

**FURTHER OBSERVATIONS ON THE STAPHYLOCOCCI,  
WITH SPECIAL REFERENCE TO THEIR HAEMOLYSINS  
AND VARIABILITY.**

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(With Plate VI and 1 Chart.)

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I.

ON THE RESULTS OF *STAPHYLOCOCCUS AUREUS* INFECTION IN RABBITS.

(a) *On the injection of vaccines, attenuated cultures and live S. aureus.*

*Agglutinins and precipitins.* Cultures of *S. aureus* isolated from infections in man were used for the inoculations. It is well known that rabbits are very susceptible to this organism, and previous inoculation with vaccines, attenuated cultures, or with carefully graduated doses of the live coccus does not confer adequate protective immunity, although the animal's serum contains immune bodies, such as agglutinins and precipitins, to a high titre.

The formation of agglutinins is more readily produced by intravenous injection of small doses of the live organism than by any other method. It is necessary to graduate the dose carefully<sup>2</sup>, or else the animal may die before

<sup>1</sup> Working during tenure of a grant given by Mr and Mrs Sannyer Atkin, in memory of their son.

<sup>2</sup> In all our experiments on the injection of live organisms, an 18-24 hours' culture on an agar slope was used; the growth was washed off with sterile normal saline, filtered through muslin, estimated, and made up to the required strength.

agglutinins or precipitins appear. The formation of agglutinins may run parallel with that of precipitins, or they may appear independently, although high titre agglutinating sera always contain precipitins.

Dudgeon and Bamforth (1925) have shown that precipitins are sometimes present in the blood of normal rabbits and we have found that agglutinins may occur also to a titre of 1 in 100.

*Haemolysins.* Some experiments were performed to find out if the sera of rabbits heavily infected with *S. aureus*, haemolyse their own red cells; this might occur from the formation of a haemolysin by the staphylococci present in the blood and tissues.

Five rabbits were inoculated intravenously with 100 million live haemolytic *S. aureus*: death occurred in from 3 to 14 days. The sera and red cells were tested 3 days after injection for the presence of haemolysins; also at intervals up to the animal's death. No haemolysin was demonstrated in any of our experiments, although the conditions were favourable for this to occur, as live organisms were present in enormous numbers throughout the body.

Furthermore, haemolytic staining of the aorta or of the endocardium was not visible in any of our rabbits which had died from a haemolytic *S. aureus* infection.

(b) *On feeding rabbits with live S. aureus.*

Two rabbits were fed every day for 4 months with carrots which had been cut up into small pieces and thoroughly sprinkled with a thick saline suspension of live *S. aureus*.

The animals gained weight steadily, and formed agglutinins for *S. aureus*, one to a higher titre than the other. The serum of one rabbit agglutinated its own strain to a titre of 1000 after 7 weeks' feeding, and 2000 after 10 weeks. Then the organism was suspended in 5 per cent. bile salt instead of saline and the feeding was continued for a further 4 weeks, but no change in the agglutinins or pathogenicity occurred. Finally, for 3 weeks it was suspended in 2.5 per cent. bile salt and 10 per cent. calcium chloride. This treatment produced no alteration in the agglutinins or ill-effects on the rabbits. The variability of the agglutinins is shown on Chart 1.

After 17 weeks' feeding on live *S. aureus*, no acute lesions had appeared, and both rabbits were killed.

The rabbit, which had the weaker agglutinins, showed healed abscesses in the right kidney, but no other lesions. Cultures from the viscera in both rabbits were sterile.

From this experiment we can deduce that *S. aureus* can be absorbed from the gut and cause septic lesions in the kidney and the formation of agglutinins in the blood. No "sensitisation" by bile salts occurred such as is claimed in the oral administration of dysentery vaccines.

(c) *On intracutaneous injection of live S. aureus and subcutaneous injection together with calcium.*

Three rabbits were inoculated with live *S. aureus*; 1. intracutaneously, 2. subcutaneously, 3. subcutaneously together with a small amount of calcium chloride. The rabbits were weighed daily; and the blood was examined weekly as to agglutinins, leucocytes and platelets.

The results may be given briefly:

1. *Intracutaneous.* First dose of 300 million live cocci, subsequent doses of 600 million every week for 12 weeks. Small closed abscesses developed at

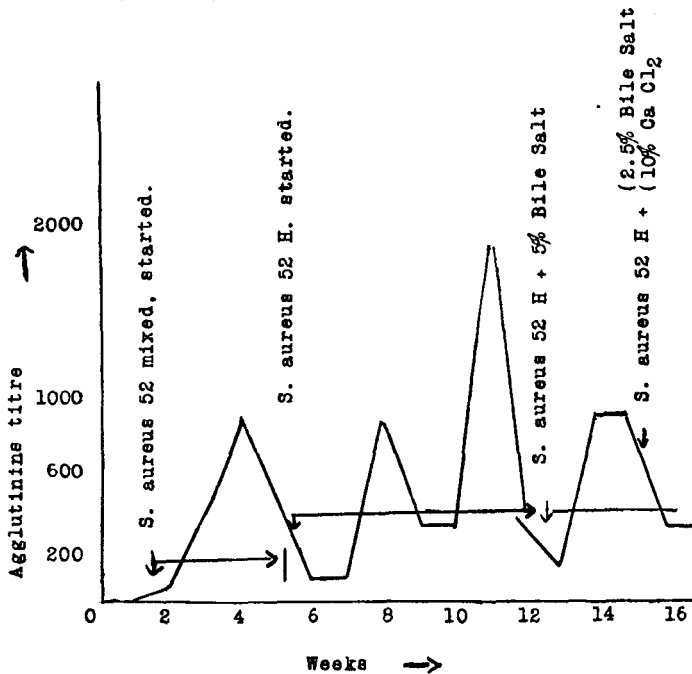


Chart 1. Feeding experiment. Rabbit X. Chart showing titre of serum agglutinins.

the sites of the injections. There were no changes in the leucocytes. The blood platelets rose to a slightly higher level, although somewhat variable. There was a steady gain in weight. Agglutinins were formed to a titre of 1000.

