STUDIES ON STAPHYLOCOCCAL HAEMOLYSINS

WITH SPECIAL REFERENCE TO THE BETA-TOXIN

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INTRODUCTION

Reference is made by Nélis, Bouckaert and Picard (1) to Arloing

and Rodet and Courment, who in 1891 and 1892 respectively investigated

"poisons" which produced a resemblance to staphylococcal infection and

which stimulated the production of suppurative lesions, in laboratory anim-

als. However, the credit for recognizing the toxigenic capacity of the

staphylococci should be given to van de Velde (2) who in 1894 reported the

discovery of a leucocidin which caused the destruction of rabbit leucocytes.

The disruption of rabbit erythrocytes caused by staphylococcal filtrates

was noted in the following year by Denys and van de Velde (3) who also dem-

onstrated the production of specific antibodies to the leucocidin by the

subcutaneous injection of filtered broth cultures of Staphylococci pyogenes.

Some years later the necrotizing, lethal and haemolytic properties of fil-

trates of certain strains of staphylococci were separately noted and des-

cribed by Nicolle and Césari (4), Parker (5), and Russ (6). Although these

several pathogenic effects of staphylococcal filtrates were reported, att-

ention was not drawn to their possible relationship to human infection, and

indeed in the ensuing years only occasional references are made to these

toxic factors. The enquiry into the Bundaberg disaster in 1928 (7) clearly

ascribed the deaths of twelve among eighteen children following the injec-

tion of diphtheria toxin-antitoxin mixtures contaminated by staphylococci

to the presence of preformed staphylococcal toxic agents. This focussed

attention again upon the pathogenic significance of these agents. Follow-

ing the Bundaberg enquiry Burnet (8) showed that a true exotoxin was pro-
duced by some strains of staphylococci grown under appropriate conditions.
Burnet also contributed valuable reports on the preparation, properties,
and interactions of staphylococcus toxin, toxoid and antitoxin (9, 10).
The clinical applications of the antigenic properties of this toxin were
soon afterwards investigated by Dolman (11, 12, 13).

The toxic effects described by the foregoing workers were at first
believed to be manifestations of a single toxic entity (8, 14, 1). However,
several workers, including Parker (15), Burky (16), Woolpert and Dack (17),
Bigger (18), and Panton and Valentine (19) found evidence suggesting that
the haemolytic, necrotizing, lethal and leucocidic effects were attributes
of separate entities; while Dolman and Kitching (20), Morgan and Graydon
(21), and Llewellyn Smith and Price (22) reported that the rabbit red cell
lysin might itself represent more than one component. Meanwhile evidence
accumulated that a fibrinolysin, a plasma-coagulase and an enterotoxin, or
food poisoning substance, might also be elaborated as distinct entities by
some strains of the staphylococci.

References in the earlier literature to the different suscepti-
bilities to lysis by staphylococcus filtrates of the red cells of various
animal species pointed to the possibility that several haemolysins might be
present in such filtrates. Glenny and Stevens (23) showed that the ability
of certain filtrates to cause a "hot-cold" lysis of a sheep cell suspension
could be correlated with an erythrogenic action upon the skin of guinea-pigs.
Unlike the rabbit cell haemolysin, which acts promptly upon cell suspensions
incubated at $37^{\circ}C.$, the sheep cell haemolysin exerts its optimal effects
when the cell suspension is exposed to refrigeration temperature after a

preliminary incubation at 37°C. This factor causing the hot-cold lysis of sheep cells and erythema on subcutaneous injection into a guinea-pig, Glenny and Stevens designated beta-toxin, and they suggested the term alpha-toxin for the lethal, necrotizing and rabbit red cell lysin of the earlier workers.

The beta-toxin of Glenny and Stevens was later shown by Dolman and Kitching (20) and by Llewellyn Smith and Price (22) to have a delayed lethal effect upon rabbits. However, although beta-toxin was shown to be obviously pathogenic, and although its antigenicity in humans was suggested by the presence of unusually high antitoxin content in random samples of human serum, and its antigenicity in rabbits clearly demonstrated by the injection of a formalinized toxin (24), its possible role in staphylococcal infections has not been investigated. Such investigations have been complicated by the fact that many toxigenic strains of staphylococci produce both the alpha and beta-toxins: thus the relative significance of each component
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in the causation of infection or the production of immunity is difficult to ascertain. Moreover, the titration of either of these components in the presence of the other was no simple matter until strains producing monovalent toxins, and hence monovalent antitoxins, became available. A more thorough appraisal of the antigenic and pathogenic properties of the beta-toxin, and of the optimal methods for its production and titration, was rendered possible by the availability in this department of a large variety of staphylococcal strains of known toxigenic capacities, including one strain which produced only the beta-toxin.

The method of beta-toxin production used throughout these studies is
  the modified Burnet method described by Dolman (11) as most suited
  for alpha-toxin production.

PRELIMINARY OBSERVATIONS ON THE MECHANISM
OF HAEMOLYSIS BY STAPHYLOCOCCAL TOXINS

In view of the fact that several puzzling features about the action of the staphylococcal toxins on animal red cells were encountered at the outset of this work, and since comparatively little seems to be known about the mechanism of haemolysis in general, numerous efforts were made to throw light on these phenomena, with only partial and tentative success. As a large proportion of the experimental work reported in this thesis involves titrations of the haemolytic power of filtrates upon red cell suspensions, a brief summary of these observations seems warranted. Ponder (25) states: "The consensus of opinion among micrurgists seems to be that the cell has an investing pellicle, contains pigment in solution, and is otherwise devoid of structure". This statement is substantiated by evidence that mobile organisms have been seen to move freely within the "ghosts" of haemolysed cells, but were unable to escape due to the presence of a surrounding membrane. Although the custom has been to regard the cell as inert and to look upon its lysis as a physico-chemical disruption of the cell membrane, it is also possible that some component of the red cell reacts specifically with staphylococcal lysins. Any conception of the mechanism of lysis must satisfactorily account for the different manifestations upon red cells of two separate and distinct lytic agents, the alpha and beta toxins.

Under these conditions alpha lysin causes a progressive, rapid lysis of rabbit erythrocyte suspensions, which cease to be opaque, and become clear red. This form of lysis can be explained as due to a progressive destruction of the cell membrane as a result of a selective adsorption of the lytic agent. In titrating the alpha lysin, various workers (8),

(20), (26), (27), have advocated the use of a 1% rabbit suspension and an incubation time of 1 hour at 37°C., the potency of a filtrate being evaluated in terms of the limiting dilution causing either complete or 50% lysis.

On the other hand, there has been no standardization of the methods of titration of the beta toxin mainly because little clinical importance has been attributed to this factor, and hence less laboratory work has been done upon its properties. The action of beta toxin is specifically upon sheep cells, (it has no apparent effect upon rabbit cells), and lysis here arises as a result of two processes: a primary exposure of toxin and cell to a temperature of 37°C., following which there is no visible change; and a secondary exposure to a lower temperature, when lysis occurs. In the beta toxin-cell reaction it would seem then that there is a progressive selective combination of the toxin with the cell membrane which is insufficient to cause disruption, but which disturbs the cell structure so that on lowering the temperature haemolysis results. In both rabbit and sheep cell haemolytic systems a "red lysis" occurs and there is no decolorization of the haemoglobin.

