A SEROLOGICAL STUDY OF THE ANTIGENS OF STAPHYLOCOCCUS PYOGENES.

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by

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INTRODUCTION.

At its outset, this thesis is concerned with serological methods of typing coagulase-producing staphylococci.

Oeding, of the University of Bergen, Norway, elaborated a method of typing to be used in epidemiological surveys; Brodie, of the University of St. Andrews, Scotland, modified this method to suit his own purposes. When the latter used his modification to type a standard series of staphylococci, the results were not similar to those obtained by the former. In Section 1 of the thesis, the historical background to serological methods of typing coagulase-producing staphylococci is reviewed. In Sections 2 and 3, the work of Oeding and of Brodie is repeated, and suggestions are drawn as to the cause of the discrepancy.

During the course of this part of the work, the question arose as to the functions of the antigens. At this time it was thought that the antigens uncovered serologically might be the counterparts of haemolysin, coagulase, enterotoxin, etc., and one of these, coagulase, was selected for further study.

In Section 4 is investigated the possible identity of coagulase and several of the various antigens uncovered by the use of the system of Brodie. When it was found that coagulase was represented by several antigens, the problem then arose of defining the number of coagulases produced by the staphylococci.

To conclude the thesis, investigations were made as to the possible value of antibodies to coagulase in the animal, and the possibility of using coagulase for the prophylaxis of staphylococcal infection.

SECTION I.

HISTORICAL INTRODUCTION.

The serological relationships of the coagulase producing staphylococci are probably natural subjects to be studied in Aberdeen, as this city was the home of Sir Alexander Ogston who was among the first to appreciate the pathogenicity of these micro-organisms. (1880[64], 1881[65], 1882[66].)

Since that time, vigorous studies have been prosecuted on this micro-organism towards the establishment of some system or systems which would allow of potentially pathogenic staphylococci being subdivided into groups or types in the hope that epidemiological studies might be undertaken. At the moment, most investigators prefer bacteriophage typing, but nevertheless, both before and subsequent to the discovery of such 'phages, many attempts have been made to establish systems of identification based on antigenic analysis. Serological typing has been investigated and reviewed by Hobbs (1948[35]), Elek (1959[26]) and Oeding (1960[57]).

First in the field were Kolle and Otto (1902[42]), and Otto (1903[61]), but the more important investigations and results are those of Hine (1922[33]), Julianelle and Wieghard (1922[36] and 1935[37]), Cowan (1938[19] and 1939[20]), Christie and Keogh (1940[17]), Hobbs (1948[35]), and Oeding (1952[54], 1953[55], 1957[56], and 1960[57]).

A concise account of the work of these and other workers is now given so that the position of studies based on antigenic analysis to date may be reasonably clear.

Kolle and Otto (1902[42]) and Otto (1903[61]) typed staphylococci by agglutination techniques using antisera produced in dogs, and found that pathogenic staphylococci belong to one serological type and non-pathogenic staphylococci to a different type.

Hine (1922[33]) - the first British investigator in this field - studied the serology of staphylococci in the hope of finding a system of classification that would permit advances in the therapy of staphylococcal infections similar to the progress that had been made in the instances of infection due to meningococci, streptococci, and pneumococci. He inoculated rabbits

intravenously with phenolated staphylococci, and estimated the titres of the antisera by agglutination tests with heat-killed, phenolated organisms. As a result, he divided (1) the pathogenic staphylococci into three types, and the (2) non-pathogenic staphylococci into two types. He also typed serologically the staphylococci recovered from boils in a female patient, and cured the condition by means of an autogenous vaccine.

Next in prominence came the studies of Julianelle and Wieghard (1922[36] and 1935[37]). These investigators prepared their antisera by injecting rabbits intravenously with heat-killed staphylococci, and confirmed the work of Hine (1922[33]) that there were three serological groups of pathogenic staphylococci. In addition, they uncovered a fourth group in which cross-reactions suggested at least the possession of common antigens. Turning to the work of Avery and Heidelberger (1923[3]), who showed that type specificity of pneumococci depended on serologically distinct capsular carbohydrates, they attempted to apply such a classification to staphylococci using polysaccharide extracts as antigens, and antisera prepared in rabbits to precipitate these (1935[38]). They thus demonstrated that, by these precipitation

tests, two large groups of staphylococci could be distinguished, but as one contained only pathogenic organisms, and the other only non-pathogens, the information, although of academic interest, was of little value in field work.

In 1938 Cowan[19] re-examined the classification proposed by Julianelle and Wieghard. Using antisera produced in rabbits by intravenous injection of heat-killed staphylococci, and the use of alcohol precipitated antigens, he confirmed the existence of the two principal groups of staphylococci. His findings, however, were complicated by the fact that he found fifteen strains which could not be adequately typed, and he therefore suggested that the classification would have to be extended. Thompson and Khorazo (1937[77]), working on the same lines, were forced to draw a similar conclusion.

In 1939, Cowan[20] returned to the problem, but now employed slide agglutination techniques with antisera produced in rabbits injected with heat-killed and formolinised staphylococci. With such antisera, cross-agglutination results were so troublesome that he was compelled to try absorption techniques on his antisera. His procedure was successful in that he was able now to

divide the coagulase producing staphylococci into three types which he designated I, II, and III. Nevertheless he found that somewhat less than a third of his strains still did not fall into any of these three types. These he placed in a fourth group. He also concluded that as the two groups which could be separated by precipitation techniques did not belong to one type each as shown by the agglutination tests, the antigens utilised were therefore dissimilar. About this time, Blair and Hallman (1936[5]), working in America, confirmed that the bulk of pathogenic staphylococci could be divided into three groups serologically.

In 1940, Christie and Keogh[17] entered the field. Working in Melbourne, Australia, they introduced six new types which they named 4,5,6,7,8, and 9. These were identified by slide agglutination tests using absorbed sera prepared in the first instance with heat-killed strains of the three Cowan types of staphylococci. It is worth noting that they did not utilise Cowan's own strains, but strains isolated locally and identified with antisera supplied by Cowan. They also pointed out that all their coagulase positive staphylococci could be agglutinated by combining the antisera of types 1 and 2, thus confirming the existence

of common antigens.

In 1948, Betty Hobbs[35] performed an extensive series of slide agglutination experiments in which she re-established the validity of the three types of Cowan and the six types of Christie and Keogh, and to these she added four new types, 10, 11, 12, and 13. She prepared her antisera in rabbits by injecting them with heat-killed staphylococci, and like the others, she required to use absorption techniques to overcome cross-reactions. With her antisera, she attempted to place these thirteen types in groups, of which she established seven. Having gone so far, she leaves open the question that she raised, namely, is there a large number of serologically distinct types, or a small number of types around which are grouped many strains showing only minor variations from the main type ?

The present phase of serological typing is based on the work of Oeding, who published his first findings in 1952[54]. His work differs from his predecessors in that he investigated not only the heat-stable antigens, but also the heat-labile antigens. In injecting his rabbits, he used formalin-killed organisms rather than heat-killed. Also, instead of enumerating types, he attempted to prepare "factor antisera" to react with one antigen only, and to make

an analysis of the antigenic patterns of staphylococci with these monovalent antisera.

In 1952 he investigated an outbreak of breast abscesses in a maternity hospital. With formalin-killed suspensions of the mastitis-producing staphylococci he injected rabbits, and absorbed the antisera with strains of staphylococci not isolated from breast abscesses. In this way he produced a factor serum which he called "e", and, by its use, he traced the course of the epidemic. In a further paper in the same year, Oeding made known the finding of a series of staphylococci from which he could prepare factor antisera which he named "a", "b", "c", "d", "e", "f", "g", "h", "i", and "k". To these he ascribed the following characteristics.

"a" was found to be a widely distributed antigen.

"b" was a weak but frequent antigen.

"d" was common to all strains examined.

"f", "h", "i", and "k" were apparently specific antigens, found to give antisera of good titre.

"g" was too weak to give useful antisera.

With these antisera, he studied the antigenic content of his available staphylococci, and placed them into major groups according to the presence in each strain of antigens reacting with the factor antisera "a", "b", "c", and "e". On this basis, he found Cowan's type I belonged to the group "abe": type II belonged to the group "ab"; and type III belonged to the group "abce". Other strains he placed in groups "abc", "b", and "bc". These major groups were further divided according to their reactions with the remaining antisera "f", "h", "i", and "k".

Later work by Hauknes and Oeding (1960[34]) showed, by gel-diffusion techniques, that factor antiserum "e" was polyvalent, and contained three antibodies, one of which they continued to designate "e", while the others were now named "m" and "n". Also, at this time, they concluded that factor antisera "f" and "g" were too weak to be of practical use.

At this stage it seemed necessary for the sake of specificity to type staphylococci according to their antigenic content of both heat-labile as well as heat-stable antigens. To study the heat-stable antigens

he preferred to autoclave the staphylococci at 120°C for 2½ hours instead of boiling them as Cowan, Hobbs, and others, had done.

All of Oeding's work thus far had been done with staphylococci harvested off nutrient agar after 24 hours incubation at 37°C. This investigator now began to study the effect of time and temperature on the rate of the development of the antibodies. He found in 1957[56] that

(a) at 37°C, with incubation for 3 - 5 hours, the cultures yielded a wide range of antigens.

(b) at 37°C, with incubation for 24 hours, the cultures (i) appeared to lose some of their antigens which were revealed by (a), and (ii) gained antigens "i" and sometimes"h".

(c) at 37°C, with incubation for 24 hours, and after autoclaving for 2½ hours at 120°C, the antigens found by (a), and lost by (b), now revealed themselves again.

Thus, the use of 5 hour and 24 hour cultures grown at 37°C and examined in the live state obviates

the need for autoclaving the suspensions, the combined findings being accepted as the antigenic structure of the staphylococcus under analysis.

At first, Oeding (1953[55]) thought that the heat-stable antigens were carbohydrate in nature, while the heat-labile antigens were protein. Later, he considered that some antigens consist of both heat-stable and heat-labile components. When he found that antiserum "e" was polyvalent, he thought that the heat-stable and heat-labile components possibly represented distinct antigens. It is now thought that in fact all the antigens are protein in nature. (Pillet et al., 1955[62]).

In 1957, Grün[31] found that animal strains often contained a further antigen that he termed "1", and that some human strains also contained this fraction.

Other workers in this field have investigated Oeding's system and have elaborated systems of their own. Among these, Brodie (1957[7]), Grün (1957[31]), Lofkvist (1957[43]), Stern and Elek (1957[72]), and Vischer (1959[78]) have all published results.

Grün[31], using Oeding's technique and strains, confirmed Oeding's results.

Vischer[78], although finding the results consistent, was not impressed with the usefulness of the method.

Lofkvist[43], devised a method of typing in which he used antisera made from Oeding's strains and his own strains. He confirmed the presence of Oeding's factor "e" in all his mastitis-producing strains, and drew up a system of classification comparable to Oeding's.

Stern and Elek's work[72] stemmed from the question of whether the antigenic complexities of the staphyloe coccus might be due to complete but unequal sharing of the various antigens. By disrupting the organisms and examining the cell walls and endoplasmic contents, they concluded, "examination of the cell walls offers no advantage over the whole cells from a serological point of view", and "..... so far as the Cowan types are concerned, the major antigens are group specific. The minor antigens are of two kinds, type specific and shared."

The question of whether the various types really differ in the composition of the antigens, or whether they all share the same antigens in unequal proportions was also posed by Mercier et al.,(1950[53]), and by Anderson and Heilesen (1951[2]).

They concluded that they found no evidence of more than three types among the heat-killed international strains, and considered further division to depend exclusively on quantitative differences.

Brodie (1957[7]) decided to use Oeding's method for typing staphylococci, but with two modifications: he preserved his strains in 8% sodium chloride broth instead of agar stabs as Oeding recommends, and, instead of using Oeding's strains to produce the antisera he used the thirteen international types for which Oeding had published formulae. His results were not similar to those obtained by Oeding, and this is discussed fully in section 2.

VALUE OF SEROLOGICAL TYPING.

There are three attractive features of serological typing of staphylococci as opposed to 'phage typing. First, there is the high proportion of strains of staphylococci that can be typed by the use of antisera. Oeding and Vogelsang (1954[59]) state that 94.6% of staphylococci in the live state can be typed by the use of antisera. In strains that do not agglutinate with antisera, autoclaving often accomplishes this and raises the proportion of strains typeable with antisera to 97%. Only 63% of strains of staphylococci can be typed by 'phages. Secondly, serological typing makes use of antigenic fractions of the cell that may be important metabolically; (the evidence for this is presented on page 99). The 'phage receptors are unknown. Thirdly, in the hands of those who utilise 'phages, it is recognised that the 'phages may lose their specificity unless appropriate precautions are taken, and the enormous number of types disclosed may make the final recognition of a strain dependant on additional criteria. These latter may be, that the organism was previously isolated from the same patient, or that a patient develops a lesion following exposure to infection by a person carrying a rather similar 'phage type of staphylococcus.

EPIDEMIOLOGICAL VALUE OF SEROLOGICAL TYPING.

Oeding (1960[57]) states, "the number of patterns, types or groups which a method is able to distinguish must not be too small or the certainty with which an epidemic strain can be picked out in patients and carriers will suffer. On the other hand, it should be considered a disadvantage if a system registers a great number of patterns unless they are well-characterised and not influenced by technical considerations. Furthermore, the number of untypeable strains must not be too large. A method must not be too complicated or it will be of limited practical value." I have commented on these points already, and it remains now to consider to what extent investigators have found serological methods of typing to be of value. Oeding (1960[57]) has suggested that the best possible results are obtained if one uses serological typing, 'phage typing, and antibiotic sensitivity patterns ("antibiograms" as Oeding calls them). As, however, this is time consuming, he has added that one can pick out suspect strains by their antibiograms, then type them either serologically or by 'phages.

Field investigations have been performed by Brodie,

Sommerville, and Wilson (1956[11]), Brodie, Jamieson, and Sommerville (1955[9]), Grün (1958[32]), Kikuth and Grün (1957[40]), Lofkvist (1957[43]), Oeding and Sompolinsky (1958[58]), Oeding and Williams (1958[60]), and Sompolinsky, Herman, Oeding and Rippon (1957[73]). In all these investigations, consistent results were obtained. "Antibiograms" were compared with serological results, and on occasion serological results were compared with results obtained by 'phage typing methods.

Reference has already been made to the work of Grün who used Oeding's strains and methods, and Lofkvist who used his own methods, and to the results obtained. The two most important investigations in this connection, however, are those of Sompolinsky, Herman, Oeding and Rippon (1957[73]), and Oeding and Sompolinsky (1958[58]).

In the first of these the authors describe the investigations in a thoracic surgery unit in which the incidence of staphylococcal sepsis in post-operative cases was 37%. The majority of strains of staphylococci recovered belonged to one type, well defined by 'phage pattern, (6, 47, 53, 77), serotype, (in the live state,

abc, in the autoclaved state, bch), and by the "antibiogram", (resistant to penicillin, streptomycin, and sulphonamides). "The source of the infection was clearly established to be two nurses of the operating team". After simple precautions, such as wearing double masks, seeing that the nose was properly covered, and always wearing gloves in the theatre, only two further cases of sepsis were found, and in neither case was the original strain of staphylococcus identified.

In the second paper, an outbreak of gastro-enteritis due to the presence of staphylococci in a potato salad is described. The results are compared with findings when ordinary "Street" strains of staphylococci and the previously discussed strains from the thoracic unit were all typed by "antibiograms", antisera, and 'phages. It is pointed out by the authors that the mixed batch of street strains, isolated in a paediatric unit, were sensitive to penicillin and best identified serologically as they were resistant to 'phages, whereas the hospital strains were best picked out by the "antibiograms" and typed with 'phages.