2. *Subcutaneous.* First dose 300 million, subsequently 600 million weekly for 6 weeks. Rabbit died from ascending paralysis a few days after the last injection. The kidneys showed old infarcts. There had been a slight gain in weight. There was a gradual rise in the leucocytes up to 16,000 per c.mm., but no relative increase in the polymorphs. The blood platelets rose from 360,000 per c.mm. to 1,600,000 per c.mm. and the agglutinins increased to 5000.

3. *Subcutaneous injection with calcium chloride.* First dose 300 million, subsequently 600 million weekly for 11 weeks. The live cocci were suspended

in 1 in 10,000 calcium chloride, 0.2 c.c. being given at each injection. Local abscesses developed at the sites of the inoculations, but did not differ in any way from the abscesses produced by the cocci without calcium. There was a steady gain in weight, a normal leucocyte count throughout, no increase in platelets, and the agglutinins rose to 10,000.

No. 1 rabbit was now given 100 million live haemolytic cocci intravenously. One month later it had to be killed owing to suppurative arthritis of the left ankle. From the date of the intravenous injection, there was a steady loss of weight, a rise in the leucocytes and in the platelets.

No. 3 rabbit received 100 million live non-haemolytic cocci intravenously. Five weeks later it developed paraplegia, due to suppurative osteomyelitis of a vertebra, with an abscess which tracked forward and pressed on the cord. Post-mortem, old infarcts were found in the kidneys. After the intravenous injection the platelets rose from 560,000 to 1,700,000 per c.mm.; the leucocytes and agglutinins did not alter, and there was a steady gain in weight.

To sum up, these experiments indicate that when the rabbit's relative immunity is sufficient to overcome a small infection, there is no change in the leucocytes, nor in the platelets: when a severe generalised infection develops then a rise in the platelets occurs which may be accompanied by a leucocytosis. A prolonged course of injections of sublethal doses of live organisms fails to confer adequate protective immunity.

(d) *On the injection of S. aureus together with colloidal silver.*

The object of these experiments was to ascertain whether colloidal silver has any inhibitory effect on pathogenicity or on the formation of immune substances.

Four rabbits were inoculated weekly with a formol-killed vaccine at the outset, and subsequently with live *S. aureus*, and two of these rabbits received weekly intravenous injections of 0.25 c.c. of 20 per cent. colloidal silver.

All four rabbits lost weight and formed agglutinins and precipitins: there was no marked difference in the titre of the sera.

Total leucocyte, differential leucocyte and reticulocyte counts were made at weekly intervals. The presence of a leucocytosis, together with a relative rise in the polymorphs, accompanied pyaemia. The immediate effect of intravenous injections of colloidal silver with *S. aureus* on the leucocytes was also noted. The blood 5 minutes after the injection showed a very marked fall in the percentage of polymorphs, which is recorded in Table I.

Films of the blood were examined also for particles of colloidal silver, but none were found free or in the cells.

From our results we have no evidence that the simultaneous injection of colloidal silver with *S. aureus* intravenously modified in any way the pathogenicity of the organism.

Table I. *The immediate effect of the intravenous inoculation of S. aureus and colloidal silver on rabbit's blood.*

Rabbits	Polymorphs	Lymphocytes
	%	%
1. Before injection ...	13	86
5 minutes after injection	1	97
2. Before injection ...	25	72
After injection ...	7	93
3. Before injection ...	44	56
After injection ...	1	97
4. Before injection ...	33	64
After injection ...	1	98
5. Before injection ...	53	45
After injection ...	2	98

As the result of injections of colloidal silver, the most marked histological changes occurred in the kidneys, which were brownish black; they showed an intense pigmentation in the connective tissue, and in the mononuclear cells between the tubules, especially of the cortex.

The connective tissue framework of reticulo-endothelial cells was strikingly outlined, and to a degree never approached by other staining methods. The glomerular tufts were free of colloidal silver, and the capsules relatively so: silver particles were not present in the epithelial cells, or lumen of the tubules.

There was a marked difference between the normal kidney tissue and the septic foci; for there was no silver, either in the centre of an abscess, or in a fairly wide zone of tissue around.

(e) *Pathological findings.*

*Kidneys.* Lesions were most constantly found in the kidneys.

(1) *Suppurative pyelonephritis.* Radiating linear abscesses occurred which have extended from capsule to pelvis, although the most extensive areas of suppuration were usually situated at the cortico-medullary junction (which is the point where the large arterioles break up into many smaller ones): the tubules may be full of septic casts.

(2) *Septic infarcts.* Wedge-shaped areas occurred in the cortex, consisting of a central necrotic focus surrounded by a zone of congestion and polymorph reaction.

(3) *Acute pyelitis.* This was sometimes the only kidney lesion found.

