EXPERIMENTS ON *STAPHYLOCOCCUS* FOOD POISONING

By F. C. MINETT

Research Institute in Animal Pathology, Royal Veterinary College, London

The observations of Jordan, Dack and others in Chicago have clearly shown the importance of staphylococci as a cause of food poisoning. Of the many foods that have been implicated cow's milk is one, and was undoubtedly responsible in the outbreaks described by Barber (1914), Crabtree & Litterer (1934) and Shaughnessy & Grubb (1936, 1937), while the observations of Ramsey & Tracy (1931) and Tanner & Ramsey (1932) were also concerned with staphylococci from milk.

The present work has special reference to the food-poisoning substance—which for convenience may be called "enterotoxin"—produced in culture by staphylococci of the cow's udder, or in milk or milk products naturally or artificially contaminated with staphylococci.

With regard to cultural methods, Jordan (1930) found that enterotoxin was produced in plain infusion broth, *p*H 7·6, within 48 hr. at 37° C., while Jordan & Burrows (1934*a*, 1935) showed that larger amounts of it could be obtained by growing the organisms on 1·0% agar under CO_2. Thus some strains which no longer produced enterotoxin in plain broth would still do so on the soft agar medium under CO_2, while some strains which formed no enterotoxin in the ordinary atmosphere would do so in an increased concentration of CO_2. Twenty food-poisoning strains in their laboratory, however, lost their enterotoxic power in time even when grown by the CO_2 method, but this power could be restored by a few transfers on a solid medium containing starch. In my experiments it was again found that under ordinary atmospheric conditions enterotoxin is formed in plain broth or in broth containing 5% sheep blood, but as a routine cultures were grown on 0·8% beef heart agar in Roux bottles incubated in an atmosphere of 20% CO_2. After 36 hr. at 37° C., 10·0 c.c. saline was added to the flask, and as much fluid as possible was pressed out of the agar through muslin, the fluid centrifuged and the supernatant passed through a small Berkefeld filter, after which, in order to destroy the α toxin which some strains produce in addition, the filtrate was placed in a bath of boiling water for 30 min. or treated with 0·3% formalin for about 36 hr. at 37° C. Stock cultures have been stored in Worth's medium at room temperature and subcultured every 3 months. Under these conditions the capacity of staphylococci to form enterotoxin was well preserved.

The following strains were used:

Strain 95, isolated from cream filling associated with food poisoning, and forwarded by the late Prof. E. O. Jordan.

Strain 18B, isolated by Dr W. M. Scott from milk in connexion with an outbreak of food poisoning due to custard.

Strains of *Staphylococcus aureus* from normal milk or from mastitis secretion; these and other cultures are designated in the text.

https://doi.org/10.1017/S0022172400011463 Published online by Cambridge University Press

In studying the formation of enterotoxin in foodstuffs, the experimental conditions were made to simulate the natural as closely as possible. The methods used are given in the appropriate sections.

Effect of enterotoxin on animals and man

The first experiments were made on cats and dogs, and later on monkeys, filtrates known to contain enterotoxin being given by the mouth. Afterwards, when Dolman's method of injecting enterotoxin intraperitoneally became available, kittens alone were used.

Experiments with monkeys

Most of the monkeys used were *Macacus rhesus*, occasionally, according to the dealer, they were Java monkeys. The filtrate to be tested was given in amounts of 20·0–30·0 c.c., usually alone but sometimes mixed with about 10·0 c.c. pasteurized milk, by means of a stomach tube, either on an empty stomach or shortly after a light feed. Close observation was then kept for 5 or 6 hr. Vomiting, single attacks or repeated, may take place at any time up to 5 or 6 hr. after feeding, but most commonly it begins after 1–3 hr. Vomiting may be sudden and without any obvious premonitory sign, or it may be preceded by such symptoms as salivation, yawning, rubbing the nose, shaking the head, grinding the teeth. Monkeys frequently regurgitate material into the mouth and then slowly swallow it, but it is doubtful if any significance should be attached to this act, since it was observed at times after unsown filtrate had been fed.

The results indicated that the propensity of monkeys to vomit after being given enterotoxin in this way varied a good deal. Thus, only seventeen out of forty-one monkeys tested with *Staphylococcus* filtrates containing enterotoxin vomited; but some of the others evinced distinct signs of abdominal pain, with diarrhoea. Definitely positive results in monkeys are, however, important, since these animals can be regarded as the best substitute for human volunteers. There is therefore much significance in the fact that vomiting was produced in monkeys by filtrates, not only of the food-poisoning strain, 95, but also of two strains of *Staph. aureus* from cases of acute and chronic mastitis respectively, and of two strains from the milk of normal cows.

Finally, feeding tests were made on nine monkeys to see whether vomiting could be produced by milk samples in which strain 95 and an udder strain, 189, had grown for 2 days at 22° C. After this time the *Staphylococcus* counts ranged from 9 to 40 millions per c.c., and from 20·0 to 25·0 c.c. of mixed milk and cream was fed to each monkey. The results were negative.