Brodie et al.,(1956[11]) demonstrated that the types of staphylococci found in the noses of nurses in a preliminary training school gradually changed when the nurses took up ward duties, so that the types which they designated "ac/-", and "ac/h" rose from 17% of all isolates to 44%, while autoagglutinable types and types named "acei" fell from 54% to 22%, indicating that some types seem to have potential for epidemic spread. In a further paper, Brodie et al., (1956[11]) found that in addition to uncovering a fourfold increase in the nasal carriage rate of staphylococci of serotype "ac/-" after one week's stay in hospital, the faecal carriage rate rose sevenfold in the case of type "ac/-", and that as this was the strain commonly associated with antibiotic resistance, the authors stress that the faecal carrier may be an important agent in hospital cross infection.

SECTION II.

CHAPTER 1.

Grün (1957[31]) prepared factor antisera to staphylococci according to the methods developed by Oeding. and using Oeding's strains (Oeding, 1953[55], 1957[56]). Brodie (1957[7]) also attempted to produce factor antisera but accepting as his standard strains the thirteen international types as described by Hobbs (1948[35]), and with certain modifications in Oeding's techniques. Grün was able to reproduce the factor antisera of Oeding, yet Brodie, employing the international types of staphylococci and accepting Oeding's antigenic formulae for them did not have the same success. Table 1 gives the antigenic formulae for these types as obtained by Brodie and by Oeding.

TABLE 1.

The formulae obtained by Brodie and by Oeding for the international types of staphylococci.

Type of Staphylococcus	Formula of Oeding	Formula of Brodie
1.	abe/i	acei/-
2.	abh/hi	-/h
3.	abc/abchi	ac/-
4.	abehk/abhi	ac/-
5.	abef/k	a/-
6.	-/(h)	a/-
7.	bcf/ci	-/-
8.	(a)bc/ci	ace/h
9.	abcfk/i	ac/h
10.	ab/abi	-/-
11.	autoagg.	auto/h
12.	(a)bc/c	ac/-
13.	(a)bc/ci	ace/-

auto. or autoagg. means that the organism is autoagglutinable.

a/- means that the organism reacts with antiserum "a" in the live state, and with none when autoclaved.

a/h means that an organism reacts in a similar manner to the above, but also with serum "h" when autoclaved.

It seemed that it might be a worthwhile project to investigate the possible reasons for these discrepancies. It was decided, therefore, to attempt

(1) to reproduce the factor antisera of Brodie.

(2) to reproduce the factor antisera of Oeding.

(3) to review the results of (1) and (2) in the light of the more recent findings of Oeding (1960[57]).

(4) to institute further investigations which might suggest themselves as a result of (1), (2), and (3).

This section deals with (1) above, and in it is discussed the strains of staphylococci used, the media employed, the methods adopted, and the results.

Further sections are directed towards setting out the methods and in carrying out the investigations in (2), (3), and (4).

METHODS AND MATERIALS.

(1) Staphylococci used.

The thirteen types of staphylococci were received from the National Collection of Type Cultures, London.

TABLE 2.

List of the types of staphylococci used and their numbers in the National Collection of Type Cultures.

Type of Staphylococcus		Catalogue number
1.	Cowan's type I	8530
2.	Cowan's type II	8531
3.	Cowan's type III	8532
4.	Christie and Keogh's type 4	6131
5.	ditto type 5	6133
6.	ditto type 6.	6134
7.	ditto type 7	6135
8.	ditto type 8	6136
9.	ditto type 9	6137
10.	Hobbs type 10	8722
11.	Hobbs type 11	8723
12.	Hobbs type 12	8725
13.	Hobbs type 13	8726

Before they were used for the preparation of antisera they had to fulfil the following criteria as suggested by Brodie (1957[7]).

1. They had to be coagulase positive by the tube method. (Mackie and M^{c}Cartney, 1960[51]).
2. They had to haemolyse horse blood agar.
3. They had to ferment mannitol in 1% peptone water.
4. They had to be able to grow in 8% sodium chloride-heart extract broth.

They were then carried in 8% sodium chloride heart extract broth, subcultures being made every two weeks. At the same time, some of the sample of each staphylococcus was preserved on Dorset's egg medium which has no added sodium chloride.

(2) <u>Heart extract agar</u>.

This was prepared as follows.

A.	(i)	Fat-free minced ox heart.	1,500 G.
	(ii)	Distilled water.	2,000 ml.

Mix these and heat to 60^{0}C for 1 hour.

B.	(i)	Bacto Difco Peptone	30G.
	(ii)	Bacto Difco Agar	60G.
	(iii)	Distilled water.	1,000 ml.

The agar and peptone were dissolved in the water, then autoclaved.

The white of 6 eggs was added to A, then B was added.

After filtering through paper pulp under pressure, acid was added to reduce the pH to 7.6. Finally, the following were added:-

0.3 G of calcium chloride.
0.3 G of potassium chloride.
7.5 G of sodium chloride.

The completed agar was stored in 1 oz. bottles and melted before use as required.

(3) Heart extract broth.

This was prepared in a similar manner to (2) above, but agar was omitted.

(4) Preparation of antisera.

The antisera were prepared according to the method of Brodie (1957[7]); that is, organisms were transferred from the salt broth to heart extract agar and grown overnight at 37°C. The resulting growth was suspended in physiological saline to which formalin had been added to bring the final concentration of formalin to 0·5%. The suspension was then diluted till its opacity matched Brown's tube no. 4 (Brown, 1919[12]). These suspensions were then injected intravenously into healthy adult rabbits twice a week, starting with a dose of 0.1 ml., and increasing to 0.2 ml., 0.4 ml., 0.8 ml., then continuing with 0.8 ml., till nine injections in all had been given. After the final injection, the animals were rested for a week and then bled out under pentobarbitone anaesthesia. The serum obtained from each was separated by centrifugation, then carbolic acid was added to give a final concentration of phenol of 0.5%.

The strains of staphylococci used were types 1,2, 3,4,7, and 10.

(5) Agglutination experiments

The antisera were tested against staphylococci which had been preserved in the 8% sodium chloride heart extract broth, then transferred to the heart extract agar for growth overnight. The suspensions tested consisted of

1. living organisms.
2. organisms killed by 0.5% formalin.
3. organisms autoclaved for $2\frac{1}{2}$ hrs. at 120^{0}C.

All suspensions were diluted to the opacity of Brown's tube no. 4. Doubling dilutions of the antisera were made, and to 0.5 ml. amounts of these were added 0.5 ml. of each bacterial suspension. All tube agglutinations were carried out at 55^{0}C in a water bath, then left to stand overnight at room temperature before being read.

(6) Absorption of the antisera.

The absorbing strain of staphylococcus was in each case grown overnight on heart extract agar, then suspended in physiological saline. The suspensions were packed by centrifugation, and the resulting deposit was added to the antiserum which was diluted 1 :10 with normal saline.

Absorption was carried out at 37^{0}C for half an hour, then the antiserum was centrifuged. It was next tested against the homologous and heterologous (absorbing) types by slide agglutination performed at room temperature for 10 minutes. If absorption was complete, carbolic acid was added to a final concentration of 0.5%. In the event of its being incomplete, absorption with a further aliquot of concentrated suspensions of staphylococci was repeated for half an hour until there was no longer any reaction with the heterologous type of staphylococcus.

Chapter 2.

In this chapter, the results of the tube agglutination experiments are set out and discussed.

TABLE 3.

Tube Agglutination results obtained after 4hours at 55°C then standing overnight at room temp., using the antisera to types 1,2,3,4,7, and 10, against formolinised and live suspensions of all thirteen types of organisms.

Type of Suspension	Type of Antiserum											
	Serum 1		Serum 2		Serum 3		Serum 4		Serum 7		Serum 10	
	Form	Live	Form	Live	Form	Live	Form	Live	Form	Live	Form	Live
1.	6400	6400	1600	1600	1600	3200	200	1600	1600	50	6400	800
2.	6400	400	12800	6400	1600	3200	400	100	800	400	100	50
3.	12800	6400	12800	3200	25600	400	25600	400	12800	3200	1600	1600
4.	3200	3200	1600	3200	0	400	400	1600	200	50	0	50
5.	800	1600	3200	1600	1600	200	1600	800	200	1600	1600	800
6.	800	100	200	0	12800	50	1600	800	3200	3200	6400	3200
7.	400	200	200	200	800	0	200	50	3200	6400	400	100
8.	400	6400	400	6400	3200	3200	400	1600	1600	6400	400	3200
9.	1600	6400	1600	1600	100	1600	1600	6400	1600	3200	6400	1600
10.	auto	auto	auto	auto	auto	auto	auto	auto	auto	auto	auto	auto
11.	auto	auto	auto	auto	auto	auto	auto	auto	auto	auto	auto	auto
12.	3200	3200	1600	1600	400	800	400	800	6400	1600	400	800
13.	1600	1600	1600	1600	100	800	1600	800	6400	1600	800	1600

TABLE 4.

Results obtained in tube agglutination experiments after 4 hours at 55°C followed by standing overnight at room temperature, using the antisera to types 1,2,3,4, 7, and 10, against suspensions of the thirteen international types of staphylococci which have been autoclaved at 120°C for 2½ hours.

Type of Suspension.	Type of Antiserum					
	Serum 1	Serum 2	Serum 3	Serum 4	Serum 7	Serum 10
1.	100	400	50	400	200	50
2.	100	1600	0	50	200	0
3.	400	100	50	200	200	0
4.	0	0	50	200	0	0
5.	50	50	50	200	100	100
6.	100	50	50	200	100	50
7.	100	100	50	200	200	50
8.	200	400	50	50	400	200
9.	0	200	50	50	100	50
10.	100	200	50	400	400	200
11.	0	200	100	0	400	0
12.	0	400	0	0	400	0
13.	0	400	0	50	400	0

Examination of the results set forth in Tables 3 and 4 shows that agglutination of living or formolinised organisms by unabsorbed antisera produces such a degree of cross-agglutination as to make the method useless for identification. The agglutination results using autoclaved organisms do not help to clarify the picture although these suggest a reduction in the number of antigens available. These findings are in agreement with the unpublished data of Brodie.

Dr. Bullen of the Rowett Institute, Bucksburn, Aberdeenshire, had become interested in the typing of staphylococci by serological methods at this time. In the initial stages of his investigations, he prepared antisera to the international types using as his test animals rabbits and hens. He kindly supplied quantities of the antisera prepared in hens so that cross-agglutination tests might be done on them. The results of these experiments are set forth in Tables 5, 6, and 7.

The results shown in Tables 5, 6, and 7 show that substitution of hen antisera for rabbit antisera in no way leads to clear results. Cross-reactions are still marked.

TABLE 5.

Results obtained in tube agglutination tests after 4 hours at 55°C followed by standing overnight at room temperature, using antisera to twelve of the international types of staphylococci prepared in hens against all thirteen types of staphylococci in the live state.

Type of Suspension.	Type of Antiserum 1	2	3	4	5	6	7	8	9	10	11	13
1.	50	100	100	0	0	200	50	100	50	100	0	0
2.	0	200	50	0	100	50	0	100	0	50	0	0
3.	50	50	50	0	0	100	50	50	0	200	0	0
4.	100	1600	50	100	50	800	50	800	1600	800	100	400
5.	100	200	100	0	50	400	50	50	100	200	50	50
6.	0	100	50	0	50	400	0	0	0	50	0	0
7.	0	50	0	0	0	50	200	0	0	0	50	0
8.	50	200	200	0	200	400	50	400	50	400	0	0
9.	0	100	50	50	100	400	100	100	50	400	0	0
10.	autoagglutinable											
11.	autoagglutinable											
12.	0	0	50	0	0	0	100	200	0	100	0	100
13.	0	200	100	0	100	50	50	200	50	200	0	100

TABLE 6.

Results obtained in tube agglutination experiments after 4 hours at 55°C followed by standing overnight at room temperature, using antisera prepared in hens to twelve of the international types of staphylococci, against formolinised suspensions of all thirteen types of staphylococci.

Type of Suspension	Type of Antiserum											
	1.	2	3	4	5	6	7	8	9	10	11	13
1.	400	200	200	200	200	400	400	400	100	200	50	50
2.	800	1600	400	200	1600	1600	1600	1600	400	800	50	200
3.	400	400	100	400	1600	1600	1600	1600	1600	1600	100	100
4.	0	50	50	0	0	50	0	0	0	50	0	0
5.	50	200	100	0	400	800	400	800	200	200	0	0
6.	200	400	100	800	200	200	100	200	100	200	50	50
7.	0	50	100	0	100	50	800	800	400	800	50	800
8.	0	800	50	100	100	0	0	0	200	800	50	100
9.	0	400	50	100	0	0	0	0	50	50	0	50
10.	autoagglutinable											
11.	autoagglutinable											
12.	50	800	800	100	200	100	400	800	100	50	50	200
13.	50	400	400	100	1600	800	800	1600	50	0	50	800

TABLE 7.

Results obtained in tube agglutination experiments using antisera to twelve of the thirteen international types of staphylococci, prepared in hens, against all thirteen types of staphylococci which were autoclaved at 120°C for 2½ hours.

Type of Suspension	Type of antisera											
	1	2	3	4	5	6	7	8	9	10	11	13
1.	50	100	200	100	50	400	0	100	400	800	100	50
2.	0	200	100	0	200	200	0	400	400	800	50	50
3.	100	200	200	100	200	400	200	200	200	800	50	100
4.	50	0	50	100	0	50	0	0	0	50	50	50
5.	100	200	200	50	200	400	100	0	200	400	50	50
6.	100	400	200	1004	400	800	0	200	200	800	50	100
7.	50	400	50	50	50	400	100	400	400	800	50	100
8.	100	400	0	100	400	200	0	800	400	800	50	100
9.	100	200	200	200	400	400	50	400	200	800	50	50
10.	100	200	400	100	200	800	200	200	400	400	200	200
11.	50	100	400	100	200	200	400	400	200	800	50	400
12.	0	200	200	50	0	100	400	400	200	400	50	400
13.	100	1600	400	200	400	800	200	400	200	1000	50	200

CHAPTER 3.

As it has been established by previous investigators and in the present investigation that unabsorbed antisera to staphylococci are useless for type differentiation, schemes of preparing factor antisera have been drawn up. This chapter is concerned with the investigations performed according to the system of Brodie (1947). Brodie studied the formulae given by Oeding for the thirteen international types and deduced from them the antisera and absorptions required to produce factor antisera. His scheme is shown in Table 8.

TABLE 8.

Table illustrating the production of factor antisera by absorption of staphylococcal antisera by various members of the international types.

Antiserum absorbed		Absorbing types		Expec.
International type	Oeding's formulae	International type	Oeding's formulae	Factor
10	ab/abi	7	bcf/ci	a
3	abc/abchi	2	abh/hi	c
1	abe/i	2	abh/hi	e
7	bcf/ci	8	abc/ci	f
		3	abc/abchi	
2	abh/hi	1	abe/i	h
1	abe/i	5	abef/k	i
4	abehk/abhi	2	abh/hi	k
		1	abe/i	

Brodie performed slide agglutination tests on the thirteen international types using these factor antisera, employing a dilution of 1/100 for living organisms, and 1/10 for organisms in the autoclaved state.

The formulae which he obtained were expressed in the same fashion as that created by Oeding; thus, a strain identified serologically as "ac/h" is so called because slide agglutinations were obtained in the live state with factor antisera "a" and "c" only, and after autoclaving, with factor antiserum "h" only. Again, a strain identified as "ac/-" means that the same reaction was found as previously with the living organism, but when autoclaved it failed to react with any of the factor antisera.

