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R. M. FRY, M.A., M.R.C.S.

LATELY DIRECTOR OF THE PUBLIC HEALTH LABORATORY, CAMBRIDGE

IN CONJUNCTION WITH

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Comparative studies on *Salmonella typhi* grown *in vivo* and *in vitro*

I. Virulence, toxicity, production of infection-promoting substances and DPN-ase activity

By A. L. OLITZKI and DINA GODINGER
Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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INTRODUCTION

The role of toxic substances produced by Enterobacteriaceae in the pathogenesis of the specific diseases is not clear. While the strains belonging to different genera (*Salmonella*, *Shigella*, *Proteus*, *Escherichia*, etc.) produce endotoxins which in the animal experiment give rise to similar symptoms, there exist striking differences in their pathogenicity and in the clinical course of infections produced by them. The cause of death in experimental infection of mice has also not yet been sufficiently cleared up.

Olitzki, Fleischhacker & Olitzki (1957) observed that in groups of non-immunized mice infected with *S. paratyphi* A in quantities corresponding to a half or a quarter of the LD_{100}, only those animals died in which the bacteria were able to multiply and to reach a high concentration, while others, in which, for still unknown reasons, the bacteria were unable to multiply, survived and acquired a protracted non-lethal infection which persisted for a month.

Olitzki & Olitzki (1958) showed that in the same group of mice infected with $2{\cdot}5 \times 10^8$ virulent *S. paratyphi* A the total bacterial count *post mortem* was approximately 10^9/animal, while in those which survived after 3 days it did not exceed 10^6/animal. Furthermore, they showed that the protecting effect of immune sera on mice infected intra-abdominally with *S. paratyphi* A was not correlated with a sterilization of the host's organs, but merely with a diminution of the bacterial count below a lethal level. A similar observation by Olitzki, Sharon & Godinger (1960) was made on mice which had been actively immunized with *S. typhi* and then infected intra-abdominally. The previous vaccination did not prevent a protracted infection of the abdominal organs which lasted at least 10–14 days; it merely inhibited the rise of the bacterial count to the lethal level of about 3×10^9 bacteria/animal. Since this rise took place within 24 hr., the lethal outcome of the infection was finally decided within this short time.

Chemotherapeutic experiments on mice were carried out by Olitzki, Sulitzeanu, Sharon & Gelernter (1961) with *S. typhi*. They showed that within a few hours after the onset of infection it was still possible to prevent deaths by relatively low doses of chloramphenicol, while at later stages of the infection when the bacterial

count in the blood and organs was already on an elevated level, higher doses and repeated administration were required in order to prevent death

Extensive chemotherapeutical experiments on mice were carried out by Olitzki & Godinger (unpublished). When the infective dose was 2×10^8 and the treatment started 2–5 hr. after the onset of the infection, then 100 % of 40 animals survived. When the treatment started 6–10 hr. after the onset of the infection then the survival rate varied in five groups of 20 mice from 10 to 30 %; its average was 23·0 %. Many treated mice died later than 24 hr. after the onset of infection and the percentage of survivors after this observed time varied from 15 to 50 %; its average was 33·0 %. Forty non-treated control animals died within 24 hr.

When the infective dose was 4×10^8 and the treatment started 2–4 hr. after the onset of the infection, then it was possible to save 83·3 % of all animals. When the treatment started after 5 hr. 50 % of the animals survived. When the treatment started 6–10 hr. after the onset of the infection then the percentage of survivors varied in five groups of 20 mice from 10–20, its average being 14·0. Thirty non-treated mice died within 24 hr. When the treatment started later than 10 hr. no more survivors were found.

When 4×10^3 bacteria suspended in a 5 % mucin suspension were injected intraperitoneally and the treatment started 8 hr. after the onset of the infection, then deaths were completely prevented or appeared 30–60 hr. after the onset of the infection. This effect of the therapy in spite of its delay can be explained by the slow increase of the bacterial count after the injection of 4×10^3 bacteria suspended in mucin. When this method was employed, the bacterial count in the abdominal cavity was only 2×10^7 after 10 hr., while 10 hr. after injection of 2×10^8 bacteria without mucin it was $2{\cdot}5 \times 10^9$, i.e. more than 100 times higher. Therefore, when the mucin method was employed and 0·5 mg. of chloramphenicol administered at suitable intervals, starting 8 hr. after the onset of the infection, then it was possible to keep the bacterial count below the lethal level for several days without causing deaths and to bring it, within 3 days, to such a low level in the abdominal cavity (about $1{\cdot}6 \times 10^3$) that the final elimination of the bacteria was possible.

The possibility of a detoxifying effect of chloramphenicol was also examined. When 4–5 mg. of acetone-dried *S. typhi* (Ty 2) were injected intraperitoneally then 19 out of 20 mice died within 24 hr. When the same bacteria were exposed to 1·0 mg./ml. chloramphenicol at 37° C. for 20 hr. then 6 out of 20 mice died within 24 hr. When acetone-dried bacteria were injected and chloramphenicol treatment of the mice started immediately, then 9 out of 20 mice died within 24 hr. However, this lowered mortality after the observation time of 24 hr. was the result of a delayed lethal effect in the chloramphenicol groups. 48 hr. after the injection of the dried bacterial substance the number of deaths in the control group was 20 and in both chloramphenicol groups 17. Therefore, complete detoxification of endotoxin by chloramphenicol could not be proved.

On the other hand, the decisive effect of the increasing concentration of bacterial substances in the host was evident and as soon as this critical concentration was reached, even a short contact determined the lethal outcome of the infection.

Additional experiments of Olitzki *et al.* (1961) showed that the living micro-organisms counted in the organs were not the only bacterial substances present in the host. By precipitation tests with specific immune sera they determined that several hours after the onset of the infection soluble antigens appeared in the organs and that their concentration increased with the progress of the infection. It seemed, therefore, that the lethal effect of the experimental infection was caused by a progressive accumulation of bacterial substances liberated from disintegrated bacteria and also from the intact living bacteria. The reversibility of these toxic effects and their curability by chemotherapeutics, even in heavily infected animals, showed that we had to deal with a toxic substance different from the classical 'exotoxins', which can act in very low concentrations, but with a substance, or a group of substances, of relatively low toxicity, which acted alone or together and determined the lethal outcome. Smith (1958) pointed out that in view of the diversity of the disease syndromes produced by Gram-negative micro-organisms it seems to be a too simple generalization to say that all these symptoms are produced by the endotoxins which under experimental conditions produce similar toxic symptoms irrespective of their origin from pathogenic or non-pathogenic organisms (e.g. *Escherichia coli*), and it may well be that in addition to the endotoxins further toxic substances may play a role. The production of additional toxins of this kind by *S. typhi* grown *in vitro* has not yet been reported, although it has been demonstrated in the case of Shiga's bacillus. Therefore it seemed to be worth while to try to find out if *S. typhi* grown *in vivo* exhibits toxic and/or aggressin-like effects which are not exerted by it when grown *in vitro*.

MATERIALS AND METHODS

Determination of virulence

Bacteria grown *in vivo* in the peritoneal cavity of infected mice were washed off with 2 ml. of physiological saline solution; the suspension was centrifuged at 1000 r.p.m. for 5 min. in order to remove blood and tissue cells. Bacteria grown in the spleen were separated from the tissues after the spleens of 80 infected mice were removed, weighed and treated as follows. With the aid of a glass homogenizer they were triturated and from the final homogeneous product a suspension was prepared which contained 100 mg./ml. spleen tissue. The tissue fragments were removed by centrifugation at 1000 r.p.m. for 5 min. and the remaining bacteria were removed from the supernatant after centrifugation at 10,000 r.p.m. for 15 min. The sedimented bacteria were then resuspended in saline and the same procedure repeated. In the final bacterial suspension tissue cells or cell fragments were not visible microscopically.

The virulence of bacteria grown *in vivo* was examined after seven passages through white mice of 20 g. weight. The bacteria were separated from the tissues as described above. In order to determine the virulence of the *in vitro* grown bacteria, the *in vivo* grown bacteria were subcultured daily on trypticase soy agar, and incubated at 37° C. for 20 hr. The virulence of both types of bacteria was tested by intra-abdominal injection into mice. The original bacterial suspension

contained 10^9 bacteria/ml. as determined by its optical density and controlled by plating of suitable dilutions. From this original suspension fourfold dilutions were prepared starting with $2 \times 10^8/0{\cdot}5$ ml. and injected intra-abdominally. The observation time was 7 days, although deaths occurred within 48 hr. after the onset of the infection.

Toxicity test

Bacteria grown *in vivo* and also those grown *in vitro* were first suspended in saline, 10 volumes of acetone added, and the suspension stored in the refrigerater till viable bacteria could no longer be demonstrated. The sediment was washed three times in cooled acetone and dried *in vacuo*. The resulting products were weighed and quantities ranging from 0·5–10·0 mg. injected into mice intra-abdominally. The observation time was 7 days, although deaths were noted within 48 hr. after the injection.

Examination of normal and infected organ extracts for pathogenizing substances

Organs of normal and infected animals were suspended in 2 ml. of distilled water per organ. These suspensions were triturated in a glass homogenizer, the product was then three times frozen at $-20°$ C. and thawed. By centrifugation at 10,000 r.p.m. for 10 min. tissue fragments and entangled bacteria were removed. In order to kill the residual living bacteria in the supernatant—about 5×10^3/ml.—the extracts were heated for 1 hr. at 50° C. When this procedure was not sufficient the extracts were heated for 2 hr. at 50° C. or for 1 hr. at 60° C.

Two series of experiments were carried out with the virulent strain Ty 2 and also the non-virulent strain O 901. In the first series tenfold decreasing quantities of bacteria were suspended in the undiluted extract and 0·5 ml. of this mixture injected intra-abdominally into mice. In the second series of experiments a constant amount of bacteria, representing the LD 30 or LD 40, was suspended in graded dilutions of the extract to be tested. The observation time was the same as in the virulence and toxicity tests.

Preparation of antisera for the examination of the antigenicity of normal and infected organs and bacteria grown under different conditions

Rabbits were immunized with spleen extracts from normal and infected mice. The extracts were prepared as for the preparation of pathogenizing substances. Without previous heating the extracts were mixed with equal amounts of Freund's adjuvants (Salk & Laurent, 1952). The bacteria grown *in vitro* were dried in acetone and 20 mg. of bacteria/ml. suspended in the adjuvant mixture. The bacteria grown *in vivo* after separation from the surrounding tissues, as described above, were then treated in the same way as those grown *in vitro*.

Groups of rabbits received injections of 1·0 ml. of the antigen–adjuvant mixtures: 0·5 ml. were given subcutaneously and 0·5 ml. intramuscularly. Four injections were given at weekly intervals. Blood was obtained 2 weeks after the last injection.

DPN-ase activity of bacteria and of normal and infected organs

100 mg./ml. of the wet organs were triturated in saline phosphate buffer solution (pH 7·2), then tissue fragments and bacteria were removed by centrifugation at 10,000 r.p.m. for 5 min. The DPN-ase activity was determined several times within a period of 20 min. contact with 0·6 μmol. DPN/ml. as described by Kaplan, Colowick & Ciotti (1951). The *in vivo*-grown bacteria were separated from the tissue fragments by the same method as used for the virulence test. The activity of both types of bacteria was tested with intact non-multiplying cells, without any disintegration procedure.

RESULTS

Virulence and toxicity tests with bacteria grown in vivo *and* in vitro *and infected organ extracts*

Table 1 shows that after seven *in vivo* passages through the abdominal cavities of mice *S. typhi* (strain Ty 2) was able to kill mice when injected intra-abdominally in amounts of $7{\cdot}5 \times 10^5$ and $1{\cdot}8 \times 10^5$, while the same dose of bacteria subcultured 10–20 times *in vitro* on trypticase agar did not exert any lethal effects. However, acetone-dried bacteria grown *in vitro* were as toxic as those grown *in vivo*, both causing a 100 % mortality when 5 mg., and a 50 % mortality when 2·5 mg. were injected intraperitoneally. Thus it became evident that the pathogenicity of the micro-organism was not correlated with toxicity. On the other hand, no toxic effect was exerted by extracts from infected organs. As soon as most of the bacteria were removed by high-speed centrifugation and any residual bacteria killed by exposure to heat at 50° C. for 1 hr., no deaths were observed after intraperitoneal injections of 0·5 ml. If any deaths were observed, these were always related to positive cultures obtained from the abdominal organs, indicating that the living bacteria were not completely removed.

Table 1. *The virulence of strain Ty* 2 *grown under different conditions for mice*

No. micro-organisms	Deaths in groups of 10 mice after intraperitoneal injection of micro-organisms grown: *in vivo*		*in vitro* No. subcultures			
	Spleen	Peritoneal fluid	1	3	10	20
$2{\cdot}0 \times 10^8$	—	—	10	9	9	7
$5{\cdot}0 \times 10^7$	—	—	9	9	8	3
$1{\cdot}25 \times 10^7$	—	10	8	8	3	3
$3{\cdot}0 \times 10^6$	8	8	6	7	2	3
$7{\cdot}5 \times 10^5$	8	8	2	1	0	0
$1{\cdot}87 \times 10^5$	3	2	0	0	0	0
$4{\cdot}7 \times 10^4$	1	0	—	—	—	—
$1{\cdot}2 \times 10^4$	0	0	—	—	—	—

Infection-promoting substances in normal infected organs

The following experiments were carried out in order to examine whether in the abdominal organs of infected mice aggressin-like substances were produced. The

experiments were carried out with the virulent Ty 2 and the non-virulent strain O 901.

In Table 2 the experiments with strain Ty 2 subcultured on trypticase agar are summarized. Undiluted organ-extracts were employed, while the bacterial suspensions were injected in tenfold-decreasing dilutions from 2×10^8 to 2×10^2 bacteria. Table 2 shows that spleen extracts from normal and also from infected mice did not enhance the pathogenicity of strain Ty 2, while such an effect was

Table 2. *The infection-promoting effect of undiluted organ extracts injected along with sublethal doses of Ty 2*

Bacteria suspended in	Extracts heated	Deaths observed in groups of 5 mice after injection of graded amounts of *S. typhi* (strain Ty 2)							
		2×10^8	2×10^7	2×10^6	2×10^5	2×10^4	2×10^3	2×10^2	0
Physiological saline solution	—	5	4	2	1	0	0	0	—
Normal liver extract	—	—	—	5	4	3	0	0	—
Liver extracts from mice infected with									
S. typhi (Ty 2)	1 hr. 50° C.	—	—	5	4	3	2	0	—
S. typhi (O 901)	1 hr. 50° C.	—	—	5	1	0	0	0	—
E. coli (O 111)	1 hr. 50° C.	—	—	4	1	0	0	0	—
S. ballerup	1 hr. 50° C.	—	—	4	2	0	0	0	—
S. hirschfeldii	1 hr. 50° C.	—	—	5	5	4	3	4	5
S. hirschfeldii	2 hr. 50° C.	—	—	5	5	4	4	4	5
S. hirschfeldii	1 hr. 60° C.	—	—	5	5	2	0	0	—
S. stanley	1 hr. 50° C.	—	—	5	5	5	5	5	5
S. stanley	2 hr. 50° C.	—	—	5	3	1	0	0	—
S. stanley	1 hr. 60° C.	—	—	5	3	1	0	0	—
Normal spleen extract	—	5	4	2	0	0	0	0	—
Spleen extracts from mice infected with									
S. typhi (Ty 2)	1 hr. 50° C.	5	4	1	0	0	0	0	—
S. typhi (O 901)	1 hr. 50° C.	—	4	1	0	0	0	0	—
E. coli (O 111)	1 hr. 50° C.	—	4	1	1	0	0	0	—
S. ballerup	1 hr. 50° C.	—	4	1	0	0	0	0	—
S. hirschfeldii	1 hr. 50° C.	—	4	2	1	0	0	0	—
S. stanley	1 hr. 50° C.	—	5	1	1	0	0	0	—

exerted by normal liver extracts. It seemed that the strongest pathogenizing effects were exerted by extracts from mice infected with *S. hirschfeldii* or with *S. stanley*, which were heated at 50° C. only. However, control experiments proved that these liver extracts still contained viable bacteria and were themselves able to kill mice. Table 3 shows the bacterial counts in extracts exposed to different temperatures. The table shows that *S. typhi* was more sensitive to heat than the other strains. After heating at 50° C. for 1 hr. its bacterial count decreased 3000-fold, while that of *S. stanley* only 133-fold. *S. hirschfeldii* proved to be the

most resistant strain, since its count decreased only 20-fold. We tried, therefore, to exclude the action of surviving bacteria by heating extracts at 50° C. for 2 hr. and also by heating them at 60° C. for 1 hr. As shown in Table 3 this latter procedure effected a complete sterilization of all extracts. This procedure seemed to be justified since even 20 bacterial cells of *S. stanley* suspended in normal liver extracts exhibited lethal effects (see Table 4).

Table 3. *Heat resistance of* Salmonella *strains in the liver extracts of infected mice*

	No. bacteria/ml. in liver extracts of mice infected with		
	S. stanley	*S. hirschfeldii*	*S. typhi* Ty 2
Not heated	$2{\cdot}0 \times 10^5$	$2{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^4$
Heated 1 hr. 50° C.	$1{\cdot}5 \times 10^3$	$1{\cdot}0 \times 10^5$	$1{\cdot}0 \times 10^1$
Heated 2 hr. 50° C.	$1{\cdot}0 \times 10^2$	$3{\cdot}0 \times 10^4$	0
Heated 1 hr. 60° C.	0	0	0

Table 4. *The infection-promoting effect of normal liver extract on* S. stanley *and* S. hirschfeldii *grown* in vitro

	Bacteria suspended in	Deaths in groups of five mice after intraperitoneal injection of							
		2×10^8	2×10^7	2×10^6	2×10^5	2×10^4	2×10^3	2×10^2	2×10^1
S. stanley	Saline	5	5	4	1	0	0	0	0
	Normal liver extract 50° C. 1 hr.	—	—	—	—	5	5	5	5
S. hirschfeldii	Saline	5	1	0	0	0	0	0	0
	Normal liver extract 50° C. 1 hr.	—	—	5	3	0	0	0	0

Table 4 also shows that the LD_{100} of *S. stanley* and *S. hirschfeldii* suspended in physiological saline was 2×10^7 and 2×10^8, respectively. However, when the bacteria were suspended in normal liver extract, the LD_{100} of *S. stanley* decreased a millionfold, and the LD_{100} of *S. hirschfeldii* only a hundredfold.

It was, therefore, concluded that the lethal action exerted by liver extracts of mice infected with *S. stanley* and heated at 50° C. for 1 hr. was exerted by a few residual bacteria. As shown in Table 2, this effect was considerably reduced by heating at 50° C. for 2 hr. or at 60° C. for 1 hr.

Since *S. hirschfeldii* was more resistant to heating, the lethal action of the liver extract from infected mice was not abolished by heating it at 50° C. for 2 hr. However, by heating the extracts at 60° C. for 1 hr. the infectivity and the lethal effect of the liver extract completely disappeared.

Table 5 summarizes the experiments carried out with the non-virulent strain O901. While the lethal dose of Ty 2 was lowered by both normal liver extracts and infected liver extracts to almost the same extent, the MLD of the non-virulent strain O901 was lowered more by infected than by normal extracts. However, only extracts of organs from mice infected with strain Ty 2 were able to exert this

effect. Organs infected with the non-virulent strain O 901 or with *S. hirschfeldii*, which possesses the same Vi-antigen as *S. typhi*, did not enhance the pathogenicity of strain O 901 more than extracts from normal organs.

Table 5. *The infection-promoting effect of undiluted extracts injected along with sublethal doses of strain* O 901

Bacteria suspended in	Extracts heated	Deaths observed in groups of 10 mice after injection of graded amounts of *S. typhi* (strain O 901)							
		10^9	2×10^8	2×10^7	2×10^6	2×10^5	2×10^4	2×10^3	2×10^2
Physiological saline solution	—	5	2	0	0	0	0	0	—
Normal liver extract	—	—	10	10	6	4	2	0	—
Liver extracts from mice infected with	—								
S. typhi (Ty 2)	1 hr. 50° C.	—	10	10	8	6	5	2	0
S. typhi (O 901)	1 hr. 50° C.	—	—	—	1	0	0	0	—
S. stanley	1 hr. 50° C.	—	—	—	1	0	0	0	—
S. hirschfeldii	1 hr. 60° C.	—	—	—	2	0	0	0	—
Extract 50 % O 901 with 50 % *S. hirschfeldii*	—	—	—	—	3	0	0	0	—
Normal spleen extract	—	—	9	7	1	0	0	0	—
Spleen extract from mice infected with									
S. typhi (Ty 2)	1 hr. 50° C.	—	10	10	9	1	0	0	—
S. typhi (O 901)	1 hr. 50° C.	—	—	2	1	0	0	—	—

In another series of experiments the action of diluted organ-extracts on constant infecting doses of strain Ty 2 and strain O 901 were examined. Some of these experiments are summarized in Table 6. They show that there exist some quantitative differences between normal and infected organs. The mortality of the mice infected with strain Ty 2 suspended in a 1/5 dilution of an extract prepared from an infected spleen was somewhat higher than that observed in those infected with strain Ty 2 suspended in normal spleen diluted 1/5. Similar differences were observed between normal liver extract and infected liver extract both diluted 1/25. In experiments with strain O 901 some differences were observed between the pathogenizing effects of normal and infected spleen extract diluted 1/5 and 1/25. In order to see whether these differences appear constantly, we infected a group of 70 mice with the same amounts of strain O 901 along with twofold dilutions of normal spleen extracts up to 1/128, and another group with the corresponding dilutions of spleen extracts from mice infected with strain Ty 2. Forty-four mice died in the first group and 46 in the second out of 70 mice. Thus, these experiments prove that diluted extracts of mice spleens infected with Ty 2 do not exhibit a greater infection-promoting activity than diluted extracts of normal mice spleens.

Since normal liver extracts could themselves evoke an infection-promoting effect, we tried to determine which of the substances usually present in the liver might act as pathogenizer. From substances which could probably play such a

role we examined sodium taurocholate and glycogen. The pathogenizing effect of the latter was already examined (1946) by Olitzki, Shelubsky & Hestrin and found to be almost inactive.

Table 6. *The infection-promoting effect of diluted organ extracts (heated at 50° C. for 1 hr.) injected along with* 2×10^6 *bacteria of strain Ty 2 and* 2×10^8 *bacteria of strain* O 901 *into white mice*

Extracts prepared from	Reciprocals of dilutions	Deaths produced in groups of 10 mice: By bacteria suspended in organ extracts: Ty 2	Deaths produced in groups of 10 mice: By bacteria suspended in organ extracts: O 901	Deaths produced in groups of 10 mice: By organ extracts without bacteria
Infected liver (strain Ty 2)	5	10	10	0
	25	6	7	0
	125	3	4	0
Infected spleen (strain Ty 2)	5	7	4	0
	25	3	4	0
	125	3	0	0
Normal liver	5	8	10	0
	25	3	7	0
Normal spleen	5	3	2	0
	25	3	0	0
Control (physiological saline solution)	—	3	4	—

Table 7. *The infection-promoting effect of glycogen and sodium taurocholate*

Injected bacteria: Strain	Injected bacteria: Quantity	Deaths in groups of 5 mice observed after injection of *S. typhi* suspended in: Physiological saline	Sodium taurocholate 1·0 %	Glycogen 10 %	Sodium taurocholate 1·0 % with glycogen 10 %
Ty 2	2×10^8	5	5	5	5
	2×10^7	4	5	4	5
	2×10^6	2	5	2	5
	2×10^5	1	5	1	5
	2×10^4	0	4	0	5
	2×10^3	—	3	—	5
	2×10^2	—	1	—	2
	2×10	—	0	—	2
	0	—	0	0	0
O 901	5×10^8	2	5	5	5
	5×10^7	0	5	3	5
	5×10^6	0	2	1	5
	5×10^5	0	1	0	5
	5×10^4	—	1	0	5
	5×10^3	—	0	0	3
	5×10^2	—	0	0	0
	0	—	—	—	—

The combined effect of in vivo *growth and the presence of organ extracts on the pathogenicity of* S. typhi

In the following experiments we examined the combined effect of *in vivo* growth and the presence of organ extracts from normal and infected mice on the pathogenicity of both strains of *S. typhi*, Ty 2 and O 901. Table 8 shows that 100 microorganisms of the *in vivo*-grown strain Ty 2 in the presence of infected liver extracts killed 3 out of 5 mice. The pathogenizing action of infected spleen extract was less marked. The enhancement of the virulence of the *in vivo*-grown strain O 901 was also less marked.

Table 8. *The effect of infection-promoting substances on the pathogenicity of* S. typhi *strains grown* in vivo

Injected bacteria		Deaths in groups of five mice after intra-abdominal injection of *S. typhi* suspended in					
				Extracts of			
		Physiological saline solution	Sodium taurocholate and glycogen	Liver		Spleen	
Strain	No.			Normal	Infected	Normal	Infected
Ty 2	10^5	1	2	5	5	1	5
	10^4	0	2	4	5	0	1
	10^3	0	1	3	4	0	1
	10^2	0	1	2	3	0	0
	10^1	0	0	2	2	0	0
	0	0	0	0	0	0	0
O 901	10^8	4	—	—	—	5	5
	10^7	0	—	—	—	1	1
	10^6	—	5	2	3	0	0
	10^5	—	4	1	2	0	0
	10^4	—	1	0	1	—	—
	10^3	—	0	0	0	—	—

The DPN-ase activity of normal and infected spleen extracts

The DPN-ase activities of normal and infected spleen extracts are summarized in Table 9, which shows that normal spleen extracts are almost inactive, containing only about 1·5 enzyme-units/mg. protein, while spleen extracts of mice infected with *S. typhi* contained 6·5–12·0 enzyme-units/mg. protein. Since other salmonella organisms such as *S. ballerup* (*Paracolobactrum ballerup*), *S. hirschfeldii* (*S. paratyphi* C) and *S. stanley* exerted similar effects and the highest activity was exhibited by *Escherichia coli*, strain O 111, this cannot be considered as a specific effect of *S. typhi*.

Furthermore, this effect was also produced by large doses of acetone-dried typhoid bacilli strain Ty 2 (4×10^{10} of acetone-dried bacterial cells of the same strain were as active as 2×10^8 living bacteria).

This effect was specific for the spleens of the infected animals. The livers of normal mice and those infected with *S. typhi*, strains O 901 and Ty 2, *S. ballerup*, *S. hirschfeldii* and *S. stanley* contained 0·4–0·8 enzyme-units/mg. of protein.

The DPN-ase activity of bacteria grown in vivo *and* in vitro

S. typhi (strain Ty 2) grown *in vitro* or *in vivo* in the peritoneal cavity did not show any DPN-ase activity. On the other hand, the bacteria grown in, and separated from, the infected spleen were highly active, containing 60 enzyme-units/10^9 cells.

Table 9. *The effect of experimental infection of white mice on the DPN-ase content of the spleen*

Injected bacteria			Protein in spleen extract	DPN μmole, after exposure to spleen extracts			DPN-ase units/mg. of protein
Strain	Quantity	Condition	(mg./ml.)	0 min.	5 min.	10 min.	
S. typhi (Ty 2)	$2{\cdot}0\times10^8$	Living	1·0	0·60	0·47	0·36	11·5
			2·0	0·60	0·35	0·18	12·0
S. typhi (Ty 2)	$4{\cdot}0\times10^{10}$	Acetone-dried	1·0	0·60	0·47	0·37	11·5
S. typhi (O 901)	$5{\cdot}0\times10^8$	Living	1·0	0·60	0·53	0·40	7·0
S. typhi (Ty 1)	$8{\cdot}0\times10^6$	Living	1·0	0·60	0·53	0·48	6·5
S. ballerup	$2{\cdot}5\times10^8$	Living	1·0	0·60	0·44	0·34	15·0
S. stanley	$1{\cdot}0\times10^7$	Living	1·0	0·60	0·52	0·43	13·0
S. hirschfeldii	$1{\cdot}0\times10^8$	Living	1·0	0·60	0·57	0·46	12·5
E. coli (O 111)	$1{\cdot}0\times10^7$	Living	1·0	0·60	0·42	0·24	18·0
Control: not infected			1·0	0·60	0·60	0·60	0
			2·0	0·60	0·57	0·53	1·5
			4·0	0·60	0·54	0·49	1·4
			8·0	0·60	0·45	0·34	1·75

Remark: 1 unit of DPN-ase decomposes 0·01 μmole of DPN within 5 min.

In order to prove whether inactive bacteria were able to absorb DPN-ase from infected spleen, 10^{10} bacteria were suspended in 1·0 ml. of spleen extract of infected mice. After contact for 30 min. at 4° C. the bacteria were removed by centrifugation and washed in saline. After contact with infected spleen extracts, the *in vitro*-grown bacteria contained 20 units, while the intraperitoneally grown bacteria contained 4·6 units/10^9 cells. None of them reached the value of 60 units/10^9 cells obtained with bacteria grown in the spleen.

Attempts to neutralize DPN-ase by immune sera

Extracts from infected spleens were exposed to immune sera obtained by immunization of rabbits with spleen-grown and acetone-dried *S. typhi* (Ty 2) and with extracts of infected organs; 0·2 ml. of immune serum/mg. of protein were added. This mixture was left for 2 hr. at 37° C. and kept overnight in the refrigerater. The original spleen extract contained 12 units/mg. of protein. This value did not decrease after the prolonged contact with any of the three tested immune sera.

Attempts were also made to neutralize DPN-ase of active bacteria separated from spleens by exposure to sera of rabbits immunized with either *S. typhi* (Ty 2) grown *in vitro* or *in vivo*; 0·2 ml. of immune serum were added to 10^9 cells. The time and temperature of contact was the same as in the experiments described

with infected spleen extracts. Then the bacteria were separated and examined for their DPN-ase activity. The original bacteria contained 42 units/10^9 cells; after the contact with the antisera against the spleen-grown and *in vitro*-grown bacteria the respective values were 59 and 63 units/10^9 cells.

Summarizing all these experiments it can be said that neither DPN-ase containing spleen extracts nor DPN-ase containing spleen-grown bacteria lost their DPN-ase activity by exposure to immune sera.

Table 10. *The DPN-ase activity of organs of normal and infected mice*

Organ	DPN-ase units/mg. of protein in organs of mice: Normal	DPN-ase units/mg. of protein in organs of mice: Infected with *S. typhi* strain Ty 2
Spleen	1·5	12·4
Pancreas	0·4	2·1
Stomach (wall)	0·4	6·6
Processus vermiformis	18·0	27·0
Sigmoid, rectum (wall)	20·0	33·0
Brain	< 1·5	< 1·5
Heart	0·1	0·1
Lungs	< 1·5	< 1·5
Liver	0·4	0·8
Kidneys	0·7	0·6
Serum	0	0
Peritoneal fluid	0	0
Duodenum, ileum (wall)	2·6	3·3
Mesenterial glands	7·7	9·4
Content of intestines 10^9 m.	26·0	25·0
Content of stomach 10^9 m.	0	0

m., micro-organisms.

The distribution of DPN-ase in the organs of normal and infected animals

The results obtained with other organs and compared with those of the spleen are summarized in Table 10. In the course of the infection with *S. typhi* a marked rise of the DPN-ase activity was observed in the spleen, the pancreas and the wall of the stomach, vermiform appendix, sigmoid colon, and rectum. Another group of organs and fluids (brain, heart, lung, liver, kidneys, the blood serum and the peritoneal fluid) was almost equally inactive in both normal and infected animals. A third group of organs from normal animals exhibited a high DPN-ase activity which was not significantly enhanced in infected animals: duodenum, ileum, and mesenterial glands. The solid contents of the intestines, consisting mainly of intestinal bacteria of normal and infected animals, were highly active, while the stomach contents of both groups of animals were inactive.

The antigens of the infected organs and the in vivo-*grown bacteria*

An anti-normal-spleen immune serum agglutinated *in vivo*- and *in vitro*-grown *S. typhi* Ty 2 up to a dilution of 1/100. The *in vitro*-grown bacteria absorbed their

homologous agglutinins, but left in the serum agglutinins for the *in vivo*-grown strain and precipitins for spleen. As shown in Table 11 the absorption with *in vivo*-grown bacteria lowered the titres for both strains and removed all anti-spleen precipitins completely, indicating that they had adsorbed spleen antigens. Normal spleen removed its own precipitins and lowered the titre for the *in vivo*-grown bacteria, indicating that their agglutination was partly effected by anti-spleen antibodies.

Table 11. *The agglutinating and precipitating properties of two anti-mouse-spleen rabbit immune sera*

		Reciprocals of titres			
		Serum absorbed with			
Immunizing antigen	Immunoreaction with antigens	Ty 2 *in vitro*	Ty 2 *in vivo*	Normal spleen	Serum un-absorbed
Normal mouse spleen	Ty 2 grown *in vitro*	5	10	100	100
	Ty 2 grown *in vivo*	100	20	50	100
	Normal spleen	10	0	0	20
Infected mouse spleen	Ty 2 grown *in vitro*	0	0	100	100
	Ty 2 grown *in vivo*	50	20	50	200
	Normal spleen	5	0	0	10

0, negative at serum dilution 1/5.

By immunization of rabbits with suspensions of infected spleens immune sera were produced which contained O-, Vi- and H-agglutinins against *S. typhi* as demonstrated by agglutination with the *in vitro*- and the *in vivo*-grown *S. typhi* strain Ty 2, and the *in vitro*-grown strains of *S. typhi* strain O 901, *S. ballerup* (Vi) and *S. stanley* (d). As shown in Table 11 the anti-infected spleen immune serum agglutinated both strains and precipitated extracts of normal and infected spleens. Absorption with the *in vitro*-grown strain removed its homologous antibodies, but left in the serum anti-spleen precipitins and anti-spleen-grown bacterial agglutinins. Absorption with the *in vivo*-grown micro-organisms lowered the titres for both the *in vitro*- and *in vivo*-grown strain and removed the anti-spleen precipitins. Absorption with spleen extracts removed the anti-spleen precipitins and lowered the agglutinin titre for the spleen-grown strain. All these experiments proved that the spleen-grown strain possessed in addition to its own antigen spleen antigens acquired by adsorption in the course of its growth in this organ.