Experiments with dogs

Dogs 6–12 months old were used, the animals being allowed to consume on an empty stomach amounts of filtrate varying as a rule from 20·0 to 40·0 c.c. mixed with an equal amount of pasteurized milk. In a preliminary

series of experiments five dogs were fed with filtrate prepared from strain 95 but only one vomited. One of the others had been given 6·0 c.c. of a filtrate which in a dose of 4·0 c.c. had produced 4 days previously violent disturbance in a human volunteer (no. 1). In this dog no symptoms were observed on the day of feeding, but 24 hr. later there were signs of slight intestinal disturbance, the stools being dull green and excessively moist. During the next 6 hr. the diarrhoea increased, the stools being very watery. Four days later the same animal was given 20·0 c.c. of another filtrate, prepared from the same stock culture of strain 95, and symptoms of intestinal disturbance were again observed 24 hr. later.

A number of tests on dogs were also made with filtrates from other strains of *Staph. aureus*, including some udder strains, but all these were negative and it seemed evident that the feeding of dogs was not a useful means of detecting enterotoxin.

Experiments with cats

In view of the statements of Tanner & Ramsey (1932), kittens 10 days to 5 weeks old were tried, 5·0–20·0 c.c. filtrate, mixed with an equal volume of pasteurized milk, being fed by means of a pipette. Twelve kittens were dosed with strain 95 filtrate but vomiting was not induced. Experiments were then made with adult cats. Three samples of filtrate prepared with strain 95 were fed in amounts of 20·0–40·0 c.c., mixed with pasteurized milk, to twenty-two cats, and of these six vomited. These filtrates were used at the same time for feeding twenty monkeys, of which seven vomited. From the results with these few animals, therefore, it seems that the susceptibility of monkeys and of adult cats is of much the same order.

After the publication by Dolman *et al.* (1936), setting out the value of the intraperitoneal route for administering enterotoxin, kittens were again brought into use, and all the results detailed below were obtained by their method. Ample evidence has been obtained that at present this is the best means of detecting enterotoxin. Kittens varying in weight from 400 to 1000 g. have been used; as a routine in the case of filtrates 3·0–4·0 c.c. amounts were given, though amounts as low as 0·25 c.c. may suffice. Most of the kittens showed three or four bouts of vomiting, usually from 20 to 60 min. after injection, and this is preceded by crying, signs of restlessness and shaking the head; in addition, urine and fluid stools are often passed. An occasional kitten shows diarrhoea only, but this has rarely been taken into account in assessing the results. Kittens which fail to vomit after a test injection almost invariably do so when retested with a filtrate of proved potency. Vomiting within 5–7 min. of injection, which might therefore have been due to mechanical causes, occurred only in quite exceptional instances. Kittens which have vomited generally die a few days later, although in the meantime their general condition has apparently become normal. On post-mortem examination of kittens which have shown repeated vomiting and diarrhoea, no obvious lesions

can be seen, and as Dolman *et al.* point out, it is wrong to speak of enterotoxin as a gastro-intestinal irritant.

Experiments on man

Owing to varying susceptibility, negative results in man with feeding experiments do not of course necessarily indicate the absence of the food-poisoning principle. On one occasion, 4·0 c.c. strain 95 filtrate diluted with 6·0 c.c. pasteurized milk was taken by an adult male (volunteer 1). This was followed by headache, nausea, repeated vomiting and frequent diarrhoea, the symptoms starting about $2\frac{1}{2}$ hr. after the feed and continuing for the next 3 hr. The following day he felt out of sorts, but afterwards his condition was normal. An adult female (volunteer 2) was ill after being given a mixture of 6·0 c.c. pasteurized milk and 6·0 c.c. filtrate prepared from a *Staphylococcus* isolated from a clinical case of mastitis in a cow (strain W). Soon after taking the mixture, the volunteer complained of headache; she vomited three times within 2 hr. and also had diarrhoea. In this case the first vomit occurred 5 hr. after dosing. An adult female (volunteer 6) took without ill effect 6·0 c.c. unsown agar filtrate with an equal volume of pasteurized milk.

Subsequently, experiments were made with naturally infected milk and cream from two cows with chronic *Staphylococcus* mastitis (191 and 118). Most of the experiments were done in two stages, the volunteers consuming a meal of fresh cream and milk from the affected quarter and a few days later a mixture of cream and milk which had been stored for 2 days at 22° C. The actual results, most of them negative, are recorded in Table II.

Production of enterotoxin by udder staphylococci and some other organisms

Jordan & Burrows (1935) have shown that enterotoxin is produced by various kinds of bacteria, when they are grown under suitable conditions.

In the experiments recorded in this section filtrates from soft agar cultures grown under CO_2 were placed in a boiling water bath for 30 min. and then injected intraperitoneally into kittens.

Staphylococci

Thirty-eight strains of *Staph. aureus* from the bovine udder were tested and of these sixteen were shown to produce enterotoxin. Seven of the thirty-eight strains were isolated from cows with acute mastitis, eight from cases of chronic mastitis, and the rest from the "normal" milk of seventeen cows; in these three groups enterotoxin production was demonstrated with four, five and seven strains respectively. Twelve strains of saprophytic (non-haemolytic) staphylococci from the milk of as many cows were tested; enterotoxin production was detected with one strain only and then only once out of three attempts.