In the present work, factor antisera of satisfactory titre were obtained using the scheme in Table 8 for all factor antisera except "h". This is normally prepared by absorbing the antiserum to type 2 with organisms of type 1 in the live state. As the type 1 organisms completely absorbed the type 2 antiserum, it was thought

that in the strain of type 1 available there may be an antigen "h". Accordingly, factor antiserum "h" was prepared by absorbing the antiserum to type 2 with organisms of types 9 and 10 in the live state, and the factor antisera so obtained were used to determine if type 1 organisms in this instance contained antigen "h". This is shown in Table 9.

TABLE 9.

Results expected when the antiserum to type 2 is absorbed by organisms of types 9 and 10.

Antiserum absorbed		Absorbing types.		
International type	Oeding's formulae	International type	Oeding's formulae	Factor expec.
2	abh/hi	9	abcfk/i	h
2	abh/hi	10	ab/abi	h

These factor antisera agglutinated organism type 1 in the live state. Hence, it was concluded that the strain of type 1 available contained antigen "h".

The investigations of Brodie (1957[7]) were now repeated using the factor antisera prepared as described for slide agglutination tests on (a) live staphylococci and (b) autoclaved staphylococci of the thirteen international types.

METHODS.

Each strain of staphylococcus was transferred from the 8% sodium chloride - heart extract broth to a heart extract nutrient agar plate, and grown for 24 hours at 37^{0}C. The growth in each case was suspended in physiological saline. Slide agglutinations tests were now performed, using the antisera diluted 1/100 with physiological saline. All suspensions were now autoclaved at 120^{0}C for 2½ hours, and similar slide agglutination tests were performed, but now the antisera were diluted 1/10.

The results are shown in Table 10, where also is shown the effect of various concentrations of antiserum. These are compared with the results obtained by Oeding and by Brodie.

TABLE 10.

Formulae obtained by Oeding, Brodie, and in the present investigation for the thirteen international types of staphylococci.

Type of Strain.	Formulae of Oeding.	Formulae of Brodie.	Formulae found at present antisera diluted as below 1/10	1/50	1/Φ00
1.	abe/i	acei/-	aceih/-	aceih/-	aceih/-
2.	abh/hi	-/h	hi/-	i/-	i/-
3.	abc/abchi	ac/-	aci/-	aci/-	-/-
4.	abehk/abhi	ac/-	aceik/-	ac(e)ik/-	-/-
5.	abef/k	a/-	ac/-	-/-	-/-
6.	-/(h)	a/-	acik/-	aci/-	c/-
7.	bcf/ci	-/-	fi/-	f/-	f/-
8.	(a)bc/ci	ace/h	acei/-	(a)cei/-	ci/-
9.	abcfk/i	ac/h	acik/-	acik/-	i/-
10.	ab/abi	-/-	ac/-	-/-	-/-
11.	auto.	auto/h	auto/-	auto/-	auto/-
12.	(a)bc/c	ac/-	c/-	c/-	c/-
13.	(a)bc/ci	ace/-	acik/-	(a)c/-	c/-

It was now decided to repeat the slide agglutination experiments using, first, the same thirteen types of staphylococci, maintained in 8% salt heart extract broth and grown for 24 hours on heart extract nutrient agar, and secondly, the thirteen types of staphylococci which had been maintained on Dorset's agar since receipt without exposure to added salt.

In this case all the antisera were used in a dilution of 1/10 for both live and autoclaved organisms.

The results are shown in Table 11.

TABLE 11.

Results obtained by typing the thirteen international types of staphylococci by the method of Brodie, and after similar procedures, but using organisms maintained on Dorset's medium.

Type of Strain.	Formulae found by Brodie's method.	Formulae found when the organisms were not exposed to added salt.
1.	acehi/-	acehi/-
2.	hi/-	hi/-
3.	aci/-	aci/-
4.	aceik/-	aceik/-
5.	ac/-	ac/-
6.	acik/-	aci/-
7.	fi/-	cfi/-
8.	acei/-	acei/-
9.	acik/-	aci/-
10.	ac/-	ac/-
11.	auto/-	auto/-
12.	c/-	acei/-
13.	acik/-	acik/-

CHAPTER 4.

DISCUSSION.

The differences between the formulae given by Oeding (1953[55]) and by Brodie (1957[7]) for the thirteen international types of staphylococci may be due possibly to one or more of the following.

1. According to Oeding, each of the thirteen types contained the antigen "b", hence Brodie was unable to prepare a factor antiserum specific for "b" when only the international types were used, as any absorption procedures would have resulted in the complete absorption of "b".

2. Brodie utilised antisera diluted 1/100 when testing live organisms, and it is shown in Table 10 (page 38) that as the antisera are diluted from 1/10 to 1/100, there is failure to demonstrate certain antigens. Thus, dilution causes failure to detect "a" in types 5 & 13, "c" in types 5 & 10, "e" in types 4 & 8, "i" in types 3, 4, & 6, and "k" in types 4, 9, & 13

Oeding (1960[57]) has suggested that when examining for the presence of weak antigens, the antisera should not be diluted further than 1/10

3. Oeding discovered that the antigen originally denoted "e" by him in fact represented three antigens which he subsequently named "e", "m", and "n". As Brodie did not utilise Oeding's strains to produce factor antisera, "e" may be undetected in some instances, (as in types 4 and 5).

4. Oeding pointed out in his review of serological methods of typing staphylococci (1960[57]) that an organism may contain an antigen which is not detected by direct agglutination tests, but which may nevertheless exhaust the antiserum specific for that antigen. Hence, antigens may be present in the international types which are not revealed by Brodie's technique.

5. Oeding used antisera prepared against his own strains of staphylococci in typing the international types, whereas Brodie used the international types for the preparation of the antisera. If any antigen in the international types proved a weak antigen, antisera of suitable strength might not contain specific agglutinins in a sufficiently high titre.

Brodie himself suggested (1957[7]) that exposure of the staphylococci to high concentrations of salt before use in the preparation of the antisera might "exercise some peculiar selectivity on the strains - a selectivity which was reflected in the antigenic formulae obtained". The results in Table 11 tend to suggest that exposure to salt appears to have altered the antigenic formulae but slightly in actual fact. It is seen, however, that the salt appears to have supressed the antigen "c" in type 7, "a", "e", and "1" in type 12, while revealing "k" in types 6 and 9. On the other hand, "k" is a weak antigen and fails to be detected not only when there is no added salt in the medium, (Table 11), but also when the specific antiserum is diluted from 1/10 to 1/100. (Table 10, page 38).

In pursuing the present investigation, it was expected that if Brodie's technique was followed exactly, results comparable with Brodie's would be obtained. In general, the results were rather similar. The differences are discussed below.

Type 1.	Brodie's formula.	acei/-
	Found at present.	aceih/-

Brodie prepared his factor serum "h" on the basis of Oeding's formulae, i.e., by absorbing the antiserum prepared against staphylococcus type 2 (abh/hi according to Oeding) with staphylococcus type 1. (abe/i). In the present instance, staphylococci of type 1 were found to absorb the antiserum to type 2 completely (see Table 9, page 36), and factor antiserum "h" had to be prepared by absorbing the antiserum prepared against staphylococcus type2 (abh/hi) with staphylococcus type 9 (abcfk/i). This represents a departure from Brodie's technique. Moreover, if instead of Oeding's formulae we substitute the formulae of Brodie,

we find that an antiserum prepared against staphylococcus type 2 (-/h) absorbed with staphylococcus type 9 (ac/h) would not be expected to yield any factor antiserum, but would be exhausted. It is possible, then, that the factor antiserum "h" prepared in the present investigation is not similar to Brodie's factor "h" antiserum.

Type 2.	Brodie's formula.	-/h
	Found at present.	hi/i

We find that in this instance that antigen "h" was found without the necessity of autoclaving, and that antigen "i" was not uncovered.

Type 3.	Brodie's formula.	ac/-
	Found at present.	aci/-

An extra antigen "i" was found in the present investigation.

Type 4. Brodie's formula. ac/-

Found at present. aceik/-

Antigens "e", "i", and "k" were found in addition to those found by Brodie. Brodie used staphylococcus type 4 in the preparation of factor antiserum "k", yet from Brodie's results this antiserum does not seem to have reacted with the homologous organism. It is possible that excessive dilution may account for this. (See types 7 and 10 for comparison.)

Type 5. Brodie's formula. a/-

Found at present. ac/-

An extra antigen "c" was found in the present investigation.

Type 6. Brodie's formula. a/-

Found at present. acik/-

In this instance, extra antigens "c" "i", and "k" were found.

Type 7. Brodie's formula. -/-

Found at present. fi/-

In this case, Brodie found that staphylococci of type 7 did not react with any of his factor antisera despite the fact that factor antiserum "f" was prepared from the antiserum prepared against staphylococcus type 7. It is possible that dilution of the antiserum was again the cause.

Type 8. Brodie's formula. ace/h.

Found at present. acei/-

In the present investigation, "i" has been gained while "h" has been lost.

Type 9. Brodie's formula. ac/h

Found at present. acik/-

Antigens "i" and "k" have been gained, but "h" lost.

Type 10. Brodie's formula. -/-

Found at present. ac/-

Antigens "a" and "c" have been gained in the present investigation. Brodie used the antiserum to staphylococcus type 10 to prepared factor antiserum "a", and with its use he was able to show the presence of factor "a" in 9 of the 13 international types, yet not in the homologous type. In table 10, page 38, it is shown that in this instance dilution of the factor antisera causes "a" and "c" to vanish, and a formula similar to that of Brodie is obtained.

Type 11. Brodie's formula. auto/h

Found at present. auto/-

Antigen "h" has been dropped.

Type 12. Brodie's formula. ac/-

Found at present. c/-

Antigen "a" has been lost.

Type 13.	Brodie's formula.	ace/-
	Found at present.	acik/-

Antigens "i" and "k" have been gained, but "e" lost.

CONCLUSIONS.

In the present investigation, the pattern of the major antigens "a", "c", and "e" and the minor antigen "i" has followed that found by Brodie fairly closely; the differences in the minor antigens "f" and "k" are thought to be the result of excessive dilution of the factor antisera. The differences in the distribution of antigen "h" have been discussed, and appear to be due to the fact that the antigen named "h" in the present investigation may be unrelated to that so named by Brodie.

It was now decided that before further comparisons could be made, antisera should be prepared according to the methods of Oeding. Even then, however, one might still expect differences, since Oeding

(a) no longer autoclaves his test organisms.

(b) does not include antigen "f" in his present scheme.

(c) might now regard an antigen previously named "e" as being "e", "m", or "n".

SECTION 3.

CHAPTER 1.

In section 2, the necessity for applying Oeding's methods to typing the international types of staphylococci was discussed.

METHODS.

(1) Staphylococci used.

Prof. Oeding sent a set of his own type strains for the preparation of antisera. These lyophilised cultures were grown on "Difco" tryptone soy agar before use, and were subsequently maintained on Dorset's egg medium. From the latter, they were grown on tryptone soy agar or nutrient agar before use.

(2) Preparation of antisera.

The method followed was that published by Oeding in 1957[56] and 1960[57], supplemented by the instruetions sent by Prof. Oeding with the sets of organisms. The bacteria were grown on tryptone soy agar for 18 hours, then suspended in 0.5% formalin. Adult rabbits

were inoculated intravenously in the doses

0.1 ml.,	0.2 ml.,	0.4 ml.,
0.4 ml.,	0.6 ml.,	0.8 ml.,
0.8 ml.,	1.0 ml.,	1.0 ml.

giving an injection on each of three successive days of three successive weeks, so that five days rest was allowed before each set of injections. After the final injection, five days rest were allowed before the animals were bled out under pentobarbitone anaesthesia. The stock sera were inactivated at 56^{0}C for twenty minutes, then merthiolate was added to a final concentration of 1/10,000 before storage in the refrigerator.

(3) Preparation of the factor antisera.

The type strains of staphylococci were grown on tryptone soy agar for 18 hours, then suspended in saline and centrifuged. The deposits were then suspended in the appropriate antisera and incubated at 37^{0}C for two hours. They were next allowed to stand overnight at 4^{0}C, then centrifuged, and the supernatant

antisera saved. When slide agglutination tests indicated that absorption was complete, the resultant factor antisera were heated to 60°C for twenty minutes, merthiolate was added to a final concentration of 1/10,000, and they were then stored in the refrigerator. Before use, completion of absorption was checked again by performing slide agglutination tests against five hour nutrient agar cultures of homologous and heterologous strains in the live state.

(4) Scheme of absorption.

Factor serum expected.	Antiserum to be absorbed.	Absorbing strains of staphylococci.
a	3647	F21 and 1503
b	2095	2253
c	3647	1503 and 3189
e	1503	3647 and Cowan I
h	17A	2095 and 1503
i	F21	1503 and 2095
k	S365	1503 and F21
m	F21	Wood 46 and Cowan III
n	Cowan III	2095 and F21

Before using these antisera, the degree of cross reaction between the strains was examined by slide agglutination tests. The results are shown in Table 12.

TABLE 12.

Table illustrating the cross reaction between Oeding's strains of staphylococci when examined by slide agglutination tests.

Type Strain	Unabsorbed antisera to Oeding's types						
	2095	1503	17	21	365	III	3647
2095	+	+	+	+	+	+	+
1503	+	+	+	+	+	+	+
17	autoagglutinable						
21	+	+	-	+	+	+	+
365	+	+	+	+	+	+	+
III	+	+	+	+	+	+	+
3647	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+
3189	+	+	+	+	+	+	+
46	(+)	(+)	(+)	(+)	(+)	+	+
2253	+	+	+	+	+	+	+
I	+	+	+	+	+	+	+

Next, the absorbed antisera (factor antisera) were examined for specificity (Table 13).

TABLE 13.

Results of using slide agglutination techniques with Oeding's strains of staphylococci and factor antisera prepared according to the method of Oeding.

Type Strain.	Factor antisera (Oeding's) a	b	c	e	h	i	k	m	n
Cowan I	+	+	+	-	-	-	+	+	-
Cowan II	-	+	-	-	-	-	-	-	-
Cowan III	+	+	+	-	-	-	-	-	+
3647	+	+	+	-	-	-	-	-	+
2095	+	+	+	-	-	-	-	-	-
1503	-	-	-	+	-	-	-	+	+
F21	-	+	+	-	-	+	-	+	-
S365	-	-	-	-	-	-	+	+	-
28	-	+	-	+	-	-	-	-	+
3189	+	+	-	-	-	-	-	+	-
Wood 46	-	-	-	-	-	+	-	-	-
2253	-	-	-	+	-	-	-	+	+

DISCUSSION.

The results set out in Table 13, page 55 are in close agreement with those of Oeding, but show disagreement in the following.

(1) Type Cowan I has gained "c". Oeding found an "e" present, but in this investigation "m" was noted, and this was formerly regarded as "e" by Oeding as previously described.

(2) Type Cowan II has lost "a".

(3) Type S365 has dropped "e", "i", and "n", but Oeding (1957[56]) reports that this strain reacts with antisera "e" and "n" weakly, and their absence in this case does not interfere with the production of factor antiserum "k".

(4) Type 28 has dropped "m".

(5) Since type 2095 contains antigens "a", "b", and "c", and since type 2253 does not contain these three antigens, absorption of the antiserum to 2095 with type 2253 does not result in a pure factor antiserum "b".

The factor antisera prepared according to the methods of Oeding were now employed to type the thirteen international types of staphylococci.

This was done by means of slide agglutination tests using the following :-

(1) Strains preserved in 8% salt heart extract broth were used after being grown on heart extract agar for 5 and for 24 hours before examination.

(2) Strains maintained on Dorset's egg medium, without exposure to added salt, were grown on nutrient agar and examined after 5 hours growth and after 24 hours growth.

(3) Strains maintained on Dorset's egg medium were grown overnight on heart extract agar, then autoclaved for 2½ hours at 120°C before examination.

