STUDIES ON FOOD-POISONING STAPHYLOCOCCI

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INTRODUCTION

Previous to the year 1930, outbreaks of microbial food poisoning were ascribed almost exclusively to members of the Salmonella (paratyphoid) group, or to Clostridium botulinum. Other microorganisms, among them members of the coliform group, streptococci, and other miscellaneous types, have occasionally been claimed to have caused food poisoning, but in most instances the published results have been open to serious criticism. With many persons, the term food poisoning came to be regarded as synonymous with food-borne Salmonella infection. However, members of the Salmonella group, or other recognized food poisoning types could not be demonstrated in a great percentage of outbreaks, although epidemiological evidence pointed definitely to certain food products as transmitting agents. In order to account for such negative findings the existence of heat-stable Salmonella toxins was postulated. Many studies have been carried out with the aim of demonstrating the existence of such toxins but have uniformly failed in their objective.

In 1914, Barber (1) demonstrated for the first time that staphylococci are capable of causing food poisoning. He found that the milk from two cows had, in several instances, led to gastro-intestinal upsets in visitors to a certain farm while native residents were apparently unaffected by the same milk. Further investigation showed that the milk from one cow, which had previously suffered from mastitis in one quarter of the udder, could be consumed with impunity while fresh, but when allowed to stand for a few hours at room temperature, gave rise to a typical gastro-enteritis when consumed by human

volunteers. He isolated two strains of staphylococci from the milk of this cow, an orange strain from the previously diseased quarter, and a white strain from the other quarters. Studies of the two strains of staphylococci demonstrated that the yellow staphylococcus was innocuous when grown in milk, but that milk inoculated with the white strain produced typical symptoms when consumed by human volunteers.

Barber's work (1) was apparently overlooked until 1930, when Dack, Cary, Woolpert, and Wiggers (16) traced an outbreak of food poisoning to a strain of hemolytic Staphylococcus aureus in a cream-filled layer cake. In this instance, they were able to reproduce a typical syndrome of food poisoning in human volunteers with sterile filtrates of the suspected strain. Subsequent to that date, many outbreaks have been described in the literature in which staphylococci have been shown to have acted as causative agents. This re-discovery of the role of staphylococci in food poisoning in 1930 served to explain the frequent failure to demonstrate any bacterial food-poisoning agents in a large percentage of the outbreaks prior to that time. Subsequent experience has shown that staphylococci are responsible for most of the food poisoning outbreaks in this country.

Certain peculiarities of the food-poisoning toxin produced by staphylococci have prevented, or hindered, its study and demonstration. In the beginning, Dack, Jordan, and co-workers (16) (17) were able to demonstrate this food-poisoning toxin only by administration of preparations to human volunteers. Subsequently, Jordan and McBroom (35) found that certain species of monkeys were susceptible to staphylococcus enterotoxin. However, monkeys appeared to vary greatly in their susceptibility to this toxin and to be relatively less susceptible than humans.

A study of the food-poisoning types of staphylococci also failed to reveal any cultural or biochemical characteristics by which they could be distinguished from the ordinary pathogenic or saprophytic types which are more or less ubiquitous. In 1935, Stone (49) developed a special gelatin culture medium on which he claimed food-poisoning staphylococci could be differentiated by their rapid proteolytic action. While this medium has some merit, various investigators have demonstrated numerous strains that are exceptions.

In 1936, Dolman, Wilson and Cockcroft (23) published a method for the detection of enterotoxin, which consisted of the intraperitoneal injection of sterile culture filtrates into kittens. Positive food-poisoning strains were indicated by lassitude, vomiting, and diarrhea, which came on usually within a period of 20-30 minutes after injection. This method appears to be specific and at present is the method of choice in identifying food-poisoning strains. However, the method is open to the objections that cats are not a readily available laboratory animal and are difficult to house and handle under laboratory conditions. Because of their breeding habits they are available only at certain periods of the year so that animals of the proper size may not always be at hand.

The need still exists for simple, inexpensive laboratory diagnostic procedures for the identification of food-poisoning staphylococci, or their enterotoxins. The following investigation has had as an objective a study of a series of food-poisoning strains in order to determine whether they possess any cultural, physiological, or biochemical characteristics by which they may be differentiated from non-food-poisoning types.

HISTORICAL

The fact that some strains of staphylococci are capable of producing a toxin, which by reason of its ability to produce gastro-intestinal disturbances when ingested by man has been called enterotoxin, has only been realized in recent years. The association of staphylococci with certain pathological processes and the fact that they produce other powerful toxins was pointed out before 1900. It is of interest, therefore, to review the early literature on staphylococci and their toxins.

Staphylococci and Staphylococcus Exotoxins

Flaum (24) presented an excellent review and discussion of the early literature dealing with staphylococci and their toxins. He pointed out that many investigators observed and associated the "spherical" bodies in the pus from abscesses and other pathological processes in the decade between 1870 and 1880, but that Koch in 1878, Pasteur in 1880, and Ogston in 1880, 1881, and 1883, were the first to establish the relationship of the organisms to the processes. He further showed that although Ogston first applied the term "staphylococci" to those organisms which occurred in grape-like clusters, Rosenbach in 1884 first isolated in pure culture on solid media two differently colored strains of staphylococci which he designated as Staphylococcus pyogenes aureus and Staphylococcus pyogenes albus.

The early history of the discovery of the exotoxins of staphylococci which follows is also taken from Flaum's (24) review.

Van de Velde, in 1894, discovered that a pleural exudate which formed in rabbits following the injection of virulent staphylococci showed evidence of marked leucocyte destruction, and this substance had a destructive action on rabbit leucocytes, even when considerably diluted. He was subsequently able to show that this substance, which was called leucocidin, was a soluble toxin formed not only in vivo, but also in vitro, in broth cultures. He showed that the toxin was destroyed by heating to 58° C. Denys and van de Velde (1895) produced an antileucocidin by injecting increasing doses of leucocidin into rabbits.

Von Lingelsheim, in 1899, confirmed the effect of staphylococcus toxin on leucocytes and also studied some of its other effects. He demonstrated the lethal action of the toxin in rabbits injected intravenously with quantities as small as 2-2.5 cc. per kilogram, and noted the necrotizing action of the toxin when injected subcutaneously.

In 1900, Kraus and Clairmont demonstrated that a bacteria-free filtrate of Staphylococcus aureus was capable of hemolyzing rabbit erythrocytes. Although they found that normal horse serum would neutralize tetanus hemolysin and cholera hemolysin they did not test the effect of horse serum on staphylococcus hemolysin.

Neisser and Wechsberg, in 1901, re-investigated leucocidin and confirmed van de Velde's observations. They developed a method for the study of leucocidin which was based on the capacity of living leucocytes to reduce methylene blue to the leuco-base, a property not possessed by the dead cells. They also made a thorough investigation of staphylococcus hemolysin, noted the varied resistance of the red blood corpuscles of different animals to its action, and succeeded in

producing an antihemolysin.

In 1906, Kraus and Pribram studied the lethal action of staphylococcus toxin and produced an antitoxin capable of neutralizing this lethal action. Nicolle and Césari (1914) reinvestigated some of the effects of staphylococcus toxins, in particular the dermo-necrotic action of the toxin when injected subcutaneously.

It will be seen from the literature as taken from the review by Flaum (24) that the early investigators had discovered all of the various manifestations of the exotoxins of staphylococci and had succeeded in producing antibodies against the various toxins. There followed then a period in which the work of the early investigators was either ignored or forgotten. From 1900 until the decade between 1920 and 1930, the lack of interest in staphylococci and their toxins is indicated by the fact that only two publications of any significance appeared.

Two events combined to renew interest in this field and the response has been so great that the literature has grown to be voluminous. The first event was the publication in 1924 by Parker (41) of a paper on the action of the dermo-necrotic toxin of Staphylococcus aureus when administered intradermally. Although this phenomenon had been observed by von Lingelsheim (1899) and Nicolle and Césari (1914), the publication by Parker (41) pointed out again that staphylococci were capable of producing exotoxins, which renewed interest in the subject and stimulated research. The other event of significance was the Bundaberg disaster (45), in which twelve of twenty-one children died following the administration of a diphtheria toxin-antitoxin mixture which had in some manner become contaminated with staphylococci. These children died after such a short incubation period

that it suggested a toxemia rather than a bacteremia. This led to further studies of the exotoxins of Staphylococcus aureus, particularly by Burnet, who took part in the investigation of the above disaster. A review of the more recent literature on this subject follows.

Burnet (7) obtained active toxins by growth of staphylococci in broth cultures in an atmosphere of ten per cent carbon dioxide, a method which was suggested by Parker, Hopkins, and Gunther (42). Burnet (8) later showed that even more active toxins could be obtained by a combination of the above method and the discovery of Bigger, Boland, and O'Meara (4) that more active toxins are produced when staphylococci are grown on an agar surface than in broth culture. Burnet's (8) technic of growing staphylococci on an agar surface in a partial atmosphere of carbon dioxide is now used almost exclusively for the production of toxins by these microorganisms. McClean (38) demonstrated that agar, as well as cellophane, filter paper, kieselguhr and kaolin probably favor toxin production by adsorbing from broth unidentified substances which inhibit toxin production.

Subsequent to Burnet's work there have appeared a great many papers dealing with the toxins of staphylococci. While Burnet (7) concluded that hemolysin, dermo-necrotic toxin, and lethal toxin were simply different manifestations of one toxic substance, evidence has been presented by several investigators to invalidate his conclusion. Bigger, Boland, and O'Meara (4) observed a lytic action on sheep and human erythrocytes which differed from the usual action in that it occurred chiefly at air temperature after the lysin and cells had been incubated for some time at 37° C., and they referred to this hemolysin as "hot-cold lysin." They also observed differences in the power of

the lysin to hemolyze the cells of different animal species. Glenny and Stevens (28) found that the hemolytic toxins of Staphylococcus aureus could be differentiated into two types which they called alpha and beta toxins. They found that the alpha toxin hemolyzed rabbit red cells at 37° C., while the beta toxin did not hemolyze rabbit corpuscles but hemolyzed sheep corpuscles when incubated at 37° C. followed by an incubation in the cold. The alpha toxin was found to produce skin necrosis when injected intracutaneously in guinea pigs and was lethal for mice when injected intravenously and intraperitoneally. The beta toxin produced a slight reddening of the skin but no necrosis on intracutaneous injection and was not lethal for mice. Flaum (24) also showed alpha toxin to have an hemolytic action on rabbit blood corpuscles and a dermo-necrotic and acute lethal effect in rabbits, while the beta toxin had a "hot-cold" lytic action on sheep blood corpuscles, an acute lethal effect in rabbits, but no dermo-necrotic action. He showed that both alpha and beta toxin acted as leucocidins, but that this activity of the beta toxin differed from the alpha in that it was more thermostable. Smith and Price (48) studied the properties of beta hemolysin and their results are in agreement with the above-mentioned investigators.

Panton and Valentine (40) found a close relationship in different toxic filtrates between hemolysin and dermo-necrotic toxin, but no relationship between these and leucocidin. Burky (6) demonstrated that while the lethal toxin is formed anaerobically or in a synthetic medium, hemolysin production is markedly inhibited under these conditions. Burnet and Freeman (9) considered leucocidin to be identical with dermo-necrotic toxin. Valentine (51) showed that while alpha

toxin is destructive to the rabbit leucocytes and not to human leucocytes, many strains of staphylococci produce a true leucocidin which is destructive to both rabbit and human leucocytes, but which is without action on red cells. Proom (43) showed that the conditions necessary for maximum yields of alpha toxin and leucocidin are different.