Streptococci

Evidence that poisonous substances may develop in food owing to contamination with streptococci has been furnished by Jordan & Burrows (1934). It was important, therefore, to enquire whether the common mastitis *Streptococcus* (*Str. agalactiae*) would behave similarly.

Fifteen β-haemolytic strains of this organism were tested, but none of them gave positive results when boiled filtrates were used, though two produced vomiting when injected in the unheated state. The unheated filtrates from four of the other strains produced no symptoms.

Bacterium coli

Strains of this organism isolated from four calves with "white scours" and from the milk of a cow with acute mastitis were tested and four of these, including the one from the cow, caused vomiting. Those giving positive results had been in stock for 4–10 months and in one case for 3 years before being tested.

PRODUCTION OF *STAPHYLOCOCCUS* ENTEROTOXIN UNDER NATURAL CONDITIONS

Barber (1914), investigating cases of food poisoning due to milk, found that he could consume without ill effect refrigerated cream from the suspected herd, whereas a mixture of milk and cream from the same milk sample after storage for 5 hr. at 28–30° C. produced gastro-intestinal disturbance, although at that time the flavour of the mixture was not noticeably changed. He was similarly affected after drinking 50·0 c.c. of sterile preserved milk which, after being sown with a white *Staphylococcus* isolated from the milk of one of the cows, had been incubated at 36·5° C. for about 8½ hr. Ramsey & Tracy (1931) showed that raw milk, inoculated with an orange *Staphylococcus* isolated from milk with a malt-like flavour, would give rise to food poisoning in man.

It was decided to investigate the formation of enterotoxin in milk and other products under experimental conditions simulating those of nature.

In milk

In preliminary experiments it was shown that enterotoxic filtrates when mixed with milk are not inactivated within 2 or 3 days at 22° C.

Tests for the production of enterotoxin were made with milk, artificially or naturally infected. With the former, two or three drops of a 24 hr. broth culture of a *Staphylococcus* known to produce enterotoxin were transferred to 20·0–25·0 c.c. milk taken from an udder free from streptococci and *Staph. aureus*, and the milk was then placed at the required temperature (usually 22° C.) for 2 or 3 days. In all cases counts were made immediately after seeding and at daily intervals during the experiment, by plating a sample of the whole milk in ox-blood agar. The milk was used in the raw state, after laboratory pasteurization (63° C., 30 min.) and after laboratory sterilization (100° C., 30 min.). When ready for test, the milk was boiled for 30 min. and injected into kittens in doses of 5·0 or 10·0 c.c. In the few instances where the milk was so acid that it clotted on boiling, the clot was broken up and the fluid expressed through muslin was injected.

Artificially contaminated milk. The formation of enterotoxin under these conditions was demonstrated with strain 18B and with two udder strains of

Staph. aureus (683 and 191). The results of the actual tests can be summarized thus. Strains 18B and 683 gave positive results with raw, pasteurized and sterilized milk after 2 or 3 days at 22° C., the milks then still being normal in appearance, whereas no enterotoxin was produced in the corresponding seeded milks kept in the cold. Strain 18B also gave positive results with pasteurized and sterilized milks held at 37° C. for 3 days (by which time the milks had curdled), and with sterilized milks at 18° C. after 2 days, when the milk was still fluid. Strain 191 gave a positive result with pasteurized milk held at 22° C. for 2 days, the milk then being normal in appearance. On one occasion strain 18B failed to grow appreciably in raw or pasteurized milk at 18° C. within 3 days and no enterotoxin had been produced. Another negative result was obtained with strain 189, using pasteurized milk kept at 22° C. for 3 days, although the strain was known to be capable at that time of producing enterotoxin in agar and had grown well in pasteurized milk. For control purposes the unseeded milk was tested after 2 or 3 days at 22° C.; sterilized milk, and as a rule pasteurized milk also, under these conditions did not induce vomiting in kittens even when injected in the unheated state, whereas the raw milk was liable to do so in the unheated state and also at times after being heated at 65° C. for 30 min. After being boiled for 30 min., however, the milk was inert.

The initial *Staphylococcus* count of the milk in these experiments commonly varied from about 0·6–1·5 millions per c.c., while after 48 hr. at 22° C. the count frequently reached 25·0–150·0 millions per c.c.

Naturally contaminated milk. In these experiments the milk used was that of cow 118, affected with chronic *Staphylococcus* mastitis in the R.H. (right-hind) quarter, from which large numbers of *Staph. aureus* were being excreted. The other three quarters of this animal were clean. In two experiments it was shown that milk freshly drawn from the R.H. quarter contained sufficient enterotoxin in 10·0 c.c. to make kittens vomit, whereas vomiting was not induced with 5·0 c.c. amounts. In one experiment milk from each of the three clean quarters failed to cause vomiting in a dosage of 10·0 c.c., either when freshly drawn or after storage for 2 days at 22° C. Milk from the R.H. quarter, after storage at 22 or 37° C. for 2 days, caused vomiting in 5·0 c.c. amounts; the same milk stored in the cold for 2 days was inert at this dose. It should be noted that after 2 days at 22° C. the R.H. milk appeared quite normal, but in one of the two experiments it coagulated when heated to 65° C.; after 2 days at 37° C. the milk had clotted. After 2 days at 22° C. final *Staphylococcus* counts in the neighbourhood of 70·0 millions per c.c. were recorded.