Slide agglutination results obtained using Oeding's factor antisera and the thirteen types of staphylococci maintained in 8% salt broth and grown for 5 hours and 24 hours on heart extract agar.

Type of Suspension	Type of antiserum																									
	a		b		c		e		h		i		k		m		n									
	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr								
1.	-	+	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-	-								
2.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
3.	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-								
4.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
5.	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-								
6.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
7.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
8.	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-								
9.	-	+	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-								
10.	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-								
11.	autoagglutinable.																									
12.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
13.	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-								

+ = agglutination in 10 mins. - = no agglutination in 10 mins. auto=autoagglutinable.

TABLE 15.

Slide agglutination results obtained using Oeding's factor antisera and the international types of staphylococci maintained on Dorset's egg medium and grown on nutrient agar for 5 hours and 24 hours.

Type of Suspension.	Type of Antiserum.																	
	a		b		c		e		h		i		k		m		n	
	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr	5hr	24hr
1.	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-
2.	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3.	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
4.	+	(+)	+	(+)	+	(+)	-	-	+	-	+	-	+	-	-	-	-	-
5.	-	-	+	+	-	-	-	-	+	(+)	-	-	-	-	-	-	-	-
6.	-	-	-	(+)	-	(+)	-	-	-	-	-	-	-	+	-	-	-	-
7.	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
8.	-	-	-	-	+	(+)	-	-	-	-	+	(+)	-	-	+	+	-	-
9.	+	auto	+	auto	+	auto	+	auto	+	auto	-	auto	-	auto	-	auto	-	auto
10.	-	auto	+	auto	-	auto	+	auto	-	auto	-	auto	-	auto	-	auto	-	auto
11.	autoagglutinable.																	
12.	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
~~13.~~																		

TABLE 16.

Results obtained using antisera prepared according to the method of Oeding to type the thirteen types of staphylococci which have been autoclaved.

Type of Suspension.	Type of factor antiserum								
	a	b	c	e	h	i	k	m	n
1.	+	+	+	-	-	-	-	+	-
2.	-	-	-	-	-	-	-	-	-
3.	-	+	-	-	-	-	-	-	-
4.	-	-	-	-	-	-	+	-	-
5.	-	-	-	-	-	-	-	-	-
6.	-	-	-	-	-	-	+	-	-
7.	-	-	-	-	-	+	-	-	-
8.	-	-	-	-	-	-	-	-	-
9.	-	-	-	-	-	-	-	-	-
10.	-	+	-	-	-	-	-	-	-
11.	-	-	-	-	-	-	-	-	-
12.	-	-	-	-	-	-	-	-	-
13.	-	-	-	-	-	-	-	-	-

+ means that there was agglutination in 10 mins.

Three sets of results are set against each other for comparison in Table 17, page 62. This table gives the results when the thirteen international types are examined by

(1) Brodie's method.

(2) Oeding's method, using organisms maintained in 8% salt broth.

(3) Oeding's method using organisms maintained on Dorset's egg medium slopes.

Following Oeding's scheme, the results obtained when the organisms were autoclaved are not shown separately, but it is noteworthy that no antigens were revealed by autoclaving that were undetected in the unautoclaved organisms when the strains maintained on Dorset's medium were examined after 5 hours incubation, or the strains maintained in 8% salt broth were examined after 24 hours incubation.

TABLE 17.

Comparative table of results using (1) Brodie's method, (2) Oeding's method, with the organisms maintained in 8% salt broth, (3) Oeding's method, with the organisms maintained on Dorset's medium, to type the thirteen international types of staphylococci.

(1) Formulae by Brodie's method	(2) Formulae by Oeding's method (salt media)	(3) Formulae by Oeding's method (Dorset's medium)
1. aceih/-	abckm	abckm
2. hi/-	b	b
3. aci/-	abc	abc
4. aceik/-	-	abchik
5. ac/-	bh	bh
6. acik/-	-	bck
7. fi/-	-	i
8. acei/-	cim	cim
9. acik/-	abceh	abceh
10. ac/-	be	be
11. auto/-	-	-
12. c/-	-	i
13. acik/-	abcm	abcm

DISCUSSION.

An examination of the results in Table 17, page 62 shows that

(1) staphylococci of types 1, 2, 3, 5, 8, 9, 10, 11, and 13 give similar formulae no matter whether they are maintained in 8% salt broth or in a medium free of added salt - Dorset's egg medium in this case - before further growth on nutrient agar, but

(2) staphylococci of types 4, 6, 7, and 12 show suppression of their antigens as revealed by Oeding's procedure (but not by Brodie's).

It is also seen from the results in Table 14, page 58, that when an organism has been maintained in 8% salt broth, its antigens are slow to develop, and in fact may not be revealed until incubation has continued for 24 hours; Few antigens are found to develop promptly - these are found in types 2 and 5 where antigen "b" is found after 5 hours incubation.

When, however, the organisms are stored on

Dorset's egg medium before being harvested from nutrient agar, it is found that while some antigens may be slow to develop (antigen "b" in type 6) in all remaining cases continued incubation for 24 hours does not result in the antigens reacting more strongly, but on the contrary reactivity is seen to decrease.

This phenomenon is called "O inagglutinability", and apparently salt has the property of slowing up both the development of antigens and of O inagglutinability.

The slowing-up of the rate of the appearance of some of the antigens may be so marked as to lead to an incomplete formula being ascribed to a strain. It is possible that it is this slowing up of the appearance of antigens that has lead to types 4, 6, 7, and 12 being given different formulae when typed according to Oeding's method but using organisms maintained in medium containing added salt and in medium free of added salt.

The points raised above are discussed further in Chapter 2, Section 3.

SECTION 3.

CHAPTER 2.

In chapter 1, section 3, it was shown that prolonged preservation of staphylococci in salt broth so delayed the appearance of certain antigens that it might cause failure to ascribe a fuller formula to certain types. This difficulty can be overcome if the following procedures are used to reveal the antigens :-

1. 18 hour cultures of the strain on tryptone soy agar may be used to inoculate nutrient agar plates. The resulting growth can be examined after 5 hours and 24 hours growth at 37^{0}C; and

2. nutrient agar cultures can be autoclaved, as this often enhances the activity of some antigens even though blocking antigens are not completely removed. Oeding (1960^{57}) states that autoclaving is a method which is of use if the strains under examination are autoagglutinable.

In this chapter, an other technique is examined, namely agglutinin absorption.

Oeding has pointed out (1960[57]) that often an antigen is weak and not able to be detected by ordinary slide agglutination techniques. Its presence, however, might be suspected if tube agglutination experiments were used, but again, these have the disadvantage of an endpoint which is inconstant, differing in each strain for each antigen. Nevertheless, these antigens not detected by slide agglutination can absorb all the homologous antibody from a factor antiserum (see page 42, para. 4). Thus a staphylococcus, failing to agglutinate with specific antiserum "a", may yet be able, when added in bulk to a known volume of this factor antiserum, to exhaust the antibody "a" content of the antiserum. This can be taken as evidence that the staphylococcus contains the antigen "a". In view of this, it seems necessary to do agglutination absorption tests in order to be sure that no antigen was undetected by the simpler tests so far performed.

METHOD.

The nine available factor antisera were examined

for their homologous titre. The staphylococci used for absorption were maintained on Dorset's egg medium and subcultured on to 4" heart extract nutrient agar plates. They were incubated at 37°C for 18 hours. The resulting growths were suspended in physiological saline and centrifuged. The deposits were added to 0.5 ml. amounts of each of the factor antisera, and absorption was allowed to take place at room temperature overnight. The antisera were then cleared of staphylococci by centrifugation, and the titre re-established by means of titration in tubes with the homologous strain.

Complete or near complete failure to agglutinate the homologous strain was taken as evidence of the exhaustion of the antiserum of its antibody.

All factor antisera "a", "b", "c", "e", "i", "k", "m" and "n" were suitable, but factor antiserum "h" was so weak that it was decided not to use it in case false or unreliable results might ensue. The other eight factor antisera were divided into 13 aliquots, each aliquot being absorbed with one strain of the thirteen international types. After such absorption, the serum

was retested against its homologous organism, and the titre, if any, recorded.

The results of this experiment are gathered together in Table 18. Any organism completely exhausting the antiserum of antibody, or at least producing a fourfold reduction in titre, is credited with being in possession of the homologous antigen. A total of 34 absorptions satisfied these criteria, and of these 23 showed complete exhaustion of the antibody content of the factor antisera.

TABLE 18.

Results of absorbing 13 aliquots of each factor antiserum, each with a different type of staphylococcus.

Type of Suspension.	Factor antisera, with reciprocals of titre							
	a	b	c	e	i	k	m	n
	640	480	240	320	320	160	960	120
1.	0	0	0	320	320	160	960	120
2.	640	60	240	320	640	80	960	60
3.	0	0	0	320	640	80	480	60
4.	0	0	0	320	640	40	960	120
5.	0	0	60	320	320	80	960	120
6.	640	0	60	320	640	40	480	120
7.	320	480	0	80	80	80	960	60
8.	320	480	0	320	80	80	0	120
9.	0	0	0	40	640	80	960	120
10.	1280	0	120	0	640	80	960	120
11.	320	480	120	320	640	80	0	120
12.	640	480	240	320	0	80	960	120
13.	0	60	0	160	640	160	960	120

This method of agglutinin absorption is not only a useful method of confirming results, but it can also be used where it is impossible to perform direct slide agglutination tests because of autoagglutinability of the strains, e.g., as in type 11. In the case of type 7, slide agglutinations performed by Oeding's technique revealed only antigen "i" however young the cultures employed were; as is seen from Table 18, this method of agglutinin absorption and back titration with the homologous organism reveals additional antigens "a" and "e".

In Table 19 are compared the results obtained in typing the thirteen international types using

(1) Oeding's method with the organisms maintained in 8% salt broth.

(2) Oeding's method with the organisms maintained on Dorset's medium.

(3) Agglutinin absorption techniques with the organisms maintained on Dorset's medium.

TABLE 19.

Comparative table showing the results obtained in typing the thirteen international types of staphylococci by direct slide agglutination and by agglutinin absorption.

	Oeding's technique, Organisms maintained In 8% salt broth	Oeding's technique, Organisms maintained On Dorset's medium	Using Agglutinin Absorption
1.	abckm	abckm	abc
2.	b	b	b
3.	abc	abc	abc
4.	-	abcik	abck
5.	bh	bh	abc
6.	-	bck	bck
7.	-	i	cei
8.	cim	cim	cim
9.	abceh	abceh	abce
10.	be	be	be
11.	-	-	m
12.	-	i	i
13.	abcm	abcm	abc

DISCUSSION.

1. Certain antigens which were revealed by Oeding's technique (using organisms maintained on Dorset's egg medium) have not been revealed by agglutinin absorption methods, namely, antigens "k" and "m" in type 1, "i" in type 4, and "m" in type 13.

2. On the other hand, certain antigens were revealed by agglutinin absorption techniques which were not uncovered using only slide agglutination tests. These are antigens "a" and "c" in type 5, "c" and "e" in type 7, and "m" in type 11.

3. Agglutinin absorption tests have failed to find a few of the antigens revealed using Oeding's technique on organisms maintained in 8% salt broth; these are, "k" and "m" in type 1, and "m" in type 13. It is also seen, however, that Oeding's technique of slide agglutination on organisms stored on Dorset's medium has revealed these.

4. Antiserum "h" could not be used for agglutinin absorption experiments as it was too weak.

5. Agglutinin absorption tests are time consuming and wasteful of antisera. They have few advantages over direct slide agglutination tests, but may be used with benefit in two instances. These are :-

(a) when the strain of staphylococcus under examination is autoagglutinable, as in type 11.

(b) where a strain contains blocking antigens that mask the other antigens, as in type 7

In Table 20, the major antigens found in the three Cowan types are set out, showing those found in the present investigations and by Oeding.

TABLE 20.

"Major" antigens found in the three Cowan types, as revealed in the present investigation and by Oeding.

Type of suspension	"Major" antigens.
Cowan type I Cowan type II Cowan type III These were obtained from the NCTC	abm + c b abc
Cowan type I Cowan type II Cowan type III These were obtained from Prof. Oeding.	abm + c b abc
Cowan type I Cowan type II Cowan type III Oeding's findings in 1960[57] and 1960[34].	abm ab abc

SECTION 4.

CHAPTER 1

In Section 1, Chapter 1, page 13, it was suggested that "serological typing makes reference to antigenic fractions of the cell that may be important metabolically". To support this claim it was decided to investigate the following.

(1) The relationship, if any, between the antigens revealed in coagulase producing staphylococci by the use of Brodie's technique, and a selected metabolic product of the staphylococcus. Coagulase was selected as being an important metabolic product for this purpose.

(2) Since in Sections 2 and 3 the inter-relationships of the antigens have been studied, it was decided to make a fresh approach and investigate the inter-relationships of the coagulases produced by the various serotypes of coagulase producing staphylococci.

This chapter is devoted to a brief outline of the history of those aspects of the functions of coagulase that are relevant to the present investigation.

Elek (1959[26]) states that Loeb (1903) was the first to postulate the existence of coagulase when he detected the action of staphylococci in causing the clotting of goose plasma.

Since then, the investigations may be said to have been :-

(1) an appreciation of the relationship between coagulase production and pathogenicity.
(2) the confirmation of the antigenicity of coagulase.
(3) the hypothesis of distinct free and bound forms.
(4) the detection of separate coagulases among various members of different 'phage groups.
(5) the possible importance of coagulase in immunity.

(1) The relationship between coagulase production and pathogenicity.

Much (1908[53]) first drew attention to the relationship between coagulase production and pathogenicity, a concept now unquestioned. Burnet (1930[13]) mentioned that pigmented types of staphylococci coagulate plasma when incubated in it for three hours, and that some white strains may do so too if cultured overnight in the plasma. The relationship between pigmentation, pathogenicity, and coagulase production was investigated by Pinner and Voldrich (1932[63]) who demonstrated that the yellow types caused haemolysis, produced alkali, liquefied gelatin, and coagulated citrated plasma. Gross (1933[30]) has discussed the accelerated clotting of the blood in patients treated surgically for osteomyelitis and carbuncles. In a long and thorough work Chapman, Berens, Peters, and Curacio (1934[16]) reported the investigation of a series of 5,000 strains of staphylococci. They showed that coagulase production, pigmentation and haemolysis were more or less parallel phenomena, and that pigmented coagulase producing strains were pathogenic for rabbits, and that coagulase-producing types which were non-pigmented were equally pathogenic, while haemolytic but non-coagulase producing types were of low virulence. They also showed that,

among these non-coagulase producing types, the haemolytic types were only slightly more pathogenic than the non-haemolytic types, and that the latter were hardly pathogenic at all. It was also noted by these investigators that if a strain produced a positive tube coagulase test, the speed of clot formation bore no relationship to the degree of virulence, i.e., all coagulase producing strains have equal potentialities for pathogenicity in the rabbit.

The most important paper in this field was that of Cruickshank (1937[18]), a Glasgow worker. Among the subjects which he investigated, he mentions coagulase production by "albus" types. As most "albus" strains were non-pathogens, and as non-pathogenicity is related to the inability to produce coagulase, investigators such as Pinner and Voldrich were puzzled by the occasional occurrence of non-pigmented coagulase producing types. This was clarified when Cruickshank described the production of coagulase by pigmented strains which gradually became non-pigmented as a result of long incubation, and he points out that these white strains were still coagulase producers and pathogenic. As a

result of this work, he recommended that coagulase production should be taken as an indication of pathogenicity because of its ease of demonstration and the constancy of the relationship. Cruickshank quoted that Gross believed coagulase to be non-antigenic, and stated that his own experience agreed also with that of Sudhues and Schimrigk (1933[74]) in considering it non-antigenic. He attempted to produce anticoagulases in rabbits and failed, or thought that he had failed, but,

(1) he tested the coagulability of blood taken from normal people and people inoculated with commercial staphylococcal toxoid. After staphylococci had been added to the plasma samples, he was unable to show any alteration in clotting times. He admitted, however, that he was unaware of the presence or absence of coagulase in the commercial toxoid used.