There are two points of special interest to be observed in renal lesions produced by *S. aureus*:

(1) An infective focus even of considerable size may resolve, and leave a large fibrotic depressed scar in the cortex: these areas were seen in the kidneys of rabbits which had recovered from the acute illness, and were examined some weeks or months later. The microscopical appearance resembled those found in chronic interstitial nephritis in man.

(2) Examination of the urine of infected rabbits may entirely fail to reveal the extent of the renal lesion. This fact is, of course, well known in the case of infective processes in the kidneys in man, especially those due to *S. aureus*.

*Heart.* Acute pericarditis and myocarditis with or without suppuration occurred: malignant endocarditis of mitral, and of mitral and aortic valves has been present.

*Osseous system.* No less than six of our rabbits died or were killed because of suppurative osteomyelitis of a vertebra causing paraplegia through compression by an abscess. Six developed suppurative arthritis, and two osteomyelitis of long bones: one had osteomyelitis of ribs.

*Nervous system.* In the regions of the spinal abscesses referred to above, lepto- and pachymeningitis were present.

In one case, we found suppuration microscopically along the central canal of the cord in the cervical and lumbar regions. The canal was dilated—filled with pus cells, and some of the small blood vessels nearby were thrombosed. Just before death, it had symptoms resembling rigors. This rabbit also had pyelonephritis.

An ascending paralysis occurred for which no cause could be found in the spinal cord, either gross or microscopical.

*Tendons.* Suppurative tenosynovitis may result from intravenous inoculation of live *S. aureus*.

*Blood vessels.* On microscopical section of all the viscera, stagnation of polymorphs is often seen in the capillaries of the lungs, heart, kidneys and in the sinuses of the liver and spleen.

## II.

### ON THE AGGLUTINABILITY OF *S. AUREUS* AND THE PRESENCE OF AGGLUTININS IN HUMAN SERA.

The agglutinability of strains of *S. aureus* depends partly on the medium upon which the organism is cultivated. In our experience plain agar (beef broth with 2.5 per cent. agar, 0.5 per cent. NaCl, reaction pH 7.4) is definitely the most satisfactory. The organisms are grown on agar slopes at 37° C. for 18–24 hours, and one or two drops of the live suspension in normal saline, according to density, are added to 1 c.c. of serum diluted as required. The reaction is read at the end of 5 hours at 52° C. In our experiments the end point of a reaction is taken as the highest dilution at which agglutination is visible to the naked eye: traces and faint traces visible with a lens are ignored, as clear-cut end-points are needed for comparison. Formolised saline suspensions of agar cultures—egg slopes—veal and beef broth cultures have been found useless for agglutination purposes, as shown in Table II.

Table II. *To show agglutinability of S. aureus.*

Culture	Rabbit serum Titre of end-point
(1) Agar—live ... ..	20,000
(2) Agar—formolised (10th subculture) ...	100
(3) Egg—live ... ..	100
(4) Veal broth—formolised (10th subculture)	100
(5) Beef broth—live (10th subculture) ...	100

Although the agglutinability of *S. aureus* grown on egg is much diminished, yet egg cultures are satisfactory for immunisation experiments, and for testing virulence.

*Immunisation.* A rabbit received intravenously five doses, at weekly intervals, of 10 million live *S. aureus* grown on egg: 1 week after the last injection, its serum agglutinated its own organism to a titre of 5000.

*Virulence.* Four rabbits, three previously inoculated for the production of agglutinins, and one normal, died from one intravenous injection of 100 million cocci from a live egg culture.

We have not attempted to find serological groups amongst strains of *S. aureus* isolated from human infections. Most strains agglutinated with

Table III. *The agglutinability of strains of S. aureus with various S. aureus antisera.*

No. of strain	Sera				
	1	2	3	4	5
55	.	0	.	.	0
26	2	.	2	.	.
23	3	.	1	.	.
15	2	.	1	.	.
9	3	.	3	3	.
6	2	.	3	3	.
1	2	.	2	3	.
2	2	.	2	.	.
4	3	.	2	.	.
8	1	1	1	.	.
11	0	.	1	.	.
38	1	.	3	.	.
50	.	2	3	.	.
45	.	0	.	.	0
46	.	0	.	.	1
53	.	3	.	.	3
54	.	3	.	.	3

samples of rabbit antisera prepared from strains of *S. aureus*, though not with the blood of patients from whom they were isolated (*vide infra*). Occasionally strains occurred which were inagglutinable with any of our sera.

The agglutination end point of a given strain of *S. aureus* varied with different antisera: the "agglutinability" of a strain may be determined by comparing its end point with several samples of antisera which had been standardised against their own organisms. In Table III are given the results of testing 17 strains of *S. aureus* with immune rabbits' blood.

The results (Table III) are expressed arbitrarily as follows: 3 signifies strong agglutination with any *S. aureus* serum as compared with the serum standardised against its own organism; 2 is intermediate; 1 is poor agglutination; 0 means inagglutinable.

*Saturation experiments.* A large number of experiments were made by allowing the serum to remain in contact with an extremely thick bacterial suspension in the ice safe, for a period of about 6 days.

The following conclusions are based on these experiments:

(1) Saturation of a serum by a strain of *S. aureus* or *S. albus* which is strongly agglutinated by it, removes all the agglutinins for every strain of *S. aureus*, including the specific strain.

(2) Saturation with an inagglutinable strain does not reduce the agglutinin content of the serum.

(3) Saturation of a serum with a strain which it only slightly agglutinates, removes the agglutinins for that strain, but leaves the specific agglutinins.

(4) In the case of staphylococci, absorption tests have no advantage over direct agglutination reactions.