However, on sheep blood agar plates the alpha toxin produces a clear decolorized area; while the beta toxin produces zones which may be either paler or darker in colour than the original blood agar, but which will not become clear except when the plate has been transferred abruptly from incubation to refrigeration temperature, in which case a clear decolorized area may develop at the margin of the diffusing beta toxin. This clearance under such circumstances is believed to represent the same phenomenon as the hot-cold lysis of sheep cell suspensions by beta-toxin. Several workers, including Bryce and Rountree (24), Naidu (28) and Kojima and

Kodama (29), have discussed the significance, in terms of the alpha and beta toxins, of the various types of haemolytic and clearance phenomena produced by strains of staphylococci grown on sheep blood agar plates. It is not proposed to review this question, but it would appear from unpublished studies by other workers in this Department that the haemolytic and clearance phenomena on blood plates cannot be regarded as simply due to the combined effects of the alpha and beta toxins.

As it was known that certain of the decolorization effects produced by staphylococcal alpha toxin on blood agar can be reproduced by hydrogen peroxide brought into contact with the blood agar, the possibilities of inducing similar bleaching of red cell suspensions by means of hydrogen peroxide were investigated.

Sufficient hydrogen peroxide was added to a sheep erythrocyte suspension to cause complete decolorization of the cells. The cells were then observed under the microscope to be still intact and unbroken. Addition of a few drops of N/1 NaOH solution, which was expected to dissolve the cell stroma, left the suspension colorless and opaque. When sheep erythrocytes which had been haemolysed with water were treated in the same manner, the decolorized cells were seen to be fragmented; while on addition of NaOH solution the suspension became completely clear, suggesting that the fragmented stroma had passed into solution. When alpha toxin is added to the intact cells already decolorized with H_2O_2, then NaOH solution is added, the suspension becomes only partially clear, suggesting that not all of the cell stroma has gone into solution. On the other hand, when beta toxin is used in place of alpha toxin, the opaque suspension becomes immediately clear on addition of the NaOH solution, indicating that the

cell stroma has been completely dissolved. These findings may be summar-
ized as follows:-

Intact cells + H_2O_2

      (suspension colorless and opaque + NaOH remains opaque
      (cells remain intact

Haemolysed cells + H_2O_2

      (suspension colorless and opaque + NaOH becomes clear
      (cells fragmented

Intact cells + H_2O_2+ alpha-toxin

      (suspension colorless and opaque + NaOH partially clear

Intact cells + H_2O_2 + beta-toxin

      (suspension colorless and opaque + NaOH completely clear

The more ready dissolution of the cell stroma by NaOH solution in
the presence of beta-toxin seems to imply that the latter combines with the
cell stroma in some manner which may render it more susceptible to dissolu-
tion, not only by NaOH solution, but also perhaps under the stress of a tem-
perature fall, such as occurs under the experimental conditions necessary
for hot-cold lysis.

TITRATION OF BETA-TOXIN BY HAEMOLYTIC METHOD

In 1909, Walbum (30) noted a delayed lytic effect of staphylo-
coccus toxin on goat erythrocytes; little or no haemolysis occurred after
two hours incubation at 37^OC., but after being left at room temperature,
a marked extension of the haemolytic end-point occurred in the toxin dilu-
tions. There was no further mention of such a phenomenon until 1927, when
Bigger, Boland and O'Meara (31) reported a similar peculiarity manifested
by sheep erythrocytes as due to the action of a staphylolysin. This lysin
acted on sheep and human cells at cold room, air and body temperature, but

the highest titres were obtained by incubation at $37^{\circ}C$. followed by exposure to air or refrigeration temperatures. Then, in 1933, Bigger (18) using sheep red cells, further described toxins which gave characteristic readings depending on the temperature and duration of four incubation periods: (1. after air temperature for one hour; 2. after $37^{\circ}C$. for one hour; 3. after air temperature for another hour; and 4. after overnight at air temperature. It is quite probable that the foregoing qualities attributed to staphylococcus filtrates are due to the beta-component which Glenny and Stevens (23) in 1935 described and demonstrated to be specifically antigenic and separate and distinct from alpha-toxin.

, Glenny and Stevens advocated reading the haemolytic titrations "about 1 hour" after tubes had been removed from the water-bath at $37^{\circ}C$. to which they were first exposed for 1 hour. Although they do not specifically so state, it appears that the second hour of exposure was to room temperature. They also state that although "it is possible to read the results of most tests about 1 hour later," ... "the final reading is taken on the following day". They claim a trace of haemolysis is the most convenient end-point. Glenny and Stevens did not have the advantage of a monovalent beta-toxin producing strain to work with, and their protocols are therefore complicated by the need to neutralize out the alpha-toxin in any filtrate under test with an appropriate antitoxin. Llewellyn Smith and Price (22) made their readings of the "hot-cold" lytic end point after 1 hour of incubation in a water bath at $37^{\circ}C$., followed by overnight standing at room temperature. They do not indicate the degree of haemolysis taken as their end-point, merely stating that "a certain percentage of red cells usually remain unlysed".

Recently Wilson (32) suggested using a preliminary incubation of 5 hours at 37°C., followed by overnight at refrigeration temperature, stating as his reason that such conditions give the maximum manifestation of beta-haemolysis. In view of the many variations proposed in the conditions for titrating beta-toxin, it was thought advisable that the effect of different conditions should be studied in order to ascertain the optimal method.

(a) Effect of Changing the Duration
of Preliminary Incubation

In this study of the haemolytic titration of beta-toxin, strain J 32, which is unusual in producing only the beta-component, was used throughout. The results set forth are representative of titrations repeated many times for confirmation.

Titration of J 32 Filtrates with Sheep Red Cells

Procedure:- 0.05 cc. of a 1/10 saline suspension of thrice-washed packed sheep erythrocytes were added to 0.45 cc. of a series of semi-dilutions of the toxin, the diluent being physiological saline. The series of tubes were incubated at 37°C. in a water bath for different lengths of time and were then placed into a water bath kept at from 7 - 10° C. by addition of ice cubes. Presence of haemolysis before cooling (if any) was noted, and the titrations were read after 1/2 to 1 hour in the water bath and again on standing overnight at refrigeration temperature. The results are recorded in Table 1.

In accordance with Wilson's contention that incubation at 37°C. for 5 hours and refrigeration overnight gives the highest titres, the results of the above titrations show that such conditions do give haemolysis in the greatest number of tubes. What Wilson does not report is that these conditions give the least number of tubes showing complete haemolysis.