(2) when comparing the coagulability of plasma taken from normal people and patients suffering from staphylococcal osteomyelitis and empyema, he found that when he added staphylococci to the plasma, there were again no differences in clotting times. More recently,

however, Lominski and Roberts (1946) found that patients suffering from deep seated staphylococcal infections have, in fact, low titres of anticoagulase; and these latter investigators have pointed out that a fixed amount of anticoagulase added to a suspension of staphylococci in plasma could neutralise a certain amount of coagulase, but the living staphylococci would continue to produce coagulase till all the anticoagulase was exhausted, and a coagulum could then be produced. Apparently, this continued production of coagulase was unknown to Cruickshank, and so led him to place a false interpretation on his work.

(2) The confirmation of the antigenicity of coagulase.

As stated above, Sudhues and Schimrigk (1935[74]) and Cruickshank (1937[18]) doubted the antigenicity of coagulase. In addition to these, Smith and Hale (1940[71]) were unable to produce in rabbits antibodies to coagulase. At first Gross (1931) believed that antigenicity could not be shown, but in 1935[30] he prepared in rabbits anticoagulases with a titre of 1/3.

That antisera to coagulase can be produced has been confirmed by the investigations of Lominski and Roberts (1940[47]), Tager and Hales (1948[76]), Rammelkamp, Hezebicks and Dingle (1950[69]), Duthie and Lorenz (1952[25]), Duthie (1952[23]), and Barber and Wildy (1958[4]).

(3) The distinction between bound and free coagulases.

Duthie (1954[24]) produced free coagulase in fluid media, and, by rupturing the cell walls of staphylococci, liberated the bound coagulase. He postulated the dissimilarity between these on the following grounds that :-

(a) plasma, treated by heat, gave a positive slide test when coagulase producing staphylococci were added, but plasma so-treated gave a negative tube test.

(b) free coagulase seemed to act on prothrombin, but bound coagulase on fibrinogen.

(c) sheep and guinea pig plasma were unsuitable for the slide test, while dog, mouse, human and rabbit plasma reacted by the slide technique: and that

(d) antibodies to free coagulase had been noted on many occasions by different investigators, that these were specific for free coagulase, that they did not react with bound coagulase, and, on the other hand, antisera prepared against the Wood 46 strain of staphylococcus inhibited bound but not free coagulase.

(4) The detection of the existence of antigenically distinct coagulases.

Rammelkamp et al. (1950[69]) found antibodies towards two types of free coagulase in the serum samples from medical students, and so argued that there might be at least two antigenically distinct free types of coagulase. In addition, Duthie (1952[25]) and Duthie and Lorenz (1952[25]) found three antigenically distinct types of coagulases in staphylococci from human sources. Nearly all the samples of human sera examined by Duthie and Lorenz reacted with one type of coagulase and not usually with more than one type, and they raised the question - to the present not finally answered - of the importance of antibodies to coagulase in promoting resistance to staphylococcal infections in humans.

Barber and Wildy (1958[4]) prepared in rabbits antisera to coagulases, using staphylococci of 'phage groups I, II, III, 3A, and 42E, and reported that the coagulases produced by staphylococci from each of these groups were antigenically distinct. They also examined 79 strains of staphylococci and found that, with two exceptions, these each produced a coagulase that reacted with one only of their antisera, and that in each case subsequent 'phage typing of the strains confirmed the correlation between the coagulase produced and the 'phage group of the staphylococci.

Thus, to date, five distinct coagulases have been shown to be produced from staphylococci. These coagulases appear to be specific antigenically, but their importance in the field of immunity remains obscure.

In addition to these findings, Tager (1948[75]) showed that coagulase is a non-dialisable protein.

(5) <u>The possible importance of coagulases in immunity</u>.

Following the successful prophylaxis against diphtheria by the parenteral administration of diphtheria toxoid, hopes were raised that staphylococcal human infections might be influenced similarly by the use of staphylococcal preparations.

Panton and Valentine (1929) "by injecting washed suspensions intradermally in rabbits and taking pus formation as their indicator effect, were able to show that repeated cutaneous infections gave rise to partial immunity to a large dose". (Elek, 1959[26]). Forssman (1935[27]) demonstrated that this type of immunity could be transmitted passively to normal rabbits.

At this time, however, interest was centred mainly on the role of alpha-toxin in infection, and Burnet (1929[13]), Kitching and Farrell (1936[41]), Ramon et al. (1936[68]) and Smith (1937[70]) found that, in rabbits, the higher the titre of anti-alpha toxin antibody, the greater the resistance to experimental staphylococcal infection. Burnet (1929[13]), Forssman (1936[28]) and Smith (1937[70]) found, however, that, when a lesion is produced, it progresses despite high levels of circulating anti-alpha toxin antibody. Downie (1937[22]) pointed out that, in such experiments, the rabbits were usually injected with a broth culture of staphylococci, and that this inoculum may contain not only alpha-toxin, but also other substances such as coagulase.

In addition to the role of alpha-toxin, interest

was also raised in the roles of leucocidin and tissue macrophages. Downie had propounded the view that, for the rabbit, alpha-toxin is identical with leucocidin. Forssman (1935[27], 1936[28]) had stressed the role of the macrophage in resisting infection. Some authorities doubt the role of toxins at all in infection: for example, Cowan (1939[21]) found that although vaccines composed of live or formolinised organisms conferred some immunity to experimental infection, a vaccine prepared from an unrelated organism (in this case Pasteurella multocida) caused similar resistance to experimental infection by staphylococci as the staphylococcal vaccines conferred. As a result, he attributed resistance brought about by the use of such vaccines to be in some measure due to non-specific factors.

Nowadays, the role of alpha-toxin is considered to be of little importance in human infections with staphylococci. It is largely due to the work of Lominski and his colleagues and to the researches of Duthie also, that interest has moved to the importance of coagulase as was originally suggested by Downie.

The possible importance of coagulase in bringing about resistance to infection by staphylococci had been raised, however, by Gross in 1933[30]. Gross noted that patients undergoing surgical treatment for carbuncles and other staphylococcal infections showed "increased coagulability of the blood". This he interpreted as being due to the liberation of coagulase into the blood stream. Cruickshank (1937[18]) felt that such patients might have antibodies to coagulase, but was unable to demonstrate this for the reasons discussed on page 79.

Lominski and Roberts (1946[47]) examined the sera of 348 patients and found antibodies to coagulase in 212; they also noted that antibodies in the sera of healthy subjects were in higher titre than in the sera taken from patients suffering from such major staphylococcal infections as osteomyelitis. They suggested that it is when the titre of anticoagulase falls that the patient is more liable to staphylococcal infection.

In 1956, Boake[6], utilising this observation, found that when rabbits were immunised with coagulase-containing preparations, their life was prolonged after experimental infection with staphylococci.

In a recent and extensive investigation, Lominski, Smith, Scott, Arbuthnott, Gray, Muir, Turner and Hedges (1962[49]) investigated the "protective values of staphylococcal products and cell components" with the aim of producing a human vaccine. They immunised rabbits with preparations rich in coagulase derived from staphylococci of 'phage groups I and III. They found that "rabbits immunised with coagulase from a 'phage group III staphylococcus were protected against challenge with group III strains, but not against challenge with group I strains. Those immunised with coagulase from 'phage group I staphylococci were not protected against challenge with group I or group III strains". They proceed to suggest that the difference in protective action afforded by the two types of coagulase may reflect a difference in the mechanism of pathogenicity of these two groups, or that an additional protective antigen was present in the group III coagulase preparation utilised.

In Section 4, chapter 2, the methods of preparing free coagulase, the strains of staphylococci employed, the method of detecting the existence of coagulase in the preparations, and the production of antisera to the coagulases, are discussed.

SECTION 4.

CHAPTER 2.

Duthie (1954[24]) has shown that free coagulase is produced in the lag phase of staphylococcal growth and is released continuously, but mainly at the first division, in heavily seeded cultures.

It was formerly difficult on occasion to demonstrate coagulase production in some strains, but Lominski, Smith, and Morrison (1953[48]), and Lominski, Morrison, and Smith (1955[45]) have shown that, in addition to producing coagulase, some strains produce a substance which inhibits the action of coagulase.

Barber and Wildy (1957[4]) and Duthie (1954[24]) have emphasised the importance of albumin for the release of coagulase, and Barber and Wildy have also indicated the usefullness of human serum in the medium. However, Lominski, O'Hea. Goudie, and Porter (1950[46]) have produced a chemically defined medium which allows of coagulase production and release.

PROCEDURES EMPLOYED IN THE PRESENT INVESTIGATIONS.

(1) Preparation of coagulase.

50 ml. volumes of "Bacto" heart extract broth with the addition of 10% Seitz-filtered human serum were inoculated with each of the thirteen international types of staphylococci taken from 8% salt heart extract broth. These cultures were incubated at 37^{0}C for five days, being frequently shaken throughout. The coagulase produced was concentrated by the method of Barber and Wildy (1958^{4}), viz., all the proteins were precipitated with two volumes of saturated ammonium sulphate, the precipitate redissolved in distilled water, and again precipitated with two volumes of ammonium sulphate; finally the deposit was taken up in distilled water.

(2) Detection of the presence of coagulase in the preparation.

It was necessary to prove the presence of coagulase in the preparation prepared as described in (1) above before proceeding further. This was difficult since, when the crude preparation was added to citrated human plasma, the clot formed immediately and as

quickly disappeared. When all further attempts to secure a clot with these crude preparations produced the same type of reaction, it was concluded that a fibrin clot had indeed formed but had so quickly changed its physical state as to be no longer visible. This result was open to interpretation as an instance of the phenomenon of coacervation.

Coacervation is that phenomenon which involves mainly hydrophilic colloids, being the separation of microscopic liquid droplets when sols of two hydrophilic colloids of opposite electric charges are mixed. These droplets may later unite to form a new viscous phase, and if this phase has the same refractive index as that of the suspending medium, then its presence is no longer visually detectable.

Since the simple tests of clot formation on admixture of coagulase behaved in such a way that they required explanation on the basis of coacervation, some other means of confirming the presence of coagulase in the preparation was sought.

If the fibrinogen content of plasma is reduced by any means, clotting is delayed in Quick's One Stage Prothrombin Test. (Quick et al., 1935[67]). If the fibrinogen content of normal plasma is affected by coagulase, then one might expect that the clotting time would again be affected in the same test. Thus, the addition of coagulase to normal plasma, prior to the performance of Quick's test, might be expected to have this result. This argument was put to the test with results that indicated that the coagulase preparations did contain coagulase.

This was done as follows. Doubling dilutions of the coagulase preparations were made, ranging from 1/2 to 1/2048. Of each dilution, 0.1 ml. was added to 0.1 ml. of human citrated plasma, and to this was added 0.1 ml. of Stayne's thromboplastin extract. The prothrombin time was noted after the addition of 0.1 ml. of 0.277% calcium chloride solution. The results are shown in Table 22. These show that the preparations made from all thirteen types of staphylococci contain coagulase, and that further investigations with these would be worth while.

TABLE 21.

Effect of adding coagulase preparations to human citrated plasma prior to performing the prothrombin time by Quick's one stage method.

Dilution of Coagulase.	Coagulase prepared from the staphylococcal types as below.												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1:2	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
1:4	NC	175	195	127	NC	NC	80	MC	NC	NC	NC	NC	NC
1:8	NC	42	35	36	75	93	29	45	70	80	90	NC	90
1:16	150	34	30	30	30	32	19	24	45	60	36	47	42
1:32	57	37	27	30	26	25	19	19	26	50	23	28	34
1:64	38	33	28	30	28	25	17	17	25	30	23	22	35
1:128	28	34	27	29	48	25	17	16	30	30	24	21	35
1:256	28	31	28	30	30	25	16	14	27	30	30	23	30
1:512	31	32	27	30	30	25	16	14	25	30	22	23	32
1:1024	30	31	28	30	30	30	15	15	25	30	27	23	30
control	30	30	30	30	30	30	15	15	25	30	30	25	30

NC means no coagulation.

15= no of seconds to achieve coagulation.

The contols used were samples of human citrated blood of varying prothrombin time, and it is seen that these can be used to detect the presence of coagulase over a wide range of prothrombin times.

(3) Preparation of antisera to the coagulase produced by the thirteen international types.

One adult rabbit was injected with one of each of the thirteen ammonium sulphate precipitated coagulases, making thirteen rabbits in all. The intravenous route was avoided for fear of causing intravascular thrombosis, and instead, the injections were given subcutaneously in the following doses in series :-

Series I.....	0.2 ml.,	0.4 ml.,	0.4 ml.,
Series II.....	0.6 ml.,	0.8 ml.,	0.8 ml.,
Series III.....	1.0 ml.,	1.0 ml.,	1.0 ml.

The injections were given on each of three successive days, allowing five days rest between each series. After the ninth injection, five days rest were again allowed, then the rabbits were bled out under pentobarbitone anaesthesia. Merthiolate was added to a final concentra-

tion of 1/10,000. All the antisera were stored in the refrigerator when not in use.

It was not found possible to prepare an antiserum to the coagulase of type 4 staphylococcus, as on four occasions it caused the death of the rabbits injected. Autopsies performed showed that death in each case was due to toxicity and not to sepsis.

SECTION 4.

CHAPTER 3.

Investigations on the coagulases of the thirteen international types of staphylococci and their anticoagulases.

Being now satisfied that coagulases had been prepared from the ammonium sulphate treated broth cultures of all thirteen staphylococcal types, their relationships to

(a) other antigens, and

(b) each other

seemed worth investigating.

(a) Relationship of the coagulase of a staphylococcal type to the other antigens of the same type.

It was attempted now to show the presence of any identity between the coagulase produced by a staphylococcus and the other antigens shown to be possessed by that type. Thus, if the coagulase of type 1 is the same as any of its antigens "a", "c", "e", or "i", then the prior exposure of any of the factor antisera to the coagulase should remove

or diminish the antibody content of the corresponding antiserum. These "neutralisation" tests were carried out by mixing factor antisera prepared by the method of Brodie (1957[7]) and the coagulase preparations made by the method of Barber and Wildy (1958[4]).

METHOD.

First, the titres of the six factor antisera were established, and the extent to which they agglutinated standardised suspensions of the thirteen international types of staphylococci.

Doubling dilutions of the six factor antisera "a", "c", "e", "f", "i", and "k" were made, and to 0.5 ml. aliquots of these were added 0.5 ml. aliquots of the suspensions of the thirteen international types of staphylococci, maintained in 8% salt broth and grown on heart extract nutrient agar for 24 hours before use. The suspensions of staphylococci were diluted to the opacity of Brown's tube no. 4.

Next, thirteen aliquots of each of the six factor

antisera were placed in test-tubes, and to 0.5 ml. amounts of each were added 0.5 ml. aliquots of the saturated aqueous solutions of the coagulase preparations. doubling dilutions of the coagulase - antiserum mixtures were made, and to these were added living suspensions of each of the thirteen types of staphylococci, diluted to the opacity of Brown's tube no. 4.

Agglutination was now allowed to take place in a water bath at 55^{0} for four hours, then the results were read after the tubes had been allowed to stand at room temperature overnight. The results are shown in Table 22.

TABLE 22.

Results obtained by agglutinating the thirteen international types of staphylococci with (1) Brodie's factor antisera, and (2) Brodie's factor antisera to which coagulase had been added.