It is highly probable, therefore, that the alpha hemolysin, dermo-necrotic, and lethal toxins are either the same or very closely associated, while alpha and beta toxins and leucocidin are separate entities.

Early investigators produced antitoxins capable of neutralizing the various toxic effects of staphylococcal exotoxins (24). Later investigators have shown that the sera of normal animals may possess the power of neutralizing staphylococcus toxins. Beumer (2) cites the work of Gross, who demonstrated that normal horse, sheep, and beef sera contained antilysins and agglutinins for staphylococci, which were not present in the sera of normal rabbits or man, and that when normal horse serum was added to broth in which staphylococci were grown, hemolysin production was inhibited. Beumer (2) confirmed the above results, but found that certain strains, by reason of their strong proteolytic activity on horse serum, were capable of producing hemolysin in its presence. Forssman (25) found that normal horse and sheep sera neutralized staphylococcus hemolysin <u>in vitro</u>, and also observed that these sera exerted a protective effect against staphylococcus infections in rabbits.

Staphylococcus Food Poisoning and Enterotoxin

The first recorded outbreak of food poisoning which can probably be attributed to staphylococci was described by Denys (19) in 1894. He described a food-poisoning epidemic in which thirty people were made ill, following the consumption of raw, or insufficiently boiled beef, in which he was able to demonstrate the presence of Staphylococcus aureus and Staphylococcus albus by cultures on agar and in broth. In 1906, Owen (39) reported an outbreak of nineteen cases of food poisoning which was attributed to dried beef infected with staphylococci. As previously mentioned, the first outbreak in which it was actually demonstrated that staphylococci were capable of causing gastro-intestinal disorders, was that reported by Barber (1) in 1914. In this instance he was able to reproduce symptoms of food poisoning by consuming milk inoculated with the suspected strain. These three reports failed to attract attention to the staphylococcus as a potential cause of food poisoning, and our knowledge of this type of food poisoning really dates back to the work of Jordan and his associates, beginning in 1930.

Dack, Cary, Woolpert, and Wiggers (16) in 1930, isolated a strain of Staphylococcus aureus from sponge cake involved in a food poisoning outbreak. Sterile veal infusion broth filtrates of the organism proved toxic when fed to human volunteers, producing symptoms identical to those shown by the victims of the original outbreak.

In the same year, Jordan (29), following the lead given by these workers, studied other strains of staphylococci from various sources, of which three were from normal human throats, one from a case of septicemia, and two from food implicated in food poisoning outbreaks.

All of these six strains were capable of producing a toxic substance which produced gastro-intestinal disturbances in human volunteers. He stated that these strains differed in their cultural characteristics and that the toxic substance in the filtrates from these strains was destroyed, or greatly weakened, by heating to 60-65° C. for thirty minutes.

In 1931, Jordan (30) reported four additional outbreaks of food poisoning in which large numbers of staphylococci were found in the incriminated foods. In each instance, strains isolated from the foods and grown in broth cultures yielded filtrates which were toxic when fed to human volunteers. MacBurney (37) reported an outbreak in which 150 people were made ill by the consumption of chocolate eclairs, the filling of which contained staphylococci, sterile filtrates of which produced severe illness in human volunteers. Jordan and Burrows (32) described five additional outbreaks of food poisoning, in each of which enterotoxin-producing staphylococci were demonstrated. Additional outbreaks have been reported by Ramsey and Tracy (44), Geiger and Gray (26), Crabtree and Litterer (14), Dack, Bowman, and Harger (15), Denison (18), Dolman (21), and many others.

In all, the victims of such outbreaks now number several thousands and in some instances single outbreaks have affected as many as 1,000 individuals. The symptomatology of staphylococcus food poisoning is quite characteristic, usually following in 3-4 hours after ingestion of the contaminated food. The outstanding symptoms are nausea, vomiting, and diarrhea, usually with a normal or slightly subnormal temperature, occasionally severe prostration and cramps, but in general followed by a rapid recovery. Fatalities from this type of food

poisoning have been very rare, death resulting only when complicated by some other pathological condition.

It is of interest to note the most common types of food products involved are cream or custard filled pastries, although milk, cream, cheese, gravy, ham, tongue, and a variety of other food products have been incriminated.

Stritar and Jordan (50) studied a series of ninety-four strains of staphylococci from various sources in order to determine whether food-poisoning strains possess any cultural, biochemical, or serological characteristics by which they can be differentiated from other pathogenic or non-pathogenic types. They concluded that food-poisoning strains agree with other members of the Staphylococcus group "in not constituting a clearly marked division." Kupchik (36) conducted a similar study and failed to find any cultural or biochemical characteristic of value in the differentiation of staphylococci of the food-poisoning type. Stone (49) developed a special gelatin medium on which he claimed food-poisoning staphylococci produced rapid liquefaction, while only slight or no liquefaction was produced by non-food-poisoning types. Dolman and Wilson (22) and Kupchik (36) failed to find this medium of differential value, having encountered food-poisoning types incapable of liquefying gelatin.

Jordan, Dack, and Woolpert (34) studied the effect of heat, storage and chlorination on the toxicity of the staphylococcus enterotoxin. They showed that the toxin was not completely destroyed by boiling for thirty minutes but was probably diminished in its toxic power, that it resisted storage at low temperatures for as long as sixty-seven days, and was not destroyed by short contact with a rather strong dose of chlorine. Woolpert and Dack (54) utilized Burnet's (8)

method of toxin production, that is, growth on a semi-solid agar medium in a partial atmosphere of carbon dioxide, for the production of the staphylococcus enterotoxin, and produced filtrates much more potent than by other methods. They also studied the relation of the enterotoxin to staphylococcus hemolysin, dermo-necrotic toxin, and lethal toxin, and found the enterotoxin to be distinct from the three latter toxins in that it was more resistant to heat and to adsorption than the other toxins, and was not neutralized by antiserum effective against the others. They noted that enterotoxin was never produced without the production of other toxins.

Jordan and Burrows (31) investigated the nature of the enterotoxin and concluded that:

1. The active principle will not distill.
2. It is not readily dialyzable.
3. It is markedly unstable to N/100 NaOH.
4. It is unstable to heat in N/100 HCl solution.
5. It is not identical with the hemolytic substance present in many filtrates, not does it produce a skin reaction.
6. It is completely removed from acid aqueous solution by extraction with ether or chloroform.
7. It may be extracted from alkaline solution with ether or chloroform, but the deleterious effect of the alkali tends to mask such removal.

It should be noted that, in the earlier papers on this subject, the toxicity of staphylococcus filtrates suspected of containing enterotoxin was determined by feeding to human volunteers. Dack, Jordan, and Woolpert (17) reported that normal animals, including monkeys, were not adversely affected when fed filtrates of known toxicity for human volunteers. Jordan and McBroom (35) utilized small monkeys of different species, usually weighing between 1-2 kilos, and found them susceptible to the enterotoxin, although to a somewhat varying degree. This variation in the susceptibility of different monkeys to the enterotoxin has also been noted by other investigators. Borthwick (5) administered toxic staphylococcus filtrates directly into the stomachs of guinea pigs and rabbits after first adjusting the reaction of the stomach to pH 7.3, and found that such animals died within five days, showing signs of acute gastro-enteritis upon autopsy. Symptoms of poisoning could also be produced by intra-rectal inoculation of the toxin when the rectum was irrigated with saline adjusted to pH 7.3. Borthwick's results have not been confirmed.

Dolman, Wilson, and Cockcroft (23) studied methods for the detection of enterotoxin in laboratory animals because they considered the use of human volunteers "not expedient," and because of the variability in the reaction of monkeys to the enterotoxin and their lack of ready availability. Because of the more or less close relationship of the cat to human beings in dietary and excretory habits, they decided upon this animal as possibly best suited for this purpose. After they had first determined that oral administration of a known toxic filtrate caused projectile vomiting and diarrhea in a cat, they found that the intraperitoneal injection of 2 cc. of a

formalinized filtrate of an enterotoxic strain would cause a characteristic syndrome in kittens, which consisted of marked lassitude and weakness, culminating in a series of paroxysms of vomiting, often within 15-30 minutes after the injection. As little as 0.5 cc. intraperitoneally of a potent filtrate would cause a severe reaction in a kitten of 350-550 grams, while as much as 3 cc. of the filtrates from innocuous strains or of formalinized broth would produce no reaction. They indicated a preference for kittens because they were relatively more sensitive than adult cats, although they indicated that the latter could be used.

Dolman, Wilson, and Cockcroft (23) prepared their toxic filtrates on a semi-solid agar medium inoculated with the strain of staphylococcus under investigation, and harvested after forty hours' incubation at 37°C. in an atmosphere of 30 per cent carbon dioxide and 70 per cent oxygen. The suspension freed from agar was sterilized by Seitz filtration. They removed the other toxic factors,namely, hemolysin, dermo-necrotic toxin, and lethal toxin, either by the addition of 0.3 per cent formaldehyde and then incubating at 37° C. until rabbit and sheep cell hemolysins were no longer detectable, or by boiling the filtrates for one-half hour prior to injection. They regarded the gastro-intestinal tract as the seat of origin of the main symptoms of staphylococcal food poisoning, but stated that enterotoxin cannot be regarded as a direct gastro-intestinal irritant since the food-poisoning symptoms are produced in kittens by intraperitoneal administration of potent filtrates, and since the gastro-intestinal tract at autopsy shows no evidence of having been subject to the action of an acute irritant.

Kupchik (36) confirmed the value of Dolman's test for the detection of enterotoxin. Rigdon (46) failed to confirm the value of the Dolman test since he was able to produce vomiting in puppies and kittens by the injection of an uninoculated control medium. He also claimed that the toxin is not thermostable, since several animals which received the heated toxin did not vomit.

In a later paper, Dolman and Wilson (22) pointed out that the materials should be brought to body temperature and injected slowly, and that under such conditions it is possible to inject, without ill effect, 5 cc. of physiological saline solution, plain broth containing 0.3 per cent formalin, or formalinized filtrate prepared from a staphylococcus strain producing no enterotoxin. They further extended their studies on the kitten test for staphylococcal enterotoxin and pointed out that in experiments involving nearly 200 kittens they found no instance of natural insusceptibility to the enterotoxin. In addition to the two methods which were described in their earlier paper (23) for separating enterotoxin from the other exotoxins of the staphylococci they added a third method, which consisted of the adsorption of toxic filtrates with a serum containing antibodies to the alpha and beta toxins but not to the enterotoxin. They further determined that the enterotoxin is antigenic, that the sera of the animals immunized with filtrates containing enterotoxin develop neutralizing properties against this antigen, and finally, described a specific flocculation reaction between the enterotoxin and its antibody.

Chapman, Lieb, and Curcio (12) studied the pathogenicity of staphylococci from food-poisoning sources as determined by their

ability to produce orange or yellow pigment, to hemolyze rabbit blood agar, to coagulate human and rabbit plasma, to produce orange or deep violet growths on crystal violet agar, to grow luxuriantly on brom thymol blue agar, and to ferment mannitol. They concluded that a typical food-poisoning staphylococcus will coagulate human and rabbit plasma, produce a positive Stone reaction, hemolyze rabbit blood agar, ferment mannitol, and grow luxuriantly on brom thymol blue agar.