A spleen-grown strain Ty 2 was separated from the tissues after seven passages. It was agglutinated by anti-*S. ballerup* immune serum 1/100, anti-O 901 immune serum 1/20, while its agglutination by *S. stanley* immune serum was negative. After seven passages through mouse spleens it had lost its agglutinability by anti-d immune serum, but it was still able to produce anti-d agglutinin in its immune serum, which contained agglutinins against *S. stanley* (d) 1/500, *S. typhi* O 901 1/5000 and *S. ballerup* (Vi) 1/200. Precipitins against infected mouse spleen were

demonstrated by the test-tube method, while the precipitins against normal spleen were only demonstrated by agar gel precipitation. Absorption by the *in vitro*-grown strain removed almost completely all Vi-agglutinins as indicated by *S. ballerup* and lowered the agglutinin titre of the *in vivo*-grown strain to 1/10. Absorption by the spleen-grown *S. typhi* Ty 2 lowered only slightly the Vi-agglutinin titre indicated by *S. ballerup* and removed the anti-infected spleen precipitins completely.

Absorption with normal spleen extract left the agglutination titre for the *in vitro*-grown strain unchanged, but lowered the agglutinin titre of the *in vivo*-grown strain to 1/10, without changing the Vi-titre indicated by *S. ballerup*. Absorption with the infected spleen extract removed Vi-antibodies almost completely.

Table 12. *The agglutinating and precipitating properties of immune serum prepared by immunization with spleen-grown* S. typhi *strain Ty* 2

Immunoreaction with antigens	Reciprocals of titres — Serum absorbed with: Ty 2 *in vitro*	Ty 2 *in vivo*	Normal spleen	Infected spleen	Serum un-absorbed
Ty 2 *in vitro*	100 (H)	500 (H)	500 (H)	100 (H)	500 (H)
Ty 2 spleen-grown	10	0	10	0	50
Infected spleen	0	0	0	0	5
Normal spleen					+
S. typhi O 901	200 (O)	2000 (O)	2000 (O)	2000 (O)	5000 (O)
S. stanley	0	500 (H)	500 (H)	0	500 (H)
S. ballerup	20 (Vi)	100 (Vi)	200 (Vi)	20 (Vi)	200 (Vi)

+, Agar gel precipitation positive.
0, Negative at serum dilution 1/5.

The agglutination by the immune serum absorbed by the spleen-grown strain with *S. stanley* which contained the d-antigen indicates the loss of H-antigen by the spleen-grown strain Ty 2. As shown in Table 12, the *in vitro*-grown Ty 2 strain and the infected spleen extract lowered the H-agglutinin titre, while the *in vivo*-grown strain was unable to do so. The agglutination with strain O 901 shows that the *in vitro*-grown strain was still able to absorb O-agglutinins, while the *in vivo*-grown strain was unable to do so; this was probably due to its absorption of the host's antigens in addition to the Vi-antigen. The agglutination with *S. ballerup* showed that the *in vitro*-grown strain was more able to absorb Vi-agglutinin than the *in vivo*-grown strain.

Thus, the agglutination experiments proved that the *in vivo*-grown strain had absorbed spleen-antigens on its surface, but lost its agglutinability by anti-H-agglutinins. Similar results were obtained by the aid of the agar-gel precipitation technique. Plate 1, fig. 1, shows results obtained with an anti-*S. typhi* immune serum produced by immunization of rabbits with the *in vitro*-grown strain Ty 2. This serum produces with its homologous strain at least six precipitation lines.

A part of these antigens was shared with strain O 901 and another part, at least 4 lines, with *S. ballerup*. Line 6, the nearest one to the serum source, was shared by all three strains. No precipitation occurred with normal or infected spleen extract.

Pl. 1, fig. 2, shows the precipitation of an anti-mouse-spleen immune serum. Opposite to the normal spleen extract appeared two sharp and one diffuse precipitation lines, while opposite to the infected spleen extract only a weak diffuse precipitation line appeared. No precipitation line appeared opposite to the sources of *Salmonella*-antigens.

Pl. 1, figs. 3 and 4, shows the reaction of anti-*S. typhi* immune serum produced by immunization of rabbits with the spleen-grown strain Ty 2. Precipitation lines appeared opposite to the sources of the bacterial antigens and the spleen antigens as well. The appearance of a precipitation line opposite to both the infected and normal spleen extracts indicates that the spleen-grown bacteria had adsorbed spleen antigens and were able to produce anti-spleen antibodies in addition to their anti-bacterial antibodies.

Pl. 1, fig. 5, shows that the soluble antigen which reacted in agar gel with the anti-spleen-grown *S. typhi* immune serum was a non-specific antigen which was shared by the *S. typhi* strains Ty 2 and O 901 and also *S. ballerup*. It was, therefore, impossible to classify it as O-, Vi- or H-antigen. It seems rather that it was an intracellular-located substance liberated *in vivo* and *in vitro* by lysis of the micro-organisms.

DISCUSSION

Clinical observations of Dennis & Saigh (1945) of typhoid patients showed that during the first week of the disease precipitable somatic antigens appeared in the serum.

Olitzki *et al.* (1961) showed that in experimental infections of white mice precipitable and toxic substances accumulated in their organs and that the action of chemotherapeutics was annihilated as soon as the number of living organisms together with these precipitable, and probably toxic, substances rose to a critical level.

In the experiments described above we studied the possibility of these substances acting as pathogenizers. In the course of this work the control experiments with organ extracts of normal mice revealed that normal liver extracts also exerted a marked pathogenizing effect on virulent and non-virulent bacteria. It was not possible to ascribe this effect to a single substance present in the liver: it seems rather that several substances are acting jointly with others as shown by the experiments with sodium taurocholate and glycogen as pathogenizers. The combined effect of mucin or agar together with insoluble particles such as kaolin on the pathogenicity of *Shigella dysenteriae* was described by Olitzki & Koch (1945) and the effect of insoluble particulate matter, viscous substance and a heparin-like fraction on *S. typhi* by Smith, Harris-Smith & Stanley (1951).

The substances described by Olitzki & Koch (1945), although originating from different sources such as agar and kaolin, exerted together the virulence-enhancing

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effect. The substances of Smith *et al.* (1951) originated from the same mucin preparation but were replaceable by other substances. The two substances described above were naturally present in the same normal organ and may be responsible for the long persistence of *S. typhi* in the liver, which has been observed in sublethal experimental infections of mice by Olitzki *et al.* (1960). Furthermore, it was shown that the pathogenicity of different *Salmonella* strains was not equally influenced by normal liver extracts. The LD_{100} of *S. typhi*, strain Ty 2, decreased from 2×10^8 to 2×10^6, of strain O 901 from 1×10^9 to 2×10^7, and that of *S. hirschfeldii* from 2×10^8 to 2×10^6. In these three strains 50–200-fold decreases of the LD_{100} were observed. On the other hand, the LD_{100} of *S. stanley* decreased from 2×10^7 to 2×10, i.e. a 10^6-fold decrease.

The additional action of the antigenic substances present in the liver was not very marked in infections with strain Ty 2. Although strain Ty 2 was able to produce in a relatively short time *in vivo* aggressin-like substances, which by experiments of Olitzki *et al.* (1961) were identified as O- and Vi-antigens, and strain O 901 was unable to do so, the injection of strain Ty 2 along with liver extracts from Ty 2-infected mice did not enhance further its pathogenicity. No differences between the LD_{100} obtained with normal and infected liver extract were observed. The respective LD_{100} of strain O 901, in the presence of liver extracts from normal and with Ty 2-infected mice, were 2×10^7 and 2×10^6, i.e. again a 10-fold decrease. It seems, therefore, that an antigen of strain Ty 2, probably its Vi-antigen, acts not only as surface antigen protecting the individual bacterium against the bacteriolytic serum action and phagocytosis, but also acts in a soluble form in the infected organs promoting the pathogenicity of bacteria devoid of this antigenic substance. It is possible that this soluble antigen acts *in vivo* by its specific reaction with circulating antibodies, which otherwise would become combined with the living micro-organisms and effect their agglutination or opsonization or mediate their lysis by complement. On the other hand, it is not clear whether this effect can be ascribed to circulating Vi-antigen alone. Liver extracts from mice infected with *S. hirschfeldii* and *S. ballerup*, both strains containing Vi-antigen, did not exert a pathogenizing effect comparable with that exerted by extracts from mice infected with *S. typhi*, strain Ty 2. Combined action of Vi- and O-antigen seems to be unlikely, since mixtures of liver extracts from mice infected with *S. hirschfeldii* together with liver extracts from mice infected with *S. typhi* strain O 901 did not exert any stronger pathogenizing effect than liver extracts of normal mice. On the other hand, it was not possible to isolate from *in vivo*-grown bacteria or from infected organ extracts additional antigens which were different from the well-known H-, O- and Vi-antigens of *in vitro*-grown bacteria.

In addition to the active production of antigens the *in vivo*-grown bacteria are able to adsorb organic substances of the host on their surface and to become 'autoantigenic'. *S. typhi* grown *in vivo* in mice produced in rabbits, in addition to anti-H-, O- and Vi-agglutinins, also anti-mouse-spleen precipitins. Another method for the detection of the absorption of host substances by micro-organisms was described by Artman & Bekierkunst (1961). They showed that lungs and

spleens of mice infected with *Mycobacterium tuberculosis* contained more diphosphopyridine nucleotidase (DPN-ase) than normal lungs, and that tubercle bacilli grown *in vivo* had absorbed this enzyme. By the experiments described above it was proved that the same process took place in the spleen of mice infected with *S. typhi*. However, this process was not restricted to the virulent strain Ty 2. Other *Salmonella* strains devoid of the Vi-antigen and a pathogenic *E. coli* strain exerted the same effect. Unpublished experiments of Olitzki & Godinger showed also that in spleens of mice infected with *Brucella abortus*, *Br. melitensis* and *Pasteurella tularensis* the same process takes place. Ajl, Rust, Woebke & Hunter (1956) found DPN-ase in plague toxin and Bernheimer, Lazarides & Wilson (1957) in streptococcal culture supernatants. The fact that an amount of 10^9 spleen-grown bacteria showed a higher enzymatic activity than the spleen itself proves that a specific absorption takes place. This absorption seemed to be restricted to living, *in vivo*-multiplying bacteria, since a 100-fold amount of dead bacteria was required in order to exert the same effect *in vivo*. It also became clear that for this specific absorption the prolonged contact between living bacteria and living host tissue was required, since absorption experiments *in vitro* did not yield bacteria possessing a DPN-ase activity as strong as that exhibited by *in vivo*-grown bacteria.

The question now arises as to how this absorption of host substances by the infecting organisms determines the further course of the infection. There exists the possibility that the coating of the invading micro-organisms with host substances inhibits the ability of the antibody-producing cells to recognize the invaders as foreign substances and to produce antibodies. Such an effect has been described by Olitzki (1935) with salmonellae coated with specific antibodies in rabbits, and is known as the 'blanketing' effect of antibodies on antigenicity. It is possible that non-specific absorption of host substances may exert a similar effect.

Another effect may be exerted by the absorbed DPN-ase when bacteria are ingested by phagocytes. The absorbed enzyme may act intracellularly on the diphosphopyridine nucleotide of the cell and may seriously interfere with the cell metabolism. This process may be of less significance in infections with extracellular-growing micro-organisms, but it may become important in infections with intracellularly multiplying organisms such as brucellae and mycobacteria. Bernheimer *et al.* (1957) observed a remarkable correlation between leukotoxicity and capacity of streptococci to produce DPN-ase.

However, in infections with strain Ty 2 of *S. typhi* which is scarcely taken up by phagocytes it seems unlikely that such a process is of significance for the progress of the infection. It seems rather that for a maximal increase of virulence three conditions must be fulfilled. This can at present be done only *in vivo*: the maximal development of the cell-bound Vi-antigen, the maximal absorption of the host's antigens and the presence of soluble bacterial substances in the suspending fluid. These conditions were fulfilled in the experiments summarized in Table 8. They effected a decrease of the LD_{100} of strain Ty 2 from 2×10^8 to 10^4 and of the LD_{100} of strain O 901 from 10^9 to 10^6, i.e. 20,000 and 1000-fold decreases, respectively.

SUMMARY

1. The virulence of *S. typhi* strains Ty 2 and O 901 injected intra-abdominally into white mice was examined. The lethal dose of strain Ty 2 grown *in vivo* was lower than that of the corresponding culture grown *in vitro*, while the lethal doses of the *in vivo*- and *in vitro*-grown strain O 901 were almost identical.

2. The extracts of infected livers and spleens were not toxic, but acted as infection-promoting substances. Extracts from normal organs exerted similar but weaker effects. Glycogen together with sodium taurocholate were powerful infection-promoting substances. The highest increases of virulence were observed when spleen-grown bacteria together with extracts from infected organs were injected.

3. Extracts from infected spleen, pancreas, stomach and lower intestines, spleen-grown *S. typhi*, other spleen-grown Enterobacteriaceae, and bacteria taken from the intestines of normal and diseased animals, exhibited high DPN-ase activity.

4. The extracts from infected organs contained soluble bacterial antigens. Their presence was demonstrated by tube precipitation, agar gel precipitation and antibody production after immunization of rabbits with extracts from infected organs with addition of adjuvants.

5. Spleen-grown bacteria were able to absorb host antigens and to produce, in addition to the known agglutinating antibodies, anti-spleen precipitins.

REFERENCES

Ajl, S. J., Rust, J. Jr., Woebke, J. & Hunter, D. H. (1956). *Fed. Proc.* **15**, 581.

Artman, M. & Bekierkunst, A. (1961). *Proc. Soc. exp. Biol., N.Y.*, **106**, 610.

Bernheimer, A. W., Lazarides, P. D. & Wilson, A. T. (1957). *J. exp. Med.* **106**, 27.

Dennis, E. W. & Saigh, A. S. (1945). *Science*, **102**, 280.

Kaplan, N. O., Colowick, S. P. & Ciotti, M. M. (1951). *J. biol. Chem.* **191**, 447.

Olitzki, A. L. (1935). *J. Immunol.* **29**, 453.

Olitzki, A. L., Fleischhacker, E. & Olitzki, Z. (1957). *J. Hyg., Camb.*, **55**, 91.

Olitzki, L. & Koch, P. K. (1945). *J. Immunol.* **50**, 229.

Olitzki, A. L. & Olitzki, Z. (1958). *Bul. Res. Council of Israel* (*Sect. E., Exp. Med.*), **7**, 105.

Olitzki, A. L., Sharon, N. & Godinger, D. (1960). *Proc. Internat. Symposium Microbiol. Standardization, Opatija*, 285.

Olitzki, L., Shelubsky, M. & Hestrin, S. (1946). *Proc. Soc. exp. Biol., N.Y.*, **63**, 491.

Olitzki, A. L., Sulitzeanu, D., Sharon, N. & Gelernter, I. (1961). *Harefuah, J. med. Assoc. Israel*, **61**, 75.

Salk, J. E. & Laurent, A. M. (1952). *J. Exp. Med.* **95**, 429.

Smith, H. (1958). *Annu. Rev. Microbiol.* **12**, 77.

Smith, H., Harris-Smith, P. W. & Stanley, J. L. (1951). *Biochem. J.* **50**, 211.

EXPLANATION OF PLATE 1

Fig. 1. Reaction of an anti-*S. typhi* (Ty 2 *in vitro*-grown) immune serum with *S. typhi*, strain Ty 2, *S. typhi*, strain O 901, *S. ballerup*, normal and infected spleen. IS, anti-Ty 2 acetone-dried immune serum; T 2, *S. typhi*, strain Ty 2 grown *in vitro*, acetone-dried; 901, *S. typhi*, strain O 901 grown *in vitro*, acetone-dried; SB, *S. ballerup*, grown *in vitro*, acetone-dried; N, normal spleen; I, infected spleen.

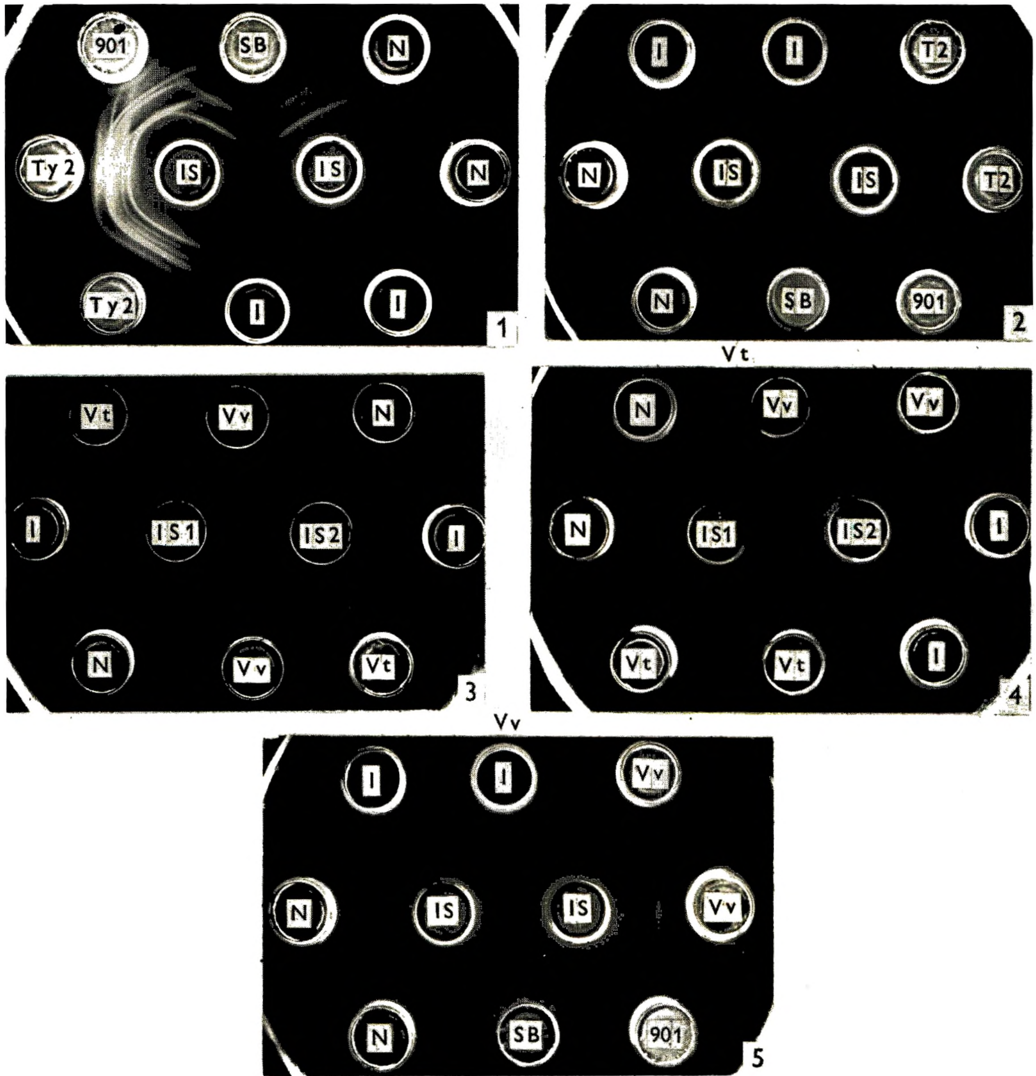
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Fig. 2. Reaction of an anti-mouse-spleen immune serum with the same antigens as used in the experiment demonstrated in fig. 1. IS, anti-mouse-spleen immune serum. The symbols of the antigens as in fig. 1.

Figs. 3, 4. Reaction of two anti-*S. typhi* (Ty 2 *in vivo*-grown) immune sera with *S. typhi* Ty 2 grown under different conditions, normal and infected spleen. IS 1 and IS 2, anti-*S. typhi* (Ty 2 spleen-grown) immune sera; Vv, *S. typhi*, Ty 2 grown *in vivo*; Vt, *S. typhi*, Ty 2 grown *in vitro*; N, normal spleen; I, infected spleen.

Fig. 5. Reactions of an anti-*S. typhi* (Ty 2 *in vivo*-grown) immune serum with *S. typhi*, strain Ty 2 grown *in vivo*, *S. typhi* strain O 901 and *S. ballerup* grown *in vitro* and acetone-dried, normal and infected spleens. IS, anti-*S. typhi* (Ty 2 spleen-grown) immune serum; 901, *S. typhi*, strain O 901 acetone-dried; SB, *S. ballerup* acetone-dried; N, normal spleen; I, infected spleen; Vv, *S. typhi*, Ty 2 grown *in vivo*.

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Comparative studies on *Salmonella typhi* grown *in vivo* and *in vitro*

II. The effect of extracts from normal and infected organs on the bactericidal serum action on strains grown *in vivo* and *in vitro*

By A. L. OLITZKI and OPHIRA KAPLAN
Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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In previous experiments of Olitzki & Godinger (1963) it was shown that *Salmonella typhi* strain Ty 2 grown *in vivo* was more virulent for mice than its corresponding culture grown *in vitro*. Extracts of infected organs acted as infection-promoting substances, while extracts from normal organs exerted similar but weaker effects. The experiments described below were carried out in order to examine whether virulent *S. typhi* grown *in vivo* is less sensitive to the bactericidal action of immune sera than the *in vitro* grown, and whether organs of normal and infected mice exert modifying effects on the bactericidal serum action, or are, in this respect, inert.

MATERIALS AND METHODS

Bacterial inocula

S. typhi strains O 901 and Ty 2, were employed. The bacteria were daily subcultured on nutrient agar and on the day of the experiment washed off from the agar slant with 5 ml. of brain-heart infusion (Difco) and pre-incubated for 1 hr. In other experiments they were washed off with 5 ml. of salt solution which contained 0·85 % of sodium chloride and 0·063 % of magnesium chloride, and used without pre-incubation. Then the number of bacteria (about 10^9/ml.) was determined photometrically and by plating. Suitable dilutions of the suspension were made in order to obtain an inoculum of 10^4 bacteria per test tube. The *in vivo*-grown bacteria were taken from spleens of infected mice, separated from the tissue as described by Olitzki & Godinger (1963), and the suitable inoculum determined by the same method as employed with the *in vivo*-grown bacteria.

Preparation of immune sera

The antisera employed were the same as used by Olitzki & Godinger (1963).

Tissue extracts

These extracts were prepared from organs of normal and infected mice according to the method described by Olitzki & Godinger (1963). 0·5 ml. of organ extract per 2·0 ml. of the total volume were added to each tube in order to examine the effect of the organ extract on the bactericidal serum action.

The bactericidal test

This test was performed according to the technique described by Felix & Olitzki (1926). Modifications of this technique are mentioned. The incubation times of the tubes which contained the bacteria exposed to immune serum and complement and of the corresponding control tubes were varied according to the conditions of the individual experiments as described below. While in the experiments of Felix & Olitzki (1926) all test tubes contained constantly 10% broth, we varied this broth concentration according to the conditions of the individual experiments.

RESULTS

The experiment presented in Table 1 shows that the bactericidal action of an anti O 901 immune serum on the homologous strain is highly enhanced by the presence of 0·1 ml./2·0 ml. broth in each test tube. This nutrient enhanced the growth of the bacteria which were not exposed to the bactericidal action of immune serum and complement but, on the other hand, it enhanced the bactericidal action when immune serum and complement were present. The experiment presented in Table 2 also shows clearly that multiplying bacteria are more sensitive to the bactericidal immune serum-complement action than non-multiplying bacteria. Table 3 shows that there exists a quantitative relationship between broth concentration, ability to multiply in it and sensitivity to the bactericidal serum action. The latter increases with the broth concentration and the improvement of the growth condition.

Table 1. *Observations on the influence of nutrient broth on the serum bactericidal action at different exposure times*

(Broth concentration, 5%. Dilution of anti-O 901-immune serum, 10^{-3}. Dilution of complement, 10^{-1}. Strain O 901 grown *in vitro*)

	Bacterial count (thousands)			
	In the presence of broth		Without broth	
Time of exposure (min.)	With antibody and complement	Without antibody and complement	With antibody and complement	Without antibody and complement
10	0·80	16·4	2·2	16·4
20	0·20	26·4	0·90	17·8
30	0·40	—	0·80	—
40	0·04	28·0	0·12	18·0
50	0	—	0·02	—
60	0	32·0	0·02	16·8
120	0	43·0	0·02	19·0
0	—	—	—	22·4

The addition of glucose and ammonium sulphate to the test tube enhanced the bactericidal serum action, although these nutrients did not effect an immediate multiplication of the micro-organisms. As shown in Table 3, the growth-enhancing

activity of the two together was approximately equivalent to a 0·025 % broth concentration. The enhancement of the bactericidal serum action in the presence of 1·0–2·0 % glucose and 0·5–1·0 % ammonium sulphate was well marked. These

Table 2. *The influence of two broth concentrations on the bactericidal action of different serum and complement dilutions*

(Strain O 901 grown *in vitro*)

Complement dilutions	Bacteria suspended in	No. bacteria after 2 hr. incubation: At antibody dilutions 10^{-2}	10^{-3}	10^{-4}	With complement only
1/10	Saline-$MgCl_2$ solution	0·56	0·80	1·20	5·20
	5 % broth added	0·02	0	0·02	0·40
	10 % broth added	0·02	0	0	0·40
1/15	Saline-$MgCl_2$ solution	0·48	1·40	1·60	7·20
	5 % broth added	0·02	0·04	0·04	1·60
	10 % broth added	0	0	0·04	0·40
Controls without complement	Saline-$MgCl_2$ solution	2·2	8·6	8·2	—
	5 % broth added	2·4	7·5	13·0	—
	10 % broth added	3·2	11·0	9·2	—

At 0 hr. inoculum, 27·0; after 2 hr. in saline-$MgCl_2$, 29·0; after 2 hr. in 5 % broth, 76·0; after 2 hr. in 10 % broth, 79·0.

Table 3. *The influence of different nutrients and varying broth dilutions on the bactericidal action of anti O-immune serum (dilution* 10^{-3}*) and complement (dilution* 1/20)

(Exposure 2 hr. at 37° C. *S. typhi* O 901)

Nutrients present and their concentrations %	Bacterial count (thousands) after exposure to the suspension fluid: With O-antibody and complement	Without antibody and complement	With antibody only	With complement only
Broth 2·5	0·04	61·0	—	—
1·0	0·19	51·9	—	—
0·05	0·37	48·8	—	—
0·025	0·48	30·4	—	—
Glucose 2 % + $(NH_4)_2SO_4$ 1 %	0·10	29·2	—	—
Glucose 1 % + $(NH_4)_2SO_4$ 0·5 %	0·13	25·9	—	—
Glucose 0·5 % + $(NH_4)_2SO_4$ 0·2 %	0·26	27·0	—	—
Saline $MgCl_2$ solution	0·56	27·5	4·4	2·1
Saline $MgCl_2$ solution 0 hr.	—	22·4	—	—

nutrients effected also a slow multiplication of the micro-organisms which became visible after an incubation at 37° C. for 18 hr. Experiments with short incubation times at 37° C., as that presented in Table 4, showed that the bactericidal serum action started after only a few minutes contact between the micro-organisms and the antibody-complement system. During this short period no increase of the bacterial count was observed in the control tubes, which contained the bacterial suspension without antibody or complement. When bacteria pre-incubated for 1 hr. in brain-heart infusion were transferred into the salt solution there was even some decrease of the bacterial count. These experiments proved that the bactericidal action of the serum was not necessarily associated with the real multiplication of the micro-organisms, but rather with the morphological and biochemical changes which took place during the lag phase, e.g. the increase of the average body length which preceded the cell division and had been described by Henrici (1923). The relationship between sensitivity to bactericidal serum action and increase in length during the lag phase is demonstrated in Table 4. It shows that the sudden increase in length during the incubation time is correlated with the sensitivity to bactericidal action which starts immediately on exposure to antibody and complement, before real cell divisions have taken place, since according to the bacterial counts in the control tubes the inoculum became doubled only after a 2 hr. incubation, meaning that one cell division was completed only after 2 hr. Therefore we concluded that the presence of nutrients may modify the bactericidal test in two ways: (1) it may speed up the increase of the bacterial count in the control tubes which do not contain antibody and complement; (2) it may enhance the bactericidal power of the antibody-complement system. It follows that experiments with normal and infected organ extracts may be influenced by their nutritional values and that the results obtained with them should be evaluated accordingly.

Table 4. *The morphological changes which occur after the transfer of* S. typhi *strain* O 901 *from a* 24 *hr. culture into two suspension fluids and the correlation of these changes with the sensitivity to bactericidal action*

(Dilution of anti-O immune serum 10^{-3}, dilution of complement 10^{-1}.)

		Changes observed after inoculation									
	Control before incuba-tion	Into broth after an incubation of (min.)					Into NaCl-$MgCl_2$ solution after an incubation of (min.)				
Observed changes		5	10	20	30	120	5	10	20	30	120
Average length (μ)	2·87	3·34	3·50	3·89	2·95	—	3·16	3·16	3·29	3·46	—
% of bacteria:											
shorter than 3·5 μ	79	58	60	42	40	—	77	77	64	57	—
3·5 μ or longer	21	42	40	58	60	—	23	23	36	43	—
Bacterial count/1000 with amboceptor and complement	17·5	1·2	0·7	—	0·07	0	2·4	1·4	—	0·6	0·4
Control without amboceptor and complement	17·5	—	—	—	—	35·0	—	—	—	—	—

Table 5 shows the effect of organ extracts of normal and infected mice on the bactericidal serum action on strain O 901. The control experiments without the bactericidal system showed that both the normal and the infected organ extracts

exerted a marked growth-promoting activity when added to the bacterial inoculum in saline or in broth.

As expected, all organ extracts from normal mice enhanced the bactericidal serum action both in saline and in broth. The extracts of organs from infected mice also promoted bacterial growth and, therefore, they enhanced also the bactericidal serum action when added to a bacterial suspension exposed to immune serum and complement in a salt solution. However, when extracts of infected organs were added to bacteria suspended in broth which itself constituted a powerful nutrient then the bactericidal action was lowered probably by soluble antigens present in infected liver, lungs and kidneys.

Table 5. *The influence of organ extracts from normal and infected mice on the growth of strain* O 901 *grown* in vitro *and the bactericidal serum action on it, anti-O immune serum dilution* 10^{-3}, *complement dilution* 1/20, *incubation* 2 *hr. at* 37° C.

	Bacterial count (thousands) in the presence of							
	Amboceptor and complement with addition of				Without amboceptor and complement with addition of			
	Infected organ extracts		Normal organ extracts		Infected organ extracts		Normal organ extracts	
Extracts prepared from	Salt solution	Broth 5 %	Salt solution	Broth 5 %	Salt solution	Broth 5 %	Salt solution	Broth 5 %
Heart	1·50	0·10	0·60	0·04	90·0	190·0	77·0	206·0
Lung	5·60	1·20	3·70	0·32	88·0	160·0	72·0	190·0
Lymphatic glands	0·06	0	0·20	0·04	110·0	150·0	48·0	140·0
Kidneys	1·30	0·12	0·36	0·04	120·0	220·0	72·0	154·0
Spleen	0·04	0·04	0·56	0·04	69·0	210·0	56·0	180·0
Liver	8·00	4·00	0·36	0·24	44·0	160·0	54·0	200·0
Peritoneal fluid	2·30	0·06	0·88	0·04	48·0	190·0	78·0	300·0
Brain	0·16	0·08	1·50	0·12	76·0	260·0	68·0	330·0
Controls without organ extract:								
0 hr.	—	—	13·0	18·0	—	—	18·0	18·0
2 hr.	—	—	12·0	0·72	—	—	26·0	120·0

If we compare in Table 5 the bactericidal effects exerted in saline and in broth, in the presence of normal extracts from heart, lungs, kidneys and liver, with that exerted in the presence of the respective extracts from infected organs, then we find that the bactericidal effect in the presence of infected organs was always lower than that observed in the presence of normal organ extracts. We repeated three times the experiments with normal and infected heart, kidneys and liver extracts and obtained the results summarized in Table 6. The results obtained with several extracts show that the majority of organ extracts of mice infected

with strain Ty 2 may exert an inhibiting effect on the bactericidal serum action on strain O 901.

Experiments with *S. typhi* strain Ty 2, grown *in vitro*, showed that this strain was less sensitive to the bactericidal action of an anti-O 901-immune serum than O 901 itself, but this resistance could be broken when 5 % broth was added and complement dilutions of 1/10 and 1/6 employed, as shown in Table 7.

Table 6. *The influence of organ extracts on the bactericidal serum action on strain* O 901 *grown* in vitro

(Incubation time 2 hr. at 37° C. Other experimental conditions as in Table 5.)

		Bacterial count (thousands) in the presence of amboceptor and complement with addition of			
		Infected organ extracts		Normal organ extracts	
Expt.	Organs	Saline-$MgCl_2$	Broth 5 %	Saline-$MgCl_2$	Broth 5 %
1	Heart	15·0	—	1·1	—
	Kidneys	8·6	—	1·4	—
	Liver	7·3	—	8·5	—
	Control without extract	16·0	—	—	—
2	Heart	16·0	—	0·5	—
	Kidneys	11·0	—	0·5	—
	Liver	16·0	—	4·4	—
	Control without extract	14·0	—	—	—
3	Heart	13·3	7·8	0·9	0·4
	Kidneys	13·8	3·6	0·5	0·08
	Liver	20·9	14·8	13·0	7·9
	Control without extract	16·0	0·3	—	—

Table 7. *The bactericidal power of anti-*O 901 *immune serum on Ty* 2 *grown* in vitro

(Incubation period 2 hr. Immune serum dilution 10^{-3}. Three dilutions of complement.)

	Bacterial count (thousands) after 2 hr. incubation with complement diluted		
Suspension fluid	1/6·7	1/10	1/20
Salt solution	100·0	110·0	122·0
5 % broth	1·7	9·6	89·0
Controls:			
Salt solution 0 hr.	—	—	120·0
Salt solution 2 hr.	—	—	160·0
Broth 5 % 2 hr.	—	—	210·0

There were no differences between the sensitivities of the *in vitro*- and the *in vivo*-grown strain Ty 2. The latter, when suspended in the salt solution, was resistant to *S. ballerup* immune serum which contained the Vi-antibody and to anti-O 901 immune serum which contained the O-antibody, but became sensitive

as soon as 5 % broth was added. In order to get a bactericidal effect, complement dilutions of 1/5 and 1/10 were employed, as demonstrated in Table 8.

Table 9 shows that all normal organ extracts added to saline or to broth promoted the multiplication of Ty 2 grown *in vivo*. The majority of the organ extracts from infected mice exerted similar growth promoting effects.

Table 8. *The bactericidal effect of anti*-S. ballerup *and anti*-O 901 *immune sera on strain Ty* 2 *grown* in vivo

Incubation period 2 hr.