<center>**TABLE 1**</center>

<center>EFFECT OF PROLONGATION OF PRELIMINARY INCUBATION AT 37°C.</center>

<center>UPON HOT-COLD HAEMOLYTIC END-POINT</center>

<center>Toxin Dilutions</center>

EXPOSURE	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/8152	C.
15' at 37°C.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
plus 30' at 10°C.	1	2	2	3	4	4	4	4	tr.	0	0	0	0	0
plus overnight refrign.	4	4	4	4	4	4	4	4	2	v. sl. tr.	0	0	0	0
25' at 37°C.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
plus 30' at 10°C.	2	2	2	3	4	4	4	4	tr.	0	0	0	0	0
plus overnight refrign.	4	4	4	4	4	4	4	4	2	0	0	0	0	0
35' at 37°C.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
plus 30' at 10°C.	2	2+	2	3	4	4	4	4	2	0	0	0	0	0
plus overnight refrign.	4	4	4	4	4	4	4	4	4	1	0	0	0	0
1 hour at 37°C.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
plus 30' at 10°C.	4	2+	1+	2	2	4	4	3	3	4	tr.	0	0	0
plus overnight refrign.	4	4	3+	4	4	4	4	4	4	4	2	0	0	0

TABLE 1 (Continued)

Toxin Dilutions

EXPOSURE	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/8152	C.
2 hours at 37°C.	2	tr.	0	0	0	0	0	0	0	0	0	0	0	0
plus 30' at 10°C.	4	4	2+	2+	2+	2+	2+	2+	2+	2	tr.	0	0	0
plus overnight refrign.	4	4	3	2+	2+	2+	2+	2+	2+	2+	1+	sl.tr.	0	0
3 hours at 37°C.	2	tr.	0	0	0	0	0	0	0	0	0	0	0	0
plus 30' at 10°C.	4	4-	2+	2	2	2	2	2	2	1+	1+	0	0	0
plus overnight refrign.	4	4	3	2	2	2	2	2	2	1+	1+	sl.tr.	v.sl. tr.	0
5 hours at 37°C.	3	tr.	tr.	tr.	sl.tr.	v.sl. tr.	0	0	0	0	0	0	0	0
plus 30' at 10°C.	4	3	2	1+	1+	1+	1+	1+	1+	1	1	1	1	0
plus overnight refrign.	4	3	2	2	2	1+	1+	1+	1+	1+	1+	1+	1	0

NOTE: In the above titrations 4 = complete haemolysis; 3 = 75%; 2 = 50%; 1 = 25%; tr. = trace; sl. tr. = slight trace; v. sl. tr. = very slight trace. Intermediate degrees of haemolysis represented by pluses and minuses.

There is what Bigger called a "spread out" end-point, whose occurrence led him to state that it was undesirable to define the Minimal Haemolytic Dose as the least amount of toxin causing 50% haemolysis.

In this laboratory we have taken 1 hour at $37^{\circ}C.$, followed by one-half hour at $10^{\circ}C.$, to be the optimal conditions under which beta-toxin may be titrated, since these are the conditions under which we found the greatest number of tubes with complete haemolysis, and also the greatest number of tubes showing some degree of haemolysis. Moreover, under such conditions, the haemolytic readings resemble closely those obtained when titrating the haemolytic effect of alpha-toxin. The extension of haemolysis after refrigeration overnight, as witnessed in the above titrations, is very constant, and therefore it is believed that the overnight reading can be dispensed with, providing there is no inhibitory phenomenon in the series of tubes. When such a zone of inhibition of lysis occurs (as e.g. in some of the first hot-cold readings given in Table 1), it is advisable to m ake the overnight reading, since this effect then disappears in the majority of cases. Such inhibition of lysis in the tubes of a series containing the higher concentrations of toxin is most often found when fresh filtrates are used. Toxins which have been stored at refrigeration temperature for over a month lose this ability and give clear-cut reactions.

Mechanism of Inhibition of Lysis
by Prolongation of Exposure Time

From Table 1, there is evidently a reduction in the haemolytic activity of beta-toxin on a sheep cell suspension when the preliminary exposure of the mixture to $37^{\circ}C.$ is prolonged from one hour to five hours. This finding, being contrary to what might have been anticipated, and certainly differing from the effect of the alpha-toxin upon a rabbit cell sus-

pension under similar conditions, was investigated in the following manner.

Preliminary observations showed that even overnight incubation of a J 32 toxin at 37°C. did not appreciably lower its haemolytic end-point; and further, that overnight incubation of a sheep erythrocyte suspension at 37°C. rendered the cells somewhat more, rather than less, susceptible to lysis by beta-lysin. Clearly, this apparent reduction in haemolytic activity of the beta-toxin is not due to a progressive detoxication during the five hours of exposure to 37°C.; nor is it due to an increased resistance of the cells to lysis. Samples of J 32, Wood and 24 strain toxins were titrated against a sheep cell suspension made by adding 5 cc. of packed sheep cells to 3 cc. of J 32 toxin diluted in 90 cc. of physiological saline. This sheep cell suspension was incubated overnight at 37°C. prior to use. It was then placed in the waterbath at 10°C. for 1/2 hour, when about 50% of the cells lysed. The unlysed cells were washed in physiological saline and resuspended. Parallel titrations of these toxins were made with a normal freshly-made sheep cell suspension of equivalent density. The readings which were made after 1 hour at 37°C., followed by 1/2 hour at 10°C., are set forth in Table 2.

This table indicates that the sheep cells exposed overnight to the action of beta-toxin at 37°C. are rendered to some degree resistant to subsequent lysis by further additions of beta-toxin. The J 32 toxin fails to give more than 50% lysis under optimal conditions in a 1:20 dilution with toxin-treated sheep cells, although it completely lysed normal sheep cells in a 1:80 dilution. Moreover, this 50% lysis extends throughout the series of dilutions, including the control tube, where toxin treated cells are used. It would appear that such treatment of the cells renders them

TABLE 2

EFFECT OF PROLONGED EXPOSURE TO BETA-TOXIN AT 37°C.

UPON LYSABILITY OF SHEEP CELL SUSPENSION

Toxin Dilutions

TOXIN	EXPOSURE		1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	Control
J 32	1 hour at 37°C.	N.C.	0	0	0	0	0	0	0	0	0
		T.C.	0	0	0	0	0	0	0	0	0
	plus 1/2 hour at 10°C.	N.C.	4	4	4	3+	2	1	tr.	0	0
		T.C.	2	2	2	2	2	2	2	2	2
24	1 hour at 37°C.	N.C.	2+	sl.tr.	sl.tr.	0	0	0	0	0	0
		T.C.	3	2+	tr.	0	0	0	0	0	0
	plus 1/2 hour at 10°C.	N.C.	4	4	4	4	4	4	4	4	4
		T.C.	4	4	4	3+	3+	3	3	2	2

NOTE: N.C. = normal sheep cell suspension

T.C. = sheep cell suspension incubated overnight with beta-toxin

liable to subsequent spontaneous lysis from the effects of adsorbed beta-toxin (which separate experiments indicated could not be removed by repeated washing of the cells with physiological saline). On the other hand, sheep cells containing such adsorbed beta-toxin remain at least partially resistant to lysis when suspended in fresh beta-toxin. The similar reduction in lysis manifested by 24 toxin (containing both alpha and beta-lysins) under these conditions indicates that this phenomenon is not only manifested when subsequent exposure is to beta-toxin of homologous strain.

This rather remarkable effect, which merits further study, must not be confused with the zones of inhibition of lysis sometimes encountered in the tubes of a titration series containing the stronger concentrations of toxin. The cause of these latter zones has so far eluded analysis.