EXPERIMENTAL

Source and Maintenance of Cultures

The strains of staphylococci used in the following investigation were isolated in all but one instance from food products involved epidemiologically in food poisoning outbreaks. The year of isolation and the food product from which each strain was isolated are listed in Table I. Although thirty-eight of the strains of staphylococci were isolated from food specimens, this collection represents only eleven different food poisoning outbreaks. While some of the cultures undoubtedly represent duplicates, it was considered advisable to retain more than one from each outbreak, either because of minor variations in color, or because some were isolated from different specimens in the same outbreak, or because toxicity tests, in some instances, had been conducted with a mixture of different strains. The results to be presented later in this discussion bear out the wisdom of the above procedure. Strain 36 was isolated in 1922 from a boil and thus differs from the other strains in origin. This strain was obtained from Mr. C. M. Brewer, Food and Drug Administration, United States Department of Agriculture, and bore the number 209, which designates the strain used widely in this country for the testing of antiseptics and germicides.

After isolation of the food poisoning cultures and study at the time of the outbreak, subcultures were prepared and held in the ice box on standard nutrient agar slants which consisted of 0.3 per cent Difco beef extract, 0.5 per cent Bacto peptone, and 1.5 per cent agar. Cultures of staphylococci isolated prior to this investigation were

TABLE I

Source of Staphylococcus Cultures and Year of Isolation

Cultures	Year isolated	Source
1-5	1935	Cream puff, apple turnover, and pastry
6-8	1935	Cream pie
9-14	1937	Chocolate eclairs
15-16	1937	Beef tongue
17-20	1935	Pickled tongue
21-22	1935	Cream pie
23-24	1936	Ice cream
25-27	1933	Vomitus from food poisoning victim
28-30	1938	Ham
31-35	1938	Ham
36	1922	Boil
37-39	1939	Ham

transferred to fresh media at irregular intervals. During the course of this investigation all strains have been transferred to fresh nutrient agar slants at least once each month.

Original Characteristics of Staphylococcus Cultures

The characteristics of the strains of staphylococci when first isolated are given in Table II. These strains were isolated and studied by different individuals at the time of the food poisoning outbreaks and since the primary object in such studies was the demonstration of the ability, or lack of ability, of the strain to produce enterotoxin, there was a considerable variation in the thoroughness with which the strains were studied. Prior to 1937, before the Stone medium and the Dolman test were in general use, it was customary to determine the fermentation reactions in certain key sugars, and to determine the hemolytic action on blood agar plates to establish the identity of the strain. After 1937, when the Stone reaction and Dolman test were adopted for use in typing food-poisoning strains, and the demonstration by several investigators that cultural reactions were of little value in determining the capacity of a given strain to produce enterotoxin, more reliance was placed upon the Stone and Dolman tests.

It will be noted that with the exception of strain 23, all of the strains on which biochemical studies were made fermented dextrose, lactose, sucrose, and mannitol with acid production, but failed to ferment raffinose, inulin, salicin, or glycerol. Strain 23, which was isolated from a sample of ice cream, failed to ferment mannitol. Strain 24, which predominated in the same sample, fermented that

TABLE II

Characteristics of Staphylococcus Strains when Isolated

Cultures	Pigment	Dextrose	Lactose	Sucrose	Mannitol	Raffinose	Inulin	Salicin	Glycerol	Hemolysis on Blood Plates	Stone Reaction
1-5	Orange	A	A	A	A	0	0	0		+	
6-8	Orange	A	A	A	A	0	0		0	+	
9-14	Orange	A*	A*	A*						+	+
15-16	Orange									+	+
17-18	Orange	A	A	A	A	0			0	+	
19	White	A	A	A	A	0			0	+	
20	Yellow	A	A	A	A	0			0	+	
21-22	Orange	A	A	A	A	0	0	0		+	
23	Orange	A	A	A	0	0	0	0		+	
24	White	A	A	A	A	0	0	0		+	
25-26	Orange									+	
27	White variant									+	
28-30	Orange									+	+
31-33	Orange									+	+
34-35	White									0	+
36	Orange									+	
37-39	Orange									+	0

A = acid formation
0 = no acid formed or negative reaction
*Carbohydrate fermentation of strain 14 only

alcohol. With the exception of strains 34 and 35, all were hemolytic on rabbit or sheep blood agar plates. Strains 34 and 35 were types of white staphylococci present in small numbers in a specimen of ham in which hemolytic Staphylococcus aureus represented by strains 31, 32, and 33 were predominant. Most of the strains isolated from food specimens produced an orange pigment at the time of isolation, and those strains which produced a white growth were always associated with pigmented types. Strain 27 was a white variant colony which was fished by the author from a blood agar plate streaked from a parent culture represented by strain 25.*

The Stone reaction was determined only on the more recently isolated strains and was positive for all strains tested except 37, 38, and 39, which were only recently isolated from a specimen of ham. Particular attention is directed to these strains which, although giving a negative Stone reaction, were demonstrated capable of producing enterotoxin.

Table III presents the results of the toxicity tests conducted with the staphylococcus strains at the time of isolation. Again there was considerable variation in procedure due to the lack of adequate methods previous to 1937. However, the animal feeding tests, in all cases in which cats were employed, produced slight or definite evidence of an ability to produce gastro-intestinal disturbances in these animals. Unfortunately, animal feeding tests were not conducted on strains 21 to 24 at the time of isolation due to the lack of suitable animals. Strains 17 to 20 and 25 to 27 were not tested for the same

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*Culture obtained from Dr. William Litterer (14).

TABLE III

Original Toxicity as Indicated by Animal Feeding and Injection

Cultures	Toxin Preparation	Toxicity
1-5	Sterile filtrate[1] composite cultures	10 cc. administered to each of 4 cats by stomach tube produced continued diarrhea in 3, with subsequent death of 2, after $3\frac{1}{2}$ and 13 days.
6-8	Milk cultures Sterile filtrate[1]	Produced slight diarrhea in cats. Injected intravenously into rabbits produced diarrhea, convulsions and death after 4-5 hours.
9-14	Custard cultures Sterile filtrates[2] composite cultures	Slight diarrhea in 3 cats fed with custard. Diarrhea in 4 cats and vomiting and diarrhea in 1 kitten by feeding.
15-16	Sterile filtrate[2]	Vomiting and diarrhea in young cats 4-5 hours after feeding.
17-20		Not tested in this laboratory.[3]
21-22		Not tested at time of isolation.
23-24		Not tested at time of isolation.
25-27		Toxic to humans.[4]
28-30	Sterile filtrate[2]	Diarrhea in 2 cats fed with 5 cc. filtrate. Vomiting in 20 minutes in 2 cats injected with 3 cc. of filtrate intraperitoneally.
31-33	Sterile filtrate[2]	Vomiting within 15 minutes and death in 48 hours in a young cat injected intraperitoneally.
34-35		Not tested at time of isolation.
36		Not tested at time of isolation.
37-39	Sterile filtrate[5]	3 cc. intraperitoneally; vomiting in cats after 20 minutes and death in 24 hours.

[1]Broth cultures grown in a partial atmosphere of carbon dioxide.

[2]Veal infusion soft agar cultures grown in a partial atmosphere of carbon dioxide.

[3]Other strains isolated from this outbreak proved toxic to rhesus monkeys by Dack, Bowman, and Harger (15).

[4]Culture obtained from Dr. William Litterer. Crabtree and Litterer (14).

[5]Culture grown on Dolman (22) medium in 25 per cent carbon dioxide.

reason, and also because similar cultures isolated from food specimens in the same food poisoning outbreak were proven toxic in other laboratories during independent investigations. Strains 34 and 35 were not tested because other strains from the same specimen were proven to be enterotoxic by the Dolman test. Strain 36 was isolated before the discovery of the role of staphylococci in food poisoning. All of the more recently isolated strains have been demonstrated to produce enterotoxin by the Dolman test.

Present Cultural and Biochemical Characteristics of Staphylococcus Strains

Those characteristics of staphylococci which in the hands of previous investigators have proven of most value in the classification of a given strain as pathogenic or as a producer of toxins were chosen for applications to the strains of staphylococci under investigation.

Chapman, Lieb, and Curcio (12) studied a series of food-poisoning cultures and concluded that like other pathogenic staphylococci they produce a yellow or orange pigment, hemolyze rabbit blood agar, coagulate human and rabbit plasma, produce orange or deep violet growths on crystal violet agar, grow luxuriantly on brom thymol blue agar, and ferment mannitol. In earlier investigations Chapman et al. (10) (11) (13) had demonstrated that pathogenic staphylococci commonly produce positive reactions to the above tests as contrasted to non-pathogenic strains which were quite variable in their reactions. Their comparison of the Stone reaction with the above tests on food-poisoning strains gave excellent correlation. A study of the tables in the work of Chapman, Lieb, and Curcio (12) indicated that the production of acid

from lactose varied from strain to strain and, while correlating fairly well with the other tests in the series, was somewhat more variable.

The methods and materials used in determining the present characteristics of the staphylococcus strains under investigation are outlined below.

Pigment production: Cultures of the staphylococci were grown on the surface of nutrient agar slants of the same composition as that used for maintaining the stock cultures. After growth for 24 hours at 37°C. and 48 hours at room temperature pigment production was judged by examination of the growth against a white background in a strong light.

Hemolysis on rabbit blood agar plates: Six per cent of citrated rabbit blood was added to veal infusion agar of the following composition:

Veal infusion	1,000 cc.
Witte's peptone	10 grams
Sodium chloride	5 grams
Agar	15 grams

Final reaction pH 7.4

The blood was added to this medium after it had been melted and cooled to 45°C. and, after gentle mixing, poured into petri dishes and allowed to harden. The surface of each plate was streaked with one loopful of a 24-hour beef infusion broth culture of one strain, the plate incubated for 24 hours at 37°C., and the results then recorded.

Coagulase production: Human blood was obtained with a sterile syringe from the median basilic vein. Coagulation was prevented by the addition to each 5 cc. of blood of 0.2 cc. of a sterile 20 per cent

solution of sodium citrate in 0.85 per cent salt solution. The blood cells were removed by centrifugation in sterile tubes. The plasma was removed from the cells by means of a sterile pipette and diluted with nine volumes of sterile 0.85 per cent salt solution. The resulting 1:10 dilution of plasma was distributed in 1 cc. quantities into small test tubes 10x75 mm. Each tube was then inoculated with one loopful of a 24-hour broth culture of a staphylococcus strain, the tubes agitated to obtain thorough mixing and placed in a water bath at 37°C. Results were read after 2, 3, and 5 hours, and were considered negative only after 24 hours' incubation. A positive coagulase test was indicated by the formation of a loose fibrinous clot which appeared in the positive cultures within three hours after inoculation.

Growth on crystal violet agar: The crystal violet agar medium was prepared as directed by Chapman (10) and had the following composition:

Agar	15 grams
Difco Proteose peptone	5 grams
Difco beef extract	3 grams
Distilled water to make . . .	1,000 cc.
pH adjusted to 6.8	
Crystal violet, Commission Certified	
0.1 per cent (based on dye content	
and not crude weight) - 3.3 cc.	

This medium was sterilized at 250°F. for 15 minutes, distributed in petri dishes, and allowed to harden. It was then inoculated by streaking a loopful of a 24-hour agar culture over about two centimeters of the surface. This heavy inoculation is necessary because of the inhibitive effect of crystal violet upon growth. Results were read after 36 hours' incubation at 37°C. Orange and deep violet colored growths were considered positive, while white, pale violet, or mottled white and

violet growths were considered negative as indicated by Chapman (10).