Immune serum	Suspension fluid	Bacterial count (thousands) after 2 hr. incubation with: Antibody and complement, diluted 1/5	Antibody and complement, diluted 1/10	Complement only, diluted 1/5	Complement only, diluted 1/10	Antibody only
S. ballerup 10^{-2}	Salt solution	1·8	1·9	2·1	1·9	1·7
	5 % broth	0·2	0·3	0·2	0·3	2·6
S. ballerup 10^{-3}	Salt solution	1·3	2·0	2·1	1·9	1·0
	5 % broth	0·08	0·08	0·2	0·3	2·4
S. typhi O 901 10^{-3}	Salt solution	2·9	3·2	1·3	1·9	3·0
	5 % broth	0·2	0·08	0·1	0·3	1·0

Controls without immune serum and complement: 0 hr., 2·3; 2 hr. salt solution, 2·8; 2 hr. broth, 4·4.

The bactericidal action was enhanced by almost all normal organ extracts independently of whether the bacteria were suspended in the salt solution or in broth. Only in one experiment with a 4 hr. incubation time did the normal liver and the normal lung extracts effect some inhibition of bactericidal action. In the experiment carried out with a 4 hr. incubation time, anti-bactericidal effects were exerted by extracts from infected heart, lung, lymphatic glands, kidneys, liver and peritoneal fluid. These effects appeared when the extracts were added either to the salt solution or to the 5 % broth solution.

In the second experiment presented in Table 9, done with a 2·0 hr. incubation, these effects of infected organ extracts were less marked. However, higher bacterial counts in comparison with normal extracts were observed in the presence of infected extract from the heart, lungs, lymphatic glands, kidneys, liver and peritoneal fluid.

DISCUSSION

The experiments described have shown that the avirulent *S. typhi* strain O 901 and the virulent strain Ty 2, grown *in vitro* or *in vivo*, are affected by bactericidal immune sera, if the conditions of the suspension fluid permit the multiplication of the micro-organisms or at least their elongation which precedes cell division. These results are in accordance with the observations made by Adler & Olitzki (1955)

Table 9. *The influence of organ extracts from normal and infected mice on the growth of strain Ty 2 grown* in vivo *and the bactericidal serum action on it*

(Immune serum dilution 10^{-3}. Complement dilution 10^{-1}.)

Incubation period and immune sera employed	Extracts prepared from	Bacterial count (thousands): In the presence of antibody and complement with addition of — Infected organ extracts — Salt solution	Broth 5 %	Normal organ extracts — Salt solution	Broth 5 %	Without antibody and complement with addition of — Infected organ extracts — Salt solution	Broth 5 %	Normal organ extracts — Salt solution	Broth 5 %
4·0 hr. anti-O 901 immune serum	Heart	16·0	2·0	0·6	6·5	160·0	200·0	240·0	329·0
	Lung	40·0	28·0	24·0	2·9	180·0	240·0	120·0	190·0
	Lymphatic glands	20·0	28·0	0·1	0·2	240·0	300·0	104·0	290·0
	Kidneys	8·9	41·0	0·7	0·3	240·0	245·0	168·0	290·0
	Spleen	2·4	0·4	5·0	0·9	180·0	200·0	210·0	160·0
	Liver	13·0	8·8	38·0	20·0	124·0	230·0	88·0	300·0
	Peritoneal fluid	32·0	18·0	9·6	0·3	24·0	180·0	192·0	300·0
	Brain	2·0	0·8	1·3	0·8	180·0	320·0	190·0	340·0
	Control without extract								
	0 hr.	—	—	17·2	17·2	—	—	17·2	17·2
	2 hr.	—	—	39·0	0·8	—	—	30·0	160·0
2·0 hr. anti-O 901 immune serum	Heart	2·4	0·2	0·2	0·02	8·8	12·0	8·6	10·8
	Lung	3·0	1·0	0·9	0·3	8·4	7·6	6·4	13·6
	Lymphatic glands	4·2	5·2	0·2	0·08	5·6	7·6	6·4	8·4
	Kidneys	1·3	1·2	0·2	0·08	8·0	6·8	7·2	9·2
	Spleen	0·2	0·02	0·2	0·1	8·4	8·8	7·0	13·0
	Liver	0·9	0·8	0·5	0·1	5·2	6·0	6·0	10·0
	Peritoneal fluid	2·9	2·1	0·6	0·08	8·4	8·0	10·0	8·0
	Brain	0·08	0·04	0·2	0·06	7·2	8·0	9·2	8·0
	Control without extract								
	0 hr.	—	—	1·2	1·2	—	—	1·2	1·2
	2 hr.	—	—	2·9	0·2	—	—	1·9	5·1

who observed that strain O901 was not attacked by active rabbit serum diluted 1/5, when the calcium and magnesium ions were removed by ethylene diamine tetra-acetate (EDTA). In all control test tubes which contained 5 % broth, inactivated immune serum and EDTA, incubated for 3 hr. at 37° C., the bacterial count was exactly the same as that of the inoculum at the beginning of the experiment. This observation proved that in absence of calcium and magnesium ions bacterial growth was stopped. Thus, the addition of EDTA inhibited both the multiplication of the bacteria and also the bactericidal action of the active serum which in the absence of EDTA was able to reduce the bacterial count within 3 hr. from 10^4 to about 10. The experiments described above confirm generally the results of the bactericidal tests obtained by Felix & Olitzki (1926), but, on the other hand, they show that the resistance of *S. typhi* to the bactericidal serum action is determined not only by the antigenic structure, e.g. presence of the Vi-antigen, but also by growth phase and the nutritional conditions of the suspension fluid during contact with the antibody-complement system. The nutrients which enhance the bactericidal action need not lead to an immediate stimulation of cell division and rise of the viable count, but may activate the processes which take place during the lag phase and which manifest themselves by an increase in cell length (Henrici, 1923), by an increase of total nitrogen (Herschey, 1938), by an initial drop and a second rise of ribonucleic acid (Wade, 1952) and by an increase of oxygen uptake (Martin, 1932). It is possible that the fixation of the antibody-complement system by the bacterial cell interferes with one of these preparatory processes which take place during the lag phase. Since resting cells of strain Ty 2 kept in the absence of any nutrient are resistant to the bactericidal serum action, it becomes clear that in the course of the *in vivo* multiplications the tissue fluids may exert a twofold effect: they may supply the necessary nutrients for the multiplication of the invader, but by the same supply of nutrients they may enhance the bactericidal serum action *in vivo* and speed up the elimination of the invader. On the other hand, it may happen that an environment poor in nutrients may prevent the multiplication of the invader, in which case its elimination by the bactericidal serum action may also be inhibited by the lack of the suitable phase which makes it sensitive to bactericidal serum action. In such a case a resting, stationary phase of the infection process may appear.

An absolute resistance of *in vivo*-grown bacteria to bactericidal serum action was not observed, and if in some experiments the *in vivo*-grown strain Ty 2 was resistant, this resistance could be broken by addition of nutrients as broth and/or organ extracts. On the other hand, it became evident that in the course of the experimental typhoid infection of the white mouse with strain Ty 2 infection-promoting substances were produced. The activity of these substances was demonstrated by their ability to lower the minimal lethal dose (Olitzki & Godinger, 1963) and, in the experiments described above, by their anti-bactericidal action. These substances seem to be partly identical with the known agglutinogens and partly identical with soluble antigens demonstrated in infected organs by the aid of the agar gel precipitation by Olitzki & Godinger (1963).

SUMMARY

The sensitivity of *in vitro*- and *in vivo*-grown strains of *S. typhi* was determined not only by their antigenic structure, but also by their growth phase. An increased vulnerability to the antibody-complement system has been found in cells during the lag and the early exponential phase, while non-multiplying cells devoid of any nutrient were almost invulnerable to the antibody-complement system. Extracts of organs from normal and infected animals may promote the growth of *S. typhi*, and, therefore, increase its vulnerability to the antibody-complement system. The majority of extracts from organs of mice infected with strain Ty 2 inhibited markedly the bactericidal action of serum on this strain and, to a lower extent, on strain O 901.

REFERENCES

ADLER, J. & OLITZKI, A. L. (1955). *Harefuah, J. med. Assoc. Israel*, **49**, 113.
FELIX, A. & OLITZKI, A. L. (1926). *J. Immunol.* **11**, 31.
HENRICI, A. T. (1923). *Proc. Soc. exp. Biol., N.Y.*, **21**, 215, 343.
HERSCHEY, A. D. (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 127.
MARTIN, D. S. (1932). *J. gen. Physiol.* **15**, 691.
OLITZKI, A. L. & GODINGER, D. (1963). *J. Hyg., Camb.*, **61**, 1.
WADE, H. E. (1952). *J. gen. Microbiol.*, **7**, 24.

J. Hyg., Camb. (1963), **61**, 31
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Demonstration of complement-fixation titres against the species-specific trachoma antigen in sera of trachoma patients

By A. L. TERZIN, M. R. FORNAZARIĆ and B. V. BIRTAŠEVIĆ
Medical Faculty and HE Military Laboratory, Sarajevo, Yugoslavia

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INTRODUCTION

It seems to be well established that the isolation of viable trachoma virus from conjunctival scrapings showing no inclusion bodies usually requires two or more blind passages and laboratory facilities not available in everyday routine (Hanna, 1962). At present, virus isolation is not, as a diagnostic method, much superior to the demonstration of inclusion bodies in conjunctival scrapings (Snyder *et al.* 1959; Sowa & Collier, 1960; Grayston, Wang, Woolridge, Yang & Johnston, 1960; Murray, Guerra, Abbot & McComb, 1962; and others). Consequently, much endeavour has been directed toward elaboration of a serological method that could be used in routine, both for the diagnosis of trachoma infections and for differentiating infections with trachoma virus from those caused by other agents of the same group (Bedsonia group) of viruses.

As indicated in a review by Bedson (1959), a good deal of valuable information has been accumulated on the antigenic composition of the psittacosis-lymphogranuloma group of infective agents.

However as yet only some of the observations bearing on the antigenic composition of psittacosis virus have been used fully in the efforts to perfect the serology of trachoma (or even the routine serology of psittacosis as practised by workers in the respective medical and veterinary branches). The species-specific antigenic activity of elementary body suspensions, first observed by Bedson in 1936 in his studies on psittacosis virus, seems to be confirmed also by recent studies on trachoma.

Numerous authors, using semipurified suspensions of trachoma virus as antigens, in agglutination or complement fixation (CF) reactions have succeeded in detecting species specific antibodies in sera of trachoma patients.

Some of these claims seem to be invalidated by apparent neglect of two facts:

(*a*) the possible presence of a group-reactive antigenic component in semipurified suspensions of elementary bodies, and

(*b*) the possible presence of group-reactive antibodies in sera of those trachoma patients who might have had previous contacts with other members of the psittacosis-lymphogranuloma group of viruses.

Nevertheless, several authors have found elementary-body antigens useful for demonstrating species-specific agglutination (Bernkopf, 1962; and others) or CF-reaction (Woolridge & Grayston, 1962; and others).

Recently we submitted additional evidence that in sera of trachoma patients group-reactive CF-titres are of no diagnostic value in respect to trachoma (Terzin & Birtašević, 1962). We reported then also, that trachoma infection did not produce group-specific CF-inhibiting antibodies in sera of men as detected by CF-inhibition reaction with group-reactive Bedsonia–anti-Bedsonia indicator system.

The present report shows that semipurified elementary body suspensions of trachoma virus, as prepared in our laboratory, may be used for detection of species-specific antibodies only in Bedsonia-negative samples of sera. The elementary body suspension was prepared according to a procedure used by Collier (1961) for the production of trachoma vaccine, and modified as described below.

MATERIALS AND METHODS

(1) *The trachoma strain and its propagation in ovo*

A strain of T'ang's trachoma virus, obtained from Dr F. B. Gordon (Naval Medical Research Institute, Bethesda, U.S.A.) has been used for the preparation of the various antigens, referred to in this report.

Several other strains were sent to us, from other laboratories, as non-lyophilized preparations, but from none of these, received after 10–14 days of journey, could viable trachoma virus be recovered.

From Dr F. B. Gordon, we received a yolk membrane suspension from the harvest of the 34th egg passage, lyophilized from SPG suspending medium, and sealed in nitrogen.

In our laboratory the strain was passed seven more times through eggs.

Prior to inoculation the eggs were incubated at about 38° C. Embryos found alive at the 7th day of age were inoculated into the yolk sack, and further incubated at 34–35° C. Yolk sacs were inoculated with 0·20 to 0·25 ml. of a well-homogenized suspension of infected yolk membranes (harvest of the 38th to 40th egg-passages), diluted 10^{-2} in plain broth, containing 500 units of streptomycin per ml. and clarified by centrifugation for 10 min. at 2000 r.p.m.

Embryos found dead 72 hr. after inoculation were discarded. At the 5th to the 7th day after inoculation, all embryos died or showed very sluggish movements. Embryos found dead or very sluggish were chilled for 2–4 hr., and harvested. Membranes showing congestion (the embryos themselves showing congestion and haemorrhages of the skin) were collected and stored in a deepfreezer until used for the preparation of antigens.

(2) *Preparation of the elementary body antigens*

All operations were conducted with materials cooled at 1°–4° C. and handled as sterile. All centrifugations at speeds below 3000 r.p.m. were done in horizontal heads, and those at higher speeds in angle heads.

The SPG solution (Bovarnick, Miller & Snyder, 1950) was adjusted to pH 7·2. The glycerol, formalin and other reagents used in these experiments, were all of analytical grade. The yolk membranes were homogenized in a Waring blender in three consecutive cycles, each of about 40 sec. (total time of grinding 2 min.).

A step-by-step description of the preparation is shown in the flow diagram (Fig. 1). In addition to the 'SPG-glycerol' and the 'SPG-formol' antigens, we prepared a batch of 'saline-formol' antigen. This saline antigen also, was prepared according to the procedure shown in the flow diagram, except that through the whole work sterile saline was used as diluent instead of the sterile SPG solution. Giemsa-

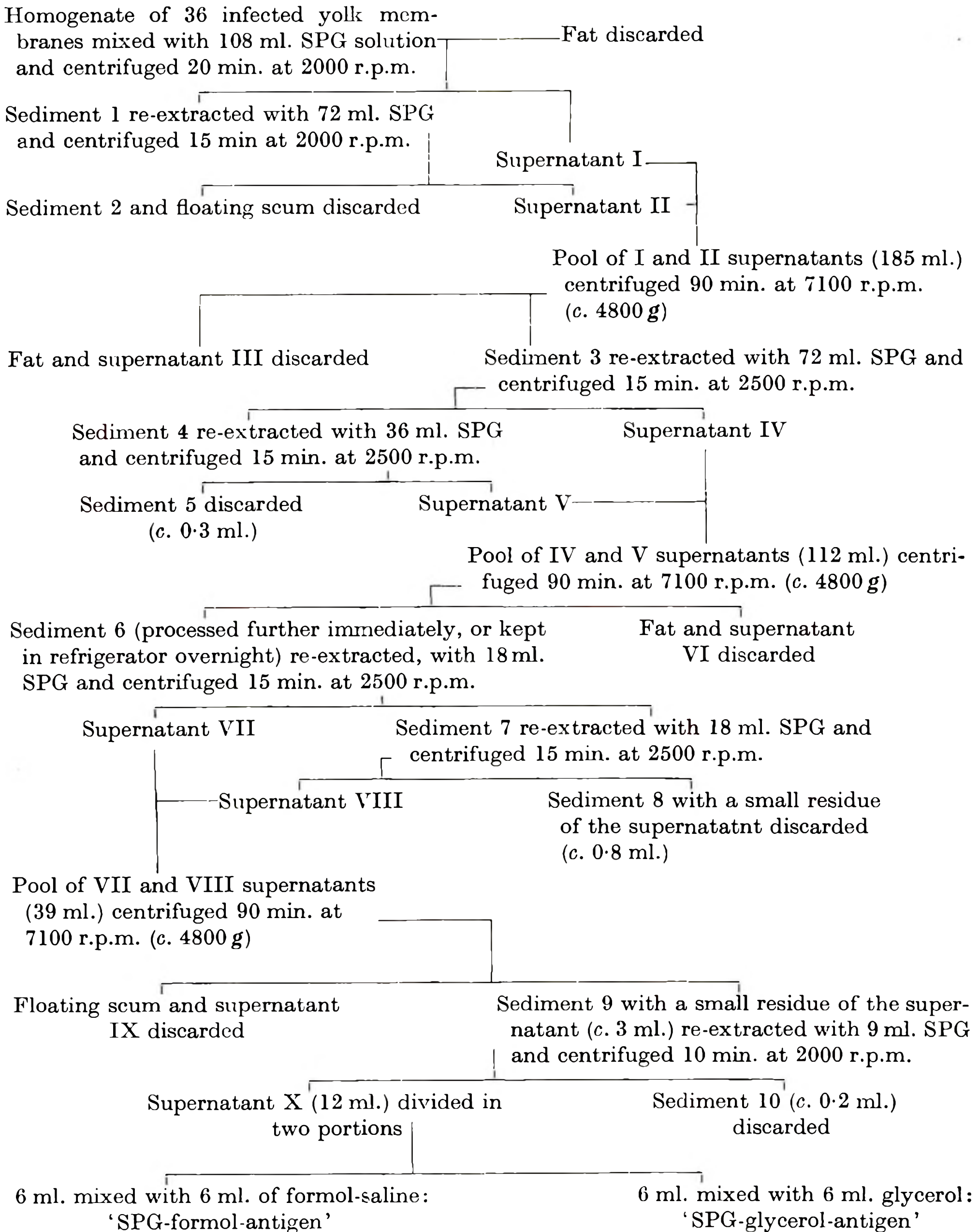

Fig. 1. Flow diagram for the preparation of elementary body antigens.

stained smears of samples from all three antigens showed under the microscope semi-purified elementary body suspensions of approximately equal density.

To both kinds of 'formol-antigen' the formalin was added in a final concentration of 0·04 %.

All three antigens were tested with more than 20 Bedsonia-negative serum specimens from patients convalescent from infections with influenza A, influenza B, or mumps viruses. None of the antigens showed fixation with any of the influenza A, B or mumps-positive, nor with Bedsonia-negative serum specimens.

(3) *Preparation and comparison of group-specific soluble antigens prepared from psittacosis and trachoma viruses*

From trachoma-infected yolk membranes we prepared the group-reactive soluble antigen, according to the procedure described previously (Terzin, Matuka, Fornazarić & Hlača, 1961).

When comparing the complement fixing activity of this antigen with that of the group-reactive soluble antigen prepared from psittacosis virus, we could detect no differences in regard to specificity, sensitivity or height of the titre against sera of men and pigeons recovered from infection with ornithosis, or against sera of rabbits and guinea-pigs immunized with psittacosis virus (Table 1).

More than 60 specimens of serum obtained from men, sheep, cattle and birds have been tested with both of the group-reactive antigens prepared from trachoma and from psittacosis virus. All serum specimens showing negative CF-reactions with the first antigen were negative also with the other and those showing positive CF-tests gave the same titre with both antigens (Table 1).

(4) *Complement fixation (CF) tests and titration of sera*

Both the technique and the reagents used in performing the CF-test were described previously (Terzin & Birtašević, 1962). All sera were titrated in twofold serial dilutions from 1/2 to 1/128 in the following sequence:

(*a*) All sera were first tested for anticomplementary activity with appropriate dilutions of the control antigen;

(*b*) all sera showing no anticomplementary activity, were tested against two dilutions of the group-reactive antigen, one containing 4 and the other 16 CF-units of the antigen;

(*c*) sera showing no CF-activity against the group-reactive Bedsonia-antigen, were tested, each against two dilutions of the SPG-formol-antigen', the 'SPG-glycerol-antigen' and the 'saline-formol-antigen'. All three antigens were used in dilutions 1/6 and 1/12, containing from 2 to 6 CF-units of the respective species-specific trachoma antigen per tube. Serum titres against antigen dilutions containing more than one sixth of an anticomplementary unit, were disregarded. Chiefly for this reason the numbers of serum specimens tested with each of the three antigens were different.

All serum specimens were obtained from the same 'Working Collective T', from cases diagnosed as trachoma by the clinical and epidemiological criteria described previously (Terzin & Birtašević, 1962). The classification of trachoma

Table 1. *Group-specific and species-specific complement fixation*

		Antigens												
					Trachoma									
		Psittacosis soluble			Soluble			SPG-formol			SPG-glycerol			
		1/256	1/512	1/1024	1/128	1/256	1/512	1/8	1/16	1/32	1/8	1/16	1/32	1/64
Anti-psittacosis serum (guinea-pig)	1/16	+++	+++	–	+++	+++	++	+++	+++	+	+++	+++	++	–
	1/32	+++	+++	–	+++	+++	–	+++	+++	–	+++	+++	+	–
	1/64	+++	+++	–	+++	+++	–	–	–	–	–	–	–	–
	1/128	+++	++	–	+++	+++	–	–	–	–	–	–	–	–
	1/256	++	+	–	++	–	–	–	–	–	–	–	–	–
	1/512	–	–	–	–	–	–	–	–	–	–	–	–	–
Bedsonia-negative trachoma serum (human)	1/4	–	–	–	–	–	–	+++	+++	–	+++	+++	++	–
	1/8	–	–	–	–	–	–	+++	+++	–	+++	+++	++	–
	1/16	–	–	–	–	–	–	+++	+++	–	+++	+++	+	–
	1/32	–	–	–	–	–	–	+++	–	–	+++	+++	–	–
	1/64	–	–	–	–	–	–	–	–	–	+++	+	–	–
	1/128	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 2. *Analysis of the duration of trachoma in various stages and the findings revealed by CF-test with three trachoma antigens*

			SPG-formol-antigen				SPG-glycerol-antigen				Saline-formol-antigen			
				Positives found				Positives found				Positives found		
Stages	Duration (years)	Mean duration (years)	No. of cases tested	No. of cases	%	Mean titres	No. of cases tested	No. of cases	%	Mean titres	No. of cases tested	No. of cases	%	Mean titres
II	1–5	3·11	18	13	72·2	1/18	5	4	80·0	1/32	13	4	30·8	1/2
III	3–11	5·33	15	12	80·0	1/20	5	5	100·0	1/42	16	7	43·8	1/3
IV	3–12	7·61	18	11	61·1	1/9	6	5	83·4	1/12	—	—	—	—
All	1–12	5·35	51	36	70·6	1/15	16	14	87·5	1/25	29	11	38·0	1/2·74

cases into stages was made according to the same criteria as described previously.

As shown in Table 2, the clinical stage is correlated with the duration of trachoma and the data on mean duration of the disease according to stages, as found in this material, are well comparable with the corresponding data found in our previous material (Terzin & Birtašević, 1962).

Fifty one serum specimens were selected which showed neither anticomplementary activity nor CF-reaction with the group-specific Bedsonia antigen. These 51 specimens were tested against experimental batches of the different species-specific trachoma antigens described in this report. For the titration of sera, if not stated otherwise, the species-specific antigens were used at the 3rd to 20th day after their preparation.

RESULTS AND CONCLUSIONS

The results seem to indicate significant differences in the incidence of CF-positive reactors, in the average CF-titres, and in the degrees of correlation between duration or stage of the diseases and the CF-titres against the various species-specific trachoma antigens. These results also seem to indicate differences between preparations in respect to stability of species-specific CF-activity, anticomplementary activity, and in other respects.

The results reported here were reproducible in the sense that from each of the SPG antigens investigated two batches at least gave identical results, and out of the 51 selected serum specimens from trachoma patients, about 30 were tested at least twice each with the same type of antigen giving comparable results at the first and subsequent titrations.

(1) *The 'saline-formol-antigen'*

As shown in Table 2, the antigen prepared by using saline as diluent, was significantly inferior to the other two antigens, both in the number of positive reactors it disclosed, and on the basis of the titres revealed with the same Bedsonia-negative sera.

(2) *The 'SPG-glycerol-antigen'*

As shown in Tables 1 and 2, when compared with the other two antigens, the 'SPG-glycerol-antigen' revealed the greatest incidence of positive reactors (about 90 % of the trachoma patients tested), showed higher antigenic titres than the other two species-specific antigens, and revealed the highest CF-titres in the Bedsonia-negative serum samples of the trachoma patients tested.

In contrast to the formol-antigens, the species-specific activity of the glycerol antigen was more stable. However, when retested several times subsequently, the antigen preserved with glycerol showed a gradually increasing anticomplementary activity. It is well known that serum samples mixed with glycerol gradually become anticomplementary. However many preparations of various viral, rickettsial, or leptospiral antigens have been found to keep well with glycerol (Cabasso, Markham & Cox, 1951; Terzin, 1953; Brand & Keil, 1955; Galton,

Powers, Hall & Cornell, 1958; and others), in our laboratory revealing no anticomplementary activity even after being stored for years.

Table 3 shows the appearance of the anticomplementary activity at various time intervals in the two types of antigen compared.

Although in several respects superior to the formol antigen, the glycerol-preserved antigen had to be abandoned because of its liability to become anticomplementary on storage.

Table 3. *Titres of anticomplementary activity of antigens*

	Days after preparation				
Antigens	2	10	20	30	130
'SPG-formol'	< 1/1	< 1/1	1/1+	1/1	1/1
'SPG-glycerol'	1/1	1/2	1/4+	1/4	1/8+

(3) *The 'SPG-formol-antigen'*

As shown in tables 1, 2 and 3 the 'SPG-formol-antigen': showed fairly high species-specific-antigenic titres as detected by CF-titration against Bedsonia-negative sera of trachoma patients, revealed both a high incidence of trachoma-positive reactors (about 70 %) and reasonably high species-specific titres in the sera of trachoma patients tested. Kept at +4° C. the SPG-formol-antigen did not show anticomplementary activity in dilutions 1/2 or higher for at least four months (later not tested).

The data presented in Table 4 indicate the CF-titres revealed with the SPG-formol-antigen in Bedsonia-negative serum samples. These titres seem to increase with increasing duration of trachoma up to the 2nd or 3rd years of the disease. After the 2nd or 3rd years of trachoma, the titres revealed show a negative correlation with the duration of the disease, at a significance level of $P = 0{\cdot}01$. For the 34 pairs of data, referring to trachoma-positive serum samples obtained from 34 patients after their second year of illness, the correlation coefficient showed to be: $r = -0{\cdot}4426$ (all data needed for this calculation may be found in Table 4).

Table 4 presents also geometrical means of the positive titres for each year separately, as well as 3 years moving averages of the positive titres, in sera of patients with disease of different duration. The 3 year moving averages shown in Table 4 represent values of geometrical means of the respective CF-titres found in subsequent 3 years periods. The trend of 3 year moving means of the CF-titres shows more clearly than the trend of the 1 year mean values the correlation existing between the average CF-titres and the duration of the disease.

When analysing the data presented in Tables 2 and 5, neither the percentage incidence of positives, nor the CF-titres were found to show significant correlation with the clinical stage of trachoma.

Also the percentage incidence of positives showed no significant correlation with the duration of trachoma (see Tables 4 and 5).

As seen from the data presented, both the clinical stage of trachoma and the heights of the CF-titres, showed significant correlation with the duration of the

Table 4. *Data indicating a correlation between CF-titres against 'SPG-formol' antigen and duration of trachoma*

Reciprocals of titres	No.	Percentage	Duration (years)											
			1	2	3	4	5	6	7	8	9	10	11	12
< 2	15	29·4	—	1	5	1	3	—	1	1	1	—	1	1
2	1	2·0	—	—	—	—	1	—	—	—	—	—	—	—
4	7	13·7	—	—	—	1	1	—	—	2	2	—	1	—
8	6	11·8	1	1	—	1	—	—	1	1	—	—	1	—
16	10	19·6	—	—	2	3	2	1	—	1	—	1	—	—
32	4	7·9	1	—	1	1	1	—	—	—	—	—	—	—
64	8	15·7	—	1	2	2	2	1	—	—	—	—	—	—
2–64	36	70·7	2	2	5	8	7	2	1	4	2	1	2	—
Geometric mean titres	36		16·0	22·6	32·0	19·0	16·0	32·0	8·0	6·7	4·0	16·0	5·7	—
3 years moving means of titres	36		19·0	25·3	23·1	20·4	18·8	17·2	10·8	6·0	6·6	6·1	8·0	—
All	51	100	2	3	10	9	10	2	2	5	3	1	3	1

Table 5. *Results of CF-tests with formalin antigen (SPG)*

(Data on 51 specimens.)

Stage	Titre	No.	Percentage	Duration (years) 1	2	3	4	5	6	7	8	9	10	11	12
II	< 2	5	27·8	—	1	4	—	—	—	—	—	—	—	—	—
	2	1	5·6	—	—	—	—	1	—	—	—	—	—	—	—
	4	1	5·6	—	—	—	1	—	—	—	—	—	—	—	—
	8	2	11·1	1	1	—	—	—	—	—	—	—	—	—	—
	16	3	16·7	—	—	2	1	—	—	—	—	—	—	—	—
	32	3	16·7	1	—	—	1	1	—	—	—	—	—	—	—
	64	3	16·7	—	1	1	1	—	—	—	—	—	—	—	—
	All	18	100·2	2	3	7	4	2	—	—	—	—	—	—	—
III	< 2	3	20·0	—	—	1	—	2	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	4	1	6·7	—	—	—	—	—	—	—	—	—	—	1	—
	8	2	13·3	—	—	—	1	—	—	—	1	—	—	—	—
	16	5	33·3	—	—	—	2	2	—	—	1	—	—	—	—
	32	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	64	4	26·6	—	—	1	1	1	1	—	—	—	—	—	—
	All	15	99·9	—	—	2	4	5	1	—	2	—	—	1	—
IV	< 2	7	38·9	—	—	—	1	1	—	1	1	1	—	1	1
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	4	5	27·8	—	—	—	—	1	—	—	2	2	—	—	—
	8	2	11·1	—	—	—	—	—	—	1	—	—	—	1	—
	16	2	11·1	—	—	—	—	—	1	—	—	—	1	—	—
	32	1	5·6	—	—	1	—	—	—	—	—	—	—	—	—
	64	1	5·6	—	—	—	—	1	—	—	—	—	—	—	—
	All	18	100·1	—	—	1	1	3	1	2	3	3	1	2	1

disease. However no correlation was found between the height of the CF-titres and the clinical stage of the disease. The percentage incidence of CF-positives showed no correlation either with the clinical stage or with the duration of the disease. The lack of significant correlation between the last three pairs of parameters, could be attributed to some of the following reasons:

(1) The number of specimens observed might be too small to allow for a statistically significant calculation.

(2) The respective pairs of parameters might be not associated causally.

(3) A proportion of the sero-positive reactors might not have been detected by the CF-test. As shown previously (Terzin, Hlača & Fornazarić, 1958), serum specimens of some hosts (e.g. pigeons), depending on the stage of the infection caused by ornithosis virus, may react positively either in CF or in CF-inhibition reaction only. One might suppose by analogy that some positive reactors against the species-specific trachoma antigen reveal just CF-titres, while others would show CF-inhibition titres only, and that the incidence of those reacting in CF-inhibition test only was restricted chiefly to certain stages of the disease. The united incidence of both types of reactors (CF and CF-inhibition positives), under the conditions assumed, certainly would show a correlation with the respective stage of trachoma. In order to verify the validity of this assumption it would be necessary to test sera of trachoma patients both by CF and CF-inhibition reaction, against species-specific trachoma-antigen and trachoma–anti-trachoma indicator systems respectively.

Although quite useful for testing Bedsonia-negative serum samples for species-specific antibodies, the 'SPG-formol-antigen' lacks many qualities of a satisfactory CF-antigen which might be recommended for use in routine. Its main disadvantages seem to be the following:

(*a*) It can be used only to test Bedsonia-negative serum samples for the presence of species-specific anti-trachoma CF-antibodies. With all psittacosis-positive or other serum samples containing group-reactive CF-antibodies against the Bedsonia antigen, the 'SPG-formol-antigen' would react positively because of its group-reactive component as shown in Table 1.

(*b*) The species-specific component of our antigens was thermolabile, a property making the 'SPG-formol-antigen' liable to deterioration.

According to the few observations made so far, the formol antigen kept at + 4° C. is gradually losing its species-specific activity, at the same time apparently gaining in its group-reactive activity.

(*c*) Due to insufficient concentration and purity the 'SPG-formol-antigen' prepared by the technique described has both a turbid appearance and relatively low working titres.

SUMMARY

Semi-purified elementary body suspensions, prepared or preserved in three different ways, have been compared as to their species-specific CF-activity as revealed both against Bedsonia-negative sera of trachoma patients (of known stage), and against psittacosis-positive sera of men and animals.

Two of these antigens (the SPG-formol-antigen and the SPG-glycerol-antigen) revealed a positive species-specific CF-reaction in 61–100 % of the Bedsonia-negative serum samples obtained from trachoma patients, with titres ranging from 1/2 to 1/128.

Various parameters (as percentage proportion of positive reactors, CF-titre, duration of the disease, clinical stage of trachoma, etc.) have been analysed for correlation, and the results obtained with the different antigens are compared with one another.

The most useful of the three antigens, showing a good species-specific CF-reactivity and no anticomplementary activity, was found to be the 'SPG-formol-antigen'. However because of its group-specific reactivity, for detection of species-specific trachoma antibodies in routine, it could be used only with Bedsonia-negative samples of sera. Because of the thermolability of its species-specific component, the SPG-formol-antigen proved to be liable to deterioration.

We have pleasure in thanking to Dr F. B. Gordon for help in procuring bibliography and the viable strain of trachoma virus. We wish to express our thanks also to Mrs R. Šepetavic and to Miss M. Krečo for their technical assistance.

REFERENCES

Bedson, S. P. (1936). Observations bearing on the antigenic composition of psittacosis virus. *Brit. J. exp. Path.* **17**, 109.

Bedson, S. P. (1959). The Harben Lectures. The psittacosis-lymphogranuloma group of infective Agents. *J. R. Inst. publ. Hlth*, **22**, 67.

Bernkopf, H. (1962). Discussion. *Ann. N.Y. Acad. Sci.* **98**, 345.

Bovarnick, M. R., Miller, J. C. & Snyder, J. C. (1950). The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. *J. Bact.* **59**, 509.

Brand, G. & Keil, A. W. (1955). Konzentrierung von Komplementbindenden Influenza-Antigen durch Glyzerindialyse. *Zbl. Bakt.* (I., Abt. Orig.), **162**, 1–2, 13.

Cabasso, V. J., Markham, F. S. & Cox, H. R. (1951). Stabilizing action of glycerine on hemagglutination of egg-adapted mumps, Newcastle disease and influenza viruses. *Proc. Soc. exp. Biol., N.Y.*, **78**, 791.

Collier, L. H. (1961). Experiments with trachoma vaccines. *Lancet*, i, 795.