The opportunity was taken with the milk of this naturally infected cow to test the relative susceptibility of kittens to enterotoxin given by the intraperitoneal route or by the mouth. Raw milk, freshly taken from the infected R.H. quarter, failed to produce vomiting at a dose of 5·0 c.c. intraperitoneally or when 50·0 c.c. was drunk. After the same milk had been stored for 2 days at 22° C. and then boiled for 30 min., however, vomiting was produced in

three kittens after intraperitoneal injection of 5·0, 2·0 and 0·5 c.c. amounts respectively. Two kittens were given by stomach tube 50·0 c.c. each of the stored milk in the unheated state with the result that one showed no symptoms while the other vomited 30 min. later.

By comparison, milk from the clean left fore-quarter failed to produce vomiting either in the fresh state or after storage at 22° C. for 2 days, when given in doses of 5·0 c.c. boiled milk intraperitoneally or when 50·0 c.c. of unheated milk was drunk.

In cheese

Cheese has been suspected in several instances of food poisoning. Levin (1917) investigated six cases which were evidently due to the consumption of "American cheese". The poisoning was apparently caused by organisms of the colon group, which were grown from the cheese and cultures of which produced vomiting on injection into cats. *Bact. coli* was also apparently responsible in the cases reported by Kathe (1937).

Small cheeses, approximately 1 lb. in weight, were made from (*a*) normal udder milk which had been sown with strain 18 B or an udder strain (0·8 c.c. culture to gallon of milk), (*b*) milk from an udder quarter affected with chronic *Staphylococcus* mastitis, (*c*) normal udder milk.

In the case of (*a*) and (*b*) the milk was allowed to remain at room temperature for about 2 or 3 days before being renneted. The cheeses were made by the standard method adopted for preparing cheeses of the Cheddar type, after which they were coated with paraffin wax and stored at temperatures of about 14° C. In testing for enterotoxin, some of the curd was taken as soon as it was ready for the press and at intervals a plug of cheese was removed with a cork borer and the cheese then resealed. 2·0 g. of the cheese (corresponding to about 20·0 c.c. of the original milk) were ground up with 5·0 c.c. distilled water, boiled for 30 min., and after cooling the fluid part squeezed through muslin. The extract thus prepared was injected into a kitten.

The results are set out in Table I. These show that enterotoxin which is produced in milk, artificially or naturally contaminated, is not destroyed during the process of cheese manufacture or during the first 2 months or so of ripening. The following comments are necessary. The milk used for preparing cheeses D and C had a malty flavour and that used for D coagulated on boiling. In cheese D the amount of enterotoxin was evidently small and could not be detected after a week's storage. Also, with most of the other contaminated cheeses the results were not invariably positive, indicating the presence of minimal amounts of enterotoxin. The result with cheese B was of course not expected. The milk used was fresh but, although it was said to have been taken from the clean quarters of cow 118, it is possible that a mistake occurred. An anomalous result was also obtained with cheese F after 67 days' storage.

In layer cake

Gravity cream from the naturally infected milk of cow 118 was stiffened by whipping with egg-white and sugar, and spread to a thickness of about 1·0 cm. between two slabs of a previously baked spongecake. The layer cake was then stored at 22° C. After 3 and 5 days slices were removed and the

Table I. *Enterotoxin in cheese*

Cheese	Milk used	Count of *Staph. aureus* (millions per c.c. milk) Initial	Final	Test of cheese after storage for days	Remarks
D	Normal, with strain 18 B	0·015	40·00	0 +, 8 −, 18 −	
G	,,	...	...	0 +, 8 +, 32 −, 55 +	
I	Normal, with strain GA	...	...	0 +, 8 −, 13 +, 32 +, 68 +	Kitten inoculated with 8-day plug showed diarrhoea and great uneasiness
A	Cow 118, R.H.	...	0·12	0 +, 8 +, 21 −, 31 +, 53 +, 106 −	
C	,,	0·025	51·00	0 +, 8 −, 12 +, 31 +, 62 −, 75 +, +, −, 110 −	Three kittens inoculated on day 75
H	,,	...	...	0 +, 12 +, 30 −, 35 +, 78 −	
B	Normal	...		0 −, 8 +, 17 −, 24 +, 26 −	
E	,,	Few colonies, not *Staph. aureus*		0 −, −, 9 −	
F	,,	,,		0 −, −, 7 −, 67 +, −, −	Kitten vomiting on day 67 was gorged with food, two other kittens negative
J	,,	...	...	0 −, 7 −, 12 −	

+ = vomit; − = no vomit. "0" refers to test of fresh curd. ... = milk not plated.