Type of	factor antisera. a		c		e		f		i		k	
organism.	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
1.	160	160	640	640	320	320	120	120	2560	640	0	0
2.	320	320	160	160	0	0	0	0	640	320	0	0
3.	80	80	1280	320	160	160	0	0	320	320	0	0
4.	0	0	80	40	0	0	640	0	0	0	80	0
5.	320	320	320	320	0	0	0	0	40	0	0	0
6.	80	0	80	0	0	0	0	0	40	0	160	0
7.	0	0	320	00	40	40	2560	0	40	40	0	0
8.	160	0	640	160	80	40	0	0	1280	1280	0	0
9.	320	160	1280	1280	160	160	0	0	640	640	0	0
10.	autoagglutinable.											
11.	autoagglutinable.											
12.	40	40	320	80	0	0	0	0	0	0	0	0
13.	0	0	320	0	40	0	0	0	80	0	0	0

Examination of these results indicates that the coagulase of each of the thirteen international types appears to be associated with the antigens shown in Table 23, and that the coagulases inter se are to some extent antigenically dissimilar.

TABLE 23.

Table showing the relationship between the coagulase of each type and the antigens recorded according to Brodie's system.

type of staphylococcus.	antigens with which the coagulase is related.
1.	i
2.	i
3.	c
4.	f
5.	-
6.	k
7.	f
8.	a
9.	-
10.	-
11.	-
12.	c
13.	c

(b) Inter-relationships of the coagulases.

By various means attempts were made to ascertain whether or not the thirteen types of staphylococci produced

1. a free coagulase common to all 13 types.
2. a coagulase different for each type.
3. a number of coagulases shared amongst the types.

The following investigations were aimed at shedding some light on this aspect of coagulase production.

(i) Gel-diffusion tests.

"Oxoid" Ion Agar was prepared in a strength of 1% and poured into 4" Petri plates. Wells were cut out with a cork borer and sealed with agar, so that each plate had a central well into which was placed one or other of the coagulase preparations. The peripheral wells were filled each with an antiserum to the range of coagulase preparations, so that each of the coagulases were tested against each of the twelve antisera. The plates were incubated at $37^{O}C$ for seven days then kept at $22^{\emptyset}C$ till lines developed.

The results were most difficult to read. There was such fusion and confusion of lines that any attempt to interpret the results was virtually impossible. This is in agreement with the findings of Oeding (1960[57]) who indicated that gel-diffusion techniques in this context are "over-sensitive".

(ii) Tests involving neutralisation of the coagulase.

It has previously been stated that the addition of undiluted crude coagulase preparations to normal human citrated plasma produced the phenomenon of coacervation. Having given some thought to the matter, it was now found that, by adding doubling dilutions of the coagulase preparations to a constant amount of citrated plasma, there was a dilution of plasma at which a relatively firm clot was obtained, and by this method the "concentration" of the coagulase could be estimated. This end point was obtained with the crude preparation usually in a final concentration of 1 : 128. Lower concentrations produced clots of varying degrees of friability.

This test was performed by making doubling dilutions of the coagulase preparations and adding 0.25 ml. volumes

to 0.25 ml. volumes of physiological saline and 0.5 ml. of human citrated plasma diluted 1/2 with physiological saline. Incubation was for 18 hours at 37^{0}C.

With this information now available, it was decided to repeat the same experiment exactly as before, keeping the coagulase concentration constant at a final dilution of 1 : 128, and substituting for the 0.25 ml. volumes of physiological saline (as underlined above) the same volume of dilution of antisera prepared against the coagulases. It was hoped that if the coagulases reacted with the added antisera, the formation of a clot would be inhibited, in other words, that the coagulase would be "neutralised". The results of these tests are given in Table 24, where the end result quoted is the concentration of antiserum immediately prior to the tube showing a firm clot, i.e., the last dilution of antiserum inhibiting clot formation.

TABLE 24.

Results obtained by inhibiting the clotting power of each coagulase by adding to it antisera prepared against the coagulases of twelve of the thirteen international types of staphylococci. Figures quoted are the reciprocals of the highest dilution inhibiting clot formation.

Type of coag.	antisera prepared against the coagulases of types											
	1	2	3	5	6	7	8	9	10	11	12	13
1	64	16	32	256	256	32	128	64	16	32	128	64
2	32	32	16	32	64	8	32	64	64	16	64	64
3	64	128	256	32	64	128	256	256	64	32	32	128
4	128	516	64	128	128	64	1024	2048	64	256	1024	1024
5	128	128	256	256	512	64	512	1024	8	8	512	128
6	128	128	128	256	128	128	512	1024	64	8	64	64
7	256	256	128	128	128	64	64	64	128	128	128	64
8	256	128	64	2048	2048	128	256	64	512	2048	128	64
9	64	64	64	64	256	32	64	32	128	1024	128	128
10	512	256	512	512	512	32	128	64	512	512	128	32
11	32	32	64	256	1024	16	64	32	256	512	64	32
12	32	32	64	128	128	16	64	32	256	256	32	32
13	32	16	64	256	256	8	64	32	128	512	32	32

Table 24 shows that the antiserum prepared to any coagulase inhibits each of the other coagulases to some extent. At this point, it was arbitrarily decided to accept as significant a result in which the coagulase was inhibited by an antiserum when the latter was diluted to the level of its own titre or one tube less in order to make allowance for experimental error. Table 25 presents a list of the coagulases and those antisera prepared against coagulases which inhibit at a significant titre.

TABLE 25.

Table showing the coagulases and their inhibition by various antisera acting at a significant titre.

Coagulase.	Antisera to the following coagulases inhibiting clotting at a significant titre
1	1,2,5,6,7,8,9,12,13.
2	1,2,5,6,9,12,13.
3	1,2,3,6,7,8,9,12,13.
4	1,2,5,6,7,8,9.11,12,13.
5	1,2,3,5,6,7,8,9,12,13.
6	1,2,3,5,6,7,8,9,12,13.
7	1,2,3,5,6,7,9,12,13.
8	1,2,5,6,7,8,9,10,11,12,13.
9	1,2,6,7,9,11,12,13.
10	1,2,3,5,6,7,8,9,10,11,12,13.
11	1,2,5,6,9,10,11,12,13.
12	1,2,5,6,9,10,11,12,13.
13	1,2,5,6,9,12,13.

It would seem from the results set forth in Table 24 that in every case any coagulase prepared from the thirteen international types is inhibited by antisera prepared against the coagulases of types 1,2,9,12,, and 13. Furthermore, each coagulase is inhibited by the antisera to coagulases prepared from either types 5 or 6, and generally both, with few exceptions, (namely, types 3 and 9). The results suggest that the coagulases produced by types 1,2, 9, 12, and 13 have a factor in common with every other type, and that this is closely related to the coagulases produced by types 5 and 6. Also, the antisera produced by types 5 and 6 are related closely to each other, at least in their reactions with the coagulases of types 1, 2,3,4,5,6,8,9,11,12, and 13, although there is some difference in their reactions with the coagulases prepared from types 7 and 10. It is therefore decided to regard the coagulases produced from types 1,2,5,6,9,12, and 13 as containing a common factor and to designate it "w".

The antisera prepared against coagulases from types 7 and 8 are seen to react in a way similar to each other in that coagulases 1,3,4,5,6,8, and 10 are inhibited by either, the coagulases prepared from types 2, 11, 12 and 13 are inhibited by neither, while the coagulases prepared from types 7 and 9 are inhibited by the antiserum to type

7 alone. It is therefoe thought that the coagulase released from types 7 and 8 are closely related or identical. Further examination of Table 24 shows that in fact the antisera react similarly in every case with the exception of their reaction with the coagulase produced by type 9, and it is proposed to regard these coagulases produced by types 7 and 8 as similar, and name them "x".

The antiserum prepared against the coagulase produced by staphylococcus type 3 is found to react with the coagulases produced by types 3, 5, 6, 7, and 10 only, so this is regarded as another possible entity to be referred to as "y".

It is seen again from Table 24 that, when a coagulase is inhibited by the antiserum to coagulase 11, it is also inhibited by the antiserum produced against the coagulase of type 10, except in the cases of coagulase produced by types 9 and 13; in these latter cases the coagulases produced by types 9 and 13 are inhibited by the antiserum to coagulase type 11, but not by the antiserum to coagulase type 10. It is consequently thought that the coagulases

produced by staphylococci of types 10 and 11 are related, although the relationship is not as close as in the previous instances. However, if we name these coagulases "z", then each of the thirteen types of staphylococci may produce a free coagulase consisting of one or more of the factors "w", "x", "y", and "z", as shown in Table 26. It must be remembered, however, that the issue is clouded by the fact that it was not found possible to prepare an antiserum to the coagulase derived from staphylococci of type 4, because every attempt to do so resulted in the death of the rabbits employed.

TABLE 26.

The coagulases produced by each of the thirteen types of staphylococci and the factors which may be common to each.

coagulase from staphylococcus type	factors.
1.	wx
2.	w
3.	wxy
4.	wxz
5.	wxy
6.	wxy
7.	wxy
8.	wxz
9.	wxz
10.	wxyz
11.	wz
12.	wz
13.	wz

As already described, (page 83), in 1958[4] Barber and Wildy prepared coagulases from staphylococci of 'phage groups I, II, III, 3A, and 42E, and found these distinct. In Table 27, the 'phage patterns of the thirteen international types of staphylococci employed are given along with a note of the coagulase type. In the present investigation, no relationship of 'phage pattern to the coagulase type was detected.

TABLE 27.

Comparison of 'phage type and coagulase type among the thirteen international types of staphylococci.

International type of staphylococcus	'Phage type	Coagulase type.
1.	52/52A/80	wx
2.	3B/3C/55/71/3A	w
3.	6/7/47/54/75/42E	wxy
4.	187	wxz
5.	3B/3C/55/71	wxy
6.	187	wxy
7.	6/47/54/75	wxy
8.	55	wxz
9.	52/6	wxz
10.	42E	wxyz
11.	3A/3B/3C	wz
12.	3A/3B/3C	wz
13.	6	wz

Thus, from Table 27, the results indicate that,

(i) staphylococci of 'phage group I have coagulases wx.

(ii) staphylococci of 'phage group II have coagulases w, wxy, wxz, and wz.

(iii) staphylococci of 'phage group III have coagulases wxy, wz, and wxyz.

(iv) staphylococci of 'phage type 3A have coagulases wz.

(v) staphylococci of type 42E have coagulases wxy, and wxyz.

(iii) Agglutination Tests.

As tests involving the neutralisation of coagulase are laborious, investigations were now made to find if the antisera to the coagulases could act as agglutinins when brought into contact with the living organisms.

The tests were performed by taking living staphylococci of the thirteen international types, maintained in heart extract broth containing 8% salt, and transferring these to heart extract agar for 18 hours incubation at 37°C. The resulting growths were suspended in physiological saline, and diluted till they matched the opacity of Brown's tube no. 4. Doubling dilutions of each antiserum were made, and the suspensions of staphylococci were set against these. Agglutination was allowed to take place in a water bath at 37°C for four hours, then the tubes were allowed to stand overnight at room temperature before being read.

The results are shown in Table 28.

These results would seem to indicate that the antibodies to the coagulases are poor agglutinins, and that this method is unsuitable for the typing of staphylococci according to their coagulases.

TABLE 28.

Results obtained when the thirteen international types of staphylococci are agglutinated by the antisera to the coagulases.

Figures given are the reciprocals of the highest dilution of antiserum completely agglutinating the suspensions.

Type of Suspension.	antisera produced against these coagulases											
	1	2	3	5	6	7	8	9	10	11	12	13
1	32	64	0	32	32	32	0	0	4	8	8	32
2	16	32	0	32	8	32	0	4	8	8	0	16
3	0	4	16	32	16	16	16	32	8	8	32	64
4	8	32	0	128	128	32	4	16	32	64	16	64
5	4	64	0	32	8	16	0	4	16	16	32	64
6	0	32	0	64	16	0	0	16	8	8	16	32
7	0	4	0	16	8	16	0	0	16	16	0	32
8	32	64	8	64	8	32	8	32	16	64	32	64
9	4	64	8	64	32	64	16	64	64	64	64	128
10	autoagglutinable											
11	autoagglutinable											
12	4	64	0	4	8	64	16	64	0	0	64	128
13	64	128	8	128	128	64	64	64	128	128	128	128

(iv) Experiments in animals.

Two experiments were performed in animals,

(1) in the first, the rabbits were inoculated with coagulase, then challenged with intravenously administered staphylococci.

(2) in the second, the rabbits were again inoculated with coagulase, and challenged with staphylococci administered by the subcutaneous route.

(1) The inoculation of rabbits with coagulase, and challenge by staphylococci administered intravenously.

In this experiment, 9 adult rabbits were used. Three were given a coagulase preparation made from a staphylococcus of Cowan type 1. Three were given coagulase from a staphylococcus of Cowan type 2, and three were given a coagulase preparation derived from a staphylococcus of Cowan type 3. The injections of coagulase were given subcutaneously, and consisted of 0.2 ml. of a saturated aqueous solution of the ammonium sulphate precipitated coagulase. Injections were administered on the first three successive days of each of two successive weeks. After this course of six injections was completed, a reinforcing dose of 0.2 ml. was given seven days later.

Seven days after this last injection, each rabbit received an intravenous inoculation of 0.2 ml. of a suspension of staphylococci in saline, diluted to the opacity of Brown's tube no. 4. These organisms had been previously washed thrice to remove traces of preformed haemolysin. It was not, however, anticipated that haemolysin formation would present any problem, as Elek (1959[26]) states, "even Julianelle found that with the methods employed in 1922, the earliest appearance of haemolysin was about six days in broth cultures."

By suitably choosing the challenge strains, it was arranged that rabbits protected by any of the three types of coagulase were challenged by all three types of staphylococci. The results are shown in Table 29.

TABLE 29.

Protective action of immunity to coagulases prepared from staphylococci of serotypes I, II, and III, as demonstrated by challenge with live organisms of the same serotypes.

Coagulase immunity to serotype :-	1	1	1	2	2	2	3	3	3
Challenge serotype	1	2	3	1	2	3	1	2	3
result	died in 6 days	killed after 3 weeks	died after 7 days	killed after 4 weeks	killed after 4 weeks	died after 1 day	died after 4 days	killed after 12 days	killed after 4 weeks
autopsy	renal abscess	renal infarct	renal abscess	renal infarct	renal infarct	renal abscess	faecal perit.	renal abscess	renal infarct

Unprotected control rabbits, inoculated with the same doses of the same organisms, died in 3 days of staphylococcal pyelonephritis.

If antibodies to coagulase are capable of stimulating resistance to experimental infection by staphylococci, one would have expected that a rabbit, inoculated with the coagulase derived from a staphylococcus of Cowan type I, would be immune to a challenge from that organism: in this instance it failed, but this might possibly be attributed to the weight of the challenge dose.

If we ignore the above for the moment, we find that a rabbit, inoculated with either the coagulase derived from a staphylococcus of Cowan type I or II, is relatively immune to a challenge by staphylococci of Cowan's type I or II, but is not immune to a challenge by staphylococci of Cowan's type III. On the other hand, a rabbit, inoculated with the coagulase prepared from a staphylococcus of Cowan's typeIII is immune to a challenge by the homologous organism, but not to a challenge from staphylococci of Cowan's types I and II.

Failure to develop immunity to attack by a particular organism is shown by the development of renal abscesses. If the animal is partially immune, such abscesses may form but fail to progress, and healing ensues with scar

formation.

A possible objection to the theory that the coagulase injections exerted a protective effect in these experiments may be raised. It may be argued that in fact the organisms produced haemolysin, and the rabbits became immune to haemolysin carried over in the coagulase. However, in reply to these we may state,

(a) Julianelle has shown that haemolysin formation does not occur before the sixth day in broth cultures (see page 117), and in the present investigations four day cultures were used.