Galton, M. M., Powers, D. K., Hall, A. D. & Cornell, R. G. (1958). A rapid macroscopic-slide screening test for the serodiagnosis of Leptospirosis. *Amer. J. vet. Res.* **19**, 71, 505.

Grayston, J. T., Wang, S. P., Woolridge, R. L., Yang, Y. F. & Johnston, P. B. (1960). Trachoma: studies of etiology, laboratory diagnosis and prevention. *J. Amer. med. Ass.* **172**, 1577.

Hanna, L. (1962). Isolation of trachoma and inclusion conjunctivitis viruses in the United States. *Ann. N.Y. Acad. Sci.* **98**, 24.

Murray, E. S., Guerra, P., Abbott, A. G. & McComb, D. E. (1962). Isolation of viruses of trachoma from patients in Ethiopia. *Ann. N.Y. Acad. Sci.* **98**, 14.

Snyder, J. C., Page, R. C., Murray, E. S., Daggy, R. H., Bell, S. D., Nichols, R. L., Haddad, N. A., Hanna, A. T. & McComb, D. (1959). Observations on the etiology of trachoma. *Amer. J. Ophthal.* **48**, 325.

Sowa, J. & Collier, L. H. (1960). Isolation of trachoma virus from patients in West Africa. *J. Hyg., Camb.*, **58**, 99.

Terzin, A. L. (1953). Glycerine as a Stabilizer of some Complement-fixing antigens of viral and rickettsial origin. *Proc. Soc. exp. Biol., N.Y.*, **84**, 215.

Terzin, A. L., Hlača, D. M. & Fornazarić, M. R. (1958). Antibodies against Bedsonia antigen in-sera of animals. *Arch. ges. Virusforsch.*, **8**, 511.

Terzin, A. L., Matuka, S., Fornazarić, M. R. & Hlača, D. M. (1961). Preparation of group-specific Bedsonia antigens for use in complement-fixation reactions. *Acta Virol.* **5**, 78.

Terzin, A. L. & Birtašević, B. V. (1962). Complement-fixation and complement-fixation-inhibition titres against the Bedsonia antigen in sera of trachoma patients. *J. Hyg., Camb.*, **60**, 21.

Woolridge, R. L. & Grayston, J. T. (1962). Further studies with a complement fixation test for trachoma. *Ann. N.Y. Acad. Sci.*, **98**, 314.

J. Hyg., Camb. (1963), **61**, 43
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Chrome typhoid vaccine

By E. E. VELLA*
The David Bruce Laboratories, East Everleigh, Marlborough, Wilts

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INTRODUCTION

Heat-killed and phenol-preserved typhoid vaccines have been in general use since their introduction by Almroth Wright, then Professor of Pathology at the Army Medical School, at the end of the last century (Wright & Semple, 1897). Details of the first two anti-typhoid inoculations in humans, published in the medical literature are recorded in the issue of the *Lancet* for 19 September 1896 (Wright, 1896).

Since then much research has been done by those interested in this field, to promote vaccines which would give protection and less side-effects; the earlier vaccines are stated to have caused ferocious reactions on occasions (Harrison, 1953).

The ideal vaccine is one that confers full life-long protection to a vaccinated individual, against a particular disease, causes no harmful local or systemic reaction upon its exhibition and administration, and is given in one small single dose, preferably by mouth.

Such ideals have not been realized as yet, but many methods and techniques have been tried, and various vaccines have been proposed, in all parts of the world. To mention only a few:

(i) Alcohol-killed and alcohol-preserved vaccine in Britain (Felix, 1941; Felix, Rainsford & Stokes, 1941).

(ii) Endotoxoid vaccine in South Africa (Grasset, 1951, numerous publications).

(iii) Acetone-dried vaccine in the U.S.A. (Landy, Gaines, Seal & Whiteside, 1954).

(iv) Other vaccines utilizing antigenic extracts such as that prepared in France (Boivin & Mesrobeanu, 1938, numerous publications; Kourilsky, Kourilsky & Boivin, 1939); in Britain (Topley, Raistrick, Wilson, Stacey, Challinor & Clark, 1937; Henderson & Morgan, 1938); and an aluminium hydroxide absorbed vaccine in Hungary (Kraus, Joo & Rethy, 1956).

In Japan, Ando & Shimojo (1957) quote the use of calcium chloride treated vaccines by Kishida, concentrated Ringer's solution by Hirano and Anzai, and alcohol-acetone vaccines by Ogunuki; Ando and other workers have advocated a vaccine in which the bacilli are killed by formalin and treated with chrome salts.

In this laboratory, experimental chrome vaccines were prepared and tested *in vitro* by agglutination reactions, and *in vivo* by active and passive mouse tests and the production of antibodies (agglutinins) in rabbits.

* Lieutenant-Colonel, Royal Army Medical Corps.

In vitro *experiments*

Organisms used: (*a*) *Salmonella typhi* Ty 2; (*b*) *Salmonella typhi* T 15; (*c*) *Salmonella typhi* T 18.

These organisms constitute the typhoid component of the current British Army typhoid-paratyphoid vaccines. The Ty 2 is a classical strain (Weil & Felix, 1920); the other two are 'wild' strains received at these laboratories, and which have been selected on account of their all-round excellent properties for vaccine production. The organisms were cultured on trypsinized-meat broth agar and harvested in physiological saline.

Agglutinatable suspensions. (*a*) Each harvest was divided in three lots, (i), (ii) and (iii), and treated in one of the following ways: (i) Formalin and chrome alum ($KCr(SO_4)_2 . 12H_2O$) were added to give final concentrations of 1 % formalin and 0·02 % chrome alum on the lines laid down by Ando (1953). (ii) Placed in a water-bath at 54° C. for 1 hr., and, after cooling, 5 % phenol was added to give a final concentration of 1 % phenol. (iii) The third lot was left untreated.

These concentrated suspensions were then washed in saline, and suspended to give a final concentration of 8000 million organisms per ml.

(*b*) In a second agglutination experiment, a harvest of *Salm. typhi* Ty 2 was divided in two lots and treated as in (*a*) (i) and (iii) above, respectively. These concentrated suspensions were diluted to 8000 million organisms per ml, and aliquot lots were subjected to heat for varying periods of time, namely 0, 10, 20, 25, 30, 35 and 40 min. in a water-bath at 60° C. and in free steam at 100° C.

Technique of agglutination. Tests were performed in round-bottomed test tubes. Mixtures of agglutinable suspensions and doubling dilutions of serum were incubated at 37° C. for 2 hr., kept in a refrigerator overnight, and read after standing for 2 hr. at room temperature (Felix & Bensted, 1954).

In vivo *experiments*

(1) The *in vitro* experiments were followed up by mouse protection tests and immunization of rabbits. For this purpose a vaccine was prepared with *Salm. typhi* Ty 2, a strain which is known to have a high content of Vi-antigen. The organism was killed by formalin (1 %) and treated with chrome alum (0·02 %). This vaccine, in a concentration of 2000 million organisms per ml. in 0·01 % merthiolate, was divided in two portions: while one portion was stored in a refrigerator at a temperature of 4–6° C., the other half was kept at a temperature of 41° C. for 1½ months to study the effects of improper and unfavourable storage conditions on this vaccine (accelerated ageing, Landy, 1953; Henderson, Peacock & Rickley, 1951).

(2) *Active mouse protection tests.* Groups of mice were immunized by subcutaneous graded doses of the chrome vaccine, namely 40, 80, 160, 320 and 640 million organisms per mouse; at the same time groups of mice were injected with a cholera vaccine, to serve as controls for non-specific immunity. The mice were challenged by an intraperitoneal injection of 80 million Ty 2 organisms, 10 days after immunization.

(3) *Antibody production in rabbits.* Six rabbits were immunized by three intravenous injections of 500 million, 1000 million and 2500 million organisms given on day 1, day 8 and day 28. Blood was collected on day 15 and day 28.

(4) *Passive mouse protection tests.* Groups of mice were passively protected by graded doses of pooled rabbit sera, viz. 0·05, 0·2 and 0·4 ml., injected intravenously. Two to three hours after passive immunization the mice were challenged by an intraperitoneal injection of 320 million *Salm. typhi* Ty 2 organisms.

(5) *Laboratory animals.* The mice (Strong A albino) and the rabbits (Porton coloured) were supplied by Allington Farm, Porton.

RESULTS

(1) The results of the agglutination reactions of the chrome suspension, compared with an untreated suspension and a phenolized suspension, are reproduced in Table 1. It is seen that, while heat-phenol damaged the Vi-antigen, the formalin-chrome treatment preserved this antigen to a marked degree.

(2) In the second experiment a clearer picture was obtained of the effects of

Table 1. *The comparative agglutination titres of* Salmonella typhi *Ty* 2, *T* 15 *and T* 18 *suspensions—untreated; heat-killed and phenolized; formalin-killed and chrome treated*

	Untreated suspension			Heat-phenol suspension			Formalin-chrome suspension			Serum controls	
Serum	Ty 2	T 15	T 18	Ty 2	T 15	T 18	Ty 2	T 15	T 18	Standard Vi susp.	Standard TO susp.
				(*a*) Not subjected to heat							
Vi	160	160	160	Trace	0	0	640	160	640	640	—
TO	0	320	160	640	640	320	0	640	320	—	640
Saline control	0	0	0	0	0	0	0	0	0	0	0
				(*b*) Steamed for 2 hr.							
TO	640	640	640	640	640	640	640	640	640	—	640
Saline control	0	0	0	0	0	0	0	0	0	—	0

Table 2. *The effect of heat on the agglutinability of formalin-chrome suspensions and untreated suspensions of* Salmonella typhi *Ty* 2

A. Formalin-chrome suspensions

	Heated at 60° C. for (min.)							Steamed at 100° C. for (min.)				
Serum	0	10	20	25	30	35	40	10	20	25	30	35
Vi	640	640	640	640	640	640	640	640	640	trace	trace	0
TO	0	0	10	20	40	40	40	320	640	640	640	640

B. Untreated suspensions.

	Steamed at 100° C. for (min.)						Serum controls	
Serum	0	10	20	25	30	35	Standard Vi susp.	Standard TO susp.
Vi	320	0	0	0	0	0	640	—
TO	160	320	640	640	640	640	—	640

heat on the Vi-antigen. It is seen from Table 2 that while heat at 60° C. for up to 40 min. (the limits of the experiment) had no destructive effect on the Vi-agglutinability and O-inagglutinability of the chrome treated suspension, steaming at 100° C. progressively damaged the Vi-antigen, at least as regards its agglutinable properties.

(3) The titre of antibodies (agglutinins) produced in rabbits showed efficient Vi-antigenicity when the chrome suspension stored at 4° C. was injected, but the suspension kept at 41° C. did not evoke Vi-antibodies. Results are reproduced in Table 3.

Table 3. *The titres of TVi and TO antibodies (agglutinins) in sera of rabbits, immunized with two or three intravenous doses of (a) chrome vaccine stored at* 4° C. *of (b) chrome vaccine stored at* 41° C.

	(a) Chrome vaccine: 4° C.			(b) Chrome vaccine: 41° C.		
	Rabbit	2nd bleed	3rd bleed	Rabbit	2nd bleed	3rd bleed
Vi titres	1	100	300	4	0	0
	2	200	300	5	0	0
	3	80	400	6	0	0
O titres in thousands	1	1·5	4·0	4	2·0	1·5
	2	2·0	1·5	5	1·5	1·5
	3	2·0	2·0	6	1·5	1·0

Readings: figures represent reciprocals of titres, standardized against TVi horse XII and TO horse sera supplied by the Central Public Health Laboratory, Colindale.

Table 4. *Passive mouse-protection tests. Number of mice surviving an intraperitoneal challenge by* 320 *million* Salmonella typhi *T* 15 *organisms, after passive immunization by graded doses of immune serum*

Serum	Chrome vaccine: 4° C.	Chrome vaccine: 41° C.
0·05 ml.	0	0
0·2 ml.	8	0
0·4 ml.	7	0

Challenge organism virulence control: 40 million organisms per mouse = 6; 80 million organisms per mouse = 2; 160 million organisms per mouse = 0.

Serum control: mice injected intravenously with 0·5 ml. 'normal' rabbit serum, not challenged = 10; mice injected intravenously with 0·5 ml. 'normal' rabbit serum, challenged as for test mice = 0.

Notes. (i) Figures for mice represent survivors from 10 mice tested in each group. (ii) 'Normal' rabbit serum refers to pooled rabbit serum free from TVi, TO and TH agglutinins.

(4) Table 4 shows the results of the passive mouse protection tests. While the serum containing Vi-antibodies (produced by the intravenous inoculation of rabbits with the chrome vaccine stored at 4° C.), gave good protection to the mice, the serum lacking the Vi-antibodies (from rabbits immunized with the chrome vaccine kept at 41° C.) failed to give protection to the test mice.

(5) The results obtained in the active mouse protection tests followed the same pattern of observations recorded in the previous two *in vivo* tests, namely that

storage of the chrome vaccine at 41° C. markedly affects its immunizing properties as seen by comparing the numbers of vaccinated mice, immunized by the chrome vaccines, surviving the challenge dose (see Table 5).

Table 5. *Active mouse-protection test. The protection afforded to mice, immunized subcutaneously by a single dose of a chrome typhoid vaccine, against an intraperitoneal challenge by* 80 *million* Salmonella typhi *Ty* 2 *organisms in saline*

Immunizing dose in millions per mouse ...	40	80	160	320	640
Chrome vaccine at 4° C.	3	3	3	6	7
Chrome vaccine at 41° C.	0	0	1	1	0
Cholera vaccine (control)	1	0	2	1	0

Challenge organism virulence control: 5 million organisms per mouse = 9; 10 million organisms per mouse = 7; 20 million organisms per mouse = 5; 40 million organisms per mouse = 1; 80 million organisms per mouse = 0.

Notes. (i) Figures for mice represent survivors from 10 mice tested in each group. (ii) Cholera vaccine was used as a control for non-specific immunity.

DISCUSSION

The chrome enteric vaccine is based on the conception that the Vi-antigen of salmonella organisms is the important immunogenic agent, and therefore any treatment in the preparation of a typhoid vaccine which tends to preserve this antigen is to be recommended.

The time-honoured method of typhoid-paratyphoid vaccine production in this country is to kill the organisms by heat and preserve the vaccine with 0·5 % phenol; it has been known, for many years now, that both heat and phenol alter or damage the Vi-antigen.

Ando & Nakamura (1950) consider the Vi-antigen as consisting of two parts, one heat-stable and the other heat-labile; the Vi-agglutinability, O-inhibition and also immunizing power (active immunity in mice and Vi- and protective antibody production in rabbits) are due to the presence of the labile part, as originally described by Felix, while the stable portion causes precipitation and feeble production of Vi- and protective antibody in rabbits.

It has been found by Japanese workers that, if typhoid bacilli are killed by formalin and treated with chrome salts, the Vi-antigen is stabilized, and, furthermore, it is claimed that a decrease in toxicity for animals occurs without impairment of the Vi- or O-antigens. It is suggested that the chrome acts as it does in the process of tanning of leather, and it combines with the surface antigen, i.e. the Vi-antigen, and produces a less soluble and a more stable structure; the antigenicity of such stable chrome vaccine with decreased toxicity was found to be very marked in animal and human experiments (Ando, Shimojo & Tadokoro, 1952).

In the series of experiments done at this laboratory the experimental chrome vaccines were tested, as stated in the introduction, by (i) *in vitro* tests, i.e. agglutination tests, and (2) *in vivo* tests, i.e. production of antibodies in rabbits, and mouse protection tests, both active and passive.

In the agglutination tests the stabilizing effect of formalin and chrome alum treatment on the Vi-antigen is shown by the O-inagglutinability of these preparations, when compared to a heat-phenol treated suspension (Table 1), and after subjection to various degrees of heat (Table 2). Formalin by itself confers no such heat-resistance on the Vi-antigen.

When the rabbits were injected with the experimental chrome vaccine (which had been properly stored at refrigerator temperatures of 4–6° C.), a good rise of Vi-antibodies was obtained, accompanying a similar high rise of O-antibodies. The estimation of circulating Vi- and O-antibodies in rabbits immunized with different typhoid vaccines has been practised for many years, and has proved to be a reliable and sensitive method of detecting any damage that may have been done to the Vi-antigen in the course of preparation of the vaccine (Felix, 1951).

Suspensions of bacteria killed by formalin, without chrome alum treatment, give rise to considerable amounts of Vi-antibodies; so it is impossible to say if treatment of the formol-killed vaccine used in this study with chrome alum had any effect *per se* on the production of Vi-antibodies as seen in Table 3 (Felix, Bhatnagar & Pitt, 1934; Kaufmann, 1935; Bensted, 1940). However, the antibodies produced by formol vaccines are somehow deficient in protective powers, as characterized by a reduced power of promoting phagocytosis and of protecting against infection with virulent strains of the typhoid bacillus (Felix & Bhatnagar, 1935; Felix & Petrie, 1938); though Ando (1953) and Nakamura found no difference between antisera produced by using living bacilli or formalin-killed bacilli. Treatment of the experimental formalin-killed vaccine with chrome alum gave rise in rabbits to antibodies which appear to have adequate protective powers (see Table 4) when tested by the passive mouse protection test.

In the active mouse protection test, carried out with a constant challenge and graded immunizing doses (Batson, 1949), good protection was obtained by vaccination with one subcutaneous injection of the chrome vaccine stored at 4–6° C. As regards mouse-protection tests, it was considered by Felix (1941) that active immunization experiments in mice do not disclose those great differences in the antigenic value of various preparations of the Vi-antigen, which are so clearly demonstrated by using the serum of vaccinated rabbits or horses in passive protection experiments with mice, or even by *in vitro* laboratory tests. Ando & Nakamura (1950) disagree with this opinion and state, 'In our experiments the mouse-method without using mucin, if ED50 (the average immunizing dose) is determined against a certain challenge dose, is shown to be sensitive enough for determining the immunizing power of vaccines. This method distinctly shows that the Vi-antigen carries out a function that the O-antigen cannot fulfil.'

It will be noted that improper storage of the experimental chrome vaccine at 41° C affected the Vi-antigen markedly, as shown by all the *in vivo* tests; but this was a severe and artificial stability test, which does not contradict the other good qualities of the chrome vaccine—namely its Vi-antigen stability and its protective properties in both active and passive mouse protection tests.

The conclusions to be drawn from this series of tests, however, have to be considered against the results of the field trials in Yugoslavia and the laboratory

assays of the vaccines used in that trial (Cvjetanovic, 1957; Yugoslavia Typhoid Commission, 1957; Edsall, Carlson, Foomal & Benenson, 1959; Standfast, 1960), in which the phenolized vaccine gave better protection to vaccinated persons than the alcoholized vaccine, notwithstanding the generally recognized preserving effect of alcohol treatment on the Vi-antigen of typhoid suspensions on one hand and the damaging effect of heat and phenol on the other (Felix, Rainsford & Stokes, 1941; Bensted, 1940; Climie, 1942; Felix & Anderson, 1951).

The Japanese workers have conducted human trials on numerous occasions with satisfactory results, and have demonstrated the rise of Vi-antibodies which was effected by the chrome vaccine, as compared with the usual lack of these antibodies when heat-killed vaccines are used. They considered the possession of the Vi-antigen and the production of Vi-antibodies to be of much importance in gauging the efficacy of typhoid vaccines, which is a reasonable view based on the observed facts that the Vi-antibody is certainly found in patients and carriers of typhoid bacilli, and bacilli isolated from patients contain this antigen (Bhatnagar, 1938; Bhatnagar, Speechley & Singh, 1938; Felix, Kirkorian & Reitler, 1935). Kaneko, Hajashi, Hiraj & Ando (1953) compared a chrome vaccine with a heated typhoid-paratyphoid vaccine and stated, 'The Vi-antibody in sera may be considered insufficient to be the direct proof of the protective immunity against the typhoid fever, but its presence should be related to some extent to the typhoid immunization and it is supposed that the chrome vaccine is the superior one for the typhoid immunization conferring higher Vi serum titres.'

The final judgement on the superiority, or even the efficacy, of the chrome vaccine will have to rest on full-scale field trials—Perry, Findlay, & Bensted (1934) remarked many years ago: 'A field trial in endemic areas of the disease would of course supply the most satisfactory evidence of the immunising power of the typhoid vaccine.'

SUMMARY

1. Experimental typhoid vaccines, treated by 1 % formalin and 0·02 % chrome alum ($KCr(SO_4)_2 . 12H_2O$), as suggested by Japanese workers were prepared and tested by the usual *in vitro* and *in vivo* tests.

2. Agglutination tests, antibody production in rabbits, active and passive mouse protection tests confirm the stability of the Vi antigen of the vaccine, if properly stored, and the good protection afforded to laboratory animals in both the active and passive mouse protection tests.

3. It is suggested that only a full-scale field trial in a typhoid endemic area can give the answer as to the real efficacy and/or superiority of the chrome vaccine over other typhoid vaccines.

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REFERENCES

ANDO, K. (1953). Studies on typhoid vaccine–chrome vaccine. *Japan. J. exp. Med.* **23**, 111–126.

ANDO, K. & NAKAMURA, Y. (1950). Studies on Vi-antigen of typhoid bacilli; on complex nature of Vi antigen and phases of typhoid bacilli. *Japan. J. exp. Med.* **20**, 737–751.

ANDO, K. & SHIMOJO, H. (1957). Antityphoid chrome vaccine. Method of its preparation and systemic reactions after its inoculation in man. *Japan. J. exp. Med.* **27**, 159.

ANDO, K., SHIMOJO, H. & TADOKORO, I. (1952). New method of preparing bacterial vaccines by use of chrome-salt (detoxified vaccine-chrome vaccine). *Japan. J. exp. Med.* **22**, 491.

BATSON, H. C. (1949). Relative significance of graded immunising and challenge doses in measuring potency of vaccines; study of mouse protection by typhoid vaccine. *J. exp. Med.* **90**, 233–53.

BENSTED, H. J. (1940). Bacterium typhosum; development of Vi-antigen and Vi-antibody. *J. R. Army Med. Cps*, **74**, 19–35.

BHATNAGAR, S. S. (1938). Vi agglutination in diagnosis of typhoid fever and typhoid carrier condition. *Brit. med. J.* ii, 1195.

BHATNAGAR, S. S., SPEECHLY, C. G. J. & SINGH, M. (1938). Vi variant of Salmonella typhi and its applications to serology of typhoid fever. *J. Hyg., Camb.*, **38**, 663–72.

BOIVIN, A. & MESROBEANU, L. (1938). Recherches sur les antigènes somatiques du bacille typhique. Sur la nature chimique des antigènes 'O' et 'Vi'. *C.R. Soc. Biol., Paris*, **128**, 5.

CLIMIE, H. (1942). Immunisation against typhoid and paratyphoid with alcohol-killed, alcohol-preserved and heat-killed, phenol-preserved vaccine. *J. Hyg., Camb.*, **42**, 411–15.

CVJETANOVIC, B. B. (1957). Field trial of Typhoid Vaccine. *Amer. J. Publ. Hlth*, **47**, 578–81.

EDSALL, G., CARLSON M., FOOMAL, S. B. & BENENSON, A. S. (1959). Laboratory tests of typhoid vaccines used in a controlled field study. *Bull. World Hlth Org.* **20**, 1017–32.

FELIX, A. (1941). New type of typhoid and paratyphoid vaccine. *Brit. med. J.* i, 391–5.

FELIX, A. (1951). Preparation, testing and standardization of typhoid vaccine. *J. Hyg., Camb.*, **49**, 280.

FELIX, A. & ANDERSON, E. S. (1951). Immunizing potency of alcohol-killed and alcohol-preserved typhoid vaccine after storage for 10 years. *J. Hyg., Camb.*, **49**, 288.

FELIX, A. & BENSTED, H. J. (1954). Proposed standard agglutinating sera for typhoid and paratyphoid A and B fevers. *Bull. World Hlth Org.* **10**, 922.

FELIX, A. & BHATNAGAR, S. S. (1935). Further observations on proporties of Vi antigen of *B. typhosus* and its corresponding antibody. *Brit. J. exp. Path.* **16**, 428.

FELIX, A., BHATNAGAR, S. S. & PITT, R. M. (1934). Observations on properties of Vi antigen of *B. typhosus*. *Brit. J. exp. Path.* **15**, 354.

FELIX, A., KIRKORIAN, K. S. & REITLER, R. (1935). Occurrence of typhoid bacilli containing Vi antigen in cases of typhoid fever and of Vi antibody in their sera. *J. Hyg., Camb.*, **35**, 421–7.

FELIX, A. & PETRIE, G. F. (1938). Preparation of antityphoid serum in horse for therapeutic use in man. *J. Hyg., Camb.*, **38**, 674.

FELIX, A., RAINSFORD, S. G. & STOKES, E. J. (1941). Antibody response and systemic reactions after inoculation of new type of TABC (typhoid-paratyphoid) vaccine. *Brit. med. J.* i, 435.

GRASSET, E. (1951). L'endoanatoxine typhoparatyphique dans la prophylaxie des infections typhoidiques; applications et resultats d'ensemble de quinze ans de vaccination (1934–48). *Rev. Immunol., Paris*, **15**, 1–19.

HARRISON, L. W. (1953). Correspondence columns. *Brit. med. J.* ii, 831.

HENDERSON, D. W. & MORGAN, W. T. J. (1938). Isolation of antigenic substances from strains of Bact. typhosum. *Brit. J. exp. Path.* **19**, 82.

HENDERSON, D. W., PEACOCK, S. & RICKLEY, J. (1951). Preservation of typhoid vaccine. *Lancet*, i, 618.

KANEKO, J., HAJASHI, R., HIRAJ, T. & ANDO, K. (1953). Results on human inoculation with typhoid chrome vaccine. *Japan. J. exp. Med.* **23**, 293–8.

KAUFMANN, F. (1935). Latest results of typhoid serology; their bearing upon production and testing of typhoid vaccines and therapeutic sera as well as upon typhoid diagnosis. *Quart. Bull. L. o. N.* **4**, 485.

KOURILSKY, R., KOURILSKY, S. & BOIVIN, A. (1939). Sur l'action immunisante chez l'homme, de l'antigène glucido-lipidique O du bacille d'Eberth. *C.R. Soc. Biol., Paris*, **131**, 190.

KRAUS, K., JOO, I. & RETHY, L. (1956). *Atti Sec. Congr. Int. Stand. Immunobiol.* (Roma 10–14 Sept.), p. 367.

LANDY, M. (1953). Enhancement of immunogenicity of typhoid vaccine by retention of Vi-antigen. *Amer. J. Hyg.* **58**, 148.

LANDY, M., GAINES, S., SEAL, J. R. & WHITESIDE, J. E. (1954). Antibody responses of man to 3 types of antityphoid immunising agents: heat-phenol fluid vaccine, acetone-dehydrated vaccine, and isolated Vi and O antigens. *Amer. J. Publ. Hlth*, **44**, 1572.

PERRY, M. H., FINDLAY, H. T. & BENSTED, H. J. (1934). Anti-typhoid inoculation; observations on immunising properties and on manufacture of typhoid vaccine. *J. R. Army Med. Cps*, **62**, 161.

STANDFAST, A. F. B. (1960). A report on laboratory assays carried out at the Lister Institute of Preventive Medicine on the typhoid vaccines used in the field study in Yugoslavia. Experiments with Vi-negative strains of Salmonella typhi. *Bull. World Hlth Org.* **23**, 37–45; 47–52.

TOPLEY, W. W. C., RAISTRICK, H., WILSON, J., STACEY, M., CHALLINOR, J. W. & CLARK, R. O. J. (1937). Immunising potency of antigenic components isolated from different strains of Bact. typhosum. *Lancet*, i, 252.

WEIL, E. & FELIX, A. (1920). Über den Doppelttypus der Rezeptoren in der Typhus-Paratyphus-Gruppe. *Z. ImmunForsch*, **29**, 24.

WRIGHT, A. E. (1896). On the association of serous haemorrhages with conditions of defective blood coagulability. *Lancet*, ii, 807–9.

WRIGHT, A. E. & SEMPLE, D. (1897). Remarks on vaccination against typhoid fever. *Brit. med. J.* i, 256.

YUGOSLAV TYPHOID COMMISSION (1957). Field and laboratory studies with typhoid vaccines. *Bull. World Hlth Org.* pp. 897–910.

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Antibodies of *Toxoplasma gondii**

By D. G. FLECK
Public Health Laboratory, Swansea

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INTRODUCTION

In the diagnosis of toxoplasma infection it is frequently impossible to attempt isolation of the organism. Resort has to be made to the examination of serum. Numerous tests have been devised. The multiplicity of such tests suggest that difficulties regarding technique or interpretation are numerous. Although the dye test and the complement-fixation test are widely used, a haemagglutination test (Jacobs & Lunde, 1957), a direct agglutination test (Fulton & Turk, 1959), a flocculation test (Siim & Lind, 1960), an agar gel diffusion (O'Connor, 1957), and a fluorescent antibody test (Goldman, 1957) have all been offered as substitutes.

The work described here is concerned with certain difficulties in performing the dye test, and with evidence that there are two antigens of *Toxoplasma gondii* capable of fixing complement. A relationship between some of the tests is suggested.

THE DYE TEST

Sabin & Feldman (1948) while looking for an *in vitro* manifestation of neutralizing antibody found that toxoplasma subjected to antiserum in the presence of fresh normal human serum lost the capacity to stain with methylene blue.

Lelong & Desmonts (1952) likened this effect to the Pfeiffer phenomenon. Toxoplasma antibody in the presence of fresh normal human serum produced a clarification of the normally granular cytoplasm of toxoplasma organisms.

Beverley & Beattie (1952) showed that the number of toxoplasma organisms in the reaction mixture modified the result of the dye test. They found that exudate removed from the mouse peritoneum more than 3 days after infection showed a high proportion of unstained organisms in the control tubes containing normal human serum but no added antibody. They considered this effect was due to antibody production in the mouse.

THE ANTIGENS OF *TOXOPLASMA GONDII*

Warren & Russ (1948), in developing the complement-fixation test for toxoplasma antibody, centrifuged the antigen they made at high speed in order to remove anticomplementary material. The complement-fixing antibody found by using this centrifuged antigen was less sensitive than the dye test.

Thalhammer (1960) used an uncentrifuged freeze-dried antigen, and obtained complement-fixing antibody titres similar to those of the dye test. It seems likely

* Part of this work was submitted as a thesis for the Degree of M.D. of the University of London.

therefore that there are two antigens both capable of producing complement-fixing antibody. It is known that at least two toxoplasma antigens exist according to the experiments of Siim & Lind (1960) and Fleck (1961).

Huldt (1958), quoting an observation by Gard, states that there is a difference between the antigens responsible for the dye test and those responsible for the complement-fixation test. Gard's observation was that dye test antibodies could not be fully neutralized with complement-fixing antigen that had been prepared by centrifugation at high speed.

Cutchins & Warren (1956) showed that complement-fixing and dye test antibody followed the inoculation into rabbits of antigen centrifuged at high speed.

PRESENT INVESTIGATION

Four experiments were performed.

(A) To confirm that the number of toxoplasma organisms in the dye test affects the antibody titre.

(B) To attempt removal by absorption of the cytoplasm-modifying substance which occurs in the peritoneal exudate of mice injected 4 or more days previously with a large number of toxoplasma organisms.

(C) To try to imitate the dye test by using detergents or other chemicals in place of antibody.

(D) To note the effect of high-speed centrifugation on toxoplasma extracts used in the complement-fixation test, and the effect these extracts have, when mixed with toxoplasma antibody in the dye test.

EFFECT OF NUMBERS OF TOXOPLASMA ORGANISMS IN THE DYE TEST

Materials and methods

Dye tests were made, using peritoneal exudate from mice inoculated 3 days previously with 0·5 ml. of a toxoplasma suspension containing 1×10^6 parasites. Serial twofold dilutions of the resultant exudates were made in saline and titrated against a standard toxoplasma antiserum. Counts of the numbers of toxoplasma organisms in each test were made with a Neubauer counting chamber.

Comparative tests with a toxoplasma suspension grown in calf-kidney tissue culture were performed. The suspensions were made by inoculating a bottle of calf-kidney tissue culture containing 5×10^6 cells with approximately 5×10^6 toxoplasma organisms. After 2 days most of the calf-kidney cells had liberated large numbers of toxoplasma organisms. These were washed twice with growth medium consisting of Hank's balanced salt solution with the addition of 0·4 % lactalbumen, 0·051 % sodium bicarbonate and 20 % bovine serum. Antibiotics were added to prevent contaminants growing in the medium. These included penicillin, streptomycin and nystatin at a concentration of 100 units/ml. together with neomycin at a concentration of 50 units/ml.

The toxoplasma organisms were resuspended in saline and counted. Serial twofold dilutions were made and titrated with standard antiserum as with the mouse exudates.

Accessory factor was obtained from normal human blood donors and consisted of serum removed from clotted blood less than 48 hr. before being stored at − 20° C. The serum was tested and shown to be free from toxoplasma antibody. No deterioration in serum stored for as long as 10 months was noted.

Results

Table 1 shows the effect of the number of organisms on the standard serum titre. The higher the number of organisms in the reacting mixture the lower the dilution at which the antiserum 50 % end-point occurs. This effect still persisted when the parasites were washed with normal fresh human serum, and when suspensions of organisms were prepared from tissue cultures. There was however considerable variation in the titres obtained with unwashed, washed and tissue culture antigens.

Table 1. *Effect of number of toxoplasma organisms on the dye test carried out with a standard antiserum*

Dye tests with unwashed toxoplasma from mouse peritoneum			Dye tests with washed toxoplasma from mouse peritoneum			Dye tests with washed toxoplasma from tissue culture		
Antigen dilutions	Antiserum dilutions (50 % end-points)	No. of orgs./ml. in millions in saline control tube	Antigen dilutions	Antiserum dilutions (50 % end-points)	No. of orgs./ml. in millions in saline control tube	Antigen dilutions	Antiserum dilutions (50 % end-points)	No. of orgs./ml. in millions in saline control tube
Neat	1/80	8·6	Neat	1/40	10	Neat	1/160	35
1/2	1/640	3·0	1/2	1/160	6·4	1/2	1/640	16
1/4	1/1280	1·9	1/4	1/320	2·9	1/4	1/640	8
1/8	1/1280	1·2	1/8	1/320	1·8	1/8	1/640	4

EFFECT OF MOUSE ANTIBODY IN MOUSE EXUDATE ON THE DYE TEST

Peritoneal exudate of mice infected with 1×10^6 toxoplasma organisms was only usable in the dye test when removed from the mouse 3 days after inoculation. If the exudates were removed from the mice 4 or more days after inoculation they showed poor staining when placed in control tubes containing accessory factor and saline only. When supernatant fluid from the centrifuged exudate of a mouse inoculated 4 days previously was absorbed overnight with a washed toxoplasma suspension at room temperature (22° C.), the supernatant did not interfere with the staining of a toxoplasma suspension. The substance appearing in the peritoneal exudate of a mouse injected 4 days previously with toxoplasma and which interferes with the dye test is precipitated with the gamma globulin protein fraction and withstands heating for 1 hr. at 56° C. It is therefore highly likely to be toxoplasma antibody and not a non-specific anti-toxoplasma factor.