Growth on brom thymol blue agar: Brom thymol blue agar was prepared as directed by Chapman, Lieb, Berens, and Curcio (13) and had the following composition:

Difco beef extract	3 grams
Difco Proteose peptone	5 grams
Lactose	10 grams
Agar	15 grams
Brom thymol blue	0.17 gram
Distilled water to make	1,000 cc.

The reaction was adjusted before sterilization to pH 9.6 with the glass electrode.

Sterilization and inoculation of this medium was the same as with the crystal violet agar medium, except that heavy inoculations were not used. The same loopful of culture used for the inoculation of the crystal violet agar was then streaked over about two centimeters of the surface of brom thymol blue agar. The ability of a strain to grow on this medium in 36 hours' incubation at 37°C. constituted a positive reaction.

Acid production from lactose and mannitol: Two methods were used for the detection of the fermentation of these two substances, but since the same results were obtained by both, only one method will be described here. The basic medium had the following composition:

Difco beef extract	3 grams
Bacto peptone	10 grams
Distilled water to make	1,000 cc.
pH adjusted to 7.0	

Ten grams of lactose or mannitol (both of which were Pfanstiehl C.P. products), and 1 cc. of a 1.6 per cent alcoholic solution of brom cresol purple were added to each liter of this basic medium. The final medium

was sterilized at 240° F. for 10 minutes and incubated at 37°C. for 24 hours before use to insure sterility. One tube of lactose broth and one tube of mannitol broth were inoculated with a small amount of the growth from a 24-hour nutrient agar culture of each organism. The tubes were incubated at 37°C. for five days and acid production recorded as positive when the color changed from purple to yellow. The other method consisted of growth of each strain on lactose or mannitol agar containing phenol red as an indicator.

Stone reaction: The dehydrated medium, Bacto Stone's extract gelatin agar, prepared according to the manufacturer's directions was used in determining the Stone reaction of the staphylococcus strains. This was prepared by suspending 7.5 grams of the dehydrated medium in 100 cc. of distilled water, maintaining constant agitation to avoid the formation of clumps. It was sterilized at 250° F. for 20 minutes. After sterilization, the medium was distributed in petri dishes which after hardening were stored in the 37°C. incubator for 24 hours in order to obtain a dry surface. One loopful of a 24-hour beef infusion broth culture was streaked on the surface of the Stone medium in such a manner as to obtain well isolated colonies. After incubation of the plates at 37°C. for twenty-four hours they were flooded with a saturated solution of ammonium sulphate and allowed to develop for five minutes before reading. The unaltered medium assumes a milky opaque appearance, while liquefaction is indicated by clear zones around the colonies. The width of the zones of gelatin liquefaction were measured against a dark background with a millimeter rule. At least two determinations were made upon each culture and the

determination which showed the greatest zone of liquefaction recorded for that particular strain. According to Stone, strains of staphylococci producing enterotoxin are surrounded by a clear zone extending at least one-eighth of an inch from the edge of the colony. His experience indicated that zones of a lesser degree were significant although not definitely positive.

The results of the application of the above tests to the thirty-nine strains of staphylococci under investigation are given in Table IV. A comparison of this table with Table II, which gives the characteristics of the same strains at the time of isolation, shows that certain changes have taken place in the cultures during the storage period subsequent to isolation.

Two strains, 4 and 20, were originally pigmented types but cultivation on artificial media has resulted in the loss of the power to produce the orange pigment. These strains are of particular interest because, as will be shown later, they are the only strains among the earlier isolations which have retained the power to produce an enterotoxic substance. As far as they can be compared, due to the lack of complete information on the fermentative powers of the strains when first isolated, no changes have occurred in the ability of the strains to ferment lactose and mannitol.

Twenty-four of the strains, or 61.5 per cent, gave positive reactions to all of the tests exclusive of the Stone reaction, agreeing with the conclusions of Chapman, Lieb, and Curcio (12) that strains of staphylococci of food-poisoning origin, in common with other pathogenic staphylococci, produce a yellow or orange pigment, hemolyze rabbit

TABLE IV

Present Cultural and Biochemical
Characteristics of Staphylococcus Cultures

Culture	Pigment	Hemolysis on Blood Plates	Coagulase	Crystal Violet Agar	Brom Thymol Blue Agar	Lactose	Mannitol	Stone Reaction Zone in mm.
1	Orange	+	+	+	+	A	A	3
2	"	+	+	0	+	A	A	1.5
3	"	+	+	0	+	A	A	2
4	White	+	+	0	+	A	A	5
5	"	+	+	+	+	A	A	5
6	Orange	+	+	+	+	A	A	3
7	"	0	+	+	+	A	A	1
8	"	+	+	+	+	A	A	3
9	"	+	+	+	+	A	A	2.5
10	"	+	+	+	+	A	A	2.5
11	"	+	+	0	+	0	A	2
12	"	+	+	+	+	A	A	2
13	"	+	+	0	+	0	A	2
14	"	+	+	+	+	A	A	2
15	"	+	+	+	+	A	A	2
16	"	+	+	+	+	A	A	2
17	"	+	+	+	+	As	A	1
18	"	+	+	+	+	As	A	2
19	"	+	+	0	+	A	A	1.5
20	White	+	+	+	+	As	A	5
21	Orange	+	+	0	+	A	A	2
22	"	+	+	0	+	A	A	2.5
23	"	0	+	+	+	A	0	0
24	White	+	+	0	+	A	A	3
25	Orange	+	+	+	+	A	A	2
26	"	+	+	0	+	A	A	3
27	"	+	+	+	+	A	A	3
28	"	+	+	+	+	A	A	2
29	"	+	+	+	+	A	A	2.5
30	"	+	+	+	+	A	A	2
31	"	+	+	+	+	A	A	3
32	"	+	+	+	+	A	A	2.5
33	"	+	+	+	+	A	A	2.5
34	White	0	0	0	+	0	A	2
35	"	0	0	0	+	0	A	2
36	Orange	+	+	+	+	A	A	0
37	"	+	+	+	+	A	A	2.5
38	"	+	+	+	+	A	A	3.0
39	"	+	+	+	+	A	A	2.5

A = acid production
As= slight acid production

blood agar, coagulate human plasma, produce orange or deep violet growths on crystal violet agar, grow luxuriantly on brom thymol blue agar, and ferment mannitol. Strain 4 differs in its ability to produce an orange or violet growth on crystal violet agar, and both strains 4 and 20 produce a white, rather than an orange or a yellow pigment, although both of these strains produce enterotoxin.

The Stone reactions are also of special interest. If the criterion of the ability of the strain to produce enterotoxin is dependent upon the production of a zone of liquefaction approximately one-eighth of an inch in width (approximately three millimeters), the Stone reactions of most of the strains in this study would indicate that they are incapable of producing enterotoxin. These strains could be considered, as pointed out by Stone, to belong to that group in which the result is significant, but not definitely positive. Three strains, 4, 5, and 20, produce wide clear zones of liquefaction at least five millimeters in width, and frequently greater. Strains 1, 6, 8, 24, 26, 27, 31, and 38 produce zones approximately three millimeters in width and would probably, therefore, be considered as enterotoxin producers. Only two strains, 23 and 36, the latter of which was not of food-poisoning origin, produced completely negative reactions on the medium. A comparison of the Stone reactions in Table IV with those given in Table II, although the reactions in the latter table are necessarily incomplete, indicates that a great many of the staphylococcus strains have lost, to some degree, their ability to liquefy gelatin. However, of the three strains which have retained the power of producing a strong Stone reaction, two have retained the power to produce enterotoxin.

In a brief study of the first twenty-eight cultures one and one-half years ago and before the start of this investigation, the Stone reaction was determined and, with the exception of cultures 2, 7, and 23, all of the cultures produced a positive Stone reaction. This seems to bear out the conclusion that these strains are gradually losing their ability to liquefy gelatin which, as will be indicated later, may be correlated with the loss of the power to produce enterotoxin.

Production of Hemolysin and Enterotoxin by Staphylococcus Strains

Burnet (7) considered that the toxic activities of staphylococcus filtrates in causing hemolysis in vitro, necrotic skin lesions in rabbits on intradermal injections, and the acute death of rabbits after intravenous injection, were all manifestations of a single antigenic substance. He introduced a method of titrating the toxin which was based upon its ability to hemolyze rabbit erythrocytes. By this method he showed that the hemolytic titer of a toxin was proportional to its toxicity for rabbits. This was confirmed by Panton and Valentine (40) and Dolman (20). Although subsequent investigators have shown that there are at least three separate toxic entities produced by staphylococci, alpha and beta hemolysin and leucocidin, it appears that the alpha hemolysin, necrotoxin and lethal toxins are either the same or very closely associated.

It was concluded, therefore, that the hemolytic titer against rabbit erythrocytes of toxins prepared from the staphylococcus strains under investigation was the best available determination by which to

judge the toxicity of these strains.

The cultural requirements for the production of toxin by staphylococci have been the subject of much research. In 1922, Walbum (52) published his fundamental work on the optimum requirements for hemolysin production by staphylococci and most of the media used by the more recent investigators are modifications based upon his findings. He pointed out among other facts that toxin production takes place best at a pH between 6 and 7; that peptone in suitable amounts increases hemolysin production; and finally, that magnesium salts have a definite stimulating effect on toxin formation. Burnet (8) combined the discoveries of earlier investigators by growing staphylococci on an agar surface in a partial atmosphere of carbon dioxide and obtained more potent toxins than obtained by either of the above methods. Burnet's technic was applied to the production of staphylococcus entertoxin by Woolpert and Dack (54) who obtained more potent enterotoxic filtrates by this procedure.

In general, meat infusion bases to which peptone, phosphate buffer, and a magnesium salt have been added, have been commonly used for production of staphylococcus toxin. More recently, however, Dolman and Wilson (22) stated that they were able to obtain higher potencies of enterotoxin by use of a "semi-synthetic" medium than those obtained by the use of meat infusion media. Because of its semi-synthetic nature the Dolman medium could be prepared and reproduced with a great deal more ease and accuracy than the veal infusion agar recommended by Woolpert and Dack (54). Therefore, it was adopted as a standard medium for the production of enterotoxin and hemolysin for this investigation.

Toxin production: The composition of the Dolman medium and the method of preparation was as follows:

Difco Proteose peptone	20 grams
Sodium chloride (NaCl)	5 grams

Dissolve in 500 cc. of distilled water.

To this was added the following salts in aqueous solution:

Dipotassium hydrogen phosphate (K_2HPO_4)	1 gram
Monopotassium dihydrogen phosphate (KH_2PO_4)	1 gram
Magnesium sulphate ($MgSO_4$)	0.2 gram
Calcium chloride ($CaCl_2$)	0.1 gram

The solution was brought to the boiling point, made up to a volume of one liter, and the pH adjusted to 7.4. The solution was again brought to the boiling point and three grams of Bacto shredded agar added and dissolved.

Gladstone (27) presented evidence which indicated that maximum hemolysin production occurs only when the surface area of the medium exposed to the gaseous environment is approximately equal to the volume of the medium in cubic centimeters. Appreciable deviation from this ratio in either direction resulted in decreased quantities of hemolysin. In view of this evidence and since it has been demonstrated by Woolpert and Dack (54) and others that those conditions favoring hemolysin production also favored production of enterotoxin, it was considered necessary to obtain an equal surface-volume ratio.