*Presence of S. aureus agglutinins in human sera.*

The blood of 49 patients sent up for Wasserman reactions was examined. These sera were tested for the presence of agglutinins with a suspension of live *S. aureus* (52).

Of these, 5 sera showed good agglutination up to an end point of 100; 5 sera contained no agglutinins; 39 gave faint traces at dilutions from 25 to 1000.

The reactions were markedly zonular, the maximum effect always occurred with serum dilutions of about 400. Heating the sera to 55° C. for 30 minutes destroyed their agglutinating power; although heating immune rabbit sera does not reduce the agglutinin content.

Another series of ten human sera were tested against the haemolytic variants of four strains of *S. aureus*, and the end points of the reactions are shown in Table IV.

Table IV.

Sus- pensions	Sera									
	1	2	3	4	5	6	7	8	9	10
52 H	400	400	0	400	400	400	0	400	0	100
60 H	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
68 H	0	0	0	0	0	0	0	0	0	0
73 H	0	0	0	1000	0	0	0	0	0	0

No. 73 H showed zonular traces with all ten sera, but 68 H was inagglutinable.

The blood of four patients who had recovered from severe *S. aureus* infections, showed no more agglutination with *S. aureus* 52 than the zonular traces given by "normal" sera: nor did two of the sera agglutinate the strains of *S. aureus* derived from their own lesions.

*Immune rabbits' sera.* The heating of these *S. aureus* sera to 55° C. for 30 minutes has no effect on the agglutinin content.

The heated and unheated sera can be kept in the ice safe, but lose their agglutinating power slowly: similarly sera preserved with 0.25 per cent. phenol. When formalin 0.1 per cent. is added to immune sera they rapidly lose their agglutinins.



## III.

## ON NON-HAEMOLYTIC VARIANTS OF HAEMOLYTIC STAPHYLOCOCCI.

Eijkman (1901) first demonstrated zones of haemolysis around colonies of organisms grown on blood agar plates: amongst other bacteria, he found that *S. aureus* showed haemolysis by this method. Niesser and Wechsberg (1901) studied the production of haemolysins by staphylococci grown in broth. Koch (1908) showed exact parallelism between haemolysis on blood agar plates, and the haemolysins produced in broth. Nogerath (1902) came to the conclusion that pathogenic staphylococci were haemolytic on rabbit's blood agar plates, whereas the non-pathogenic were not.

Since then, much work has been done to correlate the haemolysis and pathogenicity of staphylococci: and the general opinion is that *most* pathogenic staphylococci are haemolytic, and that non-pathogenic strains are not. Julianelle (1922), however, states that *all* the pathogenic varieties are potentially haemolytic: but he found no constant relation between the degrees of pigmentation, haemolysis and liquefaction of gelatin.

It is well known that strains of *S. aureus* gradually lose their colour if kept for a long time on artificial media, and it has been claimed that non-pigmented cocci can be grown from single strains of orange staphylococci. Neumann (1897) stated that from a single strain of an orange staphylococcus he produced yellow, white, and flesh-coloured variants by subculturing on an alkaline medium. Dudgeon (1908) noticed that in the crowded parts of a plate culture of *S. aureus* the colonies appeared white, but that on subculturing on to agar slopes they developed orange pigment. Bigger, Boland and O'Meara (1927) found that by subculturing a single strain of *S. aureus* in broth daily for 38 days at 37° C., or for 52 days at 22° C., white and orange cocci appeared when plated on plain agar. They also found other variants, namely rough and smooth, also viscid and non-viscid. If the subculturing was done daily on agar, the white variants appeared, but not the rough.

The results of our investigations may serve to explain many of these isolated observations by previous workers.

*Technique employed.* In the experiments described in this paper, ordinary blood agar plates were used; 20 c.c. tubes of agar were melted and cooled to 50° C. and 0.8 c.c. of sterile human oxalated blood was added, mixed, and the plates poured just before use.

If it was necessary to plate from an agar slope, a very small amount of the growth was emulsified in broth. A small loopful of this thin suspension was then spread on two blood agar plates: a single colony was plated in the same way. The plates were incubated at 37° C. for 18–24 hours, and when examined the colonies were found to be well separated. Some experiments were done in parallel, *e.g.* subculture of a single colony on agar, broth, and blood agar.

In the text, throughout this paper, *S. aureus* H signifies a haemolytic strain, and NH non-haemolytic.

It was found that when pus from an infection in man due to *S. aureus* was plated on blood agar, usually all the colonies were haemolytic. If one of the H colonies was then transferred to an agar slope, subcultured daily for 10 days at 37° C. on agar, and then plated on blood agar again, two types of colonies appeared—H and NH (Pl. VI, figs. 1 and 2). By this means we have separated the original H strain into H and NH. From these mixed plate cultures, single H and NH colonies were picked off, suspended in broth and replated directly. It was found that the colonies were pure, *i.e.* the H colonies gave only H, the NH only NH<sup>1</sup>.

From the pure H plate a single H colony was picked off on to an agar slope, and from the pure NH plate a NH colony was selected. These were subcultured for a further 10 days, and replated. Again the H colony had given rise to H and NH, but the NH to NH only.

This can apparently be done *ad infinitum*, the H colonies giving both H and NH. Experiments have been carried as far as the seventieth generation and the same results have been obtained.

The NH colonies on subculturing, produced pure NH, except on two occasions, when H colonies also appeared.