EFFECT OF SURFACE ACTIVE AGENTS ON TOXOPLASMA

Lelong & Desmonts (1952) likened the effect of antibody on toxoplasma to the Pfeiffer phenomenon. They considered that antibody lysed the parasites producing clarification of the cytoplasm. The present author thought that other substances might produce lysis of the organism and imitate the dye test.

Materials and methods

Serial twofold dilutions of various chemicals, mostly detergents, were made in physiological saline. A dye test was carried out with these dilutions instead of with test serum. One volume of diluted chemical, one volume of toxoplasma exudate and two volumes of accessory factor were mixed, and incubated in a water-bath for 1 hr. The mixtures were then stained with alkaline methylene blue for 5 min. and the parasites examined by a wet film preparation. The approximate pH of each chemical solution was measured by making a 10% solution of B.D.H. Universal Indicator in the chemical being examined.

Table 2. *Effect of various chemicals on the staining capacity of* Toxoplasma gondii

Chemical	Effect	pH of solution
Sodium lauryl sulphate	Modification at dilution 1/2	6·0
Sodium stearate	No modification with saturated solution	9·0
Teepol	Modification at dilution 1/10	7·0
Domiphen bromide	No modification found	6·5
Cetyl trimethyl ammonium bromide	Modification at dilution 1/32	7·0
Tween 80	Modification at dilution 1/4	6·0
Sorbitan mono-oleate	No modification with saturated solution	6·5
Sodium taurocholate	Modification at dilution 1/10	5·0
Sodium deoxycholate	Modification at dilution 1/10	7·0
Lysol	Lysis but no modification at dilution 1/16 or less	4·0
Phenol	Lysis but no modification at dilution 1/2 or less	4·0
Sodium azide	Modification at dilution 1/2	11·0
Sodium chloride	No modification with saturated solution	7·0

Results

Table 2 shows the effect of a range of detergents on a suspension of toxoplasma in saline containing accessory factor but no antibody. Some of the detergents and other chemicals modified the staining of the parasites so that the appearance resembled a positive result in the dye test. This effect only occurred however when the chemicals were used in very high concentrations. No correlation could be found between the type of detergent (i.e. anionic, cationic or non-ionic) and its effect.

Although such a non-specific effect is unlikely to occur in practice, dye tests should be set up with controls containing serum to be tested and toxoplasma suspension only.

THE EFFECT OF HIGH-SPEED CENTRIFUGATION ON TOXOPLASMA EXTRACTS

Complement-fixation tests

Peritoneal exudate from mice inoculated 3 days previously with a suspension of live toxoplasma was washed in physiological saline centrifuged at 3000 r.p.m. for 10 min. The deposit was then mixed with 10 times its weight of distilled water and allowed to lyse at 4° C. overnight with occasional shaking. The mixture was then frozen and thawed 4 times and centrifuged at 3000 r.p.m. for 10 min. to remove solid material. An equal volume of double strength saline was added to restore isotonicity; this mixture will be described as whole-body extract. Some of this whole-body extract was centrifuged at about 25,000 *g.* and the supernatant designated the light extract. Early experiments showed that optimum antigen dilutions were difficult to demonstrate, the antigen concentrations being too weak. The antigen solutions were then concentrated to 1/10 of their original volume by placing a cellophane dialysis sac packed with polyethylene glycol (carbowax; 20M) in the antigen solution.

Complement-fixation tests with serial twofold dilutions of extract, against fourfold dilutions of a control antiserum were made in chess board patterns on plastic plates. 2 M.H.D. of guinea pig complement was used with overnight fixation at +4° C. After fixation 2% sheep cells sensitized with 5 M.H.D. of H.I.B. were added and the mixtures incubated for 30 min. at 37° C. The mixtures were stored at 4° C. for 3 hours and the results read.

Table 3. *Complement-fixation tests (concentrated toxoplasma extracts × standard toxoplasma antiserum)*

	Extract dilutions									Control (no antigen)
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	
(1) Extract was heated and centrifuged at high speed	AC	256	64	64	64	64	32	32	32	0
(2) Extract was unheated but centrifuged at high speed	AC	AC	AC	4000	256	64	64	64	64	0
(3) Extract was heated but not centrifuged at high speed	AC	1000	1000	1000	1000	1000	1000	128	64	0
(4) Extract was not heated and not centrifuged at high speed	AC	AC	AC	AC	4000	1000	256	64	64	0

Figures indicate reciprocals of titres of standard antitoxoplasma serum showing 50% haemolysis. AC = Anticomplementary. Titres underlined are those showing a plateau of constant antibody with varying antigen concentration.

Dye-test inhibition

Concentrated extracts prepared in various ways were added to equal volumes of high titre human antisera for 1 hr. at 20° C. These mixtures were then added to the dye test and the antibody content found.

Any inhibition of antibody by the extract was noted by the fall in antibody titre. A control mixture containing saline and antibody in equal amounts was set up.

Results

Table 3 shows the results of the complement-fixation tests with extracts from infected mouse peritoneal exudates. Each experiment shows the same batch of extract treated in four different ways, i.e. heated at 56° C. for 1 hr. and centrifuged at high speed; unheated and centrifuged at high speed; heated but not centrifuged; and unheated but not centrifuged.

It can be seen that heating to 56° C. for 1 hr. removes a large proportion of the anticomplementary material.

Centrifugation produces a pattern with 50% complement fixation by antibody and extract extending over a range of three dilutions at a serum titre of 1/64. Uncentrifuged antigen produces a pattern with 50% fixation extending over a wide range at a titre of 1/1024. Heating produced only a slight fall in the sensitivity of the extract.

This suggests that there are two complement-fixing antigens of different densities. The whole body extract used as antigen gives complement-fixation titres which correlate quite closely with dye test, but the lighter extract gives complement-fixation titres much lower than those of the dye test.

Table 4 shows that mixing the light extract with antiserum and performing a dye test on the mixture does not reveal antibody inhibition. Mixing the whole body extract with antiserum and performing a dye test on the mixture does show incomplete dye-test antibody inhibition.

Table 4. *Effect of concentrated toxoplasma extracts on the dye test*

Toxoplasma extracts (concentrated with carbowax)	Standard serum dilution giving 50% end-point in dye test
(1) Heated to 56° C. for ½ hr. and centrifuged at 25,000*g*	1/2000
(2) Heated to 56° C. for ½ hr. but centrifuged at 1500*g* only	1/32
(3) Unheated but centrifuged at 25,000*g*	1/2000
(4) Unheated but centrifuged at 1500*g* only	1/16

Dye tests were performed on mixtures of equal volumes of supernatants from toxoplasma extracts and antiserum.

Control tests, containing all four forms of toxoplasma extracts without accessory factor or antiserum, were negative in the dye test.

Controls containing accessory factor and saline were also negative.

DISCUSSION

In an effort to find a simple, safe, economical substitute for the dye test, many tests for toxoplasma antibody have been devised. Many of these have produced results which, in the hands of some workers, have correlated well with the dye test, but others have shown the correlation to be poor. Whether these variations are due to differences in technique or to the measurement of different antibodies is difficult to say.

Beverley & Beattie (1952) demonstrated that the number of toxoplasma organisms in the dye test affects the end-point of the serum being measured.

That this effect is not due to soluble antigen is shown by washing the parasites and resuspending them in accessory factor. After this treatment the effect still persists.

The specificity of the dye test has frequently been impugned. Mas-Bakal (1959), however, showed that the test did not become positive in serum after the inoculation of various bacterial vaccines, viruses or protozoa into experimental animals. Kulasiri (1960) could incriminate no protozoal parasite, except toxoplasma, in the production of dye-test antibody. Feldman (1956) however demonstrated a non-specific thermolabile toxoplasma-modifying effect in animal sera. While looking for some substance which would produce a non-specific result in the dye test the author has found that many substances in high concentrations would modify the staining capacity of toxoplasma.

There is little doubt that there are at least two antigens contained in *Toxoplasma gondii*. These are demonstrated by the different antibodies they produce. It has been shown that the antigen and antibody responsible for the dye test are different from those of the haemagglutination test (Fleck, 1961). Siim & Lind (1960) stated that preliminary results of absorption experiments show evidence of existence of more than one antigen.

Warren & Sabin (1942) first demonstrated complement-fixing antigen in a rabbit brain suspension of toxoplasma. Warren & Russ (1948) produced toxoplasma antigen from infected chorioallantoic membrane; they removed the anticomplementary effect by centrifugation at 14,000 r.p.m. for 1 hr. Sabin (1949) showed that high-speed centrifugation was less likely to produce an antigen which fixed complement non-specifically in the presence of serum from patients immunized with egg vaccines. Toxoplasma antibody measured with this centrifuged complement-fixing antigen appeared later in the disease than the dye test antibody and disappeared earlier. Steen & Kass (1951) produced an antigen by lysing a toxoplasma suspension with water. This antigen was specific and was not anti-complementary. It had not been centrifuged at high speed. Thalhammer (1956) found that this antigen gave results which agreed very well with the dye test.

The present work shows that the serum titre showing 50% haemolysis in the complement-fixation test over a wide range of antigen titres, with an antigen centrifuged at high speed, differs from that shown by an antigen centrifuged at low speed. The latter material must contain a light and heavy component. The material centrifuged at high speed must contain the light component only. This light antigen was unable to inhibit the antibody responsible for the dye test.

It is suggested therefore that the soluble antigen remaining after high-speed centrifugation be called the light antigen, that the antigen which is removed by centrifugation be called the heavy antigen. Further work is needed to show the relationship of these two antigens to other tests. It may be that the heavy antigen is responsible for the dye-test antibody as well as the complement-fixation antibody described by Thalhammer (1956) and by Steen & Kass (1951). Tonjum (1962) has shown that two lines are produced when the supernatant from the peritoneal exudate of a toxoplasma-infected mouse is allowed to diffuse against immune serum in agar. Agar gel diffusion tests performed in this laboratory suggest that at least one of the lines is due to the light antigen. Preliminary work suggests that

antibody responsible for the direct agglutination test of Fulton & Turk (1959) is related to the antibody responsible for the dye test rather than that for the haemagglutination test.

SUMMARY

1. That the number of toxoplasma organisms in the dye test affects the titre of the test serum is confirmed.

2. Evidence is presented that the toxoplasma cytoplasm-modifying substance present in the peritoneal exudate of mice injected with large numbers of toxoplasma organisms more than 3 days previously is probably antibody.

3. Various chemicals including detergents were capable, in high concentration, of producing a false positive dye test.

4. Evidence is presented that there are two complement-fixing antigens, one with a low sensitivity and a low density, the other with a high density and a sensitivity close to that of the dye test. Heating the heavy antigen to 56° C. for 1 hr. removes most of its anticomplementary effects without altering its complement-fixing activity.

5. The light antigen mentioned above was capable of producing a precipitation line in agar double-diffusion experiments, but did not inhibit dye-test antibody when mixed with an equal volume of antiserum in the dye test.

6. The heavy antigen inhibited dye test antibody when mixed with an equal volume of antiserum in the dye test.

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REFERENCES

BEVERLEY, J. K. A. & BEATTIE, C. P. (1952). *J. clin. Path.* **5**, 350.
CUTCHINS, E. C. & WARREN, J. (1956). *Amer. J. trop. Med.* **5**, 197.
FELDMAN, H. A. (1956). *Ann. N.Y. Acad. Sci.* **66**, 263.
FLECK, D. G. (1961). *Nature, Lond.*, **190**, 1018.
FULTON, J. D. & TURK, J. L. (1959). *Lancet*, ii, 1068.
GOLDMAN, M. (1957). *J. exp. Med.* **105**, 557.
HULDT, G. (1958). *Acta path. microbiol. scand.* **43**, 141.
JACOBS, L. & LUNDE, M. N. (1957). *J. Parasit.* **43**, 308.
KULASIRI, C. (1960). *J. clin. Path.* **13**, 339.
LELONG, M. & DESMONTS, G. (1952). *C.R. Soc. Biol., Paris*, **146**, 207.
MAS-BAKAL, P. M. (quoted by A. POLMAN) (1959). *Trop. Geograph. Med.* **11**, 13.
O'CONNOR, G. R. (1957). *Arch. Ophthal., Chicago*, **57**, 52.
SABIN, A. B. (1949). *Paediatrics*, **4**, 443.
SABIN, A. B. & FELDMAN, H. A. (1948). *Science*, **108**, 660.
SIIM, J. C. & LIND, K. (1960). *Acta path. microbiol. scand.* **50**, 445.
STEEN, E. & KASS, E. (1951). *Acta path. microbiol. scand.* **28**, 36.
THALHAMMER, O. (1956). *Mschr. Kinderheilk*, **104**, 110.
THALHAMMER, O. (1960). *Human Toxoplasmosis*, p. 196. Copenhagen: Munksgaard.
TONJUM, A. M. (1962). *Acta path. microbiol. scand.* **54**, 96.
WARREN, J. & RUSS, S. B. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 85.
WARREN, J. & SABIN, A. B. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 11.

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Epidemiology of toxoplasmosis*

By D. G. FLECK
Public Health Laboratory, Swansea

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INTRODUCTION

Interest in toxoplasmosis has been stimulated over the last few years by the finding that this infection is widespread biologically as well as geographically. Serious clinical illness is however uncommon. Definitive diagnosis depends on isolation of the causative organism by inoculation of biopsy material into animals known to be free from toxoplasma infection. Recognition of the vegetative form of the organism is difficult histologically.

Other workers (Plaut, 1946; Tomlinson, 1945) have described toxoplasma-like organisms seen in patients dying of various diseases and accidents. It is well accepted that asymptomatic toxoplasma infection occurs.

The ability of *Toxoplasma gondii* to produce chorioretinitis and lymphadenitis is now well documented (Perkins, 1961; Siim, 1956). Suggestions are made from time to time that other syndromes may be due to toxoplasma infection. Thalhammer (1962), for example, has suggested that the frequency of infection is much greater in cases of epilepsy and mental defect than in the normal human population.

The first part of this investigation consisted in comparing the incidence of antibody in sera from a normal population with that in sera sent from suspected cases of toxoplasmosis. The normal population consisted of routine admissions to a children's hospital in which toxoplasmosis was not suspected and of children undergoing a survey for poliomyelitis antibody. Adult sera were obtained from the Welsh Regional Blood Transfusion Service.

In addition, seventy-two cases of suspected cat-scratch fever have been examined for toxoplasma antibody. Details of contact with cats and whether a scratch was sustained were recorded where possible.

Domestic pets have frequently been suggested as possible vectors of toxoplasma infection. Cats in contact with human cases have been found to be infected (Jones, Eyles & Gibson, 1957), but whether the cats or the human cases were the originators of the infection was unknown. A source of infection common to both cats and humans may have been involved. Sera from cat-scratch fever cases were examined by Jacobs (cited by Armstrong & MacMurray, 1953) with negative results.

Perkins (1961) examined cases of uveitis and found that only in patients having contact with cats was the percentage of positive dye tests greater than the percentage of negative dye tests and then not significantly so.

* Part of this work was submitted as a thesis for the Degree of M.D. of the University of London.

MATERIALS AND METHODS

All sera were stored at $-20°$ C. Dye tests were performed on sera inactivated at 56° C. for ½ hr. according to the method used by Beverley & Beattie (1952), which was similar to that of Sabin & Feldman (1948).

RESULTS

Table 1 shows that only in disorders of the eyes was the proportion of positive sera from suspected cases of toxoplasmosis significantly higher than normal; though for cases of choroiditis in the age group 51–60, the proportion was only just significant at the 5% level. Cases of mesenteric adenitis, mental defect and epilepsy gave proportions significantly lower than expected. Although disorders of the reticulo-endothelial system showed a percentage higher than normal throughout all ages, this never reached a significant level.

Table 1. *Proportion of sera positive by the dye test at* 1/16 *or more* (*Nov.* 1959–*Nov.* 1960)

(Figures in parentheses indicate percentages of sera positive at 1/16 or more.)

Age in years	Reticulo-endothelial system	Central nervous system	Jaundice	Cardio-vascular system	Choroiditis	Uveitis
0–10	32/288 (11)	9/98 (9)	3/26 (12)	0/11 (0)	24/38 (63)	8/36 (22)
11–20	30/160 (19)	0/2	2/6 (33)	1/10 (10)	54/80 (68)	18/63 (29)
21–30	25/104 (24)	5/12 (42)	0	5/12 (42)	86/144 (60)	44/101 (44)
31–40	25/76 (33)	5/13 (38)	0	5/11 (45)	75/118 (64)	43/106 (41)
41–50	17/35 (49)	1/3 (33)	1/1 (100)	7/17 (41)	39/67 (58)	33/91 (36)
51–60	10/29 (34)	6/8 (75)	1/2 (50)	3/19 (16)	24/48 (50)	26/73 (36)
Totals	139/692 (20)	26/136 (19)	7/35 (20)	21/80 (26)	302/495 (61)	172/470 (37)

Age in years	Other eye lesions	Mesenteric adenitis	Epilepsy	Mental defect	Hydro-cephalus	Normal population
0–10	4/38 (11)	0/17	10/160 (6)	1/114 (1)	5/38 (13)	15/180 (8)
11–20	7/22 (32)	1/10 (10)	0/14	1/7 (14)	0/1	13/94 (14)
21–30	9/22 (41)	0	1/3 (33)	2/2 (100)	0	15/80 (19)
31–40	6/15 (40)	0	2/4 (50)	0	1/2 (50)	20/80 (25)
41–50	6/16 (38)	0	0	0	0	28/80 (35)
51–60	11/16 (69)	0	0	0	0	23/73 (32)
Totals	43/129 (33)	1/27 (4)	13/181 (7)	4/123 (3)	6/41 (15)	114/587 (19)

Later investigations measuring the antibody content down to 1/4 for the age group 0–10 years were made during 1961. In Table 2 the results show a fall in incidence of sera positive at 1/4 or more after 6 months of age. This is due to the disappearance of passively transferred maternal antibody. A control group of the normal population at 0–6 months was not included as this would merely have reflected the antibody titres in the mothers. After 6 months the proportion of sera positive at 1/4 or more in the dye test is significantly higher than normal only in cases of choroiditis.

Table 3 analyses the cases of suspected cat-scratch fever according to their

possession of toxoplasma antibody. Although the numbers are small the cases showing toxoplasma antibody are not significantly greater than in the normal population. Of the seventy-two cases studied, forty-four gave a definite history of being scratched by a cat; of these, eight showed toxoplasma antibody titres of 1/16 or more in the dye test. Of the fifty-nine with a definite history of contact with cats, ten showed antibody titres of 1/16 or more.

Table 2. *Proportion of sera positive by the dye test at* 1/4 *or more* (*Nov.* 1960–*Nov.* 1961)

(Figures in parentheses indicate percentages of sera positive at 1/4 or more.)

Age	Reticulo-endothelial system	Central nervous system	Jaundice	Choroiditis	Other eye lesions
0–6 months	0/7	7/19 (37)	13/43 (30)	0/2	2/9 (22)
7 months to 5 years	11/55 (20)	2/21 (10)	0/2	7/16 (44)	1/23 (4)
10 years	23/129 (18)	3/11 (27)	1/4 (25)	12/19 (63)	1/14 (7)
Totals	34/191 (18)	12/51 (24)	14/49 (29)	19/37 (51)	4/46 (9)
Totals excluding 0–6 months	34/184 (18)	5/32 (16)	1/6 (17)	19/35 (54)	2/37 (5)

Age	Epilepsy	Mental defect	Hydro-cephalus	Normal population
0–6 months	15/49 (31)	2/15 (13)	19/51 (37)	—
7 months to 5 years	8/100 (8)	4/73 (5)	1/17 (6)	6/79 (8)
10 years	3/34 (9)	3/20 (15)	1/2 (50)	14/87 (16)
Totals	26/183 (14)	9/108 (8)	21/70 (30)	20/166 (12)
Totals excluding 0–6 months	11/134 (8)	7/93 (8)	2/19 (11)	20/166 (12)

Table 3. *Cases of suspected cat-scratch fever tested for toxoplasma antibody and compared with a normal population*

(Figures in parentheses indicate percentages of sera positive at 1/16 or more.)

Age in years	No. + 1/16 or more	No. negative	Proportion + 1/16 or more	Proportion of normal population + 1/16 or more
0–10	3	27	3/30 (10)	15/180 (8)
11–20	1	13	1/14 (7)	13/94 (14)
21–30	1	12	1/13 (8)	15/80 (19)
31–40	2	2	2/4 (50)	20/80 (25)
41–50	4	1	4/5 (80)	28/80 (35)
60 +	2	4	2/6 (33)	23/73 (32)
Totals	13	59	13/72 (18)	114/587 (19)

In only three cases was the dye test titre greater than 1/256. In one of these the dye test in the serum from the cat responsible for the scratch was negative; in another the dye test in the cat was positive at only 1/4. In the third case there was no definite history of scratch from the cat with which the case had been in contact. Serum from the cat could not be obtained.

It is suggested that cat scratch or contact must be an unlikely method of contracting toxoplasmosis.

DISCUSSION

Antibody to *Toxoplasma gondii* in low concentration is found in many areas of the world. This organism produces much latent infection. That it produces very much oligo-symptomatic infection is disputable.

The material presented above suggests that mental defectives and epileptics are no more likely to suffer from toxoplasmosis than the rest of the community, at least in the south of England and Wales. These results differ from those of Thalhammer (1962) but agree with those of other workers.

Feldman (1958) found congenital toxoplasmosis not to be unduly frequent in epileptic children. Cook & Derrick (1961) in Australia found that their results were in accord with those of Feldman as regards epileptic children and that toxoplasmosis was, at most, only a rare cause of congenital mental deficiency. Their results resembled those of Burkinshaw, Kirman & Sorsby (1953), who found that only 4·4 % of certified mental defectives under 15 years, in London, reacted to the toxoplasmin skin test.

Of the cases of lymphadenopathy and reticulo-endothelial disorder examined, toxoplasma infection would not seem to be a common cause. It is however, a well established one, as is shown by previous workers, and by the fact that four strains of *T. gondii* have been isolated in this laboratory from twelve lymph gland biopsies. The biopsies were in cases in which the dye test titres were more than 1/512. A dye test is indicated in a young person with lymphadenopathy, especially when the Paul-Bunnell test is negative.

Little progress has been made in elucidating the mode of transfer of *T. gondii* to man, but the investigation reported suggests that cats are unlikely vectors, at any rate by scratching.

SUMMARY

Sera sent for routine examination from hospitals in south England and Wales were examined and compared with sera from the normal population. Choroiditis was the only disease syndrome showing a significantly higher proportion of positives than normal.

Cats were shown to be unlikely vectors of toxoplasma infection, at least by scratching.

Thanks are due to many pathologists throughout the country who sent sera and details of cases. Normal children's sera were kindly sent to me by Dr A. D. Evans of the Public Health Laboratory, Cardiff, Dr A. Macrae, Colindale, and Dr Margaret Wilson, Public Health Laboratory, Sheffield. Dr Patricia Bradstreet kindly sent me details of requests for cat scratch antigen.

Mr R. Payne carried out the dye tests and Mr H. J. Godwin of the Department of Pure Mathematics, University College of Swansea, kindly checked the statistical calculations.

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REFERENCES

Armstrong, C. & MacMurray, F. G. (1953). *J. Amer. med. Ass.* **151**, 1103.
Beverley, J. K. A. & Beattie, C. (1952). *J. clin. Path.*, **5**, 350.
Burkinshaw, J., Kirman, B. H. & Sorsby, A. (1953). *Brit. med. J.* i, 702.
Cook, I. & Derrick, E. H.(1961). *Aust. Ann. Med.* **10**, 137.
Feldman, H. A. (1958). *Paediatrics*, **22**, 559.
Jones, F. E., Eyles, D. E. & Gibson, C. L. (1957). *Amer. J. trop. Med. Hyg.* **6**, 820.
Perkins, E. S. (1961). *Uveitis and Toxoplasmosis*, p. 110. London: Churchill Ltd.
Plaut, A. (1946). *Amer. J. Path.* **22**, 427.
Sabin, A. B. & Feldman, H. A. (1948). *Science*, **108**, 660.
Siim, J. Chr. (1956). *Ann. N.Y. Acad. Sci.* **64**, 185
Thalhammer, O. (1962). *Lancet*, i, 23.
Tomlinson, W. J. (1945). *Amer. J. clin. Path.* **15**, 123.

Observations on the growth of trachoma and inclusion blennorrhoea viruses in embryonate eggs

By P. REEVE AND JANICE TAVERNE
M.R.C. Trachoma Research Unit, Lister Institute of Preventive Medicine, London, S.W. 1

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INTRODUCTION

Many strains of trachoma and inclusion blennorrhoea viruses have been isolated in several countries, but their similarities appear to be more pronounced than their differences. Although serological differences between trachoma strains isolated in Saudi-Arabia (Bell, Snyder & Murray, 1959) were demonstrated by a toxin-protection test in mice, none were found between a Chinese and an Israeli strain in a neutralization test (Bernkopf, 1959). However, strains vary in biological characteristics, for instance in pathogenicity for the chick embryo, man, simians, mice and cell cultures and in their susceptibility to antibiotics.

Since 1958, seven strains of trachoma virus and four strains of inclusion blennorrhoea have been maintained in this laboratory in the yolk sac of embryonate hens' eggs. Comparison of dose-response curves obtained for these strains titrated in chick embryos revealed differences in growth characteristics.

METHODS

Virus strains

Trachoma. G 1, G 17, isolated in the Gambia, West Africa (Collier & Sowa, 1958; Sowa & Collier, 1960). Gambia 062(t), and Gambia 221(t), referred to as G062 and G221, isolated by J. Sowa (unpublished). SA 2, isolated in Saudi Arabia (Murray, Bell, Hanna, Nichols & Snyder, 1960). TE 55, isolated in China (T'ang, Chang, Huang & Wang, 1957). BOUR and ASGH, isolated in U.S.A. (Hanna, Jawetz, Thygeson & Dawson, 1960).

Inclusion blennorrhoea. LB 1 (Jones, Collier & Smith, 1959) and LB 4 (Jones, 1961) isolated in London.

Lymphogranuloma venereum (LGV). JH strain in its 264th egg passage, obtained from Dr Sylvia Reed, London Hospital.

Serial passage

Heavily infected yolk sacs were homogenized or shaken to make 10–25 % (w/v) suspensions in sucrose potassium glutamate solution (SPG) (Bovarnick, Miller & Snyder, 1950), containing 1 mg./ml. of streptomycin, and 0·2–0·5 ml. amounts injected into the yolk sacs of eggs which were then incubated at 35° C. The eggs were candled daily; yolk-sac smears made from dead embryos were stained with

Giemsa and examined for the presence of virus. The day after inoculation on which each embryo died was recorded.

Infectivity titration in eggs

Serial tenfold dilutions of virus suspension were made in SPG and 0·5 ml. of each dilution was injected into the yolk sacs of at least five 7-day embryonate eggs. Dead embryos were examined for elementary bodies and the LD 50 per ml. of the original suspension calculated (Reed & Muench, 1938).

Infectivity titrations in HeLa cells

These were done as described by Furness, Graham & Reeve (1960).

Particle counts

The elementary bodies in films of infected yolk sac suspensions were stained with Giemsa and counted under dark ground illumination (Reeve & Taverne, 1962).

RESULTS

Table 1 gives the days on which embryos died in representative titrations, and in Fig. 1 the arithmetic mean death time of each group of embryos is plotted against dose of virus inoculated expressed as a multiple of the $\log_{10}$ LD 50 (the dose killing 50 % of eggs). At least two titrations were done for strains LB 1, TE 55, G 17 and SA 2 in this laboratory; the curves shown for strain BOUR and ASGH are taken from Jawetz & Hanna (1960). Titrations of G 221 and G 1 gave points fitting the curve drawn for G 17 and BOUR; for clarity they are omitted from the figure. In many infections caused by either bacteria or viruses, log dosage, within limits, is inversely proportional to mean death time (see review by Meynell & Meynell (1958), who discuss the general characteristics of such curves). This is also true of trachoma virus (Jawetz & Hanna, 1960; Watkins, 1961) and we have observed it for all the strains tested. The dose-response curves shown in Fig. 1 are sigmoid, so that three portions can be distinguished: (1) With very large doses ($> 10^5$ LD 50 for strains TE 55 and LB 1) the curve becomes roughly parallel to the dose axis, presumably because even the largest doses take a minimum time to kill the embryo. (2) At intermediate doses, the mean death time is inversely proportional to log dose. The most probable interpretation of this is that the virus responsible for the death of the embryo increases exponentially to a critical level at which death occurs. The slope of this part of the curve is proportional to the rate of increase of the virus; since the slopes of the curves are parallel, all strains of virus examined increased at the same rate (Fig. 1). The rate estimated from the slope is 30 LD 50 per day, and the doubling time calculated on the basis of this constant is 6 hr. Jawetz, Hanna, Chino & Zichosch (1962) concluded from similar curves that strains ASGH, BOUR, APACHE and TANG in their laboratory had similar growth rates and increased by '1·3 logs of infective virus per day per g. of yolk sac'. (3) At doses < 1 LD 50 the mean death time becomes constant. Meynell & Meynell (1958) and Plus (1954) suggest that this occurs because at

Table 1. *Survival times of embryos infected with different doses of virus. Results of some representative titrations*

Strain	LD 50/ml.	Virus dilution	Day of specific death
G 17	2×10^5	10^{-1}	6, 6, 6, 7, 8
		10^{-2}	6, 7, 7, 8, 8, 9
		10^{-3}	8, 8, 8, 9, 9, 9
		10^{-4}	9, 9, 9, 9, 9, 9
		10^{-5}	9, 9, 9, 10, 11, 11, 11, 11, 12
		10^{-6}	9, 10, 11
TE 55	6×10^6	10^{-1}	4, 4, 4, 4, 5, 5
		10^{-2}	4, 4, 4, 4, 5, 5, 6
		10^{-3}	4, 4, 5, 5, 5, 6, 6
		10^{-4}	6, 6, 6, 7, 7, 8
		10^{-5}	5, 6, 6, 7, 7, 8, 8
		10^{-6}	8, 8, 9, 9, 10, 11
		10^{-7}	9, 10
SA 2	2×10^3	Undiluted	5, 5, 5, 5, 5, 6
		10^{-1}	5, 5, 5, 5, 5, 5
		10^{-2}	5, 5, 5, 6, 8, 7
		10^{-3}	7, 7, 9, 8
		10^{-4}	7, 8, 8, 8, 9
		10^{-5}	10, 10, 7
LB 1	4×10^7	10^{-2}	5, 5, 5, 5
		10^{-3}	5, 5, 5, 5, 6
		10^{-4}	6, 5, 7
		10^{-5}	5, 7, 8, 9
		10^{-6}	7, 8, 9, 9
		10^{-7}	9, 9, 9

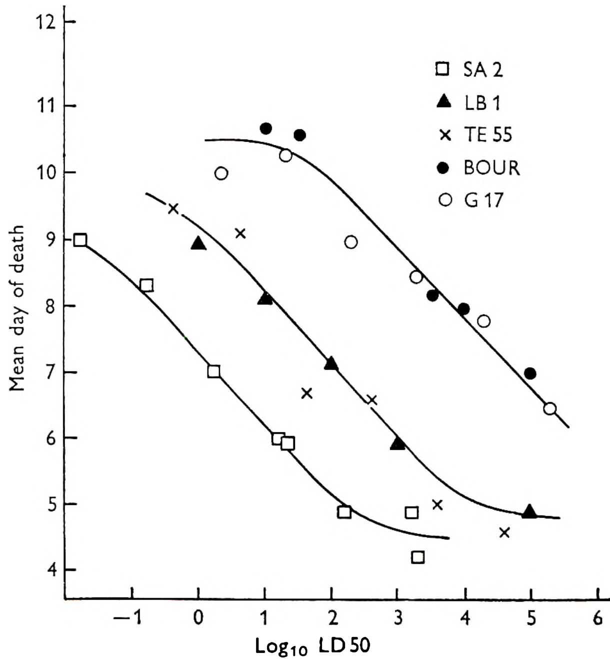

Fig. 1. Dose-response curves for different strains of trachoma and inclusion blennorrhoea in chick embryos.

doses < 1 LD 50 each response is usually caused by the multiplication of 1 particle, although the average dose may have contained many particles, each potentially capable of causing a lethal infection (Meynell & Stocker, 1957). On re-examination the data given by Jawetz & Hanna (1960) clearly fit a sigmoid curve better than a straight line.

Although the dose-response curves shown in Fig. 1 are similar in shape, for a given number of LD 50 of some strains the mean death time was less with some strains than with others. For instance, embryos receiving 10^4 LD 50 of strain

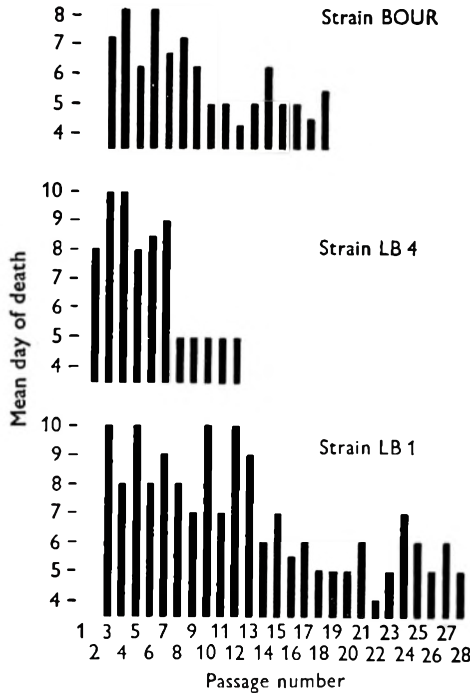

Fig. 2. The mean day of death of groups of chick embryos inoculated with three different strains of virus, showing changes occurring during routine passage.