Effect of Changes in Initial
and Subsequent Temperatures of Incubation

While Table 1 indicates that 1 hour of initial exposure at 37°C. is the most suitable period for clear-cut final readings, the possible effect upon the end-point of altering either the initial or the subsequent temperature to which titrations were exposed required further investigation. Using a J 32 filtrate, hot-cold lytic readings were made under the standard conditions already described; after 1 hour at 37°C. followed by 1 hour at room temperature (23°C.); after 1 hour at 23°C. followed by 1 hour at 10°C.; after 1 hour at 45°C. followed by 1 hour at 15°C.; and also after 1 hour at 45°C. followed by 1 hour at room temperature. A representative series of titrations are set forth in Table 3. Overnight readings are omitted, although these were made, since they show little change, in any instance, over the readings made under the test conditions.

TABLE 3

EFFECT OF CHANGES IN TEMPERATURE EXPOSURE UPON THE LYTIC ENDPOINT

Toxin Dilutions

EXPOSURE	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
1 hour at 37°C. plus 1 hour at 10°C.	4	2+	1+	2	2	4	4	3	3	4	tr.	0
1 hour at 37°C. plus 1 hour at 23°C.	4	3	1+	1+	1+	1+	1+	1+	1+	tr.+	0	0
1 hour at 23°C. plus 1 hour at 10°C.	4	4	3	tr.	tr.	0	0	0	0	0	0	0
1 hour at 45°C. plus 1 hour at 15°C.	4	4	4	4	4	4	4	4	4	4	2	0
1 hour at 45°C. plus 1 hour at 23°C.	4	4	3	2	1	1	1	1	1	tr.	0	0

The temperature fall of 30°C. from 45°C. to 15°C. approximates
that from 37°C. to 10°C. and the haemolytic end-points obtained under these
two sets of conditions are similar, the main difference between the two ser-
ies lying in the disappearance of the inhibitory zone of lysis when the
initial temperature of incubation was 45°C. However, although this aboli-
tion of the inhibitory effect might seem to point to an initial temperature
of 45°C. being preferable to one of 37°C., it seems possible that the higher
temperature renders the red cells more susceptible to lysis by any lytic
agent, so that there may be a tendency for readings made under these condi-
tions to be coloured by non-specific lysis. The fact that the first 3 tubes
of the series show some lysis after 1 hour at 45°C., before any lowering of
temperature had occurred, supports this view. When the initial temperature
was 23°C., and the subsequent temperature 10°C., the final titre given was
very much lower, thus again indicating the important influence of the init-
ial temperature upon the end-point.

Effect of Formalin on Beta-toxin

The rate of destruction of the haemolytic activity of beta-toxin
due to the effect of formalin has been reported by several workers (24, 22)
particularly from the standpoint of the antigenic capacity of the toxoided
material. Bryce and Rountree in their investigations into this question
noted that beta-toxin in the presence of 0.5% formalin is completely con-
verted into toxoid at the end of four days. Llewellyn Smith and Price,
however, stated that the action of formalin on beta-toxin is much slower
than on alpha-toxin and leaves the antigenicity almost unimpaired, while
destroying the toxicity for sheep cells. No attempt has been made hitherto
to compare the action of formalin on the various lytic factors contained in

staphylococcal filtrates of different strain origin. It was hoped that
such a study might lead to some analytical clarification of the many haem-
olytic phenomena manifested by such filtrates. Incidentally, it was hoped
that some light might be thrown on the troublesome reactions which at times
result from clinical treatment with toxoided filtrates, whose alpha-toxin
component has ceased to be detectable, but which retain residual beta-toxin.

Procedure:

25 cc. quantities of toxins from strains Wood (alpha-toxigenic
only), 24 (alpha and beta-toxigenic), Barss (alpha and beta-toxigenic) and
J 32 (beta-toxigenic only) were distributed into rubber-stoppered vials.
One set of vials received no added formalin, while to other sets 0.1%,
0.3%, 0.5%, and 1.0% formalin respectively were added. All vials were
then placed in the incubator at 37°C., samples being tested at appropriate intervals. The sheep cell titrations were read
at the end of 1 hour of incubation at 37°C.("hot-cold" sheep cell lysin).
The titres of the three lysins remaining at stated times in the unformalin-
ized toxins are set out in Table 6. In Tables 4 and 5 are given findings
for the toxins to which 0.5% and 0.1% formalin respectively had been added.
The results in toxins to which 0.3% formalin had been added were intermed-
iate between the two foregoing, while the findings with 1% added formalin
were qualitatively similar to the results in the 0.5% formalin series and
it seemed unnecessary therefore to include these.

Additional complete sets of toxins similar to the above were set
aside at room temperature and also in the refrigerator, and were likewise
submitted to titrations of residual lysins at intervals. The extensive
tabulation which would be involved in recording all these findings seems
hardly warranted, since the general effect of the lower temperatures on

TABLE 4

RATE OF DETOXICATION OF STAPHYLOCOCCAL HAEMOLYSINS AT 37 C.

(WITH 0.5% ADDED FORMALIN)

TOXINS No. Days Incubated	WOOD			24			BARSS			J 32		
	r.	h.s.	c.s.	r.	h.s.	c.s.	r.	h.s.	c.s.	r.	h.s.	c.s.
0	6000	50	60	8000	1000	8000	4500	500	4000	0	0	512
2	tr.	0	0	200	500	1500	25	130	1000	0	0	512
3	sl.tr.	0	0	32	500	1500	4	64	500	0	0	500
4	0	0	0	25	120	500	3	30	256	0	0	250
5				4	150	200	0	10	50	0	0	100
7				2	64	100		32	90	0	0	90
9				0	50	200		6	60	0	str.	60
10					50	200		6	60	0	3	60
11					16	100		3	30	0	33	30
13					3	8		3	8	0	4	25

NOTE: r = rabbit cell titre (reading is taken after 1 hour at 37°C.)
h.s.= hot sheep cell titre (reading is taken after 1 hour at 37°C.)
c.s.= "hot-cold" sheep cell titre (reading is taken after 1 hour at 37°C. followed by 1/2 hour at 10°C.)

TABLE 5

RATE OF DETOXICATION OF STAPHYLOCOCCAL HAEMOLYSINS AT 37°C.

(WITH 0.1% ADDED FORMALIN)

TOXINS	WOOD			24			BARSS			J 32		
No. Days Incubation	r.	h.s.	c.s.	r.	h.s.	c.s.	r.	h.s.	c.s.	r.	h.s.	c.s.
0	600	50	60	8000	1000	8000	4500	500	4100	0	0	512
2	1024	4	4	2048	1000	4096	2048	500	4096	0	0	512
3	512	3	4	2048	1000	4000	1024	500	4096	0	0	1024
7	128	0	0	1024	1024	4000	1024	500	4000	0	0	500
10	100	0	0	1000	1000	4000	1000	250	2000	0	0	1000
14	32	0	0	500	250	1500	500	120	1500	0	0	1500
16	32			500		800	500	64	400	0	0	1000
36	2			25	64	64	250	64	200	0	0	150
38	0			25	32	90	200	64	200	0	0	16
42				25	32	64	250	120	250	0	0	?
49				6	25	32	64	32	70	0	0	64
57				6	25	32	90	64	90	0	0	128

NOTE: Figures given represent inverse of last dilution giving 50% haemolysis under prescribed conditions of incubation.