In this way 25 different strains of *S. aureus* have been examined from boils, carbuncles, osteomyelitis, perinephric abscess, throat, intestinal and urinary sources. Of these, 20 strains were originally pure haemolytic, 3 were mixed H and NH, and 2 were pure NH.

This division into H and NH occurred at different rates in different strains of *S. aureus*. For example, No. 68 produced NH colonies after 4 subcultures, No. 75 only produced them after 21 subcultures.

When the NH variants appeared they gradually multiplied at the expense of the H; so that on daily subculturing for a long time, a pure H *S. aureus* may become pure NH, *e.g.* No. 71 became NH in 21 days. Strain 84 had been kept for 2 years with occasional subculturing on agar: at the end of this time it was still pure H; but on daily subculturing it formed NH colonies in 10 days.

Pure H strains kept for some time on the same agar slope at 22° C. or at laboratory temperature, usually showed H and NH colonies on replating. NH variants have always remained NH.

If an H colony, *e.g.* No. 81, was subcultured on agar at 37° C. every 1, 1½ or 2 hours, it remained pure H after nine transfers. The same colony subcultured *daily* for 9 days produced the usual division into H and NH. In fact, hourly subculturing seems to increase the degree of haemolysis produced by the cocci.

Besides *S. aureus*, three haemolytic strains of *S. albus*, and one haemolytic strain of a red-brown staphylococcus have been found to give the same separation into H and NH.

Subculturing in broth, however, did not result in this appearance of the NH variant; and when a haemolytic strain was grown in broth for 1 month,

Once only has a H colony given H and NH colonies upon direct replating.

NH colonies were not produced. The NH variant may appear more slowly if blood agar slopes are used for the daily subcultures instead of agar. For example, *S. aureus*, No. 85, showed H and NH colonies in 10 days on agar, but it required 4 weeks on blood agar before the NH colonies appeared.

The H colonies and the NH variants of a given strain of *S. aureus* have shown also certain morphological and cultural differences, which will be described in some detail.

*Morphology.* There were slight differences between the actual colonies. The H colonies in general were flatter and wider than the NH on the same plate; the NH colonies were dome-shaped. If they were crowded, the H colonies often ran together and became confluent; the NH were always discrete. This is shown in Pl. VI, figs. 3 and 4. In ordinary strains the H colonies were more greasy, while the NH were dry. This difference was more obvious on an agar streak, and was not the same as the mucous or viscid strains. When the original strain was sticky so were the H and NH colonies.

Another point of difference was seen when blood agar plates inoculated with mixed H and NH cocci, were incubated anaerobically for 24 hours. No haemolysis occurred; but two types of colony were present—dome-shaped, and flat colonies with a central peak. If the plates were re-incubated aerobically, after 6 hours haemolytic zones appeared around the flat colonies; the domed colonies were non-haemolytic.

*Agar slopes.* When making saline suspensions for agglutination tests, it was found that the H strains make good thick uniform suspensions which filtered through filter paper well. The NH variants were difficult to emulsify, tended to stick to the slope, filtered very thin, and showed auto-agglutination. All the NH variants were difficult to wash off the slopes, and seven of them auto-agglutinated. In this respect the NH and H cocci behaved like the rough and smooth variants of dysentery bacilli described by Arkwright (1921). The presence of a small amount of an auto-agglutinating NH variant in a series of agglutination tests may have the effect of raising the end point of the reaction.

*Pigment.* Dudgeon and Hope Simpson (1928) drew attention to the value of Dorset's egg medium in differentiating between *S. aureus* and *S. albus*, and for comparing pigmentation among strains of *S. aureus*. This medium has been used for contrasting the colour of the H cocci and their NH variants. For when the NH colonies first appeared in a given strain of *S. aureus*, if the H and NH were picked off on to Dorset's egg slopes, the H colonies were always more deeply pigmented. The NH, though an orange colour, are many shades paler.

The difference in colour between the H and NH was best seen when the egg slopes were incubated for 24 hours at 37° C. and then for another 24 hours at 22° C. The contrast between the colonies may be clearly demonstrated by spreading an egg plate at the same time as the blood agar plates. Dark and light orange colonies are seen; the light colonies may approach the citreous

lemon-yellow colour. It would, therefore, seem that the reason why a staphylococcus loses its colour after long storage on agar, is that there is an overgrowth of the paler variety.

*Liquefaction of gelatin.* The H cocci have always liquefied gelatin rapidly; the NH variants less completely and not so quickly: in 5 cases out of 20 the NH variant did not liquefy gelatin in 10 days.

*Carbohydrate fermentation.* Five strains were tested on dextrose, maltose, mannite, cane sugar, lactose, dulcitol and milk. All the H and NH variants acidified these sugars in 24 hours with the exception of dulcitol. No further experiments were attempted.

*Pathogenicity.* This was tested by inoculating pairs of rabbits intravenously with the same dose of live H cocci and the NH variant from the same strain.

*Technique.* The original *S. aureus* was subcultured until the division into H and NH occurred: from the mixed plate, single H and single NH colonies were picked off on to agar slopes for 24 hours at 37° C. These cultures were suspended in sterile saline, and made up to 200 million per c.c. One rabbit of a pair received 100 million live *S. aureus* H intravenously, the other 100 million NH variant. In two series, a third rabbit was injected with a suspension of a 24 hours' culture of the original pure H strain.