TE 55 died 5 days after inoculation while embryos receiving the same dose of the Gambian strains died 9 days after inoculation. Similarly, strain ASGH killed embryos one day earlier than the same dose of strain BOUR (Jawetz & Hanna, 1960). Their dose-response curve for strain ASGH lies between those of the Gambian strains and TE 55. The differences in behaviour in the chick embryo existing between the Gambian strains and the others are also apparent in routine passages using undiluted yolk-sac suspensions as inocula.

We analysed the records of eggs inoculated in this laboratory for each passage of each strain and noted the total numbers of embryos dying with virus in them and the day after inoculation on which they died. The total number of embryos dying without detectable virus in them, including those dying from non-specific causes, was much the same for each strain, and no strain killed more embryos within the first 48 hr. of inoculation than any other. Most embryos died on one day which was characteristic of the strain inoculated: thus, strains SA 2, LB 1,

TE 55 and also LGV killed embryos most quickly, the majority dying on the 5th day, whereas embryos inoculated with the Gambian strains G1, G17 and G221 died 3–4 days later on the 8th or 9th day. Embryos infected with Gambian strains rarely died before the 6th day and if they did, contained no virus.

Three strains changed in their growth characteristics during passage: LB1 after the 13th passage, LB4 after the 7th passage and BOUR after the 9th passage. In each case, before the change most embryos died on the 8th day, like those inoculated with Gambian strains, but thereafter the majority died on the 5th day after inoculation. With all three strains the change appears to have been abrupt (Fig. 2).

Table 2. *Infectivity titres and particle counts of different suspensions of trachoma viruses*

Virus suspension					
Strain	Pass. no.	*IFU per ml.	Particles per ml.	LD 50 per ml.	Particles per LD 50
G1	42 (*a*)	0	$3\cdot0\times10^9$	$2\cdot0\times10^2$	$1\cdot0\times10^7$
G1	42 (*b*)	0	$9\cdot5\times10^8$	$2\cdot0\times10^2$	$4\cdot5\times10^6$
G1	32	0	$2\cdot0\times10^{10}$	$9\cdot0\times10^4$	$2\cdot0\times10^5$
G221	5	0	$1\cdot5\times10^{10}$	$3\cdot0\times10^3$	$5\cdot0\times10^6$
G062	4	0	$1\cdot5\times10^{10}$	$1\cdot5\times10^4$	$1\cdot0\times10^6$
BOUR	8	0	$1\cdot5\times10^9$	$2\cdot0\times10^3$	$7\cdot5\times10^5$
BOUR	19	$1\cdot0\times10^6$	$2\cdot5\times10^8$	$1\cdot0\times10^6$	$2\cdot5\times10^2$
ASGH	16	$4\cdot3\times10^5$	$4\cdot0\times10^7$	$2\cdot0\times10^5$	$2\cdot0\times10^2$
SA2	13	ND	$8\cdot0\times10^8$	$3\cdot0\times10^5$	$2\cdot0\times10^3$
SA2	14	$2\cdot4\times10^7$	$4\cdot0\times10^9$	$4\cdot0\times10^5$	$1\cdot0\times10^4$
SA2	16	$2\cdot5\times10^6$	$1\cdot0\times10^9$	$2\cdot5\times10^6$	$4\cdot0\times10^2$
TE 55	63	$3\cdot0\times10^6$	$9\cdot0\times10^8$	$1\cdot0\times10^4$	$9\cdot0\times10^4$
TE 55	65	ND	$9\cdot0\times10^9$	$3\cdot0\times10^6$	$3\cdot0\times10^3$

* Inclusion forming units (IFU) in HeLa cells.

Particle counts

Table 2 gives results of total particle counts on suspensions of different virus strains and the ratio of particles to LD 50. Strains which had a ratio of 10^4 or less could also be titrated in HeLa cells, whereas strains with a ratio of 10^5 particles/LD 50 or more did not form inclusions in HeLa cells. The strains with the higher ratio were those for which a given dose took longer to kill chick embryos. All the strains have a higher particle/LD 50 ratio than meningopneumonitis virus, another agent of the psittacosis-lymphogranuloma group (Manire & Smith, 1959). With care to avoid heat inactivation our ratios could probably be decreased but as all suspensions were made in the same way the results are comparable. The comparatively high particle/LD 50 ratio of some strains is not entirely due to their low infectivity titres; thus, suspensions G1/passage 32 and G221/passage 5 had infectivity titres of the same order as TE 55/passage 63 and ASGH/passage 16 but much higher particle/LD 50 ratios. Figures are given for two suspensions of strain BOUR. Before passage 9 this strain contained more than 10^5 particles/LD 50 and did not form inclusions in HeLa cells. By passage 19, when the survival time of the chick embryo had decreased, it contained 10^2 particles/LD 50 and the suspension had a titre in HeLa cells of $1\cdot0\times10^6$ inclusion forming units (IFU) per ml.

DISCUSSION

Implications of the dose/death time curves

Golub (1948) observed the linear relationship between log. dose and day of death for psittacosis virus and suggested that provided large numbers of eggs were used a single dilution method could be used for titration; Jawetz & Hanna (1960) and Watkins (1961) have applied his method to trachoma virus. However, this method is valid only if the linear portion of the dose-response curve is used and if virus strains do not change their growth characteristics. Our results and those of Meynell & Meynell (1958) show that the linear relationship does not hold for doses $\leqslant$ 1 LD 50 or for very large doses ($\geqslant 10^5$ LD 50); in addition three of our strains altered their growth characteristics during passage. Furthermore, with strains killing embryos slowly there is greater variation about the mean survival time for embryos receiving the same dose and, for all strains, the less virus inoculated the greater is the scatter of embryo deaths. Our dose-response curves conform to the model proposed by Meynell & Meynell (1958) for the production of a response by the multiplication of micro-organisms for which average latent period is linearly related to logarithm of dose. The model postulates that the organisms causing the response increase *in vivo* at a constant rate so that their number rises exponentially and that the response (in this case death of the embryo) occurs when their total number reaches or exceeds a critical figure. For systems having the same growth rate, as have our strains, three situations could produce the observed regression lines:

(i) The same critical number of particles causes death, but either the strains have different lag phases before exponential growth begins or, after the lethal concentration of particles is reached, there is a delay, varying with strain, before the embryo dies. Our curves only show the exponential growth phase so cannot be used to demonstrate differences in lag phases. However, the numerous growth curves which have been published for trachoma and other viruses of the psittacosis-lymphogranuloma group are so similar (e.g. Litwin, Officer, Brown & Moulder, 1961) that such differences are unlikely.

(ii) The strains all kill when the total number of particles reaches the same critical figure though the numbers of infective particles are different.

(iii) The strains attain about the same infectivity titre but kill when the total number of particles reaches a different critical figure.

The total number of particles in an infected yolk sac from a dead embryo appears to be relatively constant, regardless of strain, and therefore on average the infectivity titres of the strains which kill more slowly would be expected to be lower than the rest, and the ratio of particles per LD 50 would be higher. Reference to Table 2 shows that the ratio of particles/LD 50 for the Gambian strains is in fact consistently higher than that for the more pathogenic strains, and the difference, which is of the order of 1000-fold, is of the magnitude which would be predicted from the growth rate and the different positions of the dose-response curves in relation to the mean death day axis. Our results therefore appear best explained by hypothesis (ii).

We do not know how the virus kills embryos, but since all strains have so much in common it seems likely that they all kill in the same way; it is possible that some strains are more virulent than others because their infective particles possess more of some lethal factor, or perhaps a lethal factor of a slightly different kind. Members of this group are known to produce a toxin (Rake & Jones, 1944; Bell, Snyder & Murray, 1959); a toxin may contribute to the death of the embryos, which show haemorrhagic lesions similar to those described for mice dying after an intravenous dose of live trachoma virus (Bell *et al.*, 1959).

It can be deduced from Fig. 1 that to kill an embryo within 24 hr. of inoculation at least 10^8 LD 50 of our most virulent strain (LB 1) would be required; and this is often more than is contained in a whole yolk sac. Therefore Watkins's (1961) argument that a toxic factor is not involved because there is no evidence that embryos die in the absence of multiplication may not be valid as, in the experiments he described, doses large enough to be immediately toxic were not administered.

Changes in behaviour on passage

T'ang *et al.* (1957) reported that 'with the increase of number of passages the lethal action of the virus for the embryos was increased', and that a steep rise in infectivity occurred between the 10th and 15th passage. We too observed some changes during passage, a given dose of three strains (LB 1, LB 4 and BOUR) now killing embryos more rapidly than originally. However, we only have the full histories of the Gambian strains: G 1, G 17 and G 221, which were received in their 3rd, 7th and 1st egg passage, have been passed in this laboratory 42, 49 and 7 times respectively and full records have been kept of the survival times. We do not have the full histories of strains which kill embryos more rapidly than the Gambian strains, i.e. TE 55, SA 2 and LGV. These may have been slow to kill the embryo immediately after isolation, and like LB 1, LB 4 and BOUR they may have increased in virulence on passage, but before we received them.

The pathogenicity of strain LB 1 for chick embryos changed in March 1959, that of LB 4 in June 1961 and of BOUR in February 1962. Since strict precautions are taken to avoid any confusion, LB 1, LB 4 and BOUR are unlikely to have become contaminated at different times with one of the strains killing embryos quickly. Furthermore, there is no evidence that any of the Gambian strains have become contaminated, although G 1 and G 17 have been passaged frequently for over 3 years. These changes are most reasonably explained by the appearance of a mutant which kills embryos more rapidly than the parent strain. The conditions of routine passage in this laboratory would especially favour its selection because high concentrations of fresh virus are inoculated into eggs with short intervals between passages.

All strains which kill chick embryos relatively quickly have the ability to induce inclusions in HeLa cells (shown for TE 55 and LB 1 by Furness, Graham, Reeve & Collier (1960), and for SA 2, LB 4, BOUR and ASGH by P. Reeve & D. M. Graham (unpublished observations)) and some have also been shown to grow in the mouse brain (Hurst & Reeve, 1960); but in their early passages strains LB 4 and BOUR

failed to infect HeLa cells (D. M. Graham & W. A. Blyth, unpublished observations). Collier (1961) noticed that after its 8th passage strain LB 1 lost the ability to infect the baboon conjunctiva and this was about the same time as the change in pathogenicity for the chick embryo occurred. There is thus some evidence for correlated changes in increased ability to kill chick embryos and to infect HeLa cells and the mouse brain, and loss of pathogenicity for the baboon. These events may also be accompanied by loss of specific antigen, as in smooth to rough variation in bacteria (Wilson & Miles, 1946). Mutation followed by selection of mutants serologically different from naturally occurring strains may account for our failure to extract specific complement-fixing antigen (Jenkin, Ross & Moulder, 1961) from virus grown in HeLa cells and to distinguish between strains by neutralization tests in HeLa cells (Reeve & Graham, 1961, and unpublished observations).

SUMMARY

When trachoma and inclusion blennorrhoea viruses were titrated in chick embryo yolk sacs and the average day of death was plotted against dose of virus inoculated, sigmoid curves were obtained. Although all strains tested had the same growth rate, a given dose of some killed embryos more quickly than others. Strains killing most rapidly had the fewest elementary bodies per LD 50 and were the only strains to form inclusions in HeLa cells. During passage in the chick embryo three strains changed in their behaviour, killing embryos faster and acquiring the ability to form inclusions in HeLa cells.

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REFERENCES

Bell, S. D., Snyder, J. C. & Murray, E. S. (1959). *Science*, **130**, 626.
Bernkopf, H. (1959). *Bull. Res. Counc. Israel*, 8E, 25.
Bovarnick, M. R., Miller, J. C. & Snyder, J. C. (1950). *J. Bact.* **59**, 509.
Collier, L. H. & Sowa, J. (1958). *Lancet*, i, 993.
Collier, L. H. (1961). *Trans. Ophthal. Soc. U.K.* **81**, 351.
Furness, G., Graham, D. M. & Reeve, P. (1960). *J. gen. Microbiol.* **23**, 613.
Furness, G., Graham, D. M., Reeve, P. & Collier, L. H. (1960). *Rev. int. Trachome*, **37**, 574.
Golub, O. H. (1948). *J. Immunol.* **59**, 71.
Hanna, L., Jawetz, E., Thygeson, P. & Dawson, C. (1960). *Proc. Soc. exp. Biol., N.Y.*, **104**, 142.
Hurst, E. W. & Reeve, P. (1960). *Nature, Lond.*, **186**, 336.
Jawetz, E. & Hanna, L. (1960). *Proc. Soc. exp. Biol., N.Y.*, **105**, 207.
Jawetz, E., Hanna, L., Chino, S. & Zichosch, J. (1962). *Proc. Soc. exp. Biol., N.Y.*, **109**, 205.
Jenkin, H. M., Ross, M. R. & Moulder, J. W. (1961). *J. Immunol.* **86**, 123.
Jones, B. R., Collier, L. H. & Smith, C. H. (1959). *Lancet*, i, 902.
Jones, B. R. (1961). *Trans. Ophthal. Soc. U.K.* **81**, 367.
Litwin, J., Officer, J. E., Brown, A. & Moulder, J. W. (1961). *J. infect. Dis.* **109**, 251.
Manire, G. P. & Smith, K. O. (1959). *J. Bact.* **78**, 523.
Meynell, G. G. & Meynell, E. W. (1958). *J. Hyg., Camb.*, **56**, 323.

Meynell, G. G. & Stocker, B. A. D. (1957). *J. gen. Microbiol.* **16**, 38.
Murray, E. S., Bell, S. D., Hanna, A. T., Nichols, R. L. & Snyder, J. C. (1960). *Amer. J. Trop. Med. Hyg.* **9**, 116.
Plus, N. (1954). *Bull. biol. Fr. Belg.* **87**, 248.
Rake, G. & Jones, H. P. (1944). *J. exp. Med.* **79**, 463.
Reed, L. J. & Muench, H. (1938). *Amer. J. Hyg.* **27**, 493.
Reeve, P. & Graham, D. M. (1961). *J. gen. Microbiol.* **27**, 177.
Reeve, P. & Taverne, J. (1962). *Nature. Lond.*, **195**, 923.
Sowa, J. & Collier, L. H. (1960). *J. Hyg., Camb.*, **58**, 99.
T'ang, F. F., Chang, H. L., Huang, Y. T. & Wang, K. C. (1957). *Chin. med. J.* **75**, 429.
Watkins, J. F. (1961). *J. gen. Microbiol.* **26**, 427.
Wilson, G. S. & Miles, A. A. (1946). *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed. London: Edward Arnold and Co.

J. Hyg., Camb. (1963), **61**, 77
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Effect of various post-irradiation treatments on ultraviolet irradiated *Shigella sonnei**

BY MITSURU NAKAMURA AND CAROL M. RAMAGE†
Department of Botany and Microbiology, Montana State University, Missoula, Montana, U.S.A.

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INTRODUCTION

Post-irradiation treatments of ultraviolet irradiated bacteria cause changes in the percentage of survivors. Roberts & Aldous (1949) listed various factors which were important in the study of ultraviolet effects upon bacteria, including conditions employed following irradiation. They reported that the recovery of ultraviolet irradiated cells was greater on a chemically defined medium than on nutrient agar. The addition of nutrient broth to the chemically defined medium caused a decrease in the number of irradiated organisms that were capable of colony formation. Alper & Gillies (1958*a*) found that there was a greater survival of ultraviolet irradiated *Escherichia coli* B on Difco nutrient agar than on Oxoid blood agar base. They reported that if the Oxoid blood agar base was dialysed the non-dialysable fraction produced good recovery of the irradiated cells comparable to that on nutrient agar. They found that chloride ion and a factor in peptone reduced the recovery of irradiated bacteria. Alper & Gillies (1958*b*) reported that an increase in the concentration of peptone in nutrient agar or the addition of sodium chloride resulted in a decrease in the numbers of organisms surviving the irradiation treatment. It was pointed out that *E. coli* had a shorter lag phase and generation time when grown in nutrient broth supplemented with sodium chloride compared to the regular nutrient broth. Growth of the organisms was more rapid on Oxoid agar than on nutrient agar. It was postulated that suboptimal growth conditions favoured recovery by preventing imbalances in the synthetic processes of the ultraviolet injured cells (Alper & Gillies, 1960). Other workers have also observed similar results (Anderson, 1949; 1951; Stein & Meutzner, 1950).

The survival of ultraviolet irradiated cells increased when the irradiated cells were held in water or saline prior to plating on the recovery medium (Hollaender & Claus, 1937). On the other hand, prolonged holding in water resulted in a decrease in the surviving fractions (Hollaender, 1943; Barner & Cohen, 1956). Charles & Zimmerman (1956) termed this phenomenon 'dark' reactivation. It was found that maximum recovery of *E. coli* B occurred within 3–5 hr. when the

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cells were held in the dark (in appropriate culture media); this was then followed by a period in which there was a decrease in viable count (Woodside, Goucher & Kocholaty, 1960). Alper & Gillies (1960) reported that the colony-forming ability of irradiated cells increased as the time interval between irradiation and contact with the growth medium increased, up to a certain level.

In the present study of the effects of various post-irradiation treatments on the survival of ultraviolet irradiated *Shigella sonnei*, some of the factors investigated were the composition of the recovery media, temperature of incubation, and variations in the time interval between irradiation and plating upon recovery media.

METHODS

Two strains of *S. sonnei* were employed in our experiments: strain 5044–59 obtained from Dr W. H. Ewing, Laboratory Branch, Communicable Disease Center, Atlanta, Georgia, and strain F-6, obtained from Dr M. Finland, Boston City Hospital, Boston, Massachusetts. The cultures were periodically checked for purity. The source of ultraviolet light was a General Electric 15 W. germicidal lamp (G15T8) which emitted waves of approximately 2537 Å. The calculated intensity value under the conditions employed was 17·2 ergs/sec./mm.^{2}. The organisms were grown in the chemically defined medium (EM) of Erlandson & Mackey (1958). A 20–22 hr. culture of *S. sonnei* grown in EM medium was washed three times by centrifugation with 0·15 M phosphate buffer and resuspended in buffer to yield an optical density (OD) reading of 0·15 on the Bausch and Lomb 'Spectronic 20' spectrophotometer (wavelength set at 575 mμ). One ml. of this suspension contained approximately 10^8 cells. Ten ml. of the EM medium were inoculated with 1 ml. of this cell suspension. The tubes were incubated at 37° C. for 20–22 hr.; the growth was harvested and the cells were washed three times and resuspended in buffer to yield an OD reading of 0·15. Five-ml. aliquots of this cell suspension were irradiated in flat-bottom Petri dishes. The samples undergoing irradiation were shaken continuously to ensure uniform exposure of the cells. One-tenth ml. aliquots were withdrawn from the Petri dishes after 15 and 30 sec. of irradiation. An aliquot removed from the suspension prior to irradiation served as the unirradiated control. The unirradiated and irradiated samples were serially diluted; 0·1 ml. aliquots were plated upon the EM medium solidified with agar, nutrient agar (Difco), and brain heart infusion agar (BHI) (Difco). These plates were inverted and incubated for 24–48 hr. and the colonies were counted on the Quebec colony counter. This experiment was done in order to determine the effect of the composition of the recovery medium on the survival of the irradiated cells. In order to determine the effect of the temperature of incubation on the survival of the irradiated cells, nutrient agar plates were inoculated with aliquots of the cell suspensions and incubated at 15, 29, 37 and 44° C. for 24 hr. This was followed by an additional incubation period of 24 hr. at 37° C. to permit growth of cells which may have survived but did not form colonies, especially at the lower temperatures of incubation. In order to determine the effect of time intervals between irradiation and plating on recovery media, aliquots of irradiated cells were held in 0·15 M

phosphate buffer for 2½, 5, and 8 hr. at 25° C. and then plated. Irradiated cells were also plated immediately to determine the change in population in the absence of any holding period. Unirradiated cells were also held at 25° C. for varying periods to determine the effect of the holding process alone. The percentage survivors after irradiation were calculated by dividing the number of viable irradiated bacteria per ml. by the number of viable unirradiated bacteria per ml. and multiplying the quotient by 100.

RESULTS

Greater recovery of irradiated *S. sonnei* occurred on the chemically defined medium than on nutrient agar or BHI agar. Recovery was slightly better on nutrient agar than on BHI agar (Fig. 1). The difference in the ability to recover colony-forming cells diminished at the higher ultraviolet dosage used. Colonies appeared larger on the BHI medium than on the EM medium; colonies on nutrient agar were intermediate in size. In order to determine whether some constituent of the EM medium favoured the recovery of irradiated cells, the ingredients of the EM medium were added singly to the BHI medium. Furthermore, ingredients of the BHI medium were added to the EM medium to learn if the components of the BHI medium inhibited the recovery of irradiated cells. Table 1 summarizes the data. The addition of thiamin, niacin, $MgSO_4$, and glucose to the BHI medium did not materially alter the ability of the medium to recover irradiated cells. However, the addition of aspartic acid may have produced a slight increase in the surviving fractions that were able to form colonies. On the other hand, the addition of BHI ingredients to the EM medium produced a significant reduction in the ability of the medium to grow the irradiated bacteria. We varied the concentration of peptone in the nutrient agar to see if the peptone concentration affected the recovery of the bacteria exposed to ultraviolet light. We tested 5, 10, and 15 g. of peptone per litre of media. There was no difference in the numbers of cells that could be recovered.

Table 1. *Effect of the composition of the plating medium on the recovery of ultraviolet irradiated* Shigella sonnei

Composition of plating medium	Percentage survivors (258 ergs/mm.^{2})
Brain heart infusion (BHI)	17·1
BHI plus thiamin	16·0
BHI plus niacin	16·7
BHI plus $MgSO_4$	17·9
BHI plus glucose	18·0
BHI plus aspartic acid	22·2
Erlandson-Mackey chemically defined medium (EM)	38·2
EM plus BHI ingredients	30·1

Since Alper & Gillies (1960) found that conditions which were more favourable for the growth of unirradiated bacteria proved to be less favourable for the recovery of irradiated organisms, we determined the growth curve of *S. sonnei* in various liquid media. The results are illustrated in Fig. 2. The lag phase was shortest in the

BHI liquid medium, longest in the EM medium, and intermediate in nutrient broth. Essentially, the growth rate of *S. sonnei* was greater and more rapid in the medium which yielded the poorest recovery of the ultraviolet-treated cells.

A post-irradiation incubation temperature of 37° C. for 24 hr. resulted in greater

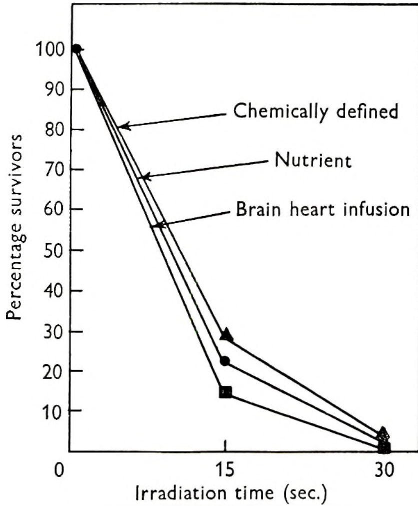

Fig. 1

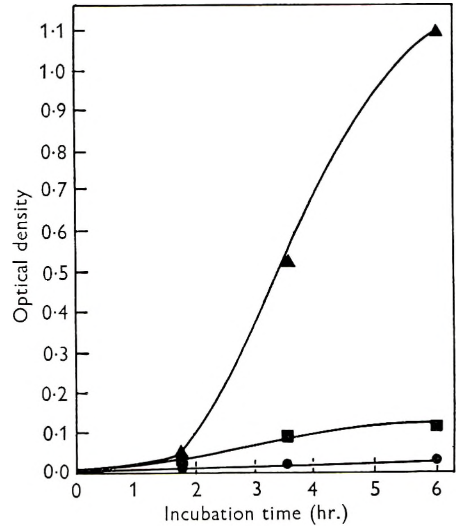

Fig. 2

Fig. 1. Recovery of ultraviolet irradiated *S. sonnei* on three different media.

Fig. 2. Growth rate of unirradiated *S. sonnei* in liquid media: ▲, Brain heart infusion; ■, nutrient broth; ●, Erlandson–Mackey chemically defined broth.

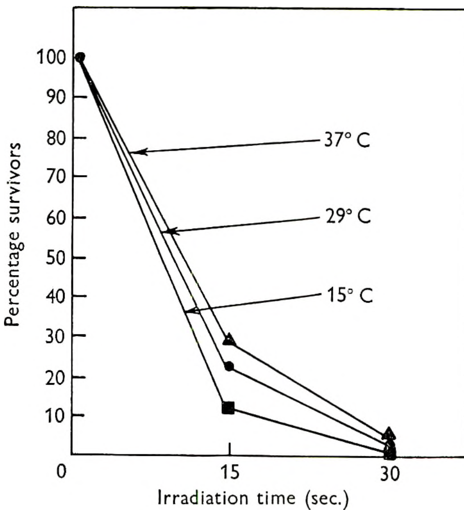

Fig. 3

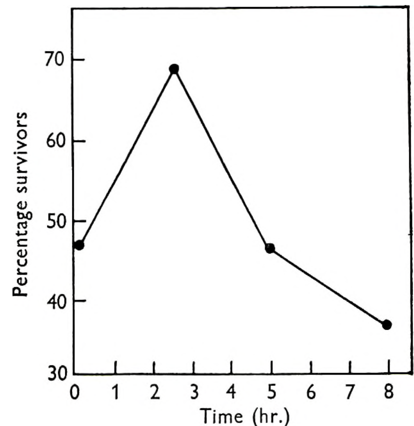

Fig. 4

Fig. 3. Survival of ultraviolet irradiated *S. sonnei* cells which were incubated at various temperatures for 24 hr. and then reincubated for 24 hr. at 37° C.

Fig. 4. Survival of ultraviolet irradiated *S. sonnei* held in buffer at 25° C. for varying periods of time prior to plating upon recovery media.

recovery of ultraviolet irradiated cells than incubation at 29 or 15° C. when the cells were exposed to the lower level irradiation (258 ergs/mm.^{2}). However, there was little difference in the ability to recover cells when exposed to the higher level of irradiation (516 ergs/mm.^{2}). Fig. 3 summarizes the results. A post-irradiation incubation temperature of 44° C. resulted in fewer colony-forming cells than at the post-irradiation temperature of 37° C.

Irradiated cells held in buffer at 25° C. for 2½ hr. possessed a greater potential for recovery from the effects of ultraviolet light than cells which were not held following irradiation or cells which were held in buffer for 5 and 8 hr. As a matter of fact the recovery of cells held for 8 hr. was poorer than cells which were plated immediately (Fig. 4).

DISCUSSION

The survival of ultraviolet irradiated *S. sonnei* varied considerably with different post-irradiation treatments. In the present study the ability to recover irradiated cells was affected by the composition of the recovery medium, the temperature of incubation following irradiation, and the time interval between exposure to ultraviolet light and plating upon recovery media. Recovery of irradiated cells was greater on simple synthetic media than on complex media. One possible explanation for this is that cell growth in the chemically defined medium may be sufficiently slower than on complex media thereby allowing a longer period of repair from the damaging effects of the irradiation. It also appeared that some component in the BHI medium actually inhibited or delayed the growth of the ultraviolet-treated cells. Although Alper & Gillies (1958*a*) found a factor associated with peptone that was inhibitory towards recovery, we did not find any differences in the ability to recover cells when the peptone concentrations varied from 5 to 15 g. per litre. However, we agree with Alper & Gillies (1960) with regard to the fact that conditions optimal for growth were unfavourable for recovery of the irradiated cells.

It is difficult to explain why cells incubated at 37° C. following irradiation permitted better recovery than cells incubated at 15 and 27° C. A reduction in the colony-forming cells when the irradiated cells were incubated at 44° C. could have been due to the fact that at this temperature the cells metabolized too rapidly to permit proper repair of injured sites. It is even conceivable that this temperature had a direct inhibitory effect upon the growth of irradiated cells.

Several suggestions have been offered to explain the increase in recovery of irradiated cells when the cells were held in a liquid medium prior to plating upon the recovery medium. Roberts & Aldous (1949) felt that during this holding period some cell 'poison' produced by the irradiation was destroyed. Alper & Gillies (1960) suggested that the increase in survival was due to the retardation of metabolism which protected the irradiated bacteria against the action of the 'peptone factor' in the plating medium. Furthermore, it has been reported that some protein synthesis was necessary for repair and that an exogenous source of nitrogen inhibited the recovery of irradiated cells (Doudney, 1959; Sawada & Suzuki, 1961). Immediate contact between irradiated cells and nutrients may

favour unbalanced growth which leads to death while holding in a liquid such as phosphate buffer before plating favours reparative processes. Billen (1957) reported that the release of 260 $m\mu$-absorbing material from irradiated cells was inhibited in the absence of exogenous metabolites. The prevention of the loss of this fraction by incubation in a nutrient-free medium might play a role in preventing death.

SUMMARY

More cells of *Shigella sonnei* were recovered on a chemically defined medium than on complex media after the cells were irradiated with ultraviolet light. The temperature of incubation affected the numbers of colony-forming cells that could be recovered. A temperature of 37° C. yielded the largest number of colonies compared with temperatures of 15, 27 and 44° C. Recovery of irradiated cells was favoured when the cells were held in buffer at 25° C. for $2\frac{1}{2}$ hr. before plating on solid media.

REFERENCES

ALPER, T. & GILLIES, N. E. (1958*a*). Dependence of the observed oxygen effect on the post-irradiation treatment of micro-organisms. *Nature, Lond.*, **181**, 961.

ALPER, T. & GILLIES, N. E. (1958*b*). 'Restoration' of *Escherichia coli* strain B after irradiation: its dependence on suboptimal growth conditions. *J. gen. Microbiol.* **18**, 461.

ALPER, T. & GILLIES, N. E. (1960). The relationship between growth and survival after irradiation of *Escherichia coli* strain B and two resistant mutants. *J. gen. Microbiol.* **22**, 113.

ANDERSON, E. H. (1949). Reactivation of ultraviolet irradiated bacteria. Abstract. *Amer. J. Botany*, **36**, 807.

ANDERSON, E. H. (1951). Heat reactivation of ultraviolet-inactivated bacteria. *J. Bact.* **61**, 389.

BARNER, H. D. & COHEN, S. S. (1956). The relation of growth to the lethal damage induced by ultraviolet irradiation in *Escherichia coli*. *J. Bact.* **71**, 149.

BILLEN, D. (1957). Modification of the release of cellular constituents by irradiated *Escherichia coli*. *Arch. Biochem. Biophys.* **67**, 333.

CHARLES, R. L. & ZIMMERMAN, L. N. (1956). Dark reactivation in ultraviolet irradiated *Escherichia coli*. *J. Bact.* **71**, 611.

DOUDNEY, C. O. (1959). Macromolecular synthesis in bacterial recovery from ultraviolet light. *Nature, Lond.*, (Suppl. no. 4), **184**, 189.

ERLANDSON, A. L. & MACKEY, W. H. (1958). Nutrition of *Shigella*: growth of *Shigella flexneri* in a simple chemically defined medium. *J. Bact.* **75**, 253.

HOLLAENDER, A. (1943). Effect of long ultraviolet light and short visible radiation (3500 to 4900 Å.) on *Escherichia coli*. *J. Bact.* **46**, 531.

HOLLAENDER, A. & CLAUS, W. D. (1937). An experimental study of the problem of mitogenetic radiation. *Bull. Nat. Res. Counc.* **100**, 75.

ROBERTS, R. B. & ALDOUS, E. (1949). Recovery from ultraviolet irradiation in *Escherichia coli*. *J. Bact.* **57**, 363.

SAWADA, F. & SUZUKI, K. (1961). Recovery of *Escherichia coli* irradiated with ultraviolet light. *J. Bact.* **81**, 288.

STEIN, W. & MEUTZNER, I. (1950). Reaktivierung von UV-inaktivierten *Bacterium coli* durch Wärme. *Naturwissenschaften*, **37**, 167.

WOODSIDE, E. E., GOUCHER, C. R. & KOCHOLATY, W. (1960). Post-irradiation responses of ultraviolet irradiated *Escherichia coli*. *J. Bact.* **80**, 252.

J. Hyg., Camb. (1963), **61**, 83
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Sources of staphylococcal infection in surgical wound sepsis*

By H. F. M. BASSETT and W. G. FERGUSON
Newcastle Regional Chest Surgery Centre, Shotley Bridge Hospital, County Durham

E. HOFFMAN and M. WALTON
Poole Hospital, Nunthorpe, Middlesbrough

ROBERT BLOWERS and CONNIE ALDERSON CONN
Public Health Laboratory, General Hospital, Middlesbrough

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There are, broadly, two ways of controlling post-operative staphylococcal wound sepsis. One is to take every possible precaution against every conceivable mode of infection. Attempts to do this are undoubtedly effective, and it is sometimes the only way of dealing quickly with a severe outbreak. But it is expensive of time, money, and temper and is so tedious that when the emergency is over, its complicated routine tends to degenerate into an ineffective ritual. Another defect of this method is that it encourages the expenditure of great effort on exotic precautions for no better reason than that someone has a bee in the bonnet about them. The other approach along which, one hopes, we are steadily moving is to determine the importance, under existing conditions, of each possible source or mode of infection and then to apply new or improved precautions against those that matter.

In this paper we report studies on the roles of the patients themselves (*self-infection*), and of the surgical team (*cross-infection*) as sources of staphylococci causing post-operative sepsis.

Self-infection has been recently much studied, with rather conflicting results. It has, however, been generally found that about half the cases of wound sepsis are caused by *Staphylococcus aureus* of the same phage type as that carried, before operation, in the patient's nose. This indicates that the nose may be an important source from which the wound is often infected; but it could mean no more than that hospitals are infested by staphylococci which, independently, infect noses and wounds. In some surveys, the former explanation was favoured by the finding that patients who were nasal carriers of *Staph. aureus* suffered sepsis much more often than those who were not carriers. In other surveys, however, this difference between carriers and non-carriers has been less evident or even absent. Moreover, patients whose nares are kept free of staphylococci by application of antibacterial creams suffer no less sepsis than untreated controls. This could be explained by the possibility that the nose—long thought of as the main site of staphylococcal

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carriage—is not the actual source of self-infection but is merely a regular concomitant of carriage in some more vital area such as the perineum (Hare & Ridley, 1958) or the skin of the operation area, from which wound infection more often occurs. In the survey at Poole Hospital we have studied wound sepsis in relation to the pre-operative presence of *Staph. aureus* not only in the nose but also on the skin of the operation area. Because the results were somewhat surprising, we planned a simplified check on them at Shotley Bridge Hospital where we also regularly swabbed the noses of surgeons and operating-room staff and compared the organisms carried by them with those cultured from septic wounds.