TABLE 6

RATE OF DETOXICATION OF STAPHYLOCOCCAL HAEMOLYSINS AT 37°C.

(WITH NO ADDED FORMALIN)

TOXINS No. Days Incubation	WOOD			24			BARSS			J 32		
	r.	h.s.	c.s.	r.	h.s.	c.s.	r.	h.s.	c.s.	r.	h.s.	c.s.
0	6000	50	60	8000	1000	8000	4500	500	4100	0	0	512
2	3000	25	32	8000	512	8000	4500	256	4000	0	0	2048
3	3000	25	32	8000	512	8000	4000	256	4000	0	0	1200
10	800	12	16	2000	400	3000	4000	256	6000	0	0	2000
18	64	4	8	512	400	1000	1024	512	2000	0	0	1000
35	2	0	0	128	2	4	1024	128	2000	0	0	512
36	0	0	0	128	2	4	1500	128	2500	0	0	500
38				128	2	4	1500	200	2500	0	0	1000
41				64	1	2	2000	128	2500	0	0	1000
42				90	0	0	2000	128	2000	0	0	1000
50				32	0	0	1000	250	2000	0	0	1000
57				16	0	0	500	64	1000	0	0	1000

the process of formalin-detoxication was simply to slow it up in respect of all three lysins under test.

From Table 4 it would appear that the haemolytic activity of_∧ all <small>filtrates from</small> four staphylococcal strains is progressively destroyed in the presence of 0.5% formalin; the alpha or rabbit cell lysin contained in Wood (alpha only) 24 and Barss disappearing much more rapidly than the beta or "hot-cold" sheep cell lysin. The beta-toxin is also progressively destroyed but at a much slower rate, and we find with these toxins, contrary to Bryce and Rountree's contention, that it is not completely destroyed at the end of 4 days. There is no indication that the lytic components of 24 and Barss filtrates are not identical. The hot-cold sheep cell titres for the Wood strain represent a simple extension of the hot sheep effect and cannot be regarded as a beta-toxin effect.

With the addition of 0.1% formalin the alpha-lysin in Wood, and the alpha and beta-lysins in 24, appear to diminish in much the same manner, though at a somewhat slower rate. All three lytic effects (rabbit cell, hot sheep,"hot-cold" sheep) of the Barss filtrate persist to greater extent than those of the 24 filtrate, although they all disappeared at the same rate in both the toxins in the series with 0.5% formalin. This seems to indicate that filtrates from Barss strain may contain some agent other than those found in 24 toxin, which contributes towards the stability of the lysins. It may also indicate that Barss filtrates may contain a higher ratio of beta-toxin to alpha-toxin than do 24 filtrates since the J 32 toxin, containing beta-lysin alone, is even more stable to 0.1% formalin than either of the other filtrates.

The series of toxic filtrates incubated at 37°C. with no addition

of formalin gives a decidedly different effect. The lysin in Wood toxin is
detoxicated in much the same manner as in the first two series, the rate of
detoxication being somewhat slower. With 24 toxin the rabbit cell lysin
disappears at a much slower rate than the hot and the hot-cold sheep lysins;
whereas in the series containing formalin, the rabbit cell lysin is de-
stroyed much more quickly than the other two lysins. The lysins of Barss
and J 32 toxins persist in the absence of formalin to a remarkable degree.
Furthermore, occasional fluctuations seem to occur in the successive titres
of some of the lysins. The explanation of these peculiar manifestations
may be that formalin has an elective affinity for the alpha-lysin. Hence,
in the absence of formalin, the alpha-lysin requires longer for detoxication
as is illustrated in the Wood and 24 toxin series. In neither of these ser-
ies does there appear to be a complicating mechanism such as must be postu-
lated to account for the fluctuations in the lytic titres of the Barss and
J 32 toxins. In the absence of formalin there is comparatively slow de-
struction of the haemolysin filtrates prepared from the Barss strain, while
the hot-cold sheep cell lytic titre of J 32 filtrate shows very little dim-
inution during the 8 weeks over which it was tested.

Incidentally, these differences in behaviour to formalin of
Barss and 24 filtrates as shown in their rates of diminution of lytic
titres may be related in some way to the peculiarly high haemolytic-combin-
ing power ratio exhibited by Barss as compared to 24 filtrates, in respect
of the rabbit cell lysin, to which Dolman and Kitching (20), and later
others, have drawn attention.

In the course of the innumerable titrations involved in the com-
pletion of these series of titrations, the complexities of the task of

TABLE 7

EFFECT OF FORMALIN UPON REMOVAL OF INHIBITORY ZONE

IN HAEMOLYTIC TITRATIONS OF J 32 FILTRATES

Amount of Added Formalin		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/514	1/1024	1/2048	1/4096
0.1%	h	0	0	0	0	0	0	0	0	0	0	0	0
	hc	1+	1	1	1+	3+	4	3+	3+	4	2	0	0
0.3%	h	0	0	0	0	0	0	0	0	0	0	0	0
	hc	4	4	4	4	4	4	4	1+	0	0	0	0
1.0%	h	4	2	0	0	0	0	0	0	0	0	0	0
	hc	4	4	4	3	0	0	0	0	0	0	0	0

NOTE: h = hot sheep cell lysin
hc= hot-cold sheep cell lysin

analyzing the properties of the different components of any one toxic fil-trate became very obvious. The J 32 filtrate, e.g., which affords the nearest approach to a monovalent toxigenic strain at present available, would seem best suited to the study of the beta-toxin. Yet even here diff-iculties arise because of the marked inhibitory zone phenomenon shown in many titrations of the hot-cold sheep lysin produced by this strain. This zoning was most marked in freshly-prepared unformalinized filtrates or in filtrates containing 0.1% formalin. Samples of the same filtrates, con-taining 0.3%, 0.5% or 1% formalin, on the other hand, showed no such inhib-itory zone. Moreover, filtrates containing the higher percentages of form-alin often showed definite, though small amounts of hot sheep cell lysin. Such observations suggest that the formalin may inactivate the substance responsible for the inhibitory zone in hot-cold sheep cell lysin titrations; and that the same or a similar inhibitory substance (likewise destroyed by formalin) may normally mask the presence in J 32 filtrates of small amounts of hot sheep cell lysin. A representative series of titrations illustrat-ing this point is given in Table 7.

Human Cell Lysin .

The fact that the erythrocytes of numerous animal species are susceptible to lysis by staphylococcal filtrates is well established. Dolman (34) pointed out that it seemed necessary to postulate the existence of numerous haemolysins in such filtrates to account for the lack of par-allelism between the haemolytic titres manifested against different animal erythrocyte suspensions by a series of filtrates. However, some of the earlier workers with staphylococcal lysins appeared not to realize that the sheep cell lysin was a different substance from the rabbit cell lysin; while more recent workers have failed to point out that the hot sheep lysin

is distinct from the hot-cold sheep cell lysin.