(b) Burnet (1929[13]), Forssman (1936[28]) and Smith (1937[70]) have all shown that while high levels of circulating anti-alpha toxin may partially protect against experimental infection, if such an infection becomes established, it always progresses despite the protective effect of the anti-alpha toxin. In our investigation, we could demonstrate healing of the renal lesions.

(c) in the present investigation, the blood of the rabbits at death was not found to contain appreciable anti-alpha toxin.

(2) Experiments in which the rabbits, after prior administration of coagulase, were challenged by staphylococci administered subcutaneously.

In this experiment, three adult rabbits, A, B, and C were vaccinated with coagulase preparations as in the last experiment, using coagulase derived from staphylococci of Cowan's types I, II, and III. Each was then challenged by the subcutaneous route, employing in each case, organisms of all three serotypes. These staphylococci were injected into the flank, and the suspensions consisted of 0.2 ml. of thrice washed organisms in physiological saline, diluted to the opacity of Brown's tube no. 4.

These results are shown in Table 30.

TABLE 30.

Results obtained when three rabbits are challenged with staphylococci of Cowan's types I, II, and III given subcutaneously, after the administration to each of single coagulase preparations.

Rabbit employed	A	B	C
Coagulase injected	Cowan type I	Cowan typeII	Cowan type III
Result of challenge with Cowan type I	no sign of inflammation	no sign of inflammation	no sign of inflammation
Result of challenge with Cowan type II	no sign of inflammation	no sign of inflammation	pus discharged in 12 days
Result of challenge with Cowan type III	no sign of inflammation	no sign of inflammation	no sign of inflammation.

DISCUSSION.

The present investigation differs from that of Lominski, Smith, Scott, Arbuthnott, Gray, Muir, Turner, and Hedges (1962[49]) in that the latter investigators utilised staphylococci of 'phage groups I and III for their experiments, whereas, in the present investigation, staphylococci of serotypes I, II, and III were used. It is known, however, that there is a close relationship between the three 'phage groups and the corresponding three groups distinguished serologically.

The results of the two investigations differ in the following respects.

(a) Lominski et al. found that the coagulase from a staphylococcus of 'phage group III protected against experimental infection by staphylococci of 'phage group III, but was ineffective in affording protection against infection by staphylococci of 'phage group I. In the present work, we found, as in the example of Lominski, that the coagulase of serotype III staphylococci protected against infection by staphylococci of serotype III, but in addition it also protected against infection by organisms of serotypes I and II when these were inoculated subcutaneously.

(b) Lominski et al. found that the coagulase from a staphylococcus of 'phage group I was ineffective in affording protection against infection by staphylococci of either 'phage groups I or III. It was found in this investigation, however, that the coagulase derived from either a staphylococcus of serotype I or II protected against subcutaneous infection by organisms of serotypes I, II, or III, and it was also found that the coagulase from a staphylococcus of serotype III gave protection against staphylococci of serotypes I and III (but not II) when the challenge dose was administered subcutaneously. However, although the coagulase of serotype III staphylococci did not protect completely against infection by organisms of serotype II, infection was delayed till the 12th day, and was of an indolent nature even then.

In other words, it was found that the administration of coagulase derived from staphylococci of serotypes I, II, or III was at least to some extent protective against the experimental infection by staphylococci of any of these serotypes, when the challenge organisms were given by the subcutaneous route.

(c) When staphylococci of serotypes I and II were injected intravenously, however, the protective effect was only

evinced by coagulase derived from staphylococci of serotypes I and II, and no protection was given by the coagulase of type III; similarly, the protective effect of the coagulase of staphylococci of serotype III was only extended to infection by staphylococci of serotype III, and not to organisms of serotypes I or II.

(d) In view of the above, it has not been found necessary to postulate that other factors besides coagulase are necessary to protect against infection by staphylococci of serotype I when the coagulase from the latter is used prophylactically.

SUMMARY.

SECTION 2.

(1) The major antigens of the thirteen international types of staphylococci ("a", "c", and "e") prepared in the present investigation, and one of the minor antigens ("i") are fairly similar in distribution to those found by Brodie.

(2) When antisera were used in a dilution similar to that used by Brodie, the minor antigens "f" and "k" were revealed occasionally only, as was also found by Brodie. When the antisera were used in a stronger concentration, these minor antigens were regularly revealed, and found to be frequently in the same strains as those recorded by Oeding.

(3) The antigen designated "h" in the present investigation differs from that so named by Brodie and Oeding.

(4) The reasons for the discrepancies between the formulae found by Brodie and by Oeding for the thirteen international strains of staphylococci are in part due to the following ;

i. Owing to the formulae of the strains used by Brodie, an antiserum to antigen "b" could not be prepared.

ii. Brodie used certain antisera in too high dilution, with consequent failure to detect certain minor antigens.

(5) While prolonged exposure of staphylococci to salt in high concentration tends to slow the rate of development of certain antigens, this is only a minor cause of the differences in the formulae of Brodie and Oeding for the thirteen international types of staphylococci.

SECTION 3.

(1) Of the nine factor antisera derived by Oeding from the use of his own strains of staphylococci, eight have been prepared successfully.

(2) The remaining factor antiserum "b" was not prepared successfully, but was polyvalent.

(3) The formulae obtained for the twelve strains of staphylococci of Oeding are in good agreement with the formulae of the latter, with the exception of antigen "b" for the reason stated in (2) above.

(4) A new method of typing staphylococci - agglutinin absorption - is described. Formulae for the thirteen international types of staphylococci obtained by the use of slide agglutination tests were compared with the formulae obtained by agglutinin absorption tests. They were similar in 6 cases. In 4 cases, Oeding's

technique of slide agglutination revealed antigens not revealed by agglutinin absorption. Hence, it is concluded that there is little risk that the use of slide agglutination techniques will cause the presence of antigens to be overlooked and a less full formula ascribed to the strain. Of the 3 instances in which the technique of agglutinin absorption revealed additional antigens, in one case the strain involved (type 11) was autoagglutinable, and in an other case the strain (type 7) exhibited unusually rapid development of blocking antigens. In instances similar to these, the technique of agglutinin absorption is useful, but otherwise it is laborious, wasteful of antisera, and only rarely reveals additional antigens.

SECTION 4.

(1) An original method of detecting the presence of staphylococcal coagulase is described, consisting of the estimation of the prothrombin

time of the plasma after its exposure to the action of the coagulase. The use of the method is illustrated when the intervention of coacervation masks clot formation.

(2) The antigenicity of coagulase in rabbits is confirmed.

(3) The free coagulases liberated by the thirteen international types of staphylococci correspond to one or other of the antigens "a", "c", "f", "i", and "k" (these being the antigens described by Brodie), but apparently not to antigen "e".

(4) The results of gel-diffusion techniques and direct agglutination tests applied to the antisera prepared against the coagulases are not informative.

(5) The results of tests involving the neutralisation of a coagulase by the antisera

prepared against the other coagulases suggest that the "coagulase" liberated by a particular strain of staphylococcus may in fact be a combination of coagulases up to four in number.

(6) The pattern of the coagulases so revealed is not related to the phage group of the staphylococcus in any of the thirteen instances studied.

(7) Rabbits immunised with a coagulase from a serotype I staphylococcus are protected to a certain extent against challenge by a staphyloe coccus of serotype II, but not III, when the challenge dose is administered intravenously. The effect of challenge by a serotype I staphylococcus was masked by the death of the rabbit.

(8) Rabbits immunised with a coagulase from a serotype II staphylococcus were protected against challenge by staphylococci of serotypes I and II when these were administered intravenously, but

not against challenge by a staphylococcus of serotype III.

(9) Rabbits immunised with coagulase from a staphylococcus of serotype III were protected against challenge by a serotype III staphylococcus when the latter was administered intravenously, but not against challenge by staphylococci of serotypes I and II.

(10) When the challenge doses of staphylococci were administered subcutaneously, the rabbits, inoculated with the coagulases from staphylococci of serotypes I and II, were protected against staphylococci of serotypes I, II, and III. When coagulase from a serotype III staphylococcus was inoculated, the rabbits were protected against staphylococci of serotypes I and III, and the lesion arising from the staphylococcus of serotype II was attenuated.

(11) Consequently, it is concluded that the antibodies to the coagulases of staphylococci have, in the rabbit at least, a protective value.

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"No scientist lives in isolation. What he is, is determined as much by his teachers.....as by his innate personality and his own efforts. (H.S.D. Garven, "On The Writing Of The M.D. Thesis").

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APPENDIX A.

On page 128, I have stated that all the factor antisera made with Oeding's strains of staphylococci according to the methods of Oeding were satisfactory with the exception of factor antiserum "b" which was polyvalent. As Oeding, up to the date of submission of this thesis, has not published the full formulae for his strains which I can quote in support of this statement, it is now necessary to show how the formulae of Oeding's strains of staphylococci were deduced from the information available in his publications and in a personal communication.

ANTIGENIC FORMULAE.

(a) Based on the scheme of factor sera preparation.

1. Strain 3647.

1a. Factor antiserum "a" is prepared by absorbing the antiserum prepared against type 3647 by types F21 and 1503. Then type 3647 contains antigen "a", but types F21 and 1503 do not.

1b. Factor antiserum "c" is made by absorbing the antiserum to type 3647 with types 1503 and 3189. Then, type 3647 contains antigen "c", but types 1503 and 3189 do not.

1c. Factor antiserum "e" is made by absorbing the antiserum to type 1503 with types 3647 and Cowan I. Then type 1503 contains antigen "e", but types 3647 and Cowan I do not.

2. Strain F21.

2a. Factor antiserum "m" is made by absorbing the antiserum prepared against type F21 with types Wood 46 and Cowan III. Then type F21 contains antigen "m", but types Wood 46 and Cowan III do not.

2b. Factor antiserum "i" is made by absorbing the antiserum prepared against type F21 with types 1503 and 2095. Then F21 contains antigen "i", while types 1503 and 2095 do not.

2c. Factor antiserum "n" is made by absorbing the antiserum made against type Cowan III with types 2095 and F21. Then, Cowan III contains antigen "n" but types 2095 and F21 do not.

2d. Factor antiserum "k" is prepared by absorbing the antiserum made against type S365 with types 1503 and F21. Then, type S365 contains antigen "k", but types 1503 and F21 do not.

2e. From 1a, type F21 does not contain antigen "a".

3. Strain 1503.

3a. From 1c, type 1503 contains antigen "e".

3b. From 1b, type 1503 does not contain antigen "c".

3c. From 2d, type 1503 does not contain antigen "k".

3d. From 1a, type 1503 does not contain antigen "a".

3e. From 2b, type 1503 does not contain antigen "i".

3f. Factor antiserum "h" is prepared by absorbing the antiserum made against type 17A by types 2095 and 1503. Then, type 17A contains antigen "h", but types 2095 and 1503 do not.

4. Strain 3189.

4a. From 1b, type 3189 does not contain antigen "c".

5. Strain 2095.

5a. Factor antiserum "b" is prepared by absorbing the antiserum made against type 2095 with type 2253. Then type 2095 contains antigen "b" and type 2253 does not.

5b. From 2c, type 2095 does not contain antigen "n".

5c. From 3f, type 2095 does not contain antigen "h".

5d, From 2b, type 2095 does not contain antigen "1".

6. Strain 2253.

6a. From 5a, type 2253 does not contain antigen "b".

7. Strain Cowan I.

7a. From 1c, Cowan I does not contain antigen "e".

8. Strain Wood 46.

8a. From 2a, type Wood446 does not contain antigen "m".

9. Strain III.

9a. From 2c, type Cowan III contains antigen "n".

9b. From 2a, Cowan III does not contain antigen "m".

10. Strain 17A

10a. From 3e, type 17A contains antigen "h".

11. Strain S365.

11a. From 2d, type S365 contains antigen "k".

This information is best gathered together and is so presented in Table A. This constitutes the information which can be deduced from the schema given for the preparation of factor antisera.

Table A.

Type	Antigens	
	(1) present	(2) absent
Cowan type I		e
Cowan type II	Not used in factor	sera preparation.
Cowan type III	n	m
3647	a, c	e
2095	b	n, h, i
1503	e	a, c, h, i, h.
17A	h	
F21	m,i	a, k, n
S365	k	
3189		c
Wood 46		m
2253		b

(b) Further antigens allocated on the basis of the publications of Oeding (1960[57]) and Hauknes and Oeding (1960[34])

Other data have been published by Oeding from time to time. In 1960[57], he states that the formulae for types Cowan I, II, and III were "abe", "ab" and "abc". Later in the same year (1960[34]) he found that the anti-serum "e" was polyvalent, and decided that type Cowan I did not possess antigen "e".

The presence of other antigens in the various types is also recorded in the same publication, and these are gathered in Table B.

Table B.

Type	Further antigens	
	(1) present	(2) absent
Cowan type I	a, b, m	e, n
Cowan type II	a, b	e, m, n.
Cowan type III	a, b, c, n	e, m
3647	n	e, m
2095		e, k, m, n
1503	e, m, n	
17A		e, m, n
F21	m	e, n
S365	(e). i, m, (n)	
3189		e, m, n
Wood 46	i	e, m, n
2253	e, m, n	

(e), and (n) = weak antigens

(c) Full antigenic formulae.

So far, antigens have been allocated to the staphylococcal strains

(1) on the basis of the schema for factor sera preparation (Table A, page A5), and

(2) on the basis of the publications of Oeding (1960[57]) and Hauknes and Oeding (1960[34]), (see Table B, page A7).

The further antigens of the strains must be deduced according to the reactions of the strains with the full range of factor antisera. These further components are given in Table C, page A9.

Table C.

Type	Further antigens identified with sera
Cowan type I	c,k
Cowan type II	none
Cowan type III	none
3647	b
2095	a, c
1503	none
17A	none
F21	b,c
S365	none
3189	a, b, m
Wood 46	none
2253	none

The full antigenic formulae based on all information so far available (from Tables A, B, and C) are now gathered together and presented in Table D.

Table D.

Type	Table A	Table B	Table C	Complete Formulae
Cowan I		a,b,m,	c,k	abckm
Cowan II		a,b		ab
Cowan III	n	a,b,c,		abcn
3647	a,c,	n	b	abcn
2095	b		a,c	abc
1503	e	e,m,n		emn
17A	h			h
F21	m,i		b,c	bcim
S365	k	(e),i,m,(n)		(e)ikm(n)
3189			a,b,m	abm
Wood 46		i		i
2253		e,m,n		emn

(e), (n) means weak antigens.

There are three points worthy of mention in these antigenic formulae.

(1) Factor antiserum "b" is prepared by absorbing the antiserum prepared against type 2095 ("abc") with type 2253 ("emn"); it is seen that absorption on this basis would yield a polyvalent antiserum reacting with antigens "a", "b", and "c".

(2) The formula of type 3189 is given as "abm"; in Tables A and B it is stated that "m" is said to be absent in this type.

(3) Antigens "e", "i" and "n" have not been found in type S365. "e" and "n" however are known to be very weak. Failure to detect these antigens does not affect the preparation of antiserum "k".

APPENDIX B.