Members of operating-room teams who were symtomless carriers or suffering from staphylococcal lesions have often been incriminated as sources of infection during sudden outbreaks of sepsis. However, many cases of infection occur during the longer non-epidemic periods, and because we knew of no recent work in which infection from the surgical team was considered during such a period, we planned to study it at Shotley Bridge Hospital.

THE INVESTIGATION AT POOLE HOSPITAL

The investigation was made in one male and one female surgical ward of a 300-bed tuberculosis hospital. The two wards are on the same floor and have the same medical but different nursing staffs. Each ward consists of 8 single-bed, 3 two-bed, and 6 four-bed rooms; thus 76 beds were concerned in the survey. There is ample isolation accommodation in a separate building, to which septic patients were moved as soon as the diagnosis was made. The operating-suite is pressure-ventilated with filtered air. The survey lasted from April 1953 to May 1956.

Methods

Precautions against infection

As a result of a severe outbreak of infection before this investigation began, the level of 'infection-consciousness' was high and there had been a recent revision of precautions against infection (Blowers, Mason, Wallace & Walton, 1955). These precautions will not be described here, except those concerning the pre-operative preparation of patients' skin and surgeons' hands.

On the evening before operation the patient's head was washed but the skin of the operation area was not treated in any way. On the day of operation the patient bathed himself or, if not well enough to go to the bathroom, was given a blanket-bath. This was finished about an hour before operation and the patient did not return to the ward or his own bed, but went to a 'preparation room' and on to a trolley laid with sterilized blankets. The skin of the operation area was shaved, cleaned with 0·5 % aqueous cetrimide solution, dried with surgical spirit then ether, and painted with 2 % iodine in 70 % ethyl or iso-propyl alcohol. The area was not bandaged and the patient was dressed in a freshly laundered operation gown and cap. He waited in the preparation room until taken to the operation theatre. There, the skin was painted sometimes with three and never less than two applications of 2 % alcoholic iodine. Each was allowed to dry before the next was

applied or before the incision was made, thus ensuring that the disinfectant had at least 3 min. in which to act.

The surgeons and nurses scrubbed their forearms and hands, with ordinary soap and hot water, for 10 min. before every operation. The hands were rinsed in surgical spirit and dried on a sterile towel before the gloves were put on.

Nose, skin, and wound swabs

Nose and back swabs were taken from each patient on the day of admission to hospital, then once each week and on the day of operation. Ordinary cotton-wool swabs on wooden sticks were moistened in 7·5 % salt-meat broth (Maitland & Martyn, 1948). One swab was used for both anterior nares and another was rubbed over the right and left subscapular areas. Each swab was broken off into salt-meat broth. After overnight incubation at 37° C., these enrichment cultures were plated on phenolphthalein-diphosphoric acid agar (Barber & Kuper, 1951) which was incubated overnight. Phosphatase-positive colonies were tested for coagulase production by the slide technique (Cadness-Graves, Williams, Harper & Miles, 1943) and strains of *Staph. aureus* were saved for bacteriophage typing in large batches.

Material from septic wounds and empyemata was collected and cultured in the ordinary way. The similarity or difference between bacteriophage-typable staphylococci was determined according to the principles suggested by Williams & Rippon (1952); untypable strains were assumed to be different from typable ones but the same as other untypables from the same patient.

Results

It was not always possible to foretell, when each patient was admitted to hospital, whether an operation would be performed; many, therefore, had nose and skin swabs taken unnecessarily. Swabs were taken from 728 patients but the following analysis concerns only 317 of them, on whom 520 operations were performed. From these, 2503 pairs of swabs were obtained with an average of 7·9 pairs from each patient, and ranging from 2 to 34. The operations were mainly lung resections and first- and second-stage thoracoplasties. Sepsis occurred after 30 (5·8 %) of the operations. There were 9 cases of intrapleural or other thoracic-space sepsis, 13 of moderate or severe parietal-wound sepsis, 2 involving an intrathoracic space and the parietal wound, and 6 of minor wound-sepsis or stitch-abscess. The sepsis rates in the whole hospital for each of the years of the survey and for the year after it (1953–57) were 3·9, 6·2, 5·4, 1·1 and 2·3 %.

Frequency of nose and back carriage

Of the 317 patients on whom operations were performed, 54 (17 %) were consistent non-carriers of *Staph. aureus* (Table 1, column 1). Of the 263 (83 %) who yielded the organism on any occasion, staphylococci were cultured from both nose and back of 186, from the nose only of 58, and from the back only of 19. From some of these, however, staphylococci of any one phage type were isolated

only once; if these organisms are regarded as being of the 'transient' rather than of the 'resident' bacterial flora, and the patients as non-carriers, carriage rates at the two sites were as shown in column 2 of Table 1. Among the regular carriers, back carriage very rarely occurred in the absence of nose carriage, but 44 (20 %) of the 219 nose carriers were not back carriers. When studying the relationship between staphylococcal carriage and wound sepsis, we have separately considered the consequences of 'carriage' and 'regular carriage' in the nose and on the skin.

Table 1. *Nose and back carriage of* Staphylococcus aureus *in* 317 *patients, based on isolations from* 2503 *weekly pairs of swabs.*

In column 1, carriage indicates that *Staph. aureus* has been isolated on one or more occasions; in column 2, regular carriage indicates that *Staph. aureus* has been isolated on two or more occasions (Poole Hospital)

	Number of patients with	
	(1) Carriage	(2) Regular carriage
Nose and back	186 (59 %)	175 (55 %)
Nose only	58 (18 %)	44 (14 %)
Back only	19 (6 %)	4 (1 %)
None	54 (17 %)	94 (30 %)
Totals	317	317

Table 2. *Reliability of single nose and back swabs taken immediately before operation* (*Poole Hospital*)

	Nose	Back
Correct	380 (80 %)	267 (56 %)
'False positive'	10 (2 %)	10 (2 %)
'False negative'	84 (18 %)	197 (42 %)
Totals	474	474

During this investigation we were only too conscious of the great labour of collecting and culturing weekly nose and skin swabs, and of phage typing the staphylococci. The possibility of conducting a similar investigation elsewhere prompted us to estimate the reliability of examining only one swab or one pair of swabs, taken immediately before each operation. We therefore compared the results of the immediate pre-operation swabs with those obtained from the weekly swabbings. A pre-operation result was assumed to be correct when it yielded staphylococci of a phage type previously isolated on any occasion from either site; or when it yielded no staphylococci from a patient whose weekly swabs had also been negative. Of the nose swabs taken immediately before the 474 operations suitable for this analysis, 380 (80 %) were thus judged to have given the correct answer (Table 2). On 10 (2 %) occasions *Staph. aureus* was isolated for the first time from the pre-operation swab; we cannot say whether these results indicated a last-minute change in the carrier state or the chance isolation of a transient strain. In the table we have recorded them as 'false positives' but recognize that some of

them may have been correct. On 84 (18 %) occasions the pre-operation nose swabs did not yield *Staph. aureus* from patients who had previously done so. These 'false negative' results are not surprising since very few of the regular carriers yielded staphylococci from every one of the weekly swabs. Thus pre-operation nose swabs gave a correct result on 80–82 % of occasions. Back swabs, similarly judged, were much less reliable, with an accuracy of only 56–58 %.

From these results we conclude that several swabs are needed for accurate determination of the carrier state. If repeated swabbing is impracticable a single pre-operation nose swab is an acceptable substitute but a single back swab is not.

Relationship between sepsis and carriage of Staphylococcus aureus

In each of the 30 cases of sepsis, *Staph. aureus* was recovered, in pure culture, from the aspirated fluid or wound. Phage comparison of these organisms with those already isolated from the nose and skin of each patient showed (Table 3) that on ten occasions the wound strains differed from those from the sites of carriage; whilst on six occasions, sepsis occurred in patients whose nose and skin had never yielded *Staph. aureus*. In these 16 cases, sepsis can be reasonably attributed to *cross-infection*. In each of the 14 other cases of sepsis, the wound

Table 3. *Frequency of sepsis after operations on carriers and non-carriers of* Staphylococcus aureus (*Poole Hospital*)

Carriers are patients from whom *Staph. aureus* was isolated from nose or back on any occasion; regular carriers are those from whom two or more isolations were obtained.

	Number of operations	Post-operative sepsis	Comparison of *Staph. aureus* from carriage-sites and wound: Same	Different
All patients	520	30 (5·8 %)	14 (2·7 %)	16 (3·1 %)
Non-carriers	78	6 (7·7 %)	—	6 (7·7 %)
Carriers: All	442	24 (5·4 %)	14 (3·2 %)	10 (2·3 %)
Nose only	87	3 (3·5 %)	1 (1·1 %)	2 (2·3 %)
Back (alone or with nose)	355	21 (5·9 %)	13 (3·7 %)	8 (2·3 %)
Regular carriers: All	379	23 (6·1 %)	13 (3·4 %)	10 (2·6 %)
Nose only	73	3 (4·1 %)	1 (1·4 %)	2 (2·7 %)
Back (alone or with nose)	306	20 (6·5 %)	12 (3·9 %)	8 (2·6 %)

staphylococci had a phage-sensitivity pattern the same as or closely related to that of staphylococci carried by the patient at some time before operation. In these 14 cases, sepsis might have been caused by *self-infection* from the nose or skin, or might have been due to cross-infection, from some external source, by staphylococci of a type that the patient was already carrying. There is no direct way of determining, for any one of these 14 cases, whether self-infection or cross-infection was responsible; but if it is assumed that carriers and non-carriers are equally susceptible to cross-infection, and that self-infection is an important additional

cause of sepsis in carriers, we should expect carriers to suffer a higher sepsis rate than non-carriers. Indeed, it can be calculated that if self-infection had been responsible for all the 14 cases in which wound strains and carriage-site strains were the same, the carriers would have had a sepsis rate about double that of the non-carriers; and that if none of them had been due to self-infection the sepsis rates of carriers and non-carriers would have been equal.

In fact (Table 3) the sepsis rates were 7·7 % for non-carriers and 5·4 % for carriers, indicating that self-infection played little or no part in these cases of sepsis. However, carriage of *Staph. aureus* on the skin of the operation area (alone or together with nose carriage) was associated with a higher sepsis rate than was nose carriage alone. This difference—which is not statistically significant—suggests that though self-infection was not a major cause of sepsis, it may have been responsible in a few cases.

Obvious cases of cross-infection, in which the nose and skin did not yield staphylococci similar to those from the wound, occurred more often in non-carriers (7·7 %) than in carriers (2·3 %). But this does not necessarily indicate that cross-infection is more frequent in non-carriers than in carriers. It is almost certainly explained by the fact that many of the cross-infections in carriers were by organisms similar to those already carried in the nose and skin and were thus not recognizable as obvious cases of cross-infection.

'Regular carriers' from whom *Staph. aureus* was isolated more than once from nose or back suffered sepsis rates that were not significantly higher than those of all carriers.

Acquisition of 'hospital staphylococci'—effect on sepsis rate

The indications that self-infection played an insignificant part in causing wound sepsis led us to wonder whether carriage of 'non-hospital staphylococci', at the time of admission to the ward, was preventing nose and skin acquisition of more virulent organisms which, if acquired, might have caused self-infection. We therefore compared the sepsis rate of patients who retained the staphylococci they were

Table 4. *Acquisition of hospital staphylococci—effect on sepsis rate (Poole Hospital)*

	Number of operations	Post-operative sepsis	Comparison of *Staph. aureus* from carriage sites and wound	
			Same	Different
Non-carriers	78	6 (7·7 %)	—	6
Carriers of strains not acquired in hospital	177	9 (5·1 %)	5	4
Carriers of strains acquired in hospital	265	15 (5·7 %)	9	6

carrying on the day of admission, with the sepsis rate of those who became carriers in hospital or whose own strains were replaced by 'hospital strains'. Table 4 shows that the sepsis rates of these two groups were very nearly the same and that neither

was greater than the sepsis rate of non-carriers. Moreover, the ratio of obvious cross-infections to possible self-infections was about the same in each group. Thus, even carriage of 'hospital staphylococci' similar to those responsible for many of the serious infections, did not increase the liability to sepsis.

All these results from Poole Hospital suggested that self-infection was very infrequent if, indeed, it was occurring at all. This unexpected finding led us to wonder whether we were observing an unusual phenomenon. We therefore decided to check it by observations at Shotley Bridge Hospital.

THE INVESTIGATION AT SHOTLEY BRIDGE HOSPITAL

This was conducted in the thoracic surgical unit of a 538-bed general hospital in a rural area. The unit occupies four modernized war-time Emergency Medical Service huts and a single-story brick building, there being 96 beds in all. At first all were in large open wards, but later two beds in each ward were separated into cubicles. There is no formal isolation unit but separate wards were used for 'clean' and for 'dirty' patients. The plenum-ventilated operating suite, of modern design, is in a separate building and has no indoor communication with the wards. The survey lasted from February 1956 to January 1960.

Methods

Precautions against infection

The methods for pre-operative preparation of patients' skin and of surgeons' hands were essentially those used in Poole Hospital. Some surgeons and nurses, however, used 2% hexachlorophene soap or a 3% hexachlorophene detergent-cream instead of ordinary soap for the pre-operative scrub, and omitted the alcohol hand-rinse.

Nose, skin and wound swabs

Because the hospital was 40 miles from the investigating laboratory and because the turnover of patients was quicker than in Poole Hospital, it was not practicable to examine weekly nose and skin swabs from each patient. We therefore relied, for determination of the pre-operative carrier-state, on one nose swab taken in the anaesthetic room. The swabs were immersed in salt-meat broth and were posted to the Public Health Laboratory, Middlesbrough, where they were incubated and subcultured. A preliminary investigation showed that *Staph. aureus* in the swabs consistently survived during the delay that this involved. The pre-operation nose swab was sometimes forgotten and patients on whom this happened have been excluded from the survey.

Wound and pus swabs were cultured in the hospital laboratory by Dr W. Stewart and *Staph. aureus* strains were sent to Middlesbrough for phage typing.

Nose swabs were taken every 3 months from all surgeons, anaesthetists, nurses, and others who worked in the operating-rooms; these swabs were treated in the same way as those from the patients.

Results

During the four years of the survey 2974 thoracic operations were performed. These were mainly lung operations for carcinoma and bronchiectasis, and heart operations for valvular and congenital lesions. Surgical sepsis due to *Staph. aureus* occurred after 75 (2·5 %) of the operations. However, full bacteriological data were available for only 2480 of the patients and it is on this smaller series that the analysis is based. Of these patients, 55 (2·2 %) suffered staphylococcal sepsis, the nature of which is shown in Table 5.

Table 5. *Nature of staphylococcal sepsis in* 55 *patients (Shotley Bridge Hospital)*

In five patients two forms of sepsis, and in one patient three forms, occurred together.

Purulent wound	27	Septicaemia	4
Empyema	18	Pyopericardium	1
Purulent drainage wound	7	Peritonitis	1
Deep wound abscess	4		

Table 6. *Frequency of* Staphylococcus aureus *sepsis in carriers and non-carriers; phage-type relationship between wound staphylococci and those carried by patients and surgical team (Shotley Bridge Hospital)*

				Wound staphylococci different from those of patient's nose but same as those from		
	Number of operations (1)	Post-operative sepsis (2)	Wound staphylococci same as those from patient's nose (3)	Surgeon (4)	Unscrubbed theatre staff (5)	Not traced (6)
All patients	2480	55 (2·2 %)	9*	12†	1	33
Carriers	1371	30 (2·2 %)	9*	9†	0	12
Non-carriers	1119	25 (2·2 %)	—	3	1	21

* Includes two untypable pairs and two that were also similar to staphylococci carried by a surgeon.

† Includes two that were also similar to strains carried by scrubbed nurses who were assisting at the operating table, and one that was carried also by an unscrubbed nurse.

Relationship between sepsis and nose carriage of Staphylococcus aureus

Of the staphylococci isolated from 55 septic lesions, 46 (84 %) differed from those carried in the patient's nose before operation, or had infected patients who were not nasal carriers. In only nine instances did the wound and nose staphylococci have similar phage sensitivity patterns and were thus possible cases of self-infection (Table 6). In two of these nine instances the nose and wound strains were both untypable and were only presumed to be the same; whilst in two others the wound strains were similar to those carried not only by the patient but also by the surgeon. The sepsis rates for carriers and non-carriers were almost exactly the same – 2·2 %.

These results confirmed the finding at Poole Hospital, that nearly every case of post-operative surgical sepsis was caused by infection from a source not in the patient himself.

Relationship of sepsis to nose-carriage of Staphylococcus aureus *by the surgical team*

During the survey, nose swabs were taken from 82 members of the surgical team. Many of these were nurses in training who worked in the unit for only a short while, but from each of the permanent members of the staff up to 15 swabs were taken. For every member of the team a card was kept, recording the result of each swabbing. Records were kept of the names of all who were in the theatre at each operation.

Whenever a case of staphylococcal sepsis occurred, the responsible organism was compared, by phage typing, with those recently recovered from the noses of all persons who were present at the operation. The results of this comparison are shown in columns 4, 5 and 6 of Table 6. On twelve occasions a surgeon, twice a scrubbed nurse, and twice an unscrubbed nurse had recently carried organisms similar to those infecting the wound and thus were possible sources of infection. On thirty-three occasions, a possible source of infection was not traced, though this failure might have been less frequent if we had swabbed the theatre team more often.

Table 7. *Relationship between probable time of infection and possible source of infection (Shotley Bridge Hospital)*

	Wound staphylococci similar to those from nose of		
	Patient	Member of surgical team	Not traced
Infection probably during operation—35 cases	6 (17 %)	11 (31 %)	18
Infection probably after operation—20 cases	3 (15 %)	2 (10 %)	15

Phage similarity between a wound strain and that carried by a surgeon by no means proves that the surgeon was the source of infection, but we further examined this possibility in another way. The clinical details of each case of sepsis were carefully considered by a surgeon who, at that time, did not know the phage-typing results. He made a decision on whether infection probably occurred at the time of operation or afterwards, in the ward. Operating-room infections were thought probable when deep sepsis occurred beneath a clean parietal wound or when unusual pyrexia began within five days of operation. Of the 55 septic cases, 35 were attributed to infection during operation and 20 to infection later (Table 7). Identity of wound strains with patients' nose strains (possible self-infection) occurred with equal frequency in both groups; but identity between wound strains and those carried by a member of the surgical team occurred in 11/35 (31 %) of the presumed operating-room infections and in only 2/20 (10 %) of the ward infections. If the similarity of the wound and the surgeons' staphylococci was mainly coin-

cidental, the frequency of phage-type similarity should have been the same in each group. That it was not suggests that a member of the surgical team was the source of infection in up to 11 of the 35 cases of sepsis.

DISCUSSION

The general conclusion from these studies is that self-infection of surgical wounds, with *Staph. aureus* from carrier sites in the patient's own body, was a much less frequent cause of sepsis than was cross-infection from external sources. This conclusion is based on the observation, in both hospitals, that the sepsis rate was no higher among nasal-carriers of *Staph. aureus* than among non-carriers; and on the finding that patients whose sites of carriage yielded *Staph. aureus* more than once before an operation suffered significantly no more sepsis than those who yielded the organism once only.

Because we found no evidence suggesting the frequent occurrence of self-infection we studied, in the second part of this investigation, one possible source of cross-infection—the surgeons and other members of the operating-room team. Of the 35 possible operating-room infections, 11 (31 %) could have come from a member of the team. We have, however, little evidence to offer concerning the routes by which these infections might have occurred. If some of the carriers amongst the staff were active 'dispersers' of staphylococci, the infecting organisms could have been airborne; but air-sampling was done during many operations in this well-ventilated theatre and *Staph. aureus* contamination of the air was very slight indeed. Glove punctures, however, were very common—about 10 % for instrument nurses and 30 % for surgeons—and since nasal carriers of *Staph. aureus* so often are skin carriers too, we think that passage of skin organisms through damaged gloves was possibly the main manner of infection. When the investigation began we did, in fact, try to keep records of all glove punctures and intended to study them in relation to actual septic incidents. Unfortunately, the recording system broke down so we were not able to make this study which, we believe, would be well worth while. Meanwhile, we feel that improved detection and prevention of glove punctures occurring during operations might prevent some sepsis of the type that we have observed.

Several studies similar to ours have indicated that self-infection is more frequent than we found it to be (Colbeck, Robertson, Sutherland & Hartley, 1959; Weinstein, 1959; Williams, Jevons, Shooter, Hunter, Girling, Griffiths & Taylor, 1959; McNeill, Porter & Green, 1961), whilst yet other studies have yielded results similar to our own (Public Health Laboratory Service, 1960; Rountree, Harrington, Loewenthal & Gye, 1960; Lowden, Vaithilingham & Milne, 1962; Moore & Gardner, 1963). The wide divergence between these findings need cause no surprise, for the frequency of self-infection may be influenced by several factors that vary from one hospital to another. For example, strains of *Staph. aureus* inhabiting different hospitals may differ in their relative affinities for carriage sites and for wounds, and in their abilities to spread from carriage sites to a wound in the same patient. Carriage of *Staph. aureus* in the nose—the basis of most of the studies—is not

accompanied by skin carriage with equal frequency in all parts of the body, so operation wounds may be more liable to self-infection in some branches of surgery than in others. Lastly, the method of pre-operative skin preparation used in a surgical unit may profoundly influence the frequency of self-infection. Thus, shaving the skin on the day before rather than on the day of operation may allow time for cocci to multiply in abrasions, so that the surgeon's knife cuts through minute abscesses instead of through near-sterile skin; disinfectants differ widely in their efficacy on skin, while surgeons differ in the time that they allow the disinfectant to act before applying the knife. In some of the reports on self-infection the methods of skin preparation and disinfection were not described but if there were differences of method they might have contributed to the differences between results.

Henderson & Williams (1961) and Stokes & Milne (1962) found that patients whose noses were kept free from *Staph. aureus* by intranasal application of anti-bacterial creams suffered no less sepsis than untreated controls. This might be held to confirm reports on the relative infrequency of self-infection; but, alternatively, it might be explained by the fact that abolition of nasal carriage does not necessarily lead to abolition of skin carriage, and by the possibility that it is skin carriage rather than nasal carriage of staphylococci that increases liability to self-infection. Our findings only partly support this possibility: the sepsis rate of patients who were nose and skin carriers was, indeed, slightly greater than that of patients who carried only in the nose, but it was still no greater than that of non-carriers.

We have no doubt that self-infection can, and sometimes does, cause serious surgical sepsis, and that precautions against it must be rigidly maintained. But over-emphasis on the frequency of self-infection may deflect attention from important risks of cross-infection. It is in the hope of preventing this that the findings reported here are presented.

SUMMARY

1. In two hospitals, post-operative staphylococcal wound sepsis occurred with equal frequency in patients who were and in those who were not nasal carriers of *Staph. aureus*.

2. Nasal carriage of *Staph. aureus* was not always associated with skin carriage at the site of operation, but even patients who were skin carriers suffered no more sepsis than non-carriers.

3. Self-infection was a less frequent cause of sepsis than was cross-infection. Other investigations of this type have revealed conflicting findings on the frequency of self-infection. These may be due to differences in the epidemiological properties of staphylococci infesting the hospitals; to different methods of pre-operative skin preparation; and to the different types of operation concerned.

4. In one of the hospitals, 31 % of probable operating-room infections were with organisms similar to those carried by some member of the surgical team. In infections thought to have occurred in the ward, only 10 % showed this similarity. In 11 of 35 presumed operating-room infections, the source may have been a member of the surgical team.

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REFERENCES

Barber, M. & Kuper, S. W. A. (1951). *J. Path. Bact.* **63**, 65.

Blowers, R., Mason, G. A., Wallace, K. R. & Walton, M. (1955). *Lancet*, ii, 786.

Cadness-Graves, B., Williams, R., Harper, G. J. & Miles, A. A. (1943). *Lancet*, i, 736.

Colbeck, J. C., Robertson, H. R., Sutherland, W. H. & Hartley, F. C. (1959). *Med. Serv. J. Canada*, **15**, 326.

Hare, R. & Ridley, M. (1958). *Brit. med. J.* i, 69.

Henderson, R. J. & Williams, R. E. O. (1961). *Brit. med. J.* ii, 330.

Lowden, T. G., Vaithilingham, P. S. & Milne, J. B. (1962). *Lancet*, ii, 752.

McNeill, I. F., Porter, I. A. & Green, C. A. (1961). *Brit. med. J.* ii, 798.

Maitland, H. B. & Martyn, G. (1948). *J. Path. Bact.* **60**, 553.

Moore, B. & Gardner, A. M. N. (1963). *J. Hyg., Camb.*, **61**, 95.

Public Health Laboratory Service (1960). *Lancet*, ii, 659.

Rountree, P. M., Harrington, M., Loewenthal, J. & Gye, R. (1960). *Lancet*. ii, 1.

Stokes, E. J. & Milne, S. E. (1962). *J. Hyg., Camb.*, **60**, 209.

Weinstein, H. J. (1959). *New Engl. J. Med.* **260**, 1303.

Williams, R. E. O., Jevons, M. P., Shooter, R. A., Hunter, C. J. W., Girling, J. A., Griffiths, J. D. & Taylor, G. W. (1959). *Brit. med. J.* ii, 658.

Williams, R. E. O. & Rippon, J. E. (1952). *J. Hyg., Camb.*, **50**, 320.

J. Hyg., Camb. (1963), **61**, 95
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A study of post-operative wound infection in a provincial general hospital

By B. MOORE
Public Health Laboratory, Exeter

and A. M. N. GARDNER
Department of Surgery, Torbay Hospital, Torquay

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INTRODUCTION

Between 1957 and 1959, staphylococcal infection in patients and nursing staff became an increasingly serious problem at the Torbay Hospital, Torquay. A preliminary survey in 1957 showed that staphylococci of the 80 phage group were widely disseminated in the main male surgical ward of the hospital, causing wound sepsis in patients and a high incidence of styes, boils and carbuncles in the nursing staff. During the ensuing year, this high incidence of infection persisted throughout the hospital, and initial efforts to deal with the problem were no more successful than those recorded from another provincial hospital about this time by Williams, Talbot & Maughan (1959). Absenteeism in the nursing staff because of skin sepsis raised serious staffing problems, the incidence of sepsis in the nursing staff being of the same order as that recorded at the London Hospital in 1958–59 by Davies (1960).

Early in 1959, as one of a number of measures to combat infection, a whole-time Infection Control Sister was appointed at the hospital (Gardner, Stamp, Bowgen & Moore, 1962). One of her earliest tasks was to organize, as part of a more comprehensive survey by a Public Health Laboratory Service (P.H.L.S.) committee (Public Health Laboratory Service, 1960), a clinical and bacteriological study of all surgical patients undergoing certain categories of operative procedure in the hospital during the first week of April, 1959. Although this limited study was all that the P.H.L.S. committee required, a more extended survey seemed desirable as a basis for infection control. Accordingly, the methods used in the initial survey were applied to a study of post-operative infection in surgical patients admitted to the Torbay Hospital in three alternate approximately quarterly periods between 1 April 1959 and the end of June 1960. The decision to make the survey an intermittent one was taken, partly to limit the necessary laboratory, clinical and clerical labour, and partly so that the Infection Control Sister could devote her energies to special problems during the intervening months.

During the period covered by the three surveys, no change occurred in the consultant surgical personnel of the hospital, in the range of operations performed, nor indeed in the general level of wound sepsis. The results of the three surveys have therefore been combined for analysis below, except where otherwise stated.

The 559 patients studied were admitted under one or other of three general surgeons, a gynaecologist or an orthopaedic surgeon. The great majority were operated on by the consultant surgeon concerned, but some patients were operated on by a surgical registrar and a few by a house surgeon.

MATERIAL AND METHODS

Selection of patients

As in the earlier P.H.L.S. study, the scope of the present surveys embraced all patients whose operation necessitated an incision through healthy skin, except those operated on primarily because of local sepsis, e.g. to incise an abscess or drain an empyema. Operations on the lower urinary tract, rectum and anus and on accidental wounds were excluded. All emergency abdominal operations were included, even though some were found to entail drainage for sepsis, e.g. an appendix abscess.

Routine investigation and records

Each patient had a nasal swab taken on the day of admission to hospital. When possible, this swab was taken before the patient reached the ward, because, particularly during the first quarterly period, a number were found to harbour staphylococci of type 80 or related types in the nose on their arrival in hospital. The original intention was that, when a week or more elapsed before the operation, the nose should be swabbed again on the day of operation. Because of acute bed shortage, however, patients were for the most part admitted to the hospital on the day before operation, and, of those included in the present study, some 80 % were operated on within 48 hr. of admission, and 95 % in less than a week. An immediate pre-operative swab was thus taken from very few patients, except during the second survey, when 106 patients were swabbed both on admission to the hospital and in the operating theatre, to see whether many of those who were not carrying staphylococci in the nose on admission became established carriers during the brief pre-operative period.

All wounds were inspected by the Infection Control Sister at the time of the first dressing, and wound swabs were taken for bacteriological examination. Dressings were, of course, not disturbed merely to permit swabbing. Where evidence of sepsis was observed, the Control of Infection Officer (A.M.N.G.) inspected the wound with the sister. Further wound swabs were collected weekly, or more often if necessary. When the patient was discharged to an outlying hospital, the sister kept in touch with the hospital matron and arranged further swabbing, or herself visited the hospital if this was thought desirable. Many patients had a final check when they paid their last visit to the out-patient clinics.

During the first month of the first survey, in April 1959, the clinical course of wound sepsis in a number of patients strongly suggested a theatre infection. Nasal swabs were therefore taken during the last week of April from all members of the theatre staff, both medical and nursing. More recently, a standard policy of swabbing theatre personnel once every 2 months has been adopted, but unfor-

tunately no further swabbing was done during this study until March 1960. Apart from the initial stages of the survey, therefore, no adequate information was available on the possible association of members of the theatre staff with wound sepsis in patients, unless they themselves were sent off duty with septic infections and thus came under closer scrutiny.

Bacteriological and clinical details on all patients were entered in a special book, and the details were in due course transferred to 6 × 4 in. Cope.-Chat. punch cards for analysis.

All swabs were sent by overnight post to the Public Health Laboratory at Exeter, as the hospital laboratory was too busy to cope with the amount of work required. When urgent bacteriological investigation was necessary in the interests of individual patients, replicate swabs were sent down to the hospital laboratory for immediate culture, and a few routine swabs collected at week-ends from patients admitted as emergencies were similarly examined at the hospital.

Laboratory methods

Nasal and wound swabs were cultured for *Staphylococcus aureus* and other pathogens by direct plating on blood agar and by broth enrichment and subculture. Wound swabs were in addition plated on to an electrolyte-deficient medium (Sandys, 1960) that inhibited the swarming of *Proteus* strains, while permitting good growth of staphylococci and of coliform organisms.

All coagulase-positive staphylococcal strains were phage-typed by the methods of Williams & Rippon (1952). The battery of phages available at the time did not include phage 81. Most of the strains described in this report as belonging to type 80 would probably now be designated 80/81.

Sensitivity tests against penicillin, streptomycin, tetracycline and chloramphenicol were done on most of the wound strains of *Staph. aureus*, on epidemiological rather than clinical grounds, and, during the second and third surveys, staphylococci were also examined for mercury sensitivity (Moore, 1960).

RESULTS

General findings

Of the 559 surgical patients investigated in the course of the three quarterly surveys, 71 (12·7 %) developed clinical evidence of post-operative wound sepsis and 51 (9·1 %) definite suppuration. The number of patients with proved staphylococcal sepsis was 48 (8·6 %).

That the incidence of wound infection remained fairly uniform throughout can be seen from Table 1, in which the findings of the three surveys are shown separately. Although no statistically significant change occurred during the period of the study in the incidence of total wound sepsis, wound suppuration or staphylococcal sepsis, the figures suggest a slowly diminishing incidence of staphylococcal sepsis, but this fall was more than offset by the increase in wound sepsis due to coliform organisms. Examination of individual patients' records shows that the increase in coliform sepsis can be largely accounted for by an 'epidemic' of

appendix abscesses that occurred during the third survey and cannot be cited as another instance of the increasing importance of hospital infection due to Gram-negative organisms.

In all, 17 patients (3 %) died in hospital during the post-operative period. Although 5 of these had septic wounds, the sepsis did not appear to be related to the cause of death.

Table 1. *Incidence of post-operative wound sepsis in the three Torbay Hospital surveys*

	1 Apr.–June 1959	2 Oct.–Dec. 1959	3 Apr.–June 1960
Total no. of patients	191	218	150
No. of septic wounds	21 (11)	28 (12·8)	22 (14·7)
No. of suppurating wounds	17 (8·9)	20 (9·2)	14 (9·3)
No. of wounds with sepsis due to:			
Staph. aureus	18 (9·4)	19 (8·7)	11 (7·3)
Coliforms	1 (0·5)	5 (2·3)	10 (6·7)

Figures in parentheses are percentages.

Analysis of sepsis due to staphylococci

Of the 48 wounds in the survey that became clinically septic and yielded staphylococci on culture, 30 were infected with organisms of the 80 group, mainly type 80 or type 52/52A/80, but including two strains of type 29/52/80. Fourteen of the 80 group infections occurred during the first survey, 9 in the second, and 7 in the third.

During the second survey, 4 apparently unrelated patients who developed wound sepsis, in November 1959, were all infected with a mercury- and antibiotic-resistant type 6/7/47/53/54 strain.

Preliminary analysis of the punch card records brought the quite unexpected finding that post-operative wound sepsis had occurred far less frequently in patients who were staphylococcal nasal carriers on admission to hospital than in non-carriers. This finding seemed so much at variance with the importance ascribed to the nasal carrier state in relation to hospital sepsis (Weinstein, 1959; McNeill, Porter & Green, 1961; Williams *et al.* 1962) that the relationship of staphylococcal nasal carriage to wound infection in the present study has been critically analysed below. An attempt has also been made to assess the incidence of self-infection, and of wound infection directly ascribable to infection by a member of the theatre staff.

Relationship of nasal carriage to wound sepsis

Of the 559 patients investigated, 538 had nasal swabs taken on admission to hospital. The incidence of subsequent staphylococcal sepsis according to the initial carrier state is shown in Table 2. Of the 153 patients who were carriers on admission, 9 (5·9 %) developed post-operative staphylococcal sepsis, while the corresponding

number among the 385 who were initially non-carriers was 36 (9·4 %). The 9 septic patients in the carrier group included, as discussed below, at least 3 whose wound infection was very probably due to the staphylococcus they carried on admission. If these are excluded, the figures for the incidence of sepsis due to staphylococci acquired in hospital become 6/153 (3·9 %) for carriers and 36/385 (9·4 %) for non-carriers.