Comparatively little interest has been shown in the human cell lysin, perhaps because it is not found present to high titres; but Roy (33) contended that the human lysin and beta-toxin were probably identical. The human cell, hot sheep cell, hot-cold sheep cell and rabbit cell lysins were titrated in toxins prepared from several strains, with a view to the possible establishment of any relationship between them, and more particularly to seek verification or otherwise of Roy's claim. The results are given in Table 8.

Several deductions from Table 8 seem warranted. First, although there is some definite extension of haemolysis in the human cell titrations after the additional 30 minutes' exposure to $10^{\circ}C.$, this extension is not comparable in degree to that which is induced in the hot-cold lysis of sheep cells by filtrates containing beta-toxin. J 32 filtrate, e.g., gives no haemolysis of human cells, either after 1 hour of incubation at $37^{\circ}C.$ or after an additional exposure for 30 minutes to $10^{\circ}C.$ On the other hand, although this sample of J 32 toxin showed no hot sheep cell lysin, it gave complete hot-cold lysis in a titre of 1:500. This fact alone is sufficient to disprove Roy's contention that the human cell lysin may represent the beta-toxin.

The possibility that the human cell lysin might be identical with the hot sheep cell lysin is more difficult to refute, from Table 8 at least. Barss and B_2 filtrates give higher human cell and also hot sheep titres than the other filtrates, while the titres of 24 and Wood filtrates show a rough parallelism of human and hot sheep cell lytic effects. Moreover, J 32 filtrate is non-haemolytic to human cells and also to sheep cells at $37^{\circ}C.$

TABLE 8

COMPARISON OF HUMAN, HOT SHEEP, HOT-COLD SHEEP AND RABBIT CELL LYTIC TITRES OF VARIOUS TOXINS

CELLS USED	STRAIN	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
HUMAN (readings after 1 hour at 37°C.)	24	tr.	tr.	tr.	tr.	0	0	0	0	0	0	0	0
	Barss	4	4	4	2+	tr.+	tr.	sl.tr.	0	0	0	0	0
	B₂	1	tr.	tr.	sl.tr.	sl.tr.	0	0	0	0	0	0	0
	Wood	3	0	0	0	0	0	0	0	0	0	0	0
	J 32	0	0	0	0	0	0	0	0	0	0	0	0
HUMAN (readings after additional 30' at 10°C.)	24	2	1	1	1	1	tr.	0	0	0	0	0	0
	Barss	4	4	4	4	4-	3	2	tr.	0	0	0	0
	B₂	2	2	1	tr.	2	2	2	tr.	0	0	0	0
	Wood	4	2	0	0	0	0	0	0	0	0	0	0
	J 32	0	0	0	0	0	0	0	0	0	0	0	0

(Continued)

TABLE 8 (Continued)

CELLS USED	STRAIN	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
SHEEP (readings after 1 hour at 37°C.)	24	4	4	4	2	tr.	0	0	0	0	0	0	0
	Barss	4	4	4	4	4	4-	4	2	tr.	0	0	0
	B₂	4	4	4	4	4	4-	2	1	tr.	0	0	0
	Wood	4	4	4-	tr.	0	0	0	0	0	0	0	0
	J 32	0	0	0	0	0	0	0	0	0	0	0	0
SHEEP (readings after additional 30' at 10°C.)	24	4	4	4	4	4	4	4	4-	4-	4-	4-	0
	Barss	4	4	4	4	4	4	4	4	4	4	4	tr.
	B₂	4	4	4	4	4	4	4	4	4	4	4-	2
	Wood	4	4	4	1	tr.	0	0	0	0	0	0	0
	J 32	4	4	4	4	4	4	4	4	3	2	0	0
RABBIT (readings after 1 hour at 37°C.)	24	4	4	4	4	4	4	4-	2	tr.	0	0	0
	Barss	4	4	4	4	4	4	4	4	4	3	tr.	0
	B2	4	4	4	4	4	4	4	4	2+	tr.	0	0
	Wood	4	4	4	4	4	4	4-	2	0	0	0	0
	J 32	0	0	0	0	0	0	0	0	0	0	0	0

But there are minor deviations from strict parallelism, too great to repre-
sent experimental error, which throw doubt upon the possible identity of the
human lysin and hot sheep cell lysin.

These titrations throw some light on certain possibilities relat-
ing to the hot sheep cell lysin. Other alternatives to the suggestion that
this is identical with the human lysin are that it represents the action at
high concentration of the hot-cold sheep cell lysin; or that it is a mani-
festation of the rabbit cell (alpha) lysin. Against the former of these
alternatives is the fact that, e.g. J 32 filtrates showed no hot sheep cell
lysis, although of high beta-toxin potency; while there is no correlation
between the respective end-points among the other filtrates. The hot sheep
cell titres of 24, Barss and B_2, for instance, were 16, 256, and 128 respec-
tively, although they all had substantially the same titres of 2-4000 hot-
cold sheep cell units. Moreover, in an experiment in which an excess of a
rabbit antitoxin prepared against J 32 toxin was added to 24, Barss, and
$B2$ filtrates, the presence of the antitoxin had no effect upon the hot
sheep cell lysin, although the hot-cold extension of lysis was prevented
in each case.

Since Wood filtrates contain a hot sheep cell lysin while J 32
filtrates are non-haemolytic for both rabbit and sheep cells at $37^{\circ}C.$,
there might possibly be a relationship between the alpha and the hot sheep
cell lysin. However, the rabbit cell titre of the other filtrates tested
in Table 8 do not run parallel to their hot sheep titres. Moreover, the
rate of detoxication of the two lytic effects by formalin is seen, on ref-
erence to Tables 4 and 5, to be decidedly dissimilar. We are then left to
conclude that the human cell lysin and the hot sheep cell lysin are each

distinct entities.

Effect of Heat upon
Human and Sheep Cell Lysins

Different degrees of stability to 56°C. manifested by the sheep

cell lysin of different strains was reported by Dolman (35). Later,

Llewellyn Smith and Price (22) also stated that the "heat stability of

beta-toxin varies from sample to sample", but claimed that with an average

sample 60-70% remains after 30 minutes at 55°C., and 5-10% after 30 minutes

at 100°C. It was decided to check these points on filtrates prepared from

strains available here. In Table 9 are shown the human and sheep cell

lytic titres of samples of various filtrates which were exposed for 5, 15,

and 30 minutes to temperatures of 56°C. and 100°C. respectively.

It is apparent that the heat stability of beta-toxin does vary

according to the strain of origin. J 32 filtrates, e.g., fall from an

initial hot-cold sheep cell titre of 800 to 250 after 30 minutes at 56°C.;

while 24 filtrate under similar conditions becomes non-haemolytic despite

an initial titre of 3000. But with no filtrate was as much as 60-70% of

the original titre left after 30 minutes at 56°C., as claimed by Llewellyn

Smith and Price. After 30 minutes at 100°C. all filtrates showed some re-

maining hot-cold lysin, but in no instance did the titre exceed 2% of the

original. Immediately, the Barss filtrate is seen to contain a relatively

high titre of human cell lysin, which proved surprisingly heat-stable after

an initial fall following 5 minutes only at 56°C. The cause of these vari-

ations in heat stability is obscure and warrants further investigation.