Table II. *Feeding experiments with human volunteers*

Exp.	Date	Milk	Amount in c.c. of cream (C.) and milk (M.) consumed	Conditions of storage	Count of *Staph. aureus* (millions per c.c. mixture)	Consumed by volunteer	Result
1	30. xi. 36	Cow 191 R.H.	5 C. + 5 M.	2 days 22° C.		1	Negative
		,,	10 C. + 10 M.	,,	13·2	2	,,
2	4. xii. 36	,,	15 C. + 15 M.	1 day ice		1	,,
		,,	15 C. + 15 M.	,,		2	Slight nausea
	7. xii. 36	,,	15 C. + 15 M.	2 days 22° C.	40·0	1	Nil definite
3	12. xii. 36	Cow 118 R.H.	60 C. + 60 M.	1 day ice		2	Vomited twice
		,,	60 C. + 60 M.	,,		1	Negative
	20. xii. 36	,,	60 C. + 60 M.	2 days 22° C.	52·0	1	,,
4	23. xii. 36	,,	50 C. + 50 M.	1 day ice		3	,,
	2. i. 37	,,	50 C. + 50 M.	2 days 22° C.	4·6	3	,,
5	21. v. 37	,,	100 M.	1 day 22° C.		1	,,
		,,	100 M.	,,		4	,,

Notes. Volunteer 3 was a boy of 14 years and volunteer 4 an adult female.

The cream mixture was consumed raw, usually instead of "lunch" and no more food was taken for 3 hr. The milk frequently developed a malty or smoky flavour after 2 days' storage.

On 16. i. 37 volunteer 1 drank 10·0 c.c. agar filtrate of strain 118 R.H. in 20·0 c.c. pasteurized milk without any symptoms resulting.

Volunteer 2 was apparently unable to tolerate a very fatty meal.

5·0 c.c. of the milk used in Exp. 5 was boiled and injected into a kitten, which vomited three times.

filling tested for enterotoxin. For this purpose, 4·0 g. filling were thoroughly shaken up with 10·0 c.c. saline at 40° C., the mixture boiled for 30 min. and centrifuged, yielding an opalescent fluid surmounted by fat. This fluid was divided into two portions, one kitten being injected with two-thirds of the amount and another kitten with the remainder. With this technique, it was found that the filling failed to induce vomiting when freshly prepared, but after 5 days' storage, by which time the cake smelt slightly sour, both kittens vomited. After 3 days' storage, when the cake tasted quite fresh, fluid corresponding to 4·0 g. filling was injected into a kitten, which vomited 40 min. later.

A test was made to see whether enterotoxin had diffused into the sponge. After 5 days' storage the filling was carefully removed from the sponge so that no visible traces remained. Slices of sponge about 0·5 cm. thick were then shaved off the internal surfaces and 8·0 g. of this were ground into a paste with 20·0 c.c. saline at 40° C., the suspension boiled for 30 min., and after cooling as much fluid as possible was squeezed through layers of muslin. The turbid fluid so obtained was centrifuged and about 5·0 c.c. fairly clear supernatant fluid injected into a kitten, which vomited twice.

Staphylococcus counts of the filling immediately after preparation, after 3 days and after 5 days at 22° C., were found to be respectively 0·2, 490 and 860 millions in the watery extract obtained from 1·0 g. filling. After 3 and 5 days' storage, therefore, the count had increased by 2450 and 4300 times.

These experiments demonstrated clearly the formation of enterotoxin in filling prepared from naturally infected cream used for making a layer cake. Cultural tests of the sponge revealed no staphylococci; the toxicity of the sponge appeared to be due therefore to diffusion of enterotoxin from the filling, and not to an outgrowth of staphylococci such as was found in somewhat analogous experiments by Dack *et al.* (1931), and by Kelly & Dack (1936).

As a control to the last experiment, gravity cream from two clean quarters of cow 118 was used for making a layer cake. Boiled extract of the filling was prepared as before. 4·0 g. of the freshly made filling were extracted and half the resulting fluid injected into each of two kittens, of which one showed no symptoms and the other vomited after 70 min. After 3 days' storage at 22° C. extracts corresponding to 4·0 g. and 2·0 g. filling were injected into two kittens, and after 5 days' storage, when the cake still tasted quite fresh, extracts corresponding to 2·6 and 1·4 g. filling were similarly injected. None of these kittens showed any symptoms. After 5 days' storage, an extract from 8·0 g. of the sponge, removed as in the previous experiment, was tested on a kitten with negative results. The result with the fresh filling was unexpected and must have been non-specific.

In meat paste

Following a report that a pot of chicken-and-ham paste had caused symptoms of food poisoning, with vomiting, in two children, the remainder

of the paste was secured for examination a few days later (in June 1937). One g. of the paste, which at this time smelt sour, was ground up in saline, centrifuged, and the supernatant milky liquid boiled for 30 min. An amount corresponding to 0·5 g. of the paste was injected into a kitten, with the result that the animal vomited 20 min. later, suffered from profuse diarrhoea, and died after 6 hr.