One experiment is quoted in full to illustrate our results. Three rabbits, *A*, *B* and *C*, weights 1600, 1500, 1770 grms. respectively, were injected with *S. aureus* No. 73. *A* received 100 million live H intravenously, *B*, 100 million original culture, *C*, 100 million NH variant. *A* was very ill in 2 days, moribund on the 4th and died with septicaemia; pyaemic abscesses were present in the heart muscle, kidneys, and throughout the subcutaneous tissues. *B* had to be killed on the 9th day because it had developed suppurative arthritis of the left ankle joint. Its blood culture was sterile. This rabbit was found to have abscesses in the heart muscle and kidneys, and pus in the ankle joint. *C* began to limp very slightly on the 14th day, and had to be killed on the 18th because it had arthritis of the right knee joint (from osteomyelitis of the head of the right tibia). Blood culture was sterile, and no other lesion could be found elsewhere either gross or microscopical.

*Pathological findings in rabbits inoculated with H cocci and their  
NH variants.*

Nine series of rabbits have been used. Their various lesions are shown in Table V.

It is evident that the H cocci are more virulent than the NH variant in that they kill quicker, and produce more widespread and severer lesions.

*Septicaemia.* Blood cultures were made under an anaesthetic at the time of death in fourteen instances.

A positive blood culture was obtained from four rabbits inoculated with H organisms, and from one with the mixed H and NH cocci, but never from rabbits injected with the NH.

Table V. *Comparative table of the lesions produced by H and NH variants of S. aureus.*

Rabbit	Strain	Days from injection to death*	Weight: gain +; loss -;	Generalised pyaemia	Pyelonephritis	Healed kidney abscesses	Myocarditis	Osteomyelitis: arthritis	Osteomyelitis of spine; paraplegia	Blood culture
A	73 H	4	-	+	+	.	+	.	.	+
B	73 original	8	-	.	+	+	+	.	.	-
C	73 NH	18	0	.	.	.	.	+	.	-
D	77 H	9	-	.	+	.	+	.	+	-
E	77 mixed	13	-	.	+	+	.	.	.	+
F	77 NH	21	-	.	.	.	.	+	.	-
G	81 H	25	-	.	.	.	.	+	.	-
H	81 original	6	-	.	+	.	.	.	.	-
I	81 NH	17	+	.	.	+	.	+	.	-
J	78 H	5	-	+	+	.	+	.	.	-
K	78 NH	R	+	.	.	.	.	.	.	-
L	52 H	18	+	.	+	+	.	+	.	+
M	52 NH	R	+	.	.	.	.	.	.	-
N	82 H	6	+	.	+	.	.	.	.	+
O	82 NH	R	+	.	.	.	.	.	.	-
P	68 H	6	0	.	+	.	.	.	.	+
Q	68 NH	R	0	.	.	.	.	.	.	-
R†	79 H	19	-	.	.	+	.	.	+	-
S†	79 NH	R	0	.	.	.	.	.	.	-
T†	83 H	10	+	.	.	+	.	+	.	-
U†	83 NH	R	+	.	.	+	.	.	.	-

\* death = rabbit died, was moribund or had to be killed owing to severity of lesions.

R = survived.

† = given second dose of 1000 million.

*Kidneys.* The H cocci produced acute and widespread suppurative pyelonephritis (73, 77, 81). The corresponding NH variants (77, 81) caused only a few small abscesses which healed; the NH variant of No. 73 did not produce any lesion in either kidney.

*Incubation period.* In eight out of nine series, rabbits injected with the haemolytic cocci died first, or had to be killed first owing to severe infection. Four rabbits which were given the NH variants had survived the H rabbits by 3-5 weeks and had been perfectly well and gaining steadily in weight all the time since the inoculations.

*Rates of growth.* The following method has been used to compare the rates of growth of the H and NH staphylococci on agar slopes. Single H and NH colonies were picked off mixed blood agar plates and grown for 24 hours at 37° C. The growths were washed off with sterile saline, filtered through muslin, estimated, and made to a strength of 1000 million cocci per c.c. 500 million ( $\frac{1}{2}$  c.c.) were then added to a series of agar slopes: the slopes were incubated with their agar surfaces horizontal. At stated intervals a known amount of saline was added to the surface growth, which was washed off completely, and the strength of the suspension estimated: from this the total number of cocci on the slope was calculated.

It would appear from the results obtained so far, that the H cocci multiply more rapidly at the outset, but that at the end of 24 hours the NH cocci are more numerous. This does not explain why the NH variants first arise from a

pure haemolytic *S. aureus*. We do know, however, that daily subculture on agar is a favourable condition for the NH to multiply at the expense of the H.

*Serological relationships.* Agglutinins. The H cocci and their NH variants when injected into rabbits give rise to agglutinins; and both are agglutinable<sup>1</sup>.

*Experiment.* Two rabbits, 20 and 21, were inoculated respectively with No. 52 H and its NH variant. The formation of agglutinins is shown in Table VI.

Table VI. Rabbits inoculated with H *S. aureus* and its NH variant.

Rabbit	<i>S. aureus</i>	Agglutinins			
		After 1st inoculation		After 2nd inoculation	
		Antigens		Antigens	
		52 H	52 NH	52 H	52 NH
20	52 H	200	50	1000	200
21	52 NH variant	—	100	2000	400

*Precipitins.* A rabbit was injected intravenously at weekly intervals with live *S. aureus* No. 52 pure NH variant. Three doses were given—10, 10, and 20 million. Precipitins were formed in the serum as shown in Table VII, so that the injection of a pure NH variant results in the formation of precipitins

Table VII.