TABLE 9

EFFECT OF HEAT UPON HUMAN AND SHEEP CELL LYSINS PREPARED FROM DIFFERENT STRAINS

TIME STRAIN	UNHEATED			HEATED TO 56 C.									HEATED TO 100 C.								
				5'			15'			30'			5'			15'			30'		
	H	HS	CS	H	HS	CS	H	HS	CS	H	HS	CS	H	HS	CS	H	HS	CS	H	HS	CS
24	2	16	3000	0	40	250	4	8	10	0	0	0	0	0	tr.	0	2	3	0	4	6
Barss	20	250	3000	8	300	900	4	60	160	4	40	120	2	20	20	3	30	60	4	6	12
B2	8	130	3500	0	40	600	1	30	120	1	tr.	30	0	20	160	0	20	80	0	8	25
J 32	0	0	800	0	0	640	0	0	160	0	0	250	0	0	160	0	0	30	0	0	15

NOTE: H = human cell titre
HS = hot sheep cell titre
CS = hot-cold sheep cell titre

Antigenic Properties of
the Beta-toxin

All workers on the beta-toxin have reported that it is antigenic. The availability in this laboratory of a high potency horse serum prepared against J 32 filtrates, and the fact that the various neutralizing powers against J 32 filtrates of the sera of workers in the laboratory have been shown to be readily titratable, supported this claim. In an endeavour to determine the readiness with which beta-antitoxin might be produced in laboratory animals, rabbits were injected with the following filtrates: Two fresh filtrates (1 and 2) and one partially toxoided filtrate (3), the titres being 1/2048, 1/1524 and 1/128 respectively. The routes and dosages of these antigens and the antibody responses are given in Table 10.

A series of 6 rabbits were used, 3 of which were given intravenous and 3 subcutaneous injections. None of the rabbits had any detectable alpha-antitoxin either before or at completion of the injections. They all showed, however, some beta-antitoxin. Rabbit 2 and Rabbit 4 died shortly after the last injections were given. Just prior to death, they showed no increase in either their alpha or beta-antitoxic titres. Although too few rabbits were used to make the results conclusive, the partially toxoided filtrate provoked a much greater antibody production in the two rabbits receiving it. Moreover, these two rabbits remained perfectly healthy throughout the immunizations, whereas one rabbit receiving each of the other two filtrates died and the second rabbit in each group lost considerable weight. This suggests the possibility that partial toxoiding of a J 32 filtrate with 0.5% formalin at refrigeration temperature may remove a considerable portion of the lethal factor, while leaving the comparatively harmless haemolytic effect and the antigenicity of the treated filtrate

TABLE 10

IMMUNIZATION OF RABBITS WITH J 32 FILTRATES

RABBIT	INTRAVENOUS			SUBCUTANEOUS		
	1	2	3	4	5	6
Hot-cold Titre of Toxins	1-2048	2-1024	3-128	1-2048	2-1024	3-128
1st injection	0.5 cc.	0.5 cc.	0.5 cc.	1.0 cc.	1.0 cc.	1.0 cc.
2nd injection 5 days later	1.0 cc.	1.0 cc.	1.0 cc.	2.0 cc.	2.0 cc.	2.0 cc.
3rd injection 4 days later	2.0 cc.	2.0 cc.	2.0 cc.	4.0 cc.	4.0 cc.	4.0 cc.
Booster dose 6 weeks later	2.0 cc.	Died	2.0 cc.	Died	4.0 cc.	4.0 cc.
Beta Antihaemolytic Units						
Original sera	160	80	80	80	80	80
7 days after booster dose	160	–	20,000	–	1,500	20,500

NOTE: The antihaemolytic units represent the number of minimal hot-cold sheep cell lytic doses that the antiserum is capable of neutralizing.

unimpaired.

Despite their high titre of antihaemolysin, rabbits 3 and 6 showed no marked resistance to intravenous injections of 3 cc. of a fresh J 32 filtrate, given to the surviving rabbits, all of whom died within 2 days. Autopsy of the rabbits dying from beta-toxin showed no characteristic pathological changes.

Skin Reaction-producing Properties of Beta-toxin

(a) In Rabbits

The effect of intradermal injections of a series of beta-toxin dilutions into the skin of normal and immunized rabbits was investigated. (Prior to being given a lethal dose of J 32 filtrate, the above series of immunized rabbits were used for skin tests.) 0.1 cc. amounts of dilutions of J 32 toxin samples of varying initial potencies were injected. The results were very irregular and unpredictable, one normal rabbit giving some detectable erythema with dilutions of toxin well beyond the hot-cold haemolytic end-point; while another rabbit gave barely discernable reactions to the undiluted toxin. Similar variations in reactivity were shown by the immunized rabbits, some having a high titre of circulating beta-antitoxin showing well-marked erythema, others having less antitoxin showing no readable reaction. It was noteworthy that in no instance did necrosis occur, even when undiluted beta-toxin was used. Moreover, the erythrogenic effect, when present, faded after about 24 hours. The conclusion was reached that the rabbit skin does not provide a suitable means of titrating beta-toxin.

(b) In Guinea-pigs

Glenny and Stevens (23) and also Llewellyn Smith and Price (22)
state that erythema is induced by the beta-toxin on intradermal injection
into guinea-pigs, and claim that the toxin and antitoxin titrations may be
made by using this erythrogenic effect on guinea-pig skin. To verify this
possibility, dilutions of several J 32 filtrates, of varying age and poten-
cy, were injected intradermally into two guinea-pigs. Although the reac-
tions given were easier to read, and of longer duration, than those obtained
in rabbits, there was no apparent parallelism between the hot-cold haemol-
ytic titres and the erythrogenic titres of the test samples. With increas-
ing dilutions, the skin reactions definitely diminished; but one non-haemol-
ytic sample of J 32 toxin appeared to be as erythrogenic as a sample contain-
ing 500 hot-cold sheep cell units. The results are shown in Table 11. Al-
though too few tests of this type were made to deny the findings of other
workers, the significance of the guinea-pig skin reactions to beta-toxin
certainly needs further study.

(c) In Humans

In this laboratory numerous attempts have in the past been made to
determine the identity of the factor in staphylococcal filtrates which
causes skin reactions on intradermal injection of human volunteers. Dolman
and Kitching (20) showed that while the beta-toxin was apparently associated
to some extent with this erythematous reaction, the latter was not propor-
tional to the sheep cell lytic titre of the toxin injected, nor to the titre
of circulating beta-antitoxin of the subject. This finding was verified,
using dilutions of J 32 filtrates.

TABLE 11

RELATIONSHIP BETWEEN HAEMOLYTIC AND ERYTHROGENIC FACTORS

IN VARIOUS BETA-TOXIN PREPARATIONS

TOXIN	Haemolytic Units	Erythrogenic Reactions			
Toxin Dilutions		1/2	1/20	1/200	1/2000
J 32 (2)	400	22 X 33 (3 X 2)	17 X 12	7 X 5	0
J 32 (3)	50	25 X 35	15 X 20	7 X 7	4 X 5
J 32 (4)	500	25 X 30	10 X 10	7 X 7	4 X 5
J 32 (5)	0	25 X 20	13 X 13	7 X 9	3 X 4

NOTE: Figures for erythrogenic reactions represent mms. of erythema measured along 2 axis at right angles.
Figures in brackets represent extent of intense erythema.