It was found that when 115 cc. of the Dolman medium was placed in a one-liter Erlenmeyer flask the resulting surface area was approximately 115-120 square centimeters. Since this container had a capacity slightly in excess of one liter, the remaining volume after introduction of the medium was almost exactly one liter which was an advantage in that it made the calculation and measurement of the carbon dioxide to be added at the time of inoculation a simple matter.

The medium was placed in flasks in 115 cc. amounts, the flasks closed with ordinary cotton plugs protected with a layer of tinfoil, and sterilized at 250°F. for 30 minutes. The sterile medium was inoculated with 2 cc. of a five-hour beef infusion broth culture of the staphylococcus under investigation, the cotton plug discarded, and the flask closed with a sterile one-hole rubber stopper through which a 7 mm. glass tube extended to within approximately 6-8 cm. of the surface of the medium. The tube was bent at right angles just above the stopper, and a bulb, blown in the tubing about one inch from the end, was packed with non-absorbent cotton to prevent the entrance of other bacteria during the process of introducing carbon dioxide and air. A rubber tube supplied with a screw clamp was slipped over the end of the glass tube for attachment to the vacuum line and to the carbon dioxide apparatus. After placing the rubber stopper in the flask the channel between the rim of the flask and the stopper was sealed with a mixture of equal parts of paraffin and petrolatum. The flask was evacuated, then attached to a carbon dioxide measuring cylinder, and 250 cc., or 25 per cent, carbon dioxide added. Air was admitted rapidly to produce a mixing of the gases but a slight negative pressure was maintained to allow for expansion of the gases at the incubation temperature of 37° C. and to hold the stopper securely in place. Incubation at 37°C. was continued for from 44-48 hours.

At the end of the incubation period the flasks were gently agitated to break up the soft agar, the total contents poured into centrifuge tubes, and centrifuged at 3,000 r.p.m. for 30 minutes. The clear supernatant fluid was decanted and sterilized by filtration through Chamberland or Berkefeld candles. Microscopic examination of

the growth in each flask was carried out at the end of the incubation period to determine freedom from external contamination. Tests were made on the filtrates to insure sterility of the toxin preparations.

Dolman kitten test: In order to eliminate the toxic effects of staphylococcus toxins other than enterotoxin, filtrates were placed in a boiling water bath for thirty minutes prior to injection. Tests for hemolytic activity of a few representative boiled filtrates showed them to be free from hemolysin for rabbit erythrocytes. Kittens of a weight ranging from 500-1,000 grams were used for most of these tests although due to a shortage of these animals it was necessary to use a few larger cats of 1,000-1,550 grams in weight. In order to compensate for this difference in weight a dosage schedule was arranged as follows:

Weight of animal	Dosage
500 - 1,000 grams	3 cc.
1,000 - 1,500 grams	4 cc.
Over 1,500 grams	5 cc.

Syringes were boiled for thirty minutes before use for injection. The filled syringes were placed in a 45°C. incubator and allowed to come to that temperature before removal for injection. The delay after removal from the incubator was so adjusted that the filtrates were approximately at body temperature at the time of injection. The animals were held securely upon their backs by an assistant and the abdomen prepared by swabbing with 70 per cent ethyl alcohol. The needle was then inserted subcutaneously and then pushed gently through the abdominal wall into the peritoneal cavity and the material injected slowly.

Tests for hemolytic activity: The technic for the titration of the hemolytic activity of the staphylococcus toxins as employed by many investigators, varies considerably in its details. The major variation in the different methods rests in the concentration of red blood cells employed. It is apparent that the sensitivity of hemolytic tests, that is, the endpoint of an hemolytic titration, decreases with increase in the concentration of red cells employed. Since it was the object in this investigation only to obtain a measure of the relative quantities of hemolysin in different filtrates, a simple sensitive test was all that was required.

Rabbit, sheep, and human blood cells were employed. Rabbit blood was obtained usually by heart puncture, but occasionally from the marginal ear vein. Sheep blood was obtained from the Bureau of Animal Industry, United States Department of Agriculture, and was usually about one week old. Fresh human blood was obtained from the median basilic vein of one individual, as needed.

To each 5 cc. of rabbit or human blood was added 0.2 cc. of a 20 per cent solution of sodium citrate in physiological salt solution to prevent coagulation. The sheep blood was defibrinated. The cells of all three species were washed in 0.85 per cent salt solution until the supernatant fluid would no longer give a test for proteins when tested with nitric acid. The washed cells were then placed in 15 cc. centrifuge tubes and packed by centrifugation at 3,000 r.p.m. for ten minutes. The packed cells were then diluted with physiological saline solution to a concentration of 20 per cent by volume, and subsequently to a 1 per cent suspension immediately before use.

In the titration suitable quantities of the toxin, or dilutions thereof, ranging from 0.5 cc. of the undiluted toxin to a 1:20,480 dilution, were placed in a series of tubes 10 x 75 mm. and the volume adjusted with physiological saline so that each tube contained 0.5 cc. To each tube was then added 0.5 cc. of a 1 per cent suspension of red blood cells thus making the total volume in each tube 1 cc. and the final concentration of red cells 0.5 per cent. After agitating each tube to obtain thorough mixing, they were placed in a water bath at 37°C. for one hour. The tubes were then removed and the degree of hemolytic action in each tube recorded, after which they were placed in the ice box at 7°C. Final readings were made and recorded after approximately twenty hours in the ice box. The hemolytic titer of each filtrate was expressed as the reciprocal of the highest dilution of toxin which produced complete hemolysis at the end of the full incubation period.

In order to test the activity of the toxic filtrates against human erythrocytes in slightly lower dilutions than obtained by the above method, a 1:1.5 dilution was prepared by adding 0.5 cc. of a 1.5 per cent suspension of human cells to a tube containing 1 cc. of the undiluted filtrate.

The results of the studies on the toxins produced by these strains of staphylococci, as measured by their hemolytic titers and their ability to produce vomiting in cats when sterile filtrates were injected intraperitoneally, are presented in Table V.

Only strains 4, 20, and 37 were proven to be capable of producing an enterotoxic substance when administered intraperitoneally into kittens or young cats. A negative result was obtained on one occasion when a

TABLE V

Hemolytic Titers* and Dolman Kitten Test of Sterile Filtrates of Staphylococcus Cultures.

Culture	Hemolytic Titer* of Filtrates against Red Blood Cells of: Rabbit	Sheep	Human	Dolman Kitten Test: Times Tested	Results
1	320	5	0	1	0
2	1280	80	<1.5**	1	0
3	320	5	0	1	0
4	2560	160	2	3	+ + +
5	160	2	0	2	0 0
6	1280	80	<1.5**	1	0
7	1280	40	0	2	0 0
8	1280	80	<1.5**	1	0
9	640	40	0	2	0 0
10	1280	20	0	1	0
11	1280	40	<1.5**	1	0
12	1280	80	<1.5**	3	0 0 0
13	1280	80	<1.5**	1	0
14	1280	40	0	1	0
15	1280	40	0	2	0 0
16	1280	40	0	1	0
17	1280	80	<1.5**	1	0
18	1280	40	1.5	1	0
19	1280	80	0	1	0
20	2560	160	2	3	+ + 0***
21	1280	80	0	1	0
22	1280	40	0	1	0
23	640	20	0	1	0
24	1280	40	0	3	0 0 0
25	640	40	0	1	0
26	640	5	0	1	0
27	80	0	0	1	0
28	—	-	-	-	Not tested
29	320	5	0	1	0
30	640	20	0	1	0
31	640	40	<1.5**	1	0
32	640	5	0	1	0
33	640	40	0	1	0
34	0	0	0	1	0
35	0	0	0	1	0
36	640	40	0	1	0
37	1280	80	2	3	+ + +
38	—	-	-		Not tested
39	—	-	-		Not tested

*Hemolytic titer expressed as reciprocal of dilution, e.g., 1280 = complete hemolysis 1:1280.

**<1.5 = 50-75 per cent hemolysis in 1:1.5 dilution.

***Adult cat, weight approximately 2 kgm., which had previously been made ill by filtrate of strain 37.

filtrate of strain 20 was injected into an adult cat of approximately two kilograms in weight, which had previously been made ill by the injection of a filtrate of strain 37. However, a subsequent filtrate produced a typical reaction in a young kitten weighing about 900 grams. Strains 7 and 9 each, on one occasion, produced vomiting in kittens but in both instances the animals did not appear acutely ill. Subsequent attempts to produce illness with filtrates of these strains met with failure.

Twenty-seven of the strains were tested only once in kittens, a procedure necessitated by the lack of sufficient animals. In all, a total of only seventeen kittens, or cats, could be obtained for this investigation. In most instances, cats in which a positive reaction had been produced were not used for subsequent tests on strains of unknown toxicity. However, Dolman and Wilson (22) demonstrated that a period of at least two weeks is necessary for the production of an immunity to the enterotoxin and stated that kittens may be used more than once for the detection of enterotoxin if tested within a two weeks' period and allowed to recover sufficiently between injections. With these precautions in mind, in some instances in which it was necessary, animals which had previously reacted positively to the injection of a filtrate, were used again after at least forty-eight hours of rest. A negative reaction in such instances was checked by the injection of one of the known toxic strains. Animals which consistently gave negative reactions were demonstrated to be susceptible to enterotoxin by the injection of a known positive filtrate.

Particular attention is directed to strains 4, 20, and 37. Strains 4 and 20, which were demonstrated in the previous experiments to have lost their ability to produce an orange pigment, but which have retained the ability to produce strong positive Stone reactions, were the only strains among the earliest isolations which have retained the ability to produce enterotoxin. Strain 37 failed to produce a positive Stone reaction when recently isolated but produced enterotoxin. More recent tests, shown in Table IV, indicate that this strain produces a questionable Stone reaction, that is, a zone less than 3 mm. in width.

A study of the hemolytic titers of these strains shows that the majority of them produce hemolysin which is more active against rabbit erythrocytes than against those of sheep or man as demonstrated by various investigators. Thirty-two of the thirty-seven strains on which the hemolytic titer was determined produced hemolysis of rabbit erythrocytes in a dilution of at least 1:320, and twenty of the strains in a dilution of 1:1280 or more. Strains 7 and 23 which failed to produce hemolysis on rabbit blood agar plates, when grown on the Dolman medium in the presence of 25 per cent carbon dioxide, hemolyzed rabbit erythrocytes in dilutions of 1:1,280 and 1:640 respectively.

Glenny and Stevens (28) showed that staphylococcus hemolysins could be differentiated into two types which they called the alpha and beta toxins. Their alpha toxin hemolyzed both rabbit and sheep cells on incubation at 37°C., while the beta toxin hemolyzed sheep cells only and these after incubation at 37°C. followed by incubation in the cold over night, the so-called "hot-cold" phenomenon of Bigger (3). Glenny and Stevens (28) observed that although sheep cells were hemolyzed by alpha toxin they were much less susceptible than rabbit cells to its action.

The hemolytic titers of the staphylococcus filtrates against sheep erythrocytes bear out the lesser susceptibility of the cells of this animal than those of the rabbit to the alpha toxin, but the titers show approximately the same relative potencies for each strain as did the rabbit cell titration.