A cultural examination of the paste showed an enormous count of staphylococci, growing as smooth white non-haemolytic colonies on ox-blood agar. Filtrate, prepared with this organism in the usual way on soft agar and then boiled, also caused vomiting in a kitten.

As a control to the above experiment, two unopened pots of similar composition and origin were examined. The paste was sterile on cultural examination and two kittens, each injected with a boiled extract corresponding to 0·5 g. paste from each pot, showed no symptoms.

Properties of *Staphylococcus* enterotoxin

An investigation of the nature and properties of enterotoxin was not one of the main objects of this work, but in an endeavour to throw some further light on the subject a certain number of experiments were carried out.

Action of heat

In the routine testing of various materials, advantage has been taken of the resistance of enterotoxin to heat by boiling the materials in a water-bath for 30 min. The effect of heat above 100° C. has not been investigated.

Action of formalin

In several experiments it has been shown that enterotoxin is resistant to low concentrations of formalin at 37° C. for a time sufficient to destroy the α toxin. Thus, in two experiments filtrates from strains 18B and an udder *Staphylococcus* were treated with formalin at a final concentration of 0·3% for 36 or 48 hr. at 37° C. The original filtrates, diluted at 1 : 1000, were haemolytic for rabbit cells; after the formalin treatment the filtrates, which in the undiluted state showed only a trace of lysis, still produced vomiting in kittens. In a third experiment, enterotoxin was inactivated by 0·3% formalin after 10 days at 37° C.

This work has confirmed—if confirmation were necessary—that enterotoxin s different from α toxin.

Action of acid and of rennet

The resistance of enterotoxin to acid and to rennet was tested as a preliminary to the work on the formation of enterotoxin in milk and cheese.

Two samples of boiled 18B filtrate were brought to *p*H 5·0 by addition of HCl and placed at 37° C. or at room temperature. In the one case, enterotoxin resisted overnight exposure at 37° C. but not one week at room temperature; in the second case, the filtrate was still active after one week at room

temperature. These tests indicated that enterotoxin sometimes resists short exposures to acid at *p*H 5·0.

The rennet powder used, obtained from Hansen's Laboratory, contained 95–98 % sodium chloride and was said to be six times the strength of ordinary concentrated liquid standard rennet, which is itself ten times stronger than the rennet ordinarily retailed. 0·24 g. of the powder was dissolved in 4·0 c.c. distilled water and 0·5 c.c. amounts transferred to a series of tubes, some of which were boiled for 10 min. 3·0 c.c. enterotoxic filtrate were then added, giving a final rennet content of 1·0 %, and the tubes were placed at 37° C. for 4–5 hr., when the contents were injected into kittens. Control tubes containing (*a*) unsown agar filtrate + unheated rennet, (*b*) 18 B filtrate + boiled rennet were included. The results of two experiments showed clearly that under these conditions rennet does not destroy enterotoxin. The unsown agar filtrate + unheated rennet had no effect on kittens. In an experiment where the period of exposure to the rennet was increased to 18 hr. at 37° C. there was still no evidence of destruction.

Action of trypsin

A formalinized filtrate containing enterotoxin, prepared from strain 18 B, was inactivated by trypsin (Liquor Trypsin Co., Allen and Hanbury) within 4 hr. at 37° C., a time which sufficed for complete digestion of a few fragments of ox fibrin. The haemolytic toxin was likewise destroyed by this treatment.

Antigenic power

Exp. 1. An attempt was made to immunize groups of kittens by giving them subcutaneous enterotoxin (formalinized filtrate) prepared from strain 18 B and an udder strain, GA. The injections were badly tolerated and finally only three out of fifteen kittens remained alive, viz. kitten K 1 (18 B) and K 2 and 3 (GA). Each of these kittens had received a total of 20·0–24·0 c.c. enterotoxin given at 8 or 9 injections at 7–9-day intervals in doses rising from 0·25 to 5·0 c.c. At 10–14 days after the final dose, test injections were given intraperitoneally as follows.

K 1. 5·0 c.c. GA—some uneasiness but no vomiting. A control kitten given GA filtrate in the same dose vomited four times. Ten days later K 1 was given 3·0 c.c. 18 B enterotoxin and did not vomit. The control kitten which received 2·0 c.c. of the same filtrate vomited three times. Ten days later still, K 1 was injected with 4·0 c.c. *coli* enterotoxin and vomited an hour later. A control which received 3·0 c.c. of *coli* enterotoxin vomited six times.

K 2 and 3. 5·0 c.c. GA—no vomiting. Two control kittens which were given 4·0 c.c. of the same filtrate vomited repeatedly. Seven days later K 2 and 3 were given 3·0 c.c. 18 B enterotoxin and each kitten vomited on three occasions.

This experiment suggested the production of resistance to enterotoxins of 18 B and of an udder *Staphylococcus* following injections of filtrate from 18 B.

The resistance set up by enterotoxin from the udder strain could however be broken down by the stronger 18B enterotoxin.