(2) J 32 toxin stored at refrigerator temperature for 1 year
(3) J 32 toxin stored at refrigerator temperature for 6 months with 0.5% formalin
(4) J 32 toxin freshly made
(5) J 32 toxin stored at 37°C. with 0.5% added formalin until no trace of sheep cell lysin remained

Again, Darrach, Fulton and Lamont-Havers all attempted in this laboratory without success to isolate a human skin-reaction-producing factor from staphylococcal toxins. In an effort to determine whether small amounts of residual alpha-lysin, of human cell lysin, or of hot or hot-cold sheep cell lysin, or some combination of these factors, might account for the human skin reactions, several series of injections were made using toxoided filtrates of the Barss, B_2, and 24 strains as well as untreated filtrates of the Wood and J 32 strains. These experiments showed consistently greater reactions brought on by the Barss and B_2 filtrates, some volunteers showing large erythematous areas covering as much as 80 X 100 mms. in extent, with considerable tend rness and oedema. One individual, who had proved almost wholly resistant to previous injections of other filtrates, showed definite reactions to 0.1 cc. of the Barss and B_2 filtrates injected in 1:100 dilution. It would appear, therefore, that filtrates of these two strains contain the skin-reaction-producing substance to high degree. Table 12 shows the titre of the various lysins present in these filtrates and also in J 32 and 24 filtrates injected simultaneously into a group of six volunteers, whose average reactions to injections of 0.1 cc. of 1:100 dilutions of these filtrates is summarized.

Several smaller scale tests of the kind shown in Table 12 also demonstrated that the peculiar capacity of B_2 and Barss filtrates to cause human skin reactions cannot be correlated with the presence of either free alpha or beta-toxin; or with the human cell or hot sheep cell content. The nature of the substance must remain for the present undetermined, but the type of reaction it causes resembles a bacterial toxin rather than an allergin, although Bryce and Burnet (36) claim that the reaction is an aller-

TABLE 12

LYTIC TITRES OF FILTRATES AND THEIR AVERAGE SKIN-REACTION-PRODUCING PROPERTIES

IN HUMAN VOLUNTEERS

Filtrate	Human Cells	Rabbit Cells	Sheep Cells Hot	Sheep Cells Hot-Cold	Average Skin Reaction
B2	8	1	8	32	++++
Barss	3	3	16	40	++++
24	8	1	16	32	+
J 32	0	0	0	50	+

NOTE: 0.1 cc. amounts of 1:100 dilutions in physiological saline injected
intradermally.
Similar injections of broth gave no reactions.

ic response. The reaction appears only after a definite interval of sever-
al hours, it then progresses to a maximum intensity after 36 hours, when it
slowly fades, leaving a discoloured and perhaps even desquamating skin sev-
eral days later. The desirability of avoiding the use of filtrates of this
type in products for human use is evident, unless it can be shown that the
factor is a desirable antigen and can be suitably detoxified by some means.

COMMENTS

The main comment to be made on this work is perhaps that in the
field of staphylococcus haemolysins, things are not as simple as might be
hoped and desired. There would seem to be no doubt of the existence of a
distinct entity which causes hot-cold lysis of sheep cells and evokes ery-
thema in the skin of rabbits, guinea-pigs, and humans; and for which Glenny
and Stevens' term "beta-toxin" would seem to be fitting, to distinguish it
from the alpha-toxin. The capacity to produce beta-toxin has been shown by
several workers to be a property of numerous strains of staphylococci, in-
cluding those of human origin. The 24 strain, e.g., was originally iso-
lated from a human finger infection. Bryce and Rountree (24) and also
Minett (3) claim that staphylococci of animal origin, especially of bovine
origin, are predominantly beta-toxin producers. The beta-toxigenic strains
may be identified by their property of causing a wide zone of partial blood
decolorization on sheep blood agar according to Dolman (35), Naidu (28),
Bryce and Rountree (24), and Kojima and Kodama (29).

The antigenic properties of beta-toxin have been confirmed, as has
the fact that it can be partially detoxified by addition of formalin, and
still remain antigenic. Whether these antigenic properties should be app-

lied to human use, by incorporating toxoided filtrates from beta-toxigenic
strains in staphylococcus toxoids, must await clinical trial of the effi-
cacy of such preparations. It may be argued that the capacity to produce
hot-cold sheep cell lysis and erythema in the skin of rabbits, guinea-pigs,
or even human beings is insufficient evidence of the pathogenicity for man
of the beta-toxin. However, although incapable of causing the rapid death
of numerous species of laboratory animals, as does the alpha-toxin when in-
jected intravenously, the beta-toxin will kill rabbits within a few days,
and sometimes within a few hours. Moreover, other workers in this labora-
tory have recently shown that on intraperitoneal injections of beta-toxin
into kittens and cats, violent vomiting and diarrhoea may occur, the anim-
als frequently dying within 2 or 3 days, and often overnight. That such
toxic effects may occasionally play a part in human clinical infections
with beta-toxigenic staphylococci can hardly be doubted.

The antigenic and pathogenic properties of the hot sheep cell
lysin, the human cell lysin and the human skin erythrogenic factor present
in high degree in Barss and B_2 filtrates require further study. Such stud-
ies, however, are bound to present many complications because of the fact
that these substances are frequently present together in a given filtrate.
Monovalent toxigenic strains if encountered would greatly facilitate study
of the separate toxic entities.

SUMMARY

1. Optimal conditions for the assay of the hot-cold sheep cell lysin present in some staphylococcal filtrates were found to be 1 hour of incubation at 37°C. followed by 30 minutes exposure to an ice water bath at 10°C. The period and temperature of preliminary exposure and also of the subsequent exposure to a lower temperature, had considerable influence upon the end-point.

2. The hot-cold sheep cell lysin is distinct from the hot sheep/ cell lysin, and also from the human cell lysin.

3. The hot-cold sheep cell lysin can be detoxified by incubation with formalin, leaving a product which is capable of evoking an antibody response in rabbits.

4. The hot-cold sheep cell lysin undergoes considerable destruction by exposure to 56°C. for 30 minutes, while only a small percentage of the original lytic power remains after 30 minutes at 100°C. Filtrates from different strains exhibited variations in heat stability.

5. Intradermal injections of beta-toxin containing filtrates into rabbits, guinea-pigs, and humans showed no correlation between the hot-cold sheep cell lytic titre and the degree of skin reaction. However, the beta-toxin undoubtedly does evoke an erythrogenic reaction which is of mild degree and is rather evanescent. Some rabbits and human beings show resistance to this erythrogenic factor.

6. Rabbits immunized with beta-toxin failed to acquire any significant resistance to the lethal effect of beta-toxin, which killed immunized and normal rabbits within two days, without significant pathological changes.

7. No parallelism was noted between the hot-cold lysin and the erythrogenic factor for guinea-pig skin, contrary to the findings of other workers.

8. Partially detoxified filtrates of Barss and B2 strains contain a potent erythrogenic factor for human skin, which could not be interpreted as due to either the hot or hot-cold sheep cell lysins, the human cell lysin, or to small amounts of residual rabbit cell lysin (alpha-toxin).

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