Roy (47) and Flaum (24) investigated the action of staphylococcus toxins on human erythrocytes and both concluded that the human red cell hemolysin is probably identical with the beta toxin of Glenny and Stevens (28). Roy (47) among others, has pointed out that beta toxin cannot be titrated by its hemolytic action on sheep cells in the presence of a greater quantity of alpha toxin since sheep cells are also susceptible to lysis by the latter toxin. Although the identity of beta toxin and the human lysin cannot be considered as definitely established, it was considered advisable to study the activity of these staphylococcus filtrates against human erythrocytes.

The filtrates of the strains studied possessed little activity against human erythrocytes as shown in Table V. Eight strains produced a + + + hemolysis reaction, a + + + + reaction representing complete hemolysis, in a 1:1.5 dilution of the filtrates. Three strains produced complete hemolysis at a dilution of 1:2, and one strain complete hemolysis at a dilution of 1:1.5.

Strains 4 and 20 differed from others in the collection in that they both produced a slightly greater quantity of hemolysin for all three types of cells, 1:2,560, 1:160, and 1:2 against rabbit, sheep, and human erythrocytes respectively.

With the exceptions of strains 4 and 20, strain 37 produced hemolytic titers equal to that produced by any of the other strains

against rabbit (1:1,280) and sheep cells (1:80) and even equalled the former strains in its action against human cells (1:2).

Other sterile filtrates of strains 4, 20, and 37, were prepared on different occasions. Filtrates of strains 4 and 20 consistently produced highly potent hemolysins for rabbit cells, frequently hemolyzing these cells in a dilution of 1:5,120, and on one occasion a filtrate of strain 4 was active in a dilution of 1:10,240. Filtrates of strain 37 were never active against rabbit cells in dilutions greater than 1:1,280. Filtrates of strains 2, 5, 24, 30, and 36 prepared on other occasions seldom exceeded the original titers shown in Table V. Strain 2, 24, and 36 each on one occasion attained titers of 1:2,560, 1:1,280, and 1:2,560 respectively, but filtrates of strains 4 and 20 prepared at the same time were active in dilutions of 1:10,240 and 1:5,120 respectively.

Studies on the Differentiation of Enterotoxin-Producing Staphylococci with Normal Horse Serum

During the course of this investigation an article by Beumer (2) was located which presented the results of a study on the action of normal horse serum on staphylococcus toxin. Beumer cited the work of Gross who had demonstrated that: (1) the introduction of human or rabbit hemoglobin into broth favored the production of hemolysin by staphylococci, while horse, sheep or beef hemoglobin had no such action; and (2) the fresh normal serum of the last three species possessed the power of preventing the formation of hemolysin by staphylococci when introduced into broth. The latter activity of horse, sheep, and beef serums was attributed by Gross to their greater content of antihemolysins and normal agglutinins, as demonstrated by the following figures, which

were presented by that author and cited by Beumer (2):

Serums	Antihemolysin	Agglutinins
Man	0	1:400
Rabbit	0	1:400
Horse	1:50	1:2,000
Sheep	1:20	1:2,000
Beef	1:40	1:2,000

Gross also noted a marked agglutination of staphylococci in broth containing horse serum, a phenomenon which did not occur in broth to which rabbit or human serum was added. Beumer (2) also stated that Nélis and Poncelet, Ramon, Richou and Descazeaux, Descazeaux and Richou, and Ramon, Richou, Nicol and Lupu have pointed out that fresh normal horse serum normally possesses the power of neutralizing staphylococcus hemolysin in vitro. Forssman (25) studied the antilytic action of the normal sera from four horses and four sheep and found all of them to contain considerable or even large quantities of these substances.

Beumer (2) confirmed the results of Gross in regard to neutralizing action of horse serum on the hemolytic activity of staphylococcus toxin on rabbit cells in vitro. However, he demonstrated that the ability of horse serum to prevent the formation of staphylococcus toxin, when added to the broth in which the organism was grown, was not general, but depended upon the particular strain employed. He studied the production of staphylococcus toxin in a broth prepared according to the directions of Walbum (52) which had the following composition:

Veal	250 grams
Peptone	10 grams
NaCl	1 gram
$MgSO_4$ (M/1 solution)	1 cc.
Distilled water to make . . .	1,000 cc.

Adjusted after sterilization to pH 7.4.

He stated that incubation in this medium for five days at 37°C. was a sufficient period to obtain active toxins. To the above medium he added 10 per cent of normal horse serum.

His study of three strains showed that all of them produced active toxin in the ordinary Walbum broth, but that in broth to which 10 per cent horse serum was added, toxin production by two of the strains was completely inhibited, while the third strain "Jean," produced a toxin of an hemolytic titer equal to that in the ordinary broth.

He further demonstrated that the hemolytic titer produced by this strain was diminished appreciably only when the concentration of horse serum was increased to 50 per cent, and stated that even in pure serum traces of hemolysin were produced. Attempts to adapt his "sensitive" strains by growth in serum broth led him to the conclusion that the "sensitivity" of a strain to horse serum was a stable character which could not be changed. When incorporated in an agar medium horse serum did not inhibit formation of hemolysin. A study of the mechanism of the inhibition of hemolysin formation led Beumer (2) to the conclusion that the strain "Jean" by reason of strong proteolytic action on horse serum was able to destroy its antitoxic power, an activity which was lacking in his other strains. He demonstrated the intense proteolytic activity of the strain "Jean" by growth on coagulated horse serum.

In view of the evidence which Stone (49) and others have presented, although disputed by other investigators, that enterotoxic-staphylococci differ from other strains of this organism by reason of their greater proteolytic activity on a special gelatin medium, Beumer's (2) investigations suggested a study of the effect of

enterotoxin-producing staphylococci on normal horse serum and the effect of the latter upon hemolysin production by these strains.

One and one-half liters of horse serum were obtained through the courtesy of the Bureau of Animal Industry, United States Department of Agriculture. Blood was drawn from a normal horse, allowed to clot and the serum removed, no special precautions being taken to prevent contamination. The serum was stored in the ice box over night and received the following day, within twenty-four hours from the time the whole blood was drawn. Immediately upon receipt the serum was sterilized by filtration through a five inch Berkefeld "V" candle and distributed in sterile 300 cc. Erlenmeyer flasks. The serum in each flask was checked for sterility.

A. Proteolytic action of staphylococcus cultures on horse serum.

Seventy parts of horse serum were diluted with thirty parts of sterile 10 per cent Difco beef extract, and 10 cc. amounts of the mixture were transferred by means of a sterile pipette into sterile petri dishes. The petri dishes were then placed in an Arnold sterilizer and slowly heated until the horse serum had firmly coagulated. The final medium was similar to the Stone medium, therefore, with a final concentration of 3 per cent of beef extract, and horse serum substituted for the gelatin and agar. "Touch" colonies were made by transferring a small amount of growth from a nutrient agar slant culture of each strain to the surface of the medium, care being exercised not to break through the surface. Plates were then incubated at 37°C. for 24 and 48 hours and the results were recorded after each interval.

The proteolytic activity of the staphylococcus strains on coagulated horse serum are recorded in Table VI. The results of the

TABLE VI

Proteolytic Activity of Staphylococcus Strains on Coagulated Horse Serum

Culture	Zone of Proteolysis	Culture	Zone of Proteolysis
	mm.		mm.
1	±	21	1
2	2	22	1
3	1	23	±
4	3L	24	2
5	4L	25	0
6	1	26	1
7	0	27	±
8	±	28	±
9	±	29	±
10	±	30	±
11	±	31	0
12	1	32	0
13	±	33	0
14	±	34	±
15	0	35	±
16	0	36	±
17	±	37	±
18	±	38	±
19	1	39	±
20	4L		

0 = no zone
± = very slight zone
L = liquefaction

forty-eight hour readings only are given here but they differ little from the twenty-four hour results. As is readily seen by inspection of this table, only strains 4, 5, and 20 were actively proteolytic on coagulated horse serum and they not only produced clear zones around the colony but liquefied the medium. It will be noted that these three strains were also the only strains in the group which produced strong positive Stone reactions (Table IV).

Other methods were tested to improve on the above procedure but met with failure. When agar was added to the medium and the amount of horse serum reduced to 50 per cent, proteolytic activity could not be demonstrated with any of the strains. The Dolman medium for toxin production from which the agar was omitted and further modified by the addition of 70 per cent horse serum, gave results similar to those presented in Table VI, but on this medium proteolysis was not very pronounced.

B. Inhibition of hemolysin production by horse serum.

Since a study of the staphylococcus cultures indicated that strains 4 and 20, both of which had been shown to produce enterotoxin, were capable of actively breaking down horse serum, while strain 37, though capable of producing enterotoxin, was devoid of such activity, the next step indicated was a determination of the ability of normal horse serum to inhibit hemolysin production by these strains.

Preliminary studies were conducted by growing a few selected strains in Walbum veal infusion broth, prepared as directed by Beumer (2) and in the Dolman medium modified only in that the agar was omitted. A determination of the hemolytic titers produced by the strains in

these media indicated the Dolman broth to be slightly superior to the Walbum broth for hemolysin production. Neither medium appeared to present optimum conditions for toxin production.

A preliminary experiment was conducted with Dolman broth of the following composition:

Difco Proteose peptone	20 grams
NaCl	5 grams
K_2HPO_4	1 gram
KH_2PO_4	1 gram
$MgSO_4$	0.2 gram
$CaCl_2$	0.1 gram
Distilled water to make	900 cc.

The pH was adjusted to 7.4, the broth distributed in 45 cc. amounts in 100 cc. Erlenmeyer flasks, and sterilized at 250°F. for 20 minutes. To one set of flasks of the above medium 5 cc. of sterile horse serum was added to produce a final concentration of 10 per cent, while to another set 5 cc. of sterile distilled water was added. One flask from each set was inoculated with each of a few selected strains of staphylococci and incubated for five days at 37°C. At the end of this period the broth cultures were centrifuged until clear and the hemolytic titer of the supernatant fluid determined against rabbit erythrocytes by the method described earlier in this paper (pp. 37-38).

The results obtained are presented in Table VII. In this medium only strain 4 showed the ability to produce hemolysin in the presence of horse serum. Strains 20 and 30 both produced an appreciable titer of rabbit cell hemolysin in the absence of horse serum, but strains 6 and 24 produced only very slight quantities, and strain 37 none whatever.

TABLE VII

Hemolysin Production by Selected Staphylococci in Dolman Broth and in Dolman Broth + 10 per cent Horse Serum

Culture	Hemolytic Titer* - Rabbit Erythrocytes	
	Dolman Broth	Dolman Broth + 10 per cent Horse Serum
4	40	80
5	2	0
6	1.5	0
20	20	0
24	1.5	0
36	40	0
37	0	0

*Hemolytic titer expressed as reciprocal of highest dilution producing complete hemolysis.

A similar experiment which differed from that just cited only in that the concentration of horse serum was increased to 20 per cent, and the incubation period increased to eight days, gave the following results (Table VIII):

TABLE VIII

Hemolysin Production in Dolman Broth and in Dolman Broth + 20 per cent Horse Serum

Culture	Hemolytic Titer* - Rabbit Erythrocytes	
	Dolman Broth	Dolman Broth + 20 per cent Horse Serum
4	40	160
5	10	0
20	10	160
36	20	0
37	0	0

*Hemolytic titer expressed as reciprocal of highest dilution producing complete hemolysis.