Exp. 2. Two groups of four rabbits were given subcutaneous followed by intravenous injections of 18B and GA enterotoxins respectively. The total amount of enterotoxin given was 23·0–25·0 c.c. in ten injections at weekly intervals and in doses ranging from 0·25 to 5·0 c.c. The animals were bled before the first and also 10 days after the last injection and the four batches of sera separately pooled. In testing the sera for neutralizing properties, enterotoxin and serum were mixed and allowed to stand for an hour at 37° C. before being injected intraperitoneally into kittens. The results were as follows. 18B enterotoxin was neutralized when mixed with an equal volume of the corresponding immune serum but not by normal serum or by GA immune serum. When 18B enterotoxin was mixed with one-quarter of its volume of 18B immune serum, neutralization was not achieved. GA enterotoxin was neutralized when mixed with one-quarter of its volume of the corresponding serum or of 18B immune serum but not by this amount of normal serum.

Antibodies, therefore, are clearly produced in rabbits but, as in Exp. 1, the GA enterotoxin was less active than that of 18B. Dolman & Wilson (1938) and Davison *et al.* (1938) have also stated recently that enterotoxin is antigenic.

Diffusion of enterotoxin from the bacterial cell

While filtration experiments had indicated that enterotoxin freely diffuses into the culture medium, it was judged necessary to ascertain whether any of it remains intimately bound to the cell during growth and if so whether enterotoxin behaves as an endotoxin in the sense of Boivin and his associates.

Exp. 1. Two strains of *Bact. coli* known to produce enterotoxin were selected. Roux flasks of soft agar were sown with these strains and incubated in air at 37° C. (*a*) for 20 hr., and (*b*) for 36 hr. With (*a*) the surface growth was removed with saline, centrifuged, the deposit washed twice and resuspended in saline at 0·2 g. per c.c. This was then mixed with an equal volume of $N/2$ trichloracetic acid, allowed to stand for 3 hr. in the cold, centrifuged, and the supernatant dialysed for 36 hr. in a 5·0 % collodion sac against running water.

With (*b*) 5·0 c.c. saline was added to each flask, the juice was expressed from the agar, the fluid centrifuged, the supernatant treated with trichloracetic acid and dialysed as before.

By injecting kittens with the dialysates from (*a*) and (*b*) it was found that all the enterotoxin was contained in the agar. Clearly, also, the enterotoxin had not been destroyed by treatment with trichloracetic acid.

Exp. 2. Although staphylococci are not known to produce an endotoxin of the kind envisaged by Boivin, an experiment similar to the preceding was carried out with strain 18B. The result was similar to that of Exp. 1.

Dialysis experiments

Strain 18B filtrates of proved haemolytic and enterotoxic activity were divided into two parts. To one part formalin was added to destroy the haemolysin and each portion was then dialysed for 24–48 hr. at cool laboratory temperature in sacs of 5·0 and 0·5 % collodion against small amounts of saline. Usually about 15·0 c.c. filtrate was put in the sac, which was in contact with a similar amount of saline. A trace of toluene was added to the non-formalinized filtrate if the sac was to stand as long as 48 hr.

Judging from the fact that in two experiments with different filtrates as much as one-third of the dialysate after 24 hr. standing produced merely diarrhoea or delayed vomiting in kittens, it is surmised that even with the more permeable sac enterotoxin is only slightly diffusible. After 48 hr. dialysis, symptoms were produced more promptly. In comparable experiments, only a trace of haemolysin had diffused through the more permeable sac in 48 hr., while none at all could be detected in dialysates from 5·0 % collodion sacs. The sac contents were tested for haemolysin and enterotoxin at the same time as the dialysates.

DISCUSSION

There are good grounds for believing that the substance which produces gastro-intestinal disturbance in kittens after intraperitoneal injection is the same as that which produces food poisoning in man, and most of the present experiments have been carried out on this assumption.

There is experimental evidence to show that human beings vary considerably in their susceptibility to enterotoxin. In the few human beings tested I have been unable to set up poisoning with natural materials (milk and cream) containing enterotoxin and large numbers of staphylococci. Barber (1914) and Ramsey & Tracy (1931) were able to do so in experiments on themselves, indicating high individual susceptibility and/or production of rather large amounts of enterotoxin by their strains. With regard to monkeys, although these animals are less susceptible than man (Jordan & Burrows, 1935), they certainly have their uses when the results are positive. As to the suitability of kittens, when tested by the intraperitoneal route, there can be no question. Over 300 kittens have been used in this work and this experience has confirmed that they are uniformly and highly susceptible, provided they are over 400 g. in weight. Care should be taken, however, that at the time of injection they are not gorged with food.

With regard to the practical importance of *Staphylococcus* food poisoning, Dolman (1934) thinks that relatively few *Staphylococcus* strains produce enterotoxin, and this explains the comparative rarity of this form of poisoning. Now that the more delicate kitten test is available, it seems more likely that enterotoxin is produced by very many parasitic strains but that the amounts are usually insufficient to cause symptoms in persons of average susceptibility.