Precipitin antigen	Precipitin end-point	
	Before inoculation	After 3rd inoculation
52 H ... ..	Nil	1/100
52 NH variant	Nil	1/100
85 H ... ..	Tr/50	1/100

both for the NH variant and for the H strain, and also for another strain of *S. aureus*.

It has been stated elsewhere that if a culture of *S. aureus* is agglutinated by a serum, then saturation of that serum with *S. aureus* removes all agglutinins. As may be expected, therefore, no serological difference between an H strain and its NH variant was found by saturation experiments.

*Haemolysin in centrifuged suspensions.* Haemolytic strains of *S. aureus* were grown on agar for 8–24 hours at 37° C., washed off with distilled water, and shaken for  $\frac{1}{2}$ –1 hour. The suspensions were then centrifuged at high speed for about an hour until the supernatant fluid appeared clear of organisms. When this supernatant fluid was pipetted off and made up to a salt concentration of 0.85 per cent. by adding a small amount of hypertonic saline, it was found to be haemolytic to human, rabbit's, and sheep's red cells; titres of 200 were obtained. The lytic action occurred within a few minutes of incubating the mixture of red cells and lysin at 37° C. This is conclusive evidence that the haemolysis is not due to cocci present in the mixture, but to an active lysin. Bigger, Boland, and O'Meara (1927) were the first to record a haemolytic action by this method.

<sup>1</sup> As NH variants tend to auto-agglutinate after growing on agar, another medium was used to obtain a uniform suspension. If NH colonies are grown on inspissated blood serum slopes they give perfectly uniform agglutinable suspensions when washed off with saline.

## DISCUSSION.

Are all haemolytic staphylococci really mixed cultures? or are the non-haemolytic variants an attenuated form produced by artificial changes in their environment?

It is a fact that the large majority of strains of *S. aureus* isolated from acute infective processes are pure haemolytic, but all are potentially able to produce non-haemolytic variants.

Haemolytic and non-haemolytic colonies may occasionally be found when the pus from a patient is plated on blood agar. This has happened three times in our series from a parotid abscess, from a chronic abscess of thigh, and from a case of chronic osteomyelitis. We have obtained pure non-haemolytic strains twice: once from the urine, and once from a blood culture.

Supposing a colony were composed of both H and NH cocci, it would appear haemolytic on a blood agar plate, but if this colony was suspended in saline, it is reasonable to expect that, on replating, some non-haemolytic colonies would be found. This has occurred on one occasion out of the large number of instances that this experiment has been made. Cultural investigation from a single coccus has not yet been undertaken.

The NH variants are produced *in vitro*; are they produced by staphylococci dividing *in vivo*? When rabbits were injected with a pure H strain, either pure haemolytic or mixed H and NH were recovered from its lesions, but if a rabbit is injected with a pure NH variant, only NH cocci are recovered. In Table VIII the results of these experiments are recorded.

Table VIII. *Inoculation of rabbits and recovery of H S. aureus and NH variants.*

Rabbits injected with	Recovered from lesions		
	Pure H	H and NH	Pure NH
Pure H 12	8	4	0
Mixed H and NH 7	2	3	2
Pure NH 4	Nil	Nil	4

A pure H strain of *S. aureus* will give rise to haemolytic cocci and to non-haemolytic variants both *in vitro* and *in vivo*. A pure NH variant probably always remains NH; and, although it may kill an animal quickly, it never regains the power of haemolysis.

Thus, all the evidence accumulated so far indicates that these NH cocci are true variants arising from originally pure H strains of staphylococci.

## CONCLUSIONS.

1. Rabbits inoculated with *S. aureus* readily produce precipitins and agglutinins in the blood, but protective immunity has not yet been established.

2. Colloidal silver, when injected intravenously with *S. aureus*, is taken up by the reticulo-endothelial cells. It does not reduce the virulence of the organisms, nor modify the formation of immune substances. The polymorphs almost entirely disappear from the peripheral blood immediately after an injection of colloidal silver with *S. aureus*.