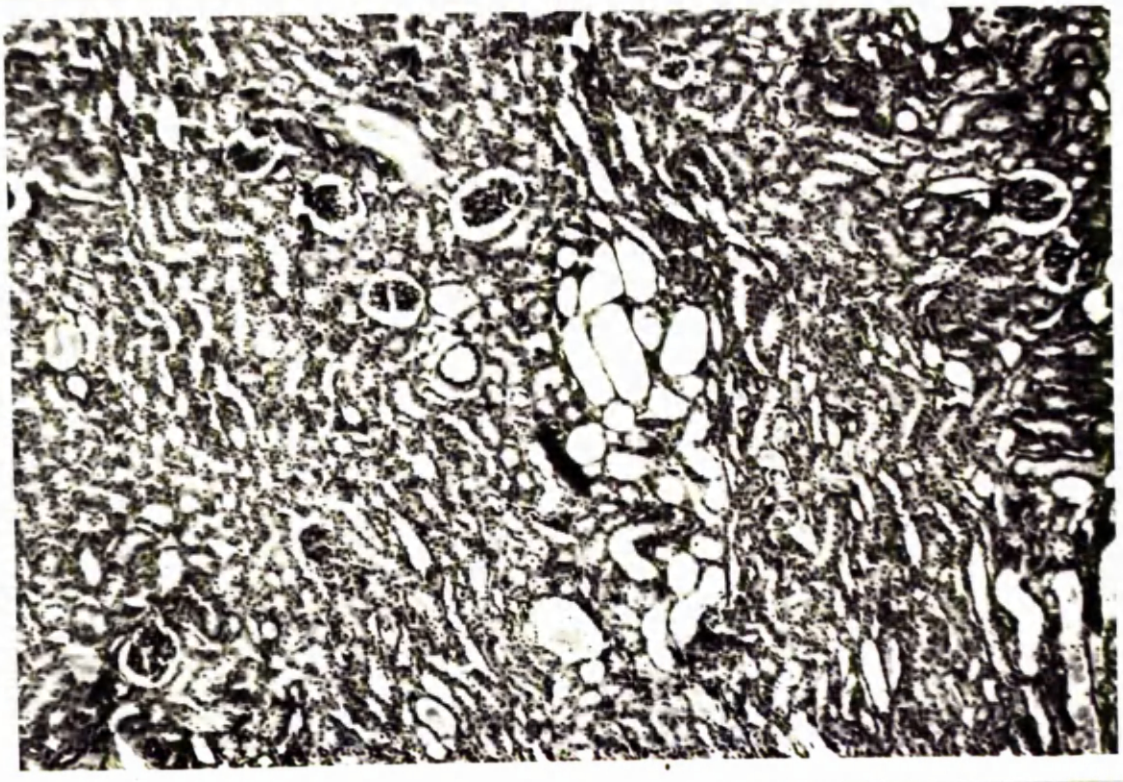
This appendix consists of microphotographs of kidneys removed at autopsy from the rabbits inoculated with coagulase prepared from the various staphylococcal serotypes, then subjected to challenge by intravenously administered staphylococci (see page 116).

Microphotographs of the kidneys from the following rabbits are not shown, as, in each case, autopsy revealed gross pyelonephritis with abscess formation.

(1) The rabbits inoculated with coagulase from serotype I staphylococci, and challenged with staphylococci of serotypes I and III.

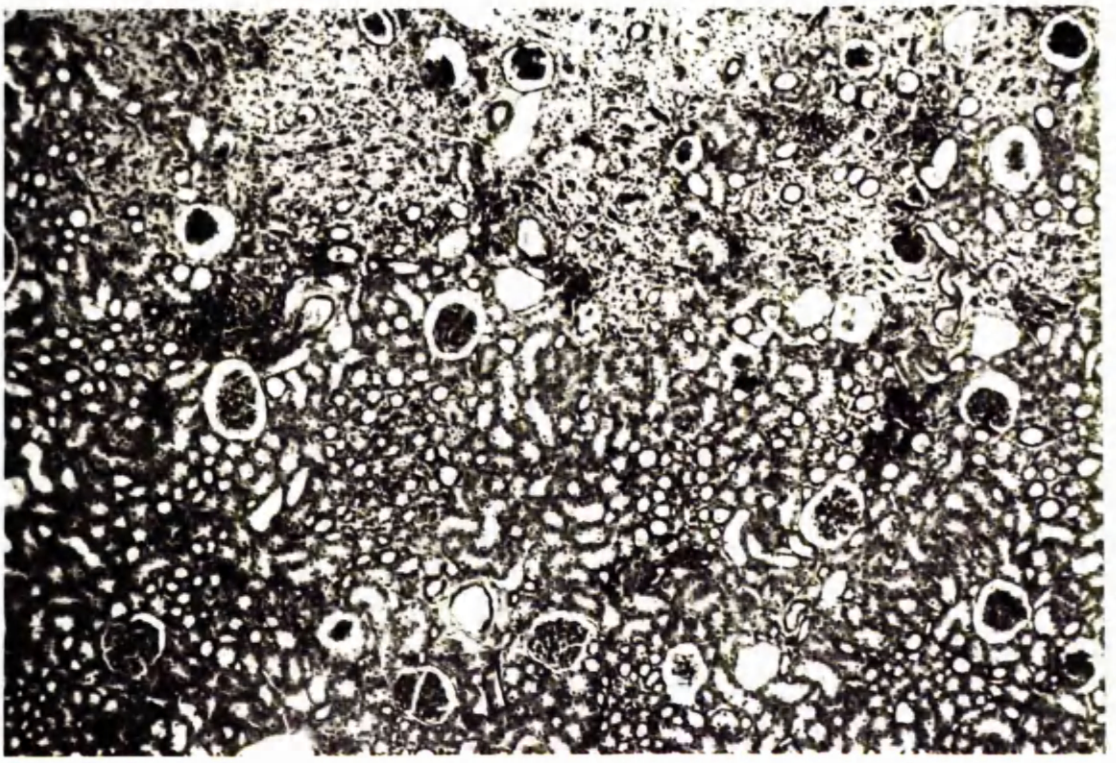
(2) The rabbits inoculated with coagulase prepared from a staphylococcus of serotype III, and challenged with staphylococci of serotypes I and II.

All microphotographs are enlarged 12 times.



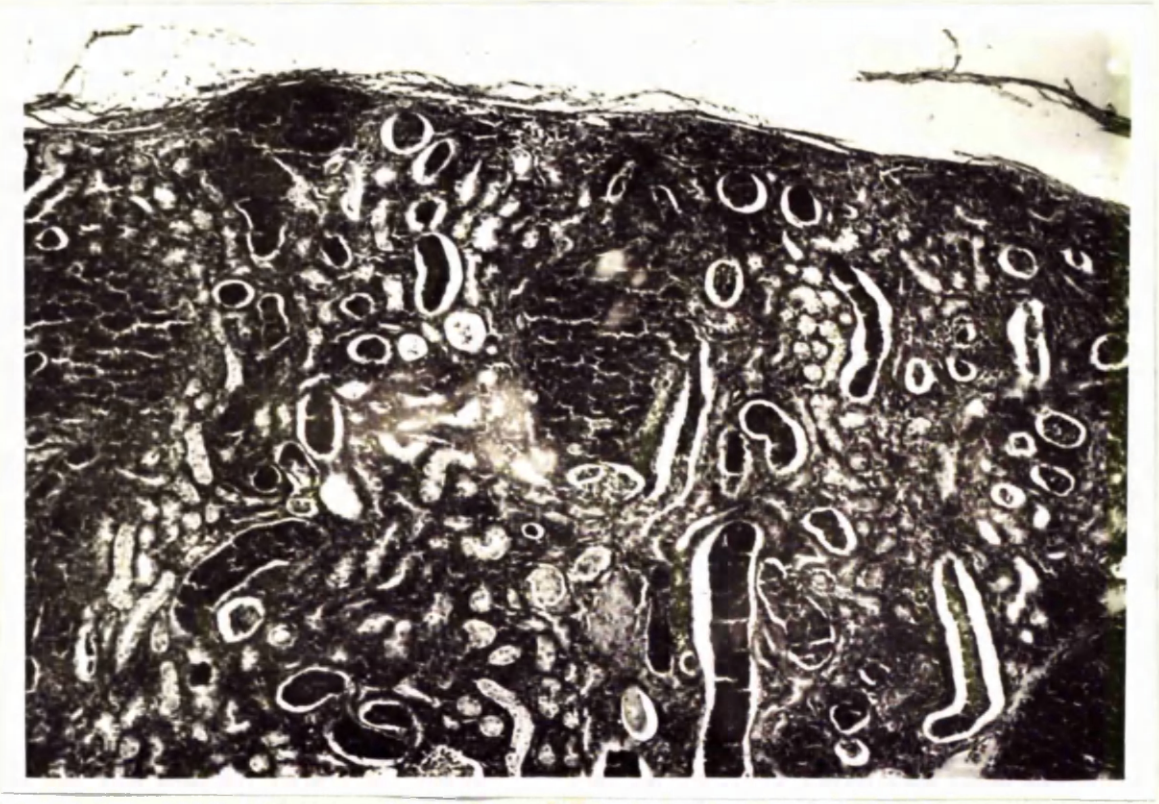
Rabbit inoculated with coagulase prepared from a staphylococcus of serotype II, then challenged with staphylococci of serotype I.

Much of the parenchyma is normal, and no abscess formation or round cell infiltration is seen. Infarction of certain glomeruli, however, has led to areas of tubular atrophy and dilatation.



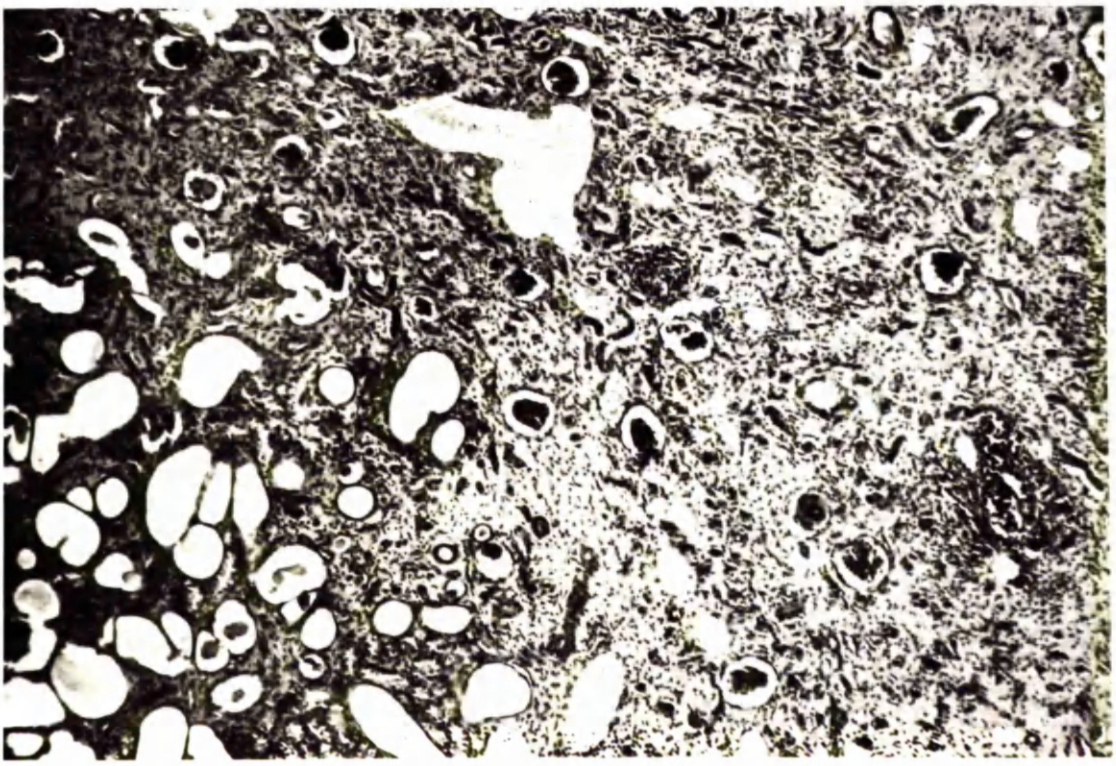
Rabbit inoculated with coagulase prepared from a staphylococcus of serotype II, and challenged with a staphylococcus of serotype II.

Parenchymal damage is slight in this instance, consisting of patchy oedema and round cell infiltration.



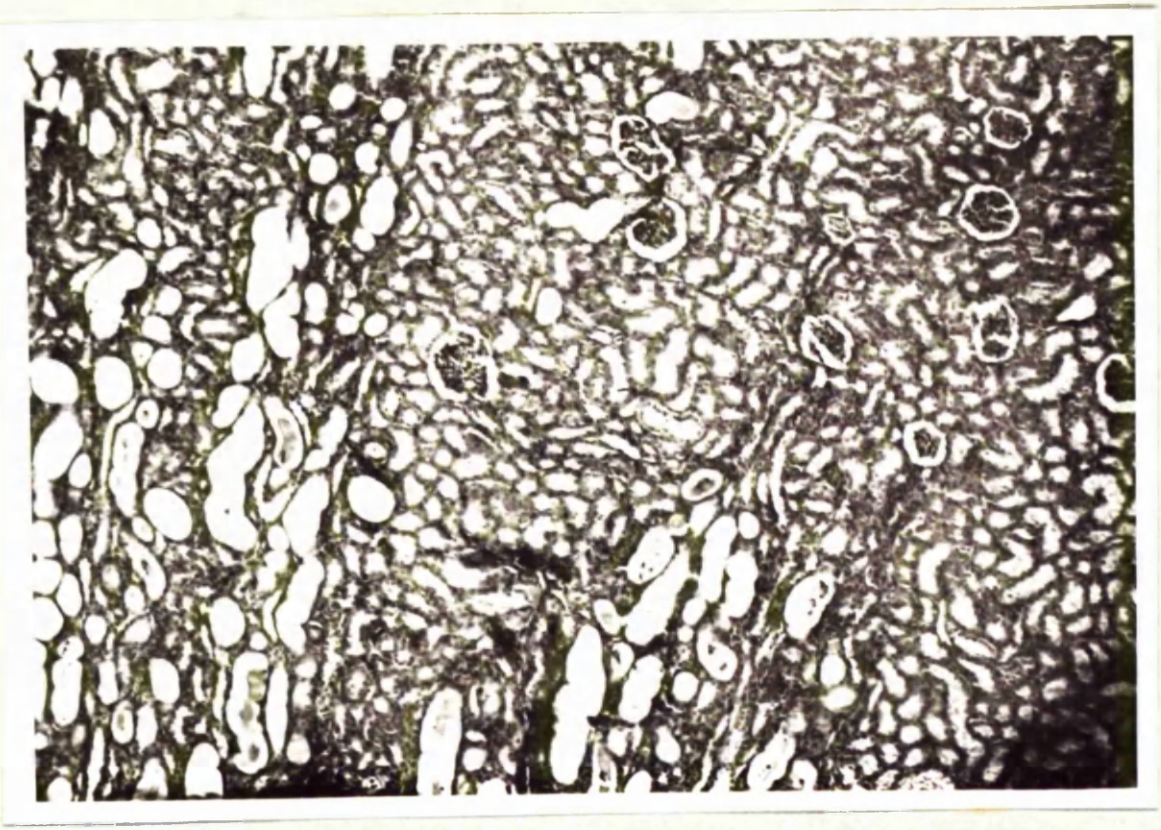
Rabbit inoculated with the coagulase prepared from a staphylococcus of serotype II, and challenged with a staphylococcus of serotype III.

Widespread abscess formation is seen.



Rabbit inoculated with the coagulase derived from a staphylococcus of serotype III, and challenged with a staphylococcus of serotype III.

Parenchymal abnormality is present, and consists of patchy oedema and foci of round cells, together with glomerular infarction resulting in tubular dilatation and atrophy; in contrast to the last example (see page B5) no suppurative change is found.



Rabbit inoculated with the coagulase derived from a staphylococcus of serotype I, and challenged with a staphylococcus of serotype II.

Parenchymal damage is relatively slight, consisting of glomerular atrophy in a patchy distribution which has caused some tubular atrophy and dilatation.