In view of the findings of Jordan and Burrows (1935), there is also the possibility that by growth in starchy foods the enterotoxin-forming capacity of bacteria may be enhanced. As to the source of contamination of the food, there has perhaps been a tendency to overestimate the importance of human agency (coughing or sneezing, cases of furunculosis); the present observations emphasize that food-poisoning staphylococci need not be derived from man or from a disease source but may be introduced with the food itself and particularly with raw milk.

As to the nature of enterotoxin, the substance has properties recalling those of the soluble toxins, (*a*) in its free diffusibility in culture, from which it can be readily separated by filtration, (*b*) in its low diffusibility through collodion membranes, and (*c*) in exciting the production of antibodies. Whether these antibodies are specific has not been definitely established. So far as is known, enterotoxin differs from some other well-known toxins only in its relatively low toxicity when injected into laboratory animals, e.g. rabbits, mice, and in its relatively high resistance to heat. Obviously, much more work, particularly from the chemical point of view, is required before one can say much about the nature of the product.

Summary

1. Feeding tests on monkeys (*Macacus rhesus*), dogs and cats are unsatisfactory for detecting the presence of enterotoxin, owing to the variable susceptibility of these animals by the oral route.

2. Using Dolman's method, in which the material is injected intraperitoneally into kittens, the production of enterotoxin has been demonstrated by: (*a*) sixteen out of thirty-eight strains of *Staph. aureus*, isolated from cases of acute or chronic mastitis or from normal udder milk; (*b*) four out of five strains of *Bact. coli*, mostly from calves with "white scours". No enterotoxin was obtained from fifteen strains of *Str. agalactiae* from mastitis in cows.

3. The formation of enterotoxin under natural conditions has been observed: (*a*) In udder milk seeded with *Staph. aureus* or naturally contaminated with that organism and stored at atmospheric temperatures (18 and 22° C.). The substance remains active in cheese prepared from such milk. (*b*) In layer cake made with cream naturally contaminated with *Staph. aureus*.

4. A small outbreak of poisoning due to potted meat paste was shown to be caused by a non-haemolytic *Staphylococcus*.

5. A few feeding experiments on man with milk or cream, in which food-poisoning staphylococci had grown, were negative, but on one occasion a *Staphylococcus* from a case of mastitis yielded a culture filtrate which caused symptoms of food poisoning.

6. Enterotoxin has the following properties. It is resistant to heat (95° C., 30 min.), to low concentrations of formalin sufficient to destroy the haemolytic toxin, to acid (*p*H 5·0), and to rennet, but is destroyed by trypsin.

It diffuses freely into the culture medium but only slightly through collodion. It is antigenic. Its properties are such that enterotoxin can be classed as a bacterial exotoxin.

ACKNOWLEDGEMENTS. My thanks are due to the Directors of United Dairies Limited for defraying the cost of this work, and to Messrs E. B. Anderson and L. J. Walker of the United Dairies Central Laboratories for their assistance, including the making of the experimental cheeses. I am also indebted to the late Prof. E. O. Jordan and to Dr W. M. Scott for sending me cultures of food-poisoning staphylococci.

REFERENCES

BARBER, M. A. (1914). *Philipp. J. Sci.* **9**, 515.
CRABTREE, J. A. & LITTERER, W. (1934). *Amer. J. publ. Hlth*, **24**, 1116.
DACK, G. M., WOOLPERT, O., NOBLE, I. & HALLIDAY, E. G. (1931). *J. prev. Med.* **5**, 391.
DAVISON, E., DACK, G. M. & CARY, W. E. (1938). *J. infect. Dis.* **62**, 219.
DOLMAN, C. E. (1934). *J. infect. Dis.* **55**, 173.
DOLMAN, C. E. & WILSON, R. J. (1938). *Canad. publ. Hlth J.* **29**, 35.
DOLMAN, C. E., WILSON, R. J. & COCKCROFT, W. H. (1936). *Canad. publ. Hlth J.* **27**, 489.
JORDAN, E. O. (1930). *J. Amer. med. Ass.* **94**, 1648.
JORDAN, E. O. & BURROWS, W. (1934). *J. infect. Dis.* **55**, 363.
—— —— (1934*a*). *Amer. J. Hyg.* **20**, 604.
—— —— (1935). *J. infect. Dis.* **57**, 121.
KATHE (1937). *Z. Bakt.* **140**, 71.
KELLY, F. C. & DACK, G. M. (1936). *Amer. J. publ. Hlth*, **26**, 1077.
LEVIN, W. (1917). *J. Lab. clin. Med.* **2**, 761.
RAMSEY, R. J. & TRACY, P. H. (1931). *Proc. Soc. Exp. Biol., N.Y.*, **28**, 390.
SHAUGHNESSY, H. J. & GRUBB, T. C. (1936). *J. infect. Dis.* **58**, 318.
—— —— (1937). *Canad. publ. Hlth J.* **28**, 229.
TANNER, F. W. & RAMSEY, R. J. (1932). *Amer. J. med. Sci.* **184**, 80.

(*MS. received for publication* 11. VI. 38—Ed.)