Inspection of Table VIII shows that strains 4 and 20 not only produce hemolysin in the presence of 20 per cent horse serum, but actually increased quantities of hemolysin were produced as compared with the plain Dolman broth. Strains 5 and 36, which produced hemolysin in the plain broth, failed to produce measurable quantities in the presence of horse serum. Strain 37 in these experiments failed to produce hemolysin in the presence or absence of horse serum. This strain appears to be different in that it produces hemolysin only when grown in a partial atmosphere of carbon dioxide.

This was demonstrated by an experiment in which a few strains of staphylococci were grown for five days at 37°C. in an atmosphere of 20 per cent carbon dioxide, in flasks of plain Walbum broth and also in this broth to which had been added sufficient horse serum to produce a concentration of 10 per cent. The results of this experiment are presented in Table IX.

TABLE IX

Hemolysin Production by Staphylococci
Grown in Atmosphere of 20 per cent Carbon Dioxide in
Walbum Broth and Walbum Broth + 10 per cent Horse Serum

Culture	Hemolytic Titer* - Rabbit Erythrocytes	
	Walbum Broth	Walbum Broth + 10 per cent Horse Serum
4	320	160
5	10	0
6	640	160
20	160	320
24	40	80
36	320	160
37	320	1280

*Hemolytic titer expressed as reciprocal of highest dilution producing complete hemolysis.

Although, as demonstrated in Table VIII, horse serum inhibited the production of hemolysin by certain strains, the results presented in Table IX demonstrate the stimulating effect of a partial atmosphere of carbon dioxide on hemolysin production by most of the strains studied in spite of the presence of horse serum. It is apparent that the stimulating effect of carbon dioxide is greater than the inhibiting effect of horse serum on the production of hemolysin by these strains. The results are also notable in that they demonstrate the great stimulating effect of horse serum upon hemolysin production by strain 37, a producer of enterotoxin, when grown in an atmosphere of carbon dioxide.

After a study of the results on the effect of horse serum on selected strains, it was decided to extend the observations to a larger group of cultures. Since growth in Dolman broth containing 20 per cent horse serum appeared to show the best differentiation between the enterotoxic and non-enterotoxic strains, the same procedure was adopted for this experiment.

In the preparation of the Dolman broth the quantity of the distilled water was reduced to compensate for the added horse serum.

Nineteen strains selected as representative of the entire collection were grown at 37°C. in Dolman broth plus horse serum and, as a control, in the same medium without horse serum. Samples were removed from each culture at intervals of 2, 4, 6, 10, and 15 days. After centrifugation, the clear supernatant fluid of each sample was titrated for hemolytic activity against rabbit erythrocytes by the method previously described (pp. 37-38).

The results are presented in Table X. In this table the hemolytic titers of each culture after incubation for 2, 8, and 15 days only are given since the other intervals did not show significant differences.

TABLE X

Hemolysin Production by Representative Staphylococcus Strains Grown in Dolman Broth and in Dolman Broth + 20 per cent Horse Serum

Culture	Hemolytic Titer* - Rabbit Erythrocytes					
	Dolman Broth			Dolman Broth + 20 per cent Horse Serum		
	2 days	8 days	15 days	2 days	8 days	15 days
4	10	20	40	2	80	40
5	2	2	10	0	0	0
6	0	0	±	0	0	0
7	0	0	±	0	0	0
9	0	0	±	0	0	0
10	0	0	0	0	0	0
12	0	0	±	0	0	0
16	0	0	±	0	0	0
19	0	0	±	0	0	0
20	10	10	20	0	160**	160
23	0	0	±	0	0	0
24	5	2	20	0	0	0
26	0	0	±	0	0	0
27	0	2	2	0	0	0
30	0	0	5	0	0	0
33	0	0	±	0	0	0
36	40	80	80	0	0	0
37	10	0	±	0	0	0
38	0	0	±	0	0	0

± = at least 50 per cent hemolysis in lowest dilution tested, 1:2.

*Hemolytic titer expressed as reciprocal of highest dilution producing complete hemolysis.

** = while strain 20 was negative after two days, the hemolytic titer was 1:40 after four days, and 1:160 after six days.

A study of Table X reveals that only strains 4 and 20 were capable of producing hemolysin when 20 per cent horse serum was present in the medium. This result confirmed the preliminary experiments cited (Table VIII). While only seven of the strains produced any appreciable amount of hemolysin in the plain Dolman broth, most of the strains produced at least traces of hemolysin in the medium after fifteen days' incubation. Strain 37 is again of interest in that it produced a low hemolytic titer in plain Dolman broth after two days, which disappeared on longer incubation and failed completely to produce hemolysin in the presence of horse serum.

C. Agglutination of staphylococci by normal horse serum.

The previous experiments have demonstrated that two of the three strains of enterotoxin-producing staphylococci differ from other strains as indicated by their ability to produce a strong positive Stone reaction, to liquefy coagulated horse serum, and to produce rabbit erythrocyte hemolysin when grown in Dolman broth containing 20 per cent normal horse serum. The third enterotoxin-producing strain (37) differed from those two (4 and 20) in that it was not actively proteolytic on Stone's medium and coagulated horse serum. Since this strain was shown to produce hemolysin only when grown in an atmosphere of carbon dioxide, a direct comparison could not be made of the ability of the three strains to produce hemolysin in the presence of horse serum. However, when grown in a partial atmosphere of carbon dioxide, the considerable increase in titer of rabbit erythrocyte hemolysin produced by strain 37 in broth containing horse serum as compared with that produced in broth without horse serum, indicated a relationship to the other enterotoxic strains. Therefore, other means were sought by which the similarity or dissimilarity of the three enterotoxic strains could be established.

Normal horse serum, as was indicated in the discussion of Beumer's (2) investigations, has been shown to contain normal agglutinins for staphylococci. Since strains 4, 20, and 37 had shown certain differences from other strains in their behavior in the presence of horse serum, it appeared possible that they might also differ in their relation to the normal agglutinins of this serum.

Agglutination reactions were conducted with six selected cultures which included strains 4, 20, and 36, the enterotoxin producers, and strains 7, 9, and 36, which do not produce enterotoxin. The technic was as follows:

Antigen: The growth from twenty-four hour nutrient agar slant cultures of each strain was suspended and diluted with 0.85 per cent salt solution to approximately the same turbidity as determined by visual examination in a strong artificial light. Antigens prepared from strains 4 and 20 were filtered through paper before use to remove any clumps which could not be completely suspended. The other strains produced suspensions free of clumps.

Horse serum: The normal horse serum used in these tests was from the same lot as that used in earlier experiments.

Serial dilutions of the horse serum ranging from 1:10 to 1:5,120 were prepared and placed in 0.5 cc. quantities, in a series of agglutination tubes 10 x 75 mm. Antigens of the respective strains were added in 0.5 cc. quantities to produce a total volume of 1 cc. in each tube. The incubation time and temperature were varied and are recorded with individual experiments. The results of these tests were recorded so that + + + + indicates complete agglutination, 0 no agglutination, while + + +, + +, +, and ± indicate approximately 75, 50, 25, and less than 25 per cent agglutination, respectively.

In the first experiment the agglutinations were carried out at 37°C. for twenty-four hours. The results of this experiment are given in Table XI.

TABLE XI

Agglutination of Staphylococcus Strains
by Normal Horse Serum

Culture	37°C. - 24 hours Serum Dilutions										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	C
4	±	±	+	++	+++	±	±	0	0	0	0
20	±	±	++	+++	++	+	±	±	0	0	0
37	±	±	+	++	++	±	0	0	0	0	0
7	++++	++++	++++	++++	++++	++++	++	+	±	0	0
9	++++	++++	++++	++++	++++	+++	+	0	0	0	0
36	++++	++++	++++	++++	++++	+++	++	±	0	0	0

C = control tube containing 0.5 cc. antigen and 0.5 cc. of 0.85 per cent salt solution.

The results presented in Table XI showed very definitely that the enterotoxic staphylococci differed from the other strains in their resistance to agglutination by normal horse serum. In the lower serum dilutions strains 4, 20, and 37 showed a definite growth, which was lacking in the other strains. This fact suggested that the serum agglutinins were destroyed by growth of the enterotoxic strains in the lower dilutions, while in the higher serum dilutions in which there was no evidence of growth by these strains, partial agglutination occurred. However, the fact that strains 7, 9, and 36 were completely agglutinated at higher serum dilutions than strains 4, 20, and 37, and that strain 37 had previously been found apparently devoid of proteolytic activity on horse serum suggested that the difference in agglutinability was probably due to some inherent characteristic.

Two additional series of agglutination tests were set up in which the factor of growth was minimized by alteration of the incubation time and temperature. One series was incubated at 37°C. for three hours, the other at 55°C. for three hours, followed by storage of both series at 7°C. over night after which readings were made. The technic of these tests were otherwise identical with the previous experiment.

The following table (Table XII) presents the results of this experiment.

TABLE XII

Difference in Agglutinability of Enterotoxic and Non-Enterotoxic Staphylococci by Normal Horse Serum

	37°C.-3 hours, 7°C. over night						55°C.-3 hours, 7°C. over night					
	Serum Dilution						Serum Dilution					
Culture	1:10	1:20	1:40	1:80	1:160	C	1:10	1:20	1:40	1:80	1:160	C
4	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	++	+	+	0	0	0
37	0	0	0	0	0	0	0	0	0	0	0	0
7	++++	+++	+	±	0	0	0	0	0	0	0	0
9	+++	+++	±	0	0	0	++	++	±	0	0	0
36	++++	+++	+	0	0	0	+	+	0	0	0	0

C = control tube containing 0.5 cc. antigen and 0.5 cc. of 0.85 per cent salt solution.

The results presented in Table XII demonstrate that enterotoxin-producing staphylococci (strains 4, 20, and 37) resist agglutination by normal horse serum when the tests are carried out at 37°C. for three hours followed by prolonged incubation at 7°C. Under the same conditions

the non-enterotoxic strains were completely, or almost completely, agglutinated in 1:10 dilutions of the serum. With this incubation no evidence of growth occurred in the horse serum dilutions or in control suspensions in 0.85 per cent salt solution. The tests carried out at 55°C. for three hours followed by incubation at 7°C. failed to show any significant difference between the strains.

The results of the experiments on the agglutination of staphylococci by normal horse serum strongly suggest that a fundamental difference exists between those strains which are capable of producing enterotoxin and those which are devoid of this power.

DISCUSSION

The results of these studies of thirty-eight strains of staphylococci isolated from specimens of food incriminated in food poisoning outbreaks and one strain from a pathological process, reveal several points worthy of discussion.

Most authorities agree that the strains of pathogenic staphylococci usually produce an orange pigment. It will be recalled that all but one of the strains which were regarded as of etiological significance at the time of isolation, produced an orange pigment. The results of this study show that the majority of the strains have retained this character. Two strains, numbers 4 and 20, while originally orange pigmented types, now produce white growths on nutrient agar slants. These two strains, with strain 37 which was only recently isolated, were the only strains which produced enterotoxin as determined by the Dolman kitten test.

The most outstanding point of interest revealed by the comparison of the original with the present cultural and biochemical characteristics of the strains is the loss of the ability by most of them to actively liquefy gelatin, as judged by their Stone reaction. Only three strains, two of which produce enterotoxin (4 and 20), showed marked proteolytic action on the Stone medium. The remaining strains, while exhibiting some degree of activity on the Stone medium, were by comparison with the three former strains only weakly proteolytic.

The fermentative ability of the strains, as judged by their fermentation of lactose and mannitol, does not appear to have altered during storage.