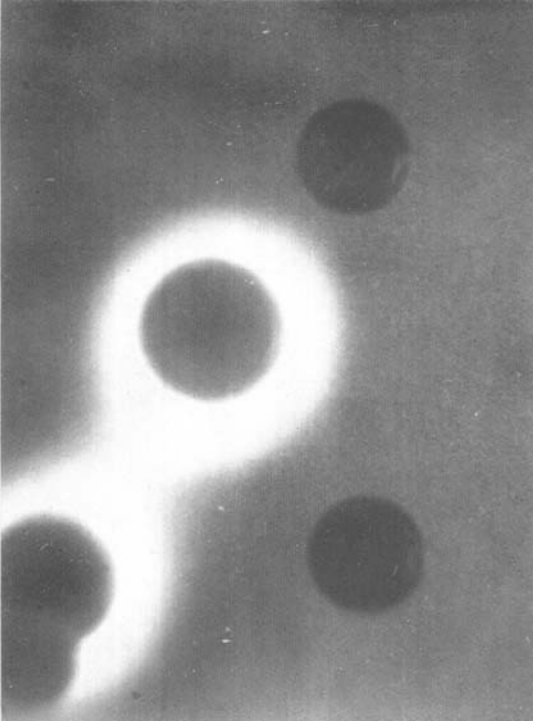


Fig. 1

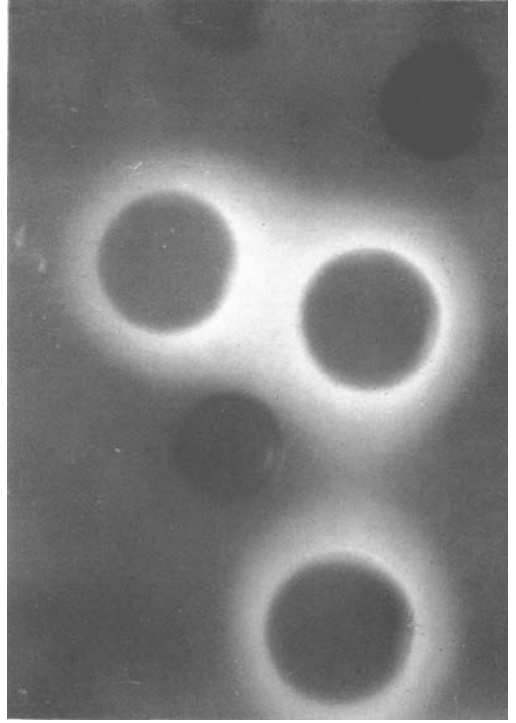


Fig. 2

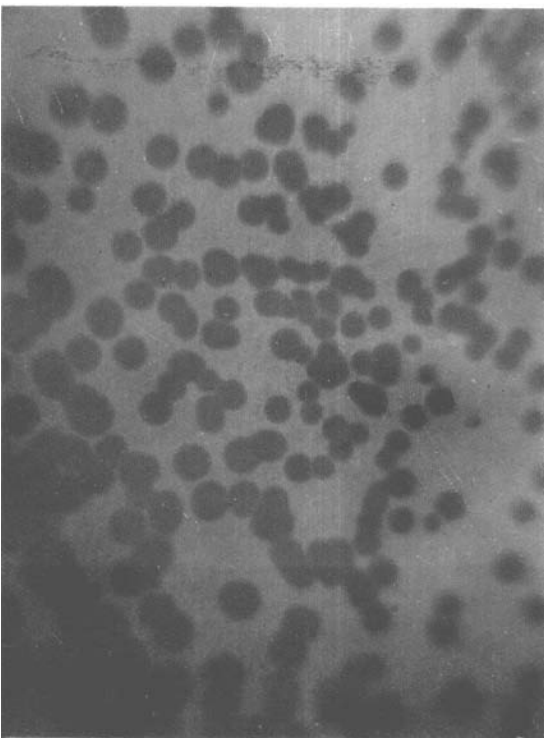


Fig. 3

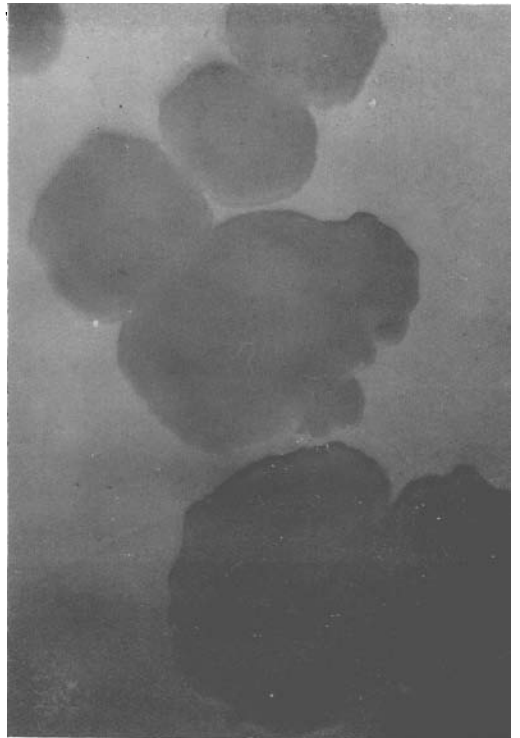


Fig. 4



3. Rabbits fed on *S. aureus* develop specific immune substances, and evidence of a general infection was forthcoming.

4. Intradermal injection of live *S. aureus* results in the formation of agglutinins.

5. A small amount of calcium chloride injected with *S. aureus* does not modify local or general lesions.

6. In a rabbit, which is resisting an *S. aureus* infection, the blood platelet count remains normal. When this resistance is broken down by a fatal infection, the count may rise to 2–3 times the normal value.

7. "Normal" human sera agglutinate a live *S. aureus* suspension to a slight extent. The blood of patients during severe *S. aureus* infections, or when convalescent from them, have not shown any greater agglutination than the normal sera—either to their own or to other strains.

8. Cultures of *S. aureus* derived from different sources, vary greatly in their agglutinability; this is independent of other properties, such as colour, haemolysis, etc.

9. Saturation experiments have no advantage over ordinary agglutination tests in determining specific grouping of *S. aureus*.

10. All haemolytic staphylococci produce non-haemolytic variants, when subcultured daily on agar at 37° C. for about 10 days.

11. The NH variant differs from the haemolytic strain in diminished pathogenicity, less pigmentation, and in slow liquefaction of gelatin.

12. The H and NH also differ in their rate of growth, and because the NH auto-agglutinates.

13. It is suggested that attempts should be made to prepare efficient *S. aureus* vaccines and antisera from haemolytic strains.

14. The pathological lesions met with in rabbits inoculated with *S. aureus* are similar to those which occur in man.

#### DESCRIPTION OF PLATE VI.

Figs. 1, 2. H and NH colonies from 10th subculture of a pure haemolytic *S. aureus*: seen by transmitted light on a blood agar plate.

Figs. 3, 4. Plates of H and NH cocci of the same degree of crowding, showing that the NH colonies (Fig. 3) are discrete, but the H colonies are confluent (Fig. 4).

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