						2×10^5				
Proteus 6600*	240	6000	240	9×10^4	1800	55×10^4	600	75×10^4	240	$2\times$
	2·4	3000	2·4	35×10^3	18	3×10^5	6	4×10^4	2·4	$2\times$
Ps. pyocyanea 8223	20	10	19	13×10^4	125	2×10^6	500	5×10^5	—	—
	8·5	0·5	8·5	4×10^4	18	35×10^4	10	16×10^3	15	10^6
	—	—	—	—	3·5	15×10^5	—	—	—	—
Ps. pyocyanea 6626*	600	22×10^4	600	45×10^4	800	5×10^5	1200	165×10^3	600	$45\times$
	6	11×10^4	6	14×10^4	8	7×10^5	12	11×10^4	6	$35\times$
Staph. aureus 8223	6·5	85×10^3	8·5	33×10^4	5·5	3×10^4	670	3×10^5	—	—
	—	—	—	—	—	—	6·7	55×10^4	4·5	$6\times$
Staph. aureus 6587*	80	26×10^4	80	44×10^4	90	7×10^4	180	4×10^5	80	$85\times$
	0·8	3×10^5	0·8	4×10^5	0·9	11×10^4	1·8	15×10^4	0·8	$3\times$
Str. pyogenes 8075	18	7250	1·85	6500	10	12×10^4	—	—	—	—
	2·2	10×10^3	—	—	2	1200	—	—	2	$12\times$
Str. pyogenes 6398*	6	22×10^3	6	26×10^3	80	13×10^4	8·2	39×10^3	6	$65\times$
	0·06	22×10^3	0·06	3×10^4	0·8	11×10^4	0·08	29×10^3	0·06	$13\times$
Str. pyogenes 6549*	4	2×10^4	4	23×10^3	—	—	0·05	21×10^3	4	$55\times$
	0·04	16×10^3	0·08	25×10^3	0·4	3950	0·0005	25	0·04	$65\times$

* In some of the tests, the viable bacteria at 0 hr. were calculated from counts of the suspensions that we inoculated into serum, slough suspension, blister fluid, and nutrient broth.

† 0 = No bacteria found on culture of 0·08 ml. (4 drops) of undiluted serum, blister fluid, etc.

Ps. pyocyanea and Proteus was variable. When the serum was heated at 56° C. for 1 hr., *E. coli* grew in it, though not so well as streptococci and staphylococci and not so well as they grew in slough suspension or in broth. All the bacterial strains grew in slough suspension, but the Gram-negative bacilli grew in this medium (as they grew in broth) more vigorously than the streptococci or the staphylococci. The pattern of growth in blister fluid was similar to that which was found in fresh

serum, but the strains of *Ps. pyocyanea* and Proteus showed a greater tendency to grow in blister fluid than in fresh serum.

DISCUSSION

Our experiments support the view put forward by Wright and his colleagues (1918) that some bacteria, including streptococci and staphylococci, will grow even from small inocula in fresh serum, while others, such as the intestinal coliform bacilli, fail to grow in fresh serum. Strains of other Gram-negative bacilli (*Ps. pyocyanea* and Proteus) gave variable results, sometimes approaching or resembling the Gram-positive cocci in their ability to grow in fresh serum.

Wright and his colleagues considered that coliform bacilli and other 'serosaprophytes' failed to grow in fresh serum because they were unable to metabolize the components of this medium; they showed that tryptic digestion of fresh serum converted it into a good culture medium for such organisms, and attributed the change to the release of products of proteolysis which would support the growth of these bacteria. We found that tryptic digestion and heating at 56° C. for 1 hr. both had this effect on serum. Both methods of treatment deprived serum of complement activity and of bactericidal effects associated with the properdin system; it seemed reasonable, therefore, to attribute the effect (or at least part of it) to the inactivation of these bactericidal proteins by heating or proteolysis. But while bacteria which were killed by fresh serum would grow in serum that had been heated or trypsinized, still better growth was obtained in serum which had been treated in both of these ways. This may have been due to a more complete destruction of the bactericidal components by the double treatment, or to the elimination of bactericidal effects combined with a positive improvement in growth-promoting properties—perhaps through the breakdown of protein molecules to smaller units. The relevance of the bactericidal powers of serum and exudate to the natural defence of wounds against infection is of interest. In a fresh wound, exudate which has this property is likely to destroy small inocula of intestinal coliform bacilli, which must be common contaminants. Bacteria which can grow readily in fresh exudate would be expected to destroy its complement and properdin by the action of their own proteolytic enzymes and by stimulating leucocytic infiltration; but small inocula of such organisms are likely to fall prey to phagocytes, specific antibodies and other components of the defence machinery.

If the bactericidal (and perhaps the antitryptic) powers of serum play any part in defence, they are more likely to do so in clean operation wounds or in small traumatic wounds than in burns or severe lacerations. The presence of slough in a wound is well known to enhance the risks of infection. Our observation that Gram-negative bacilli appeared to be at an advantage in slough suspensions while Gram-positive cocci were favoured by fresh serum and exudate is consistent with the frequent predominance of *Ps. pyocyanea*, Proteus and other Gram-negative bacilli during the first two or three weeks before the separation of slough, and with the subsequent predominance of *Staph. aureus* and (when it gains access) *Str. pyogenes* on granulating and unhealed surfaces.

SUMMARY

Six strains of *Str. pyogenes* and four strains of *Staph. aureus* were found to grow well from large or small inocula in fresh human serum; four strains of *E. coli* failed to grow from large inocula and were killed when the inocula were small. *Ps. pyocyanea*, Proteus and *Str. viridans* sometimes grew and sometimes failed to grow in fresh serum.

In serum which had been treated with trypsin or heated at 56° C. for 1 hr. the Gram-negative bacilli grew readily, and growth was still better in serum which had been heated and then trypsinized. Heating at 56° C. and treatment with proteolytic enzymes were shown to destroy complement and to inactivate the bactericidal activity of fresh serum against a Shigella highly sensitive to the properdin system. It is suggested that these changes might be enough to explain the conversion of serum by proteolytic enzymes into a suitable medium for the growth of coliform bacilli. This change might be brought about by bacterial or leucocytic enzymes. Fibrinolytic serum from a cadaver was found to retain at least a part of its complement and bactericidal activity.

Strains of *E. coli*, *Ps. pyocyanea*, Proteus, *Staph. aureus* and *Str. pyogenes* were tested for growth in burn slough suspension and blister fluid. In slough suspension all strains grew well, but the Gram-negative bacilli grew rather more vigorously than the Gram-positive cocci. In blister fluid *E. coli* was killed and other strains grew.

These findings are discussed in relation to wound sepsis.

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