Winslow, Rothberg, and Parsons (53) found that staphylococci which failed to liquefy gelatin tended to be less active fermenters than the liquefiers. They also stated that the white chromogens in this group were less pathogenic, less active in gelatin liquefaction, and slightly less vigorous in their fermentative reactions than the orange types.

The white strains 4 and 20 which actively liquefy gelatin, ferment lactose and mannitol, and produce enterotoxin appear to differ from the white strains studied by Winslow, Rothberg, and Parsons (53).

It is apparent from the results obtained that there is some relationship between the proteolytic activity of most of the strains and their ability to produce enterotoxin. Since strain 5 is exceptional in that it is actively proteolytic but does not produce enterotoxin, and strain 37 exceptional in that it is not actively proteolytic but produces enterotoxin, it is necessary to conclude that some fundamental character of a strain other than, but perhaps associated with, proteolytic activity may govern the ability to produce enterotoxin.

Burnet's (7) conclusion that the hemolytic titer of filtrates of staphylococci as determined against rabbit erythrocytes is a reliable measure of toxin production, seems to be borne out by this study. The hemolytic titer of sterile filtrates of strains 4 and 20, when grown in Dolman's medium in a partial atmosphere of carbon dioxide, as tested against both rabbit and sheep erythrocytes, were consistently higher than those produced by other strains grown under the same conditions. Strain 37 produced hemolytic titers, which, while not as high as those produced by strains 4 and 20, attained as high a level as that produced by any of the other strains.

The hemolysin produced by these strains of food-poisoning origin was of the alpha type which, as shown by Glenny and Stevens (28), produces hemolysis of both rabbit and sheep erythrocytes at 37°C. It is impossible to determine the presence of small amounts of beta hemolysin in the presence of an excess of alpha hemolysin. The probable identity of beta hemolysin with the lysin which is active against human cells, was established by Roy (47) and Flaum (24). The toxins which they studied hemolyzed human cells in the cold after a preliminary incubation at 37°C.

In the study on the hemolytic activity of filtrates it was pointed out that strains 4, 20, and 37 produced complete hemolysis of human erythrocytes at a dilution of 1:2. Several other strains, while producing partial hemolysis of human erythrocytes, were not effective at this dilution. Although the hemolytic titrations of strains 4, 20, and 37 against human cells is not appreciably higher than those produced by other strains, the results indicate a further difference between the enterotoxic and non-enterotoxic strains. The human lysin produced by these strains differed from that discussed by Roy (47) and Flaum (24) in that it produced hemolysis of human erythrocytes at 37°C.

Rigdon (46) studied the Dolman kitten test and found that the injection of as little as 5 cc. of sterile broth would produce vomiting in kittens and dogs, while at the same time demonstrating that the injection of boiled filtrates of enterotoxic staphylococci often failed to produce symptoms of food poisoning in the animals. Dolman and Wilson (22) state that if precautions are taken to warm the filtrates to body temperature and inject them slowly, false positive reactions do not occur.

During the course of these studies filtrates were injected into kittens or young cats fifty times, and on only two occasions were false positive reactions obtained. Re-injection of these filtrates with strict observation of Dolman's precautions resulted in negative reactions. In other cases in which positive reactions were obtained, repeated injections of other sterile filtrates of the same strain produced positive results indicating the test to be specific.

As indicated previously in this discussion, only three strains, 4, 20, and 37, could be demonstrated by the Dolman test to produce enterotoxin. A direct comparison of these results with those given in Table III cannot be made because the toxicity of every individual strain was not determined when originally isolated, and further, because the toxicity of some strains was determined by feeding tests in which evidence of toxicity was indicated only by the production of diarrhea. The following facts collectively indicate that at least some of the strains from each source were capable of producing enterotoxin when originally isolated:

1. They were isolated from food specimens incriminated by epidemiological evidence in outbreaks in which the symptoms and incubation periods were characteristic of staphylococcus food poisoning.

2. They were isolated, frequently in pure culture, from food specimens in which no other known food-poisoning types were present.

3. Filtrates fed or injected intraperitoneally demonstrated some evidence, and in many cases, definite evidence of toxicity.

Bearing in mind the limitations imposed by the lack of data on kitten tests with individual strains at the time of isolation, a comparison of the toxicities of the strains at the time of isolation and during this investigation clearly demonstrates that many of these strains have lost the power to produce enterotoxin during the relatively long storage period on laboratory media. This is in agreement with the findings of Jordan and Burrows (33) and other investigators.

The study of the relation of food poisoning staphylococci to normal horse serum was prompted by the observations of Beumer (2). He showed that while hemolysin production of most pathogenic staphylococci was inhibited when grown in broth containing normal horse serum, certain strains did not exhibit this property. He presented evidence that the ability of certain strains to produce hemolysin in the presence of horse serum was due to the destruction of the normal antilysins by the proteolytic breakdown of the serum proteins by these strains. In view of the evidence presented by Stone (49) that food poisoning staphylococci are more actively proteolytic to gelatin than other strains, it appeared possible that they might also be more actively proteolytic against horse serum than non-food-poisoning types. A study of this collection of staphylococci showed that the same strains which produced strong positive Stone reactions would also liquefy coagulated horse serum. Since strain 37, which produced enterotoxin was not proteolytic on Stone's medium or coagulated horse serum, and strain 5 was actively proteolytic on both but failed to produce enterotoxin, it appeared that the proteolytic activity of a strain was of no value in the interpretation of its ability to produce enterotoxin.

A determination of the ability of normal horse serum to prevent hemolysin production by these strains produced more promising results. The proteolytic, enterotoxin-producing strains were found to be capable of producing greater quantities of hemolysin in broth containing horse serum than in control broth not containing the latter substance. The proteolytic, non-enterotoxic strain 5 produced small amounts of hemolysin in the absence of horse serum and no hemolysin when horse serum was present in the broth. None of the other strains in the collection were capable of producing hemolysin in the presence of horse serum, while most of them produced at least traces of hemolysin in control broth. Enterotoxic strain 37 failed to produce hemolysin in the presence or absence of horse serum when grown in air, but when grown in a partial atmosphere of carbon dioxide produced much greater quantities of hemolysin in the presence of horse serum than in its absence.

It appeared from these results that some enterotoxic strains could be differentiated from non-enterotoxic strains by their ability to produce hemolysin for rabbit erythrocytes in the presence of normal horse serum, but that certain enterotoxic strains, by reason of their more fastidious growth requirements, could not be separated by this method. This led to a study of the effect of normal horse serum upon the microorganism itself rather than upon the toxins produced.

When the effect of normal horse serum was tested upon suspensions of living organisms by means of the agglutination reaction the results showed a marked difference between the enterotoxic and non-enterotoxic strains. In those tests in which the tubes were incubated at 37°C. for twenty-four hours, agglutination occurred at much higher dilutions

of the horse serum than in those incubated at 37°C. for three hours and then at 7°C. over night. With the former conditions of incubation the enterotoxic strains showed marked growth in the lower serum dilutions with production of a diffuse turbidity, whereas in the higher dilutions where no apparent growth took place, only incomplete agglutination of the finely granular type occurred. The strains which did not produce enterotoxin showed complete agglutination in the serum dilutions from 1:10 to 1:160 or 1:320 with the formation of large aggregates or clumps.

Incubation at 37°C. for three hours followed by storage in the ice box over night resulted in lower agglutinin titers, but such incubation accentuated the difference between enterotoxic and non-enterotoxic strains. Enterotoxic strains were not agglutinated in a 1:10 dilution of horse serum, while two of the non-enterotoxic strains were completely agglutinated in a dilution of 1:10 and the other, almost completely in this dilution.

With a full realization of the limitations imposed upon any interpretation of the above results by reason of the fact that only one horse serum and a few staphylococcus strains were studied, the significance implied by these results is of great importance. The results strongly suggest the probability that a fundamental difference exists between enterotoxic and non-enterotoxic strains of staphylococci in their agglutinability by normal horse serum.

A consideration of our general knowledge of the serological relationships of microorganisms, and in particular between organisms within a group or genus, would indicate that this difference depends primarily upon a difference in antigenic structure. Food-poisoning staphylococci do not appear to differ essentially in their cultural

and biochemical properties from ordinary pathogenic types, and the difference between them may rest in the possession, by the food-poisoning types, of a different surface antigen. However, Stritar and Jordan (50) demonstrated by agglutination and agglutinin adsorption tests that the food-poisoning staphylococci are a heterogeneous group, and from their work it would seem that a difference in antigenic structure cannot be considered the explanation of the phenomenon.

Whatever the mechanism may be, if more extensive studies reveal that the phenomenon is common to all enterotoxic staphylococci and is produced by other normal horse serums, it will offer a simple and rapid method for typing food-poisoning strains. That such a test is needed is beyond dispute since the simplest and most reliable physiological method, the Dolman kitten test, possesses disadvantages which limit its general usefulness.

SUMMARY AND CONCLUSIONS

1. Thirty-eight strains of staphylococci isolated from specimens of foods involved in eleven outbreaks of food poisoning, and one strain of pathological origin, were studied.

2. The original cultural, biochemical, and physiological characteristics of the strains, as far as could be determined from the available data, indicated that with two exceptions the food-poisoning strains commonly produced an orange pigment, hemolysis of rabbit or sheep blood agar, and were active in their ability to ferment carbohydrates, liquefy gelatin, and produce symptoms of food poisoning in cats.

3. The present cultural and biochemical characteristics as determined in this investigation demonstrates that storage of the strains under laboratory conditions has resulted in the loss of the ability by the majority of the strains to actively liquefy gelatin, which is correlated with the loss of the power to produce enterotoxin.

4. Two strains, 4 and 20, which lost their ability to produce an orange pigment, retained their power to produce a positive Stone reaction (gelatin liquefaction), and to produce enterotoxin. These strains also produced greater quantities of toxin than the other strains as measured by the production of hemolysin for rabbit erythrocytes.

5. A third strain (5) produced a strong positive Stone reaction but failed to produce enterotoxin.

6. A recently isolated strain (37) failed to produce a positive Stone reaction but produced enterotoxin.

7. The three strains (4, 5, and 20) which produced strong positive Stone reactions also liquefied coagulated normal horse serum, while strain 37 failed to do so.

8. The enterotoxic strains 4 and 20 produced greater quantities of hemolysin for rabbit erythrocytes in broth containing 20 per cent normal horse serum than in its absence.

9. The addition of 20 per cent normal horse serum to broth inhibited the production of hemolysin for rabbit erythrocytes by all of the strains except the enterotoxin-producing strains 4 and 20. The third enterotoxic strain, 37, failed to produce rabbit cell hemolysin when grown in air, but when grown in a partial atmosphere of carbon dioxide produced greater quantities of hemolysin in the presence of 20 per cent normal horse serum than in its absence.

10. When the effect of normal horse serum was tested on representative strains by means of the agglutination reaction, it was found that enterotoxic strains were agglutinated poorly or not at all while non-enterotoxic strains were completely agglutinated at relatively high serum dilutions, when the tests were incubated at 37°C. for 24 hours, or at 37°C. for 3 hours, followed by storage over night at 7°C.

11. While no definite conclusion can be drawn from the study of such a small series of strains, it is suggested that the characteristic of a strain, as indicated by its failure to agglutinate in normal horse serum or dilutions thereof, may prove to be a simple and rapid method for the detection of food-poisoning staphylococci.

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