Table 2. *Incidence of staphylococcal sepsis in patients who were nasal carriers on admission to hospital and in non-carriers*

	Carriers	Non-carriers
Total no.	153	385
No. of wounds with staphylococcal sepsis	9 (5·9 %)	36 (9·4 %)
Septic wounds with staphylococci acquired in hospital	6 (3·9 %)	36 (9·4 %)

Table 3. *Incidence of staphylococcal sepsis according to sex and carrier state on admission to hospital*

	Total no.	Wounds with staphylococcus sepsis
Males		
Carriers	46	3 (6·5 %)
Non-carriers	159	16 (10·1 %)
Females		
Carriers	108	6 (5·6 %)
Non-carriers	225	20 (8·9 %)

The unexpected excess of wound sepsis in non-carriers raised the question whether there might not be some material difference between the carrier and the non-carrier group that made them not truly comparable. One such difference was in the sex distribution in both groups. The staphylococcal carrier rate on admission to hospital was found to be somewhat lower in males than in females, and thus the proportion of males in the carrier group (30·6 %) was lower than among the non-carriers (42 %). The incidence of sepsis according to sex and carrier state was therefore calculated and is shown in Table 3.

The table shows a remarkable similarity between the sexes in the relative excess of sepsis in non-carriers. The difference in sepsis incidence can thus not be explained in terms of differences in sex distribution in the carrier and non-carrier groups.

Another possibility was that the nature of the operations undergone by the carrier and non-carrier groups happened to differ significantly, so as to make the non-carriers more susceptible to wound sepsis, not by virtue of their non-carrier state, but because the operations done on them were more conducive to subsequent wound sepsis than those done on the carrier group. This again was excluded as an adequate explanation of the differences found. The percentage composition of the carrier and non-carrier groups in respect of 14 major categories of operative procedure is shown diagrammatically in Fig. 1. The only major difference between the

two groups was in the number of hernia operations, which totalled 47 (13 %) in the non-carrier group and only 9 (5·9 %) in the carrier group. As, however, the incidence of sepsis after hernia was far lower than for the series as a whole, this difference between the two groups would tend to under-estimate the difference in sepsis rates rather than exaggerate it.

Again, there was the possibility that, for some reason or other, the wounds of non-carriers had been exposed to a heavier load of staphylococcal contamination

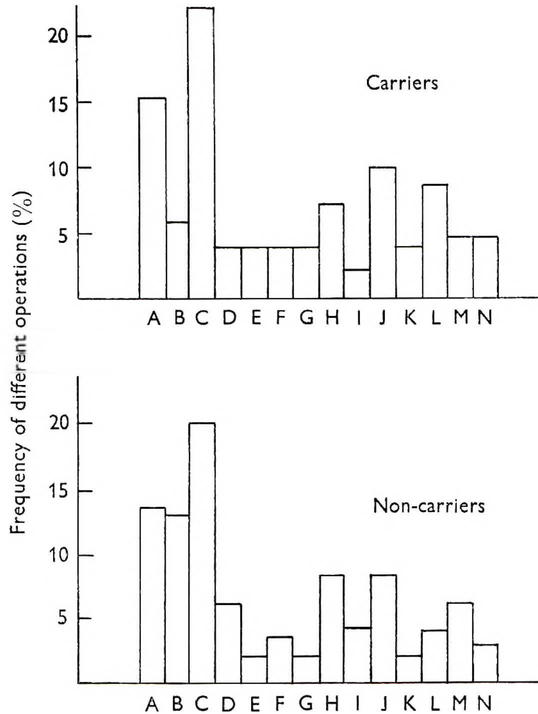

Fig. 1. Relative frequency of different operations on carriers and non-carriers. A, gynaecological; B, hernia; C, appendix; H, gallbladder; J, gut resection; L, bone-pinning.

than those of carriers. This was impossible to exclude with any confidence, but some evidence against it was available. Many wounds in both groups were infected with staphylococci although not clinically septic. The combined figures of clinical and subclinical infection with staphylococci might be expected to reflect in some measure the relative exposure to staphylococci of wounds in the two groups. The actual figures were closely similar for both groups, namely 38/153 (24·8 %) for the carrier group, and 87/385 (22·6 %) for the non-carriers. For what this evidence is worth, therefore, it suggests no major difference in exposure to staphylococcal contamination between the two groups.

Finally, the comparability of the carrier and non-carrier groups was tested in respect of age distribution. This was of some importance because susceptibility to wound sepsis has been shown to increase with age, irrespective of other factors (Lidwell, 1961), while the staphylococcal nasal carrier rate diminishes with advanc-

ing age (Williams, 1947). One might, therefore, observe the increased sepsis rate in non-carriers through inclusion in the non-carrier group of a relatively higher proportion of more susceptible older people.

The decline in the staphylococcal carrier rate with advancing age was confirmed in the present survey, e.g. 35 % of those under 40 were staphylococcal nasal carriers on admission to hospital, as compared to 25 % of those over 40. The age-specific staphylococcal wound sepsis rate still showed an excess, however, in each major age group among those who were non-carriers on admission to hospital. The sepsis incidence in those over and under 40, for instance, in relation to carrier state is shown in Table 4.

Table 4. *The incidence of staphylococcal wound sepsis in younger and older patients according to nasal carrier state*

Age	Carriers	Non-carriers
Under 40	2/57 (3·5 %)	6/105 (5·7 %)
Over 40	7/92 (7·6 %)	30/269 (11·2 %)

Ages of patients were determined in retrospect from hospital records. The total of 523 patients in this table represents those who were swabbed on admission to hospital and whose ages had been entered in the case notes.

Table 5. *Incidence of staphylococcal sepsis according to nasal carrier state on admission to hospital and presence or absence of post-operative drain*

	Wounds with drain		Wounds without drain	
	Total no.	No. with staphylococcal sepsis	Total no.	No. with staphylococcal sepsis
Carriers	39	3 (7·7 %)	114	6 (5·3 %)
Non-carriers	102	21 (20·6 %)	273	15 (5·5 %)

No information available on presence or absence of drain in 10 non-carriers.

The higher incidence of staphylococcal sepsis in those over 40 is of course not a simple age effect, as in general the major operative procedures were carried out on older patients.

The foregoing analysis would seem to indicate that the excess of staphylococcal wound sepsis in those who were non-carriers on admission to hospital cannot be explained in terms of differences between the carrier and non-carrier groups in age, sex, operative procedure undergone or exposure to staphylococcal contamination.

A study of the individual patients' records provided a useful pointer to the kinds of operation that contributed most to the excess of sepsis in non-carriers. It was clear that, as reported by previous workers (e.g. Gillespie, Alder, Ayliffe, Bradbeer & Wypkema, 1959; Public Health Laboratory Service, 1960), wounds requiring post-operative drainage had shown a higher incidence of sepsis than wounds not drained. The incidence of sepsis in drained and non-drained wounds was therefore calculated for carriers and non-carriers. First of all, the proportions of wounds requiring drainage were very similar for the two groups, namely 39/153 (25·5 %)

and 102/375 (27·2 %) for carriers and non-carriers respectively. No information was available as to whether drains had been inserted after operations on 10 non-carriers; hence the total of 375 instead of 385. Analysis of the incidence of staphylococcal sepsis in terms of the staphylococcal carrier state and the presence or absence of a drain is shown in Table 5. This shows that the excess of sepsis in the non-carrier group was concentrated in those patients whose wounds required a post-operative drain. If the closely similar sepsis figures in the other three categories of the table are pooled, the difference between these groups and that of 'drained non-carriers' is highly significant (χ^2 with Yates correction = 21·73; $P < 0{\cdot}001$).

Incidence of wound sepsis in carriers according to phage group of staphylococcus carried on admission to hospital

McNeill *et al.* (1961), and Williams *et al.* (1962) reported higher wound sepsis rates in patients who acquired staphylococci in the nose after admission to hospital or who changed the type of staphylococcus carried while in hospital, than in those who remained non-carriers or continued to harbour their own initially carried strain throughout.

Whether acquisition of staphylococci in the nose while in hospital predisposes to or determines wound sepsis, or merely reflects the contaminated environment to which the patient has been exposed, will be discussed later. In the context of the present study, however, the phage group distribution of staphylococci carried in the noses of patients on admission to the Torbay Hospital is of some interest for two reasons. First, the interval between the patient's admission to hospital and the operation was usually so brief that the likelihood of pre-operative acquisition of staphylococci in the nose and of sufficient multiplication of the acquired strains to establish a dangerous carrier state would seem remote. Certainly, none of the 106 patients in the second Torbay Survey, who were swabbed both on admission to hospital and in the operating theatre, showed any evidence of such a precocious development of a profuse nasal carrier state in the brief interval before operation. To this extent, therefore, the influence of acquired nasal staphylococci on the incidence of sepsis must be far more tenuous in a hospital such as the Torbay Hospital than it might be in a teaching hospital, where according to, e.g. Table III of Williams *et al.* (1962), the average stay of surgical patients in the hospital before operation was probably considerably longer than at Torquay.

In another respect, however, the Torquay material provides an interesting counterpart to the surveys of McNeill *et al.* (1961) and of Williams *et al.* (1962). Presumably because of the high incidence of infections due to the 80 group at Torquay from 1957 onwards, 38 (7·1 %) of the 538 patients who were swabbed carried a staphylococcus of the 80 group in the nose on admission to hospital. In this group of 38 patients, 5 (13·2 %) developed post-operative staphylococcal wound sepsis as compared to a sepsis rate of only 2·6 % in patients who carried other phage types of staphylococcus in the nose when admitted to hospital. Thus, patients who had acquired staphylococci of the epidemic 80 group in the nose before admission to hospital suffered an incidence of staphylococcal sepsis of an order

very similar to that experienced by those patients in the St Bartholomew's Hospital surveys who acquired a staphylococcus in the nose while in hospital. Even with this small group of 5 patients, however, in only 1, as discussed below, could the post-operative sepsis be confidently ascribed to the strain carried on admission to hospital.

These findings on the relationship between the staphylococcal nasal carrier state and the incidence of wound sepsis are discussed below in the context of other work on this subject. First, however, the incidence of wound sepsis due to self-infection with staphylococci carried on admission to hospital, and of wound infection associated with the operating theatre, is assessed in the following sections.

Self-infection

As shown in Table 2, 9 of the 153 patients who were staphylococcal nasal carriers on admission to hospital developed post-operative staphylococcal wound sepsis. In one of these nine patients, the sepsis was almost certainly the result of self-infection. The patient in question yielded a nasal staphylococcus of phage type 187 on admission to hospital on 22 May 1959 and had a total hysterectomy on the following morning. The wound discharged a large amount of pus when the sutures were removed 9 days later, and staphylococci of the same phage type were isolated. The only other occasion during this survey when an organism of this type was isolated from any patient was a month later, when a type 187 staphylococcus was isolated from a clinically healthy wound.

A second female patient, also admitted for hysterectomy, in December 1959, was found to have a staphylococcus of an unusual group III pattern in her nose on admission, and her wound showed a slight sero-purulent discharge due to organisms of the same phage pattern 8 days later.

As mentioned above, 5 patients who carried staphylococci of the 80 group in the nose on admission to hospital developed post-operative staphylococcal sepsis, but in only 1 could self-infection be deemed highly probable, and this man had just been an in-patient in a medical ward of the hospital. Of the remaining 4, two carried type 80 staphylococci in the nose on admission and their wounds yielded staphylococci of the closely related type 52/52A/80. Though these may have been instances of self-infection, staphylococci of type 52/52A/80 were so widely distributed in the hospital at the time that other sources of infection, whether in the theatre or in the wards, could not be excluded. Finally, one patient whose nose yielded type 52/52A/80 staphylococci developed wound sepsis due to a type 80 strain, and another who also carried type 52/52A/80 in her nose developed a type 52 wound infection. Technically, either might have been due to self-infection but a doubt remains.

Infection associated with the operating theatre

Gillespie, Alder, Ayliffe, Powell & Wypkema (1961) were of the opinion that, although the nasal carriage of staphylococci by the nursing staff was not of epidemiological significance in the wards, this was not so of carriers working in the theatre. The first Torbay Hospital survey, from April to June 1959, furnished

clear evidence in support of this view. The high incidence of septic infections in the nursing staff during 1958 has already been mentioned. A high staphylococcal carrier rate in the theatre staff was therefore to be expected, but the theatre personnel had not been recently swabbed when the survey started. By the last week of April, however, a number of patients had developed staphylococcal wound sepsis under circumstances that pointed strongly to an operating theatre infection, and all the surgical and nursing personnel of the theatre were accordingly swabbed during that week. Correlation of the swabbing results with the incidence of wound sepsis showed that two appendicectomy patients with severe type 52/52A/80 wound sepsis had both been operated on by a house surgeon who was found to be a heavy nasal carrier of staphylococci of this type, and who also had an external otitis infected with this organism. The same house surgeon had assisted at another operation on a third patient, whose nephrectomy wound became septic and again yielded staphylococci of type 52/52A/80. Two further cases of severe wound sepsis, operated on within 3 days of each other during the 2nd week of April, had staphylococci of type 29/52/80 in their wounds, a relatively stable phage pattern in our experience and associated with two of the most severe cases of wound sepsis seen in the course of another survey, in Exeter, in 1958. The junior theatre sister who took part in the operation on one of the Torquay cases proved to be a nasal carrier of type 29/52/80 staphylococci. She had been on sick leave, suffering from boils, on two occasions during the previous year. She may well have infected the 2 patients in question.

Apart from these 5 patients, whose wounds were very probably infected directly by theatre personnel at a time when the hospital staff showed a high nasal carrier rate of epidemic staphylococci, several other patients who developed post-operative wound sepsis were clinically theatre infections with deep suppuration and constitutional symptoms. The precise mode of infection could not be established, however, partly because the theatre staff had not been swabbed at the relevant time, and partly because most of these infections were with organisms known to be widely disseminated in the wards, and therefore possibly brought to the theatre on the person of the patient concerned.

Since August 1960 all members of the theatre staff—medical, nursing and technical—have been swabbed routinely every 2 months. Very few carriers of epidemic staphylococci have been found, and only 3 in the past 18 months. The incidence of post-operative sepsis, and of skin infections in the nursing staff, have also fallen considerably during the past 2 years.

DISCUSSION

In three British papers published in recent years (Williams, Jevons *et al.* 1959; McNeill *et al.* 1961; Williams *et al.* 1962), the acquisition of nasal staphylococci of hospital types by patients admitted to surgical wards has been singled out as an important determinant of post-operative wound infection. An alternative view, as expressed, for example, by Rountree, Harrington, Loewenthal & Gye (1960), would deem it equally likely that, where patients acquire ward strains of

staphylococcus at about the same time in their noses as in their wounds, both nose and wound have been infected from some other source.

The distinction is of practical moment in deciding what preventive measures are most likely to reduce the incidence of post-operative sepsis. If the nose is the main source of wound staphylococci, suppression of the nasal carrier state should materially lower the incidence of wound sepsis. Gillespie (1958), reviewing the earlier findings of the Bristol investigators, noted that, where 'nasal prophylaxis' was applied in two surgical wards, as a sole precaution, there had been a marked fall in the nasal carrier rate but a far smaller effect on the incidence of wound sepsis. He suggested that, to control cross-infection in surgical patients, it might be necessary to deal with both nasal carriers and the infected open wound, while at the same time blocking some of the more obvious routes of ward cross-infection, such as contaminated blankets or baths. More recently, the controlled trials of Henderson & Williams (1961) and of Stokes & Milne (1962) confirmed the ineffectiveness of nasally applied antibiotics in reducing the incidence of wound sepsis.

The findings of the Torbay Hospital survey, as discussed in the foregoing pages, and of Bassett *et al.* (1963), are in sharp contrast to those of the St Bartholomew's Hospital group (Williams *et al.* 1962). In the Torbay Hospital study, the presence of a staphylococcus in the nose on admission to hospital was almost a guarantee of freedom from wound sepsis rather than a predisposing cause, and Bassett and his colleagues also report a lower incidence of wound sepsis in nasal carriers than in non-carriers. Can these conflicting findings be reconciled on closer examination?

One source of confusion has been the use of the term 'nasal carrier' in a number of quite different senses. Thus, in discussing the source of infection of surgical wounds, Williams, Blowers, Garrod & Shooter (1960) cite the papers of Weinstein (1959), Williams *et al.* (1959), and Colbeck, Robertson, Sutherland & Hadley (1959) as evidence that nasal carriers of *Staph. aureus* suffer wound infection more often than non-carriers, and the investigations of the Public Health Laboratory Service (1960) and of Bassett *et al.* (then in preparation) as having yielded different results. Of these papers, those of Colbeck *et al.* (1959) and of the Public Health Laboratory Service (1960) refer to the staphylococcal nasal carrier state of patients on admission to general hospitals for surgery. Knowledge of staphylococcal carriage at this point of time permits later assessment of whether or not the hospital bears responsibility for cross-infection of a patient's wound with the bacterial flora of other patients, or whether it has simply failed to exclude the patient's own flora from the depths of his wound. For Williams *et al.* (1959), on the other hand, the nasal carrier group includes not only those who harbour staphylococci in the nose on admission to hospital, but also those who acquire nasal staphylococci while in hospital. Weinstein (1959) uses the term in yet another sense. His patients were not swabbed at all on admission to the thoracic surgical unit concerned, but, on average, about 4 months later, when they were about to be operated on. For him, therefore, the nasal carrier state reflects a 4 months' interaction of the patient with an environment contaminated with hospital staphylococci, particularly of the 80/81 group. Some confusion is inevitable until a great deal more has been learned about the nature of the nasal carrier state. Meanwhile, the use of the term merely

reflects the successful isolation of a staphylococcus from a nasal swab at a particular time, and some caution is required in correlating such success with the variables of hospital infection.

As a prelude to a wider discussion of the sources of post-operative infection in surgical wounds, some comments are called for on the paper by Williams *et al.* (1959), the first to state a detailed case for ascribing sepsis in surgical patients to nasal staphylococci acquired in hospital. The argument centres essentially on the finding that the incidence of post-operative staphylococcal wound sepsis in the surveys described was 2 % in 342 patients who were never nasal carriers of staphylococci and 7·1 % in the 380 who carried at some time. The authors reached the conclusion that the nose was often the source of the sepsis, and that the incidence of sepsis might be reduced if acquisition of staphylococci in the nose could be prevented. Alternative interpretations of the main findings were discussed, however, namely (i) that the lesion had infected the nose, or (ii) that both nose and lesion were infected separately from some other source. The first was thought unlikely, because in only very few patients were the staphylococci not found in the nose until after the development of sepsis. This argument is suspect on two counts. First, nasal swabbing of patients was done only once a week, while swabs from septic wounds were presumably taken when the sepsis was observed. In a later paper, Williams *et al.* (1962) describe the assumptions and conventions that had to be adopted in estimating the day of acquisition of a nasal staphylococcus because of this interval between successive swabbings. Their analysis of the priority in time of nasal over wound infection was analysed in terms of days rather than weeks. The conclusions would have been correspondingly more compelling if nasal swabs had been taken daily rather than at weekly intervals. Even, however, if one accepts prior infection of the nose as having occurred, the argument from priority of nasal acquisition is not entirely convincing. If a patient is placed in a heavily contaminated environment, and is in due course operated on, his nose is almost bound to become infected with a staphylococcus present in large numbers in the ward before his wound becomes infected with the same strain, partly because of the respiratory function of the nose which makes it an effective sampler of the ward contamination, partly because of the interval between admission and operation, and also because the wound will presumably normally be protected from ward contamination except during dressings.

Prior infection of the nose is thus compatible with separate infection of both nose and lesion from some other source. This latter possibility was also discussed by Williams and his colleagues, but dismissed on the grounds that 'to explain the differing experience of nasal carriers and non-carriers one would have to postulate some general staphylococcal "diathesis" which made those who became nasal carriers also more likely to become infected in their wound or elsewhere. In this case one would hardly expect the nose and lesion to show the same type of staphylococcus with such frequency.' An alternative argument to that of invoking a staphylococcal diathesis would be simply to regard the acquisition of a nasal staphylococcus as a sensitive indicator of exposure to staphylococci in the ward. Those who acquired a nasal staphylococcus would *ipso facto* be more exposed to

wound contamination. Whether or not one would 'expect nose and lesion to show the same type of staphylococcus with such frequency' requires further scrutiny. If one regards the acquisition of a nasal staphylococcus as an indication of exposure to staphylococcal contamination, phage typing of the nasal staphylococcus permits the more precise inference that the patient has been exposed to contamination with that particular phage type, and to this extent one would expect that his wound might also become contaminated with the same type of staphylococcus. Apart from this, however, reference to the paper in question shows that the close parallelism of staphylococcal phage types in nose and wound was observed only with tetracycline-resistant epidemic strains of staphylococcus. To assess the significance of the acquisition of antibiotic-resistant staphylococci, of the same phage type, in nose and wound at about the same time, would require knowledge of the degree of contamination of the ward at the relevant time with epidemic strains of different phage types. This information is not given in the 1959 paper, but in an earlier paper by the same group and discussing work done in the same series of studies, Shooter *et al.* (1958) make special mention of nine profuse 'staphylococcal broadcasts' of individual phage types of staphylococcus in the ward under investigation. Several of the broadcasts could be attributed to patients, with infected lesions such as a tracheotomy wound or infected urine, that were particularly likely to disperse staphylococci. It seems probable, therefore, that in the foregoing studies, as in others described in the literature, e.g. by Barber & Warren (1962), environmental contamination at any one time tended to be caused by one phage type of epidemic staphylococcus that was widely disseminated in the ward. If this was so, it would again increase the likelihood that the wound and nose of a given patient acquired the same strain of epidemic staphylococcus, independently, from the contaminated environment.

A more serious criticism of these studies of Williams *et al.* (1962) remains to be mentioned. Before ascribing high and low sepsis rates to the presence or absence of a nasal carrier state, it is material to inquire whether the carriers and non-carriers were comparable in other respects, e.g. as regards age or the nature of the operations undergone. The necessary information is not given in the paper in question. In the three male wards studied, however, there were especially large numbers of patients undergoing rectal operations in ward A, vascular operations in ward B and urinary tract operations in ward C. Table III of Williams *et al.* (1962) would seem to point to a lack of comparability in some material respect between those patients who, on the one hand, changed strains or acquired nasal staphylococci while in hospital and had a high sepsis rate, and those in the two low sepsis groups, on the other hand, who were never carriers or who were carriers on admission and kept their own strains. Only 5·5 % of the high sepsis group had a total stay in hospital of 1–10 days, compared to 56·0 % of the non-carriers and 38·5 % of those who carried the initially isolated strain throughout. This difference cannot be accounted for merely by the longer stay in hospital of the septic patients in the high sepsis group, as these comprise no more than about 12 % of the total. It would seem to be explicable only in terms of some quite major difference between the two groups, presumably in the operations undergone. If this was so, the validity

of ascribing the differences in sepsis rates to the nasal carrier state seems questionable on these grounds alone.

Despite these comments, few would dispute the importance of the nasal carrier state in the general context of staphylococcal infection; differences of opinion in this field are largely a matter of varying emphasis. Whether or not a particular patient develops post-operative wound sepsis will depend in the long run on the interplay of two major factors, namely (i) his susceptibility to infection—still largely a qualitative concept—and (ii) the possible access to his wound of a sufficient number of virulent staphyloccoci to give rise to wound sepsis. These two determining elements are discussed in turn in the following paragraphs.

The patient's susceptibility to infection

Not all patients admitted to surgical wards develop post-operative wound infection. Certain factors such as age, the nature and duration of operation and the presence or absence of a drain have long been known to predispose to sepsis, but many such factors are intercorrelated and their independent effects are difficult to assess. Lidwell (1961) applied the method of multiple regression analysis to the records from twelve hospitals collected by the Public Health Laboratory Service (1960). This showed that age, sex, duration of operation, length of incision, and the insertion of a drain were all associated with increased risk of post-operative sepsis. Certain operations appeared to carry a relatively higher basic risk of sepsis than others, and Lidwell showed that this accounted almost entirely for the differences in sepsis rates observed in the various hospitals investigated during the Public Health Laboratory Service study. Clearly, therefore, any comparison between the findings of different surveys of wound infection must be approached with some caution unless the populations concerned can be appropriately matched for such factors as those discussed by Lidwell. Equally, the linking of high or low sepsis rates with other characters such as a nasal carrier state is somewhat hazardous unless the influence of some of the more obvious factors has first been excluded.

In the Torbay Hospital study, the curious association of a low sepsis rate with nasal carriage on admission to hospital was observed during scrutiny of the first survey records. Attempts to explain this finding in terms of non-comparability in some other respect of the carrier and non-carrier groups were quite unsuccessful. Thus, one is faced with the problem whether in Torquay at the time in question carriers admitted to hospital for surgery were indeed more resistant to staphylococcal infection than non-carriers admitted at the same time. As already mentioned, the difference in sepsis rates between the two groups apparently concerned patients whose wounds had been drained rather than those with clean-stitched wounds. This finding is largely a matter for record rather than discussion, in the absence of previous data. Curiously, the ratio of the incidence of sepsis in non-carriers and carriers is very similar to that between the proportion, in Rountree & Barbour's study (1951), of non-carrier nurses who picked up a hospital staphylococcus in the nose on entering the wards and of carrier nurses similarly infected. The mechanism of exclusion of hospital strains from the noses of carriers was not explained. The

present findings raise the query whether it might not have an immunological basis rather than a quasi-mechanical one as generally assumed.

A minor finding that might be related to the Torquay results is mentioned in the Public Health Laboratory Service report, namely that, of the 106 patients in the surveys in question who carried nasal staphylococci of phage group II on admission to hospital, only one showed a post-operative wound cross-infection. Possibly a scrutiny of the incidence of cross-infection in nasal carriers of different staphylococcal phage types might throw further light on this subject.

Whatever the explanation of the Torquay findings, clearly staphylococcal nasal carriage on admission to hospital was not, in the series of 559 patients investigated, attended by an increased risk of post-operative wound sepsis, but by a diminished risk. Bassett *et al.* (1963) interpret the findings of their two hospital surveys as indicating that self-infection of wounds was of minor importance, but that cross-infection probably occurred with equal frequency in carriers and non-carriers, the apparent excess of cross-infection in non-carriers being due to the impracticability of demonstrating cross-infection in carriers whose noses and wounds harboured the same phage type of staphylococcus. In the Torquay survey, however, the total incidence of sepsis in those who were staphylococcal nasal carriers on admission to hospital was so low that the existence of an increased resistance to sepsis in this group seems a reasonable inference.

Development of wound sepsis in relation to the infecting dose of staphylococci

The occurrence of post-operative staphylococcal wound sepsis in a given patient is conditioned by the possibility of access to the wound of an adequate number of pathogenic staphylococci. The infecting dose required will presumably vary with the patient's susceptibility, and access of these organisms to the wound will depend on various factors, including the physical breach in the skin barrier caused by the operative incision or by the continuing presence of a drain in the wound during the post-operative period.

Little is known of the initial stages in the establishment of the subcutaneous staphylococcal lesion, but the experimental work of Elek & Conen (1957) suggested that the minimum pus-forming dose for virulent staphylococci by subcutaneous injection was of the order of 2–8 million organisms. As natural infection with the minimum pus-forming dose seemed to them highly improbable, these authors concluded that special circumstances were required to enable the ordinary infecting dose to reach the minimum pus-forming level. One such circumstance was the presence of a foreign body such as a suture, which in their experiments reduced the minimum pus-forming dose several thousandfold. Another was tissue trauma or devitalization, and surgeons stress the importance of this factor. Howe (1956), for instance, maintained that the 'localized suppuration commonly called wound infection is a septic breakdown of devitalized tissue, blood clot or serum'.

Although one might object that the experimental techniques of Elek & Conen must have failed in some way to simulate natural infection, or that the cultures they used were not as virulent as hospital staphylococci—naturally their experimental strains used on human volunteers were not fully antibiotic-resistant, for

instance—nevertheless it is pertinent to consider briefly a few quantitative aspects of some of the accepted modes of wound infection.

The work of Hare and his colleagues, and particularly of Hare & Cooke (1961), showed that the nasal carrier without skin or wound infection usually disseminates remarkably few staphylococci, whereas patients with skin infections contaminate their clothing and environment profusely. Much of the existing literature on wound infection is compatible with the suggestion that the skin may sometimes serve as an enrichment medium for staphylococci, and could well provide wound-infecting doses of the order required in Elek & Conen's experiments, whether from the person of the surgeon with a septic infection or on the patient's susceptible wound area. Williams & Miles (1949), for instance, found that, in patients with industrial wounds of the hand, staphylococcal wound contamination was associated with skin, but not with nasal, carriage of staphylococci. They interpreted this finding as meaning that direct transfer of staphylococci from nose to wound was rare, and that nasal carriage predisposed to wound contamination, only by initiating carriage in one or more discrete areas of skin, which might or might not become the site of wounding. Some remarkable instances of the carriage of *Staph. aureus* on clearly definable areas of apparently normal skin in persons who were not nasal carriers were cited by Thomas (1961), who mapped out the distribution of skin flora using a sticky tape method. Again, the notoriously epidemic group 80 strains are known to cause skin lesions as well as wound infection. Finally, the common view, first expressed by Barber & Burston (1955), that nasal carriers among hospital staff become dangerous to patients only when they develop boils or other skin lesions, might be explained in terms of increasing skin contamination with staphylococci, without postulating an increase in strain virulence.

A careful quantitative study of staphylococcal skin carriage, both deep and superficial, in an adequate series of surgical patients, and particularly in established nasal carriers of epidemic staphylococci, might well show that some of these patients were operated on through or near localized areas of skin staphylococcal carriage.

How would the foregoing emphasis on skin carriage influence the interpretation of all the relevant evidence on the genesis of wound infection? The acquisition of nasal staphylococci *per se* would be looked on only as evidence of exposure to ward contamination. It would become an indirect determinant of wound infection, in the patient concerned, only where skin carriage followed. The time required for the development of skin carriage might lie in the pre-operative period, as in the Torbay Hospital and Public Health Laboratory Service studies, and also in those of Weinstein (1959) and McNeill *et al.* (1961). All four reported a similar increased incidence of self-infected wound sepsis in persons who were nasal carriers of epidemic strains on admission to hospital or in nasal carriers who had been in hospital for a long time before operation. The development of skin carriage might also follow a long post-operative sojourn in hospital, and explain some instances of late wound sepsis.

To explain wound sepsis in terms of contamination acquired from the environment would appear more difficult. Rountree & Beard (1962) give strong circum-

stantial evidence for the view that some patients acquire both nose and wound infection from ward bedding. Can one envisage the source of an adequate infecting dose of staphylococci in the ordinary ward environment? Two relevant points may be cited here. First, several reports (e.g. Shooter *et al.* 1958; Barber & Warren, 1962; Rountree & Beard, 1962) have indicated that, in surgical wards, very large numbers of epidemic types of staphylococcus may be disseminated from time to time, probably from patients with heavy skin or wound contamination or with staphylococcal lung or urinary tract infection. Rountree & Beard mention unpublished evidence suggesting that these strains can persist for long periods on various types of textile. Increased resistance to drying may therefore explain in part the success of these strains in establishing themselves in hospitals.

Contaminated blankets and sheets have been looked on as a source of staphylococcal broadcasts, during bedmaking, or from friction between blankets and counterpanes (Rubbo, Stratford & Dixson, 1962). The work of Elek & Conen raises a further possibility that has apparently not been investigated, namely that particles of wool or cellulose fluff contaminated with staphylococci gain entry into wounds, at operation or in the ward, and initiate wound sepsis by acting as foreign bodies in the same way as the suture material in Elek & Conen's experiments. One particular observation made during other studies in a surgical ward at Torquay seemed to lend support to some such mechanism. Two apparently unrelated cases of severe wound sepsis occurred in the ward with an interval of several days between them. It then transpired that for various reasons both patients and their beds had been moved round the ward many times—one of them eight times and the other five times. Only once were they in contiguous positions in the ward, namely on the night before the second patient's operation. This seemed to point strongly to massive contamination of the person or clothing of the second patient just before he went to the operating theatre and to direct access of contaminated fluff to his wound.

SUMMARY

1. A survey of post-operative wound infection was done in 1959–60 on 559 surgical patients admitted to a provincial general hospital.

2. Clinical evidence of post-operative wound sepsis was observed in 71 (12·7 %), suppuration in 51 (9·1 %) and staphyloccocal wound sepsis in 48 (8·6 %).

3. Seventeen of the patients died in hospital. Although 5 of these had septic wounds, the sepsis did not appear to have been the cause of death.

4. Contrary to some reported findings, the post-operative wound sepsis rate was considerably lower in patients who were staphylococcal nasal carriers on admission to hospital than in non-carriers. Nine out of 153 carriers (5·9 %) developed wound sepsis and 36 out of 385 non-carriers (9·4 %). When allowance is made for 3 highly probable self-infections, the incidence of wound cross-infection was 3·9 % in carriers and 9·4 % in non-carriers.

5. The excess of sepsis in non-carriers could not be explained in terms of different age or sex distribution in carrier and non-carrier groups, nor by differences in the types of operation undergone or in degree of exposure to staphylococcal contamination.

6. The excess of sepsis in non-carriers was accounted for by the patients whose wounds had drains rather than by clean-stitched wounds.

7. Those carriers who harboured a staphylococcus of the 80/81 group in the nose on admission to hospital had a higher incidence of wound sepsis than carriers of other phage types or staphylococcus.

8. Three probable instances of wound self-infection occurred, and in the early stages of the survey at least 5 wound infections were probably directly caused by two members of the theatre staff carrying staphylococci of the 80/81 group in the nose and with existing skin sepsis or a recent history.

9. The survey findings and a study of the literature suggested: (*a*) that the acquisition of a nasal staphylococcus in hospital was probably, as a rule, evidence of exposure to staphylococcal contamination and not a determinant of wound sepsis, unless the patient also became a skin carrier; (*b*) that a small proportion of patients are self-infected, some are directly infected by theatre personnel, and the wounds of other patients are directly or indirectly contaminated by staphylococci from the ward environment; (*c*) that wool or cotton fluff contaminated with staphyloccoci may cause wound sepsis by falling into open wounds and as foreign bodies induce a significant reduction in the minimum pus-forming dose of staphylococci; (*d*) that before ascribing high or low sepsis rates to factors such as the nasal carrier state, the relevant groups should be shown not to differ materially in respect of other factors known to influence the incidence of wound sepsis.

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REFERENCES

BARBER, M. & BURSTON, J. (1955). Antibiotic-resistant staphylococcal infection. *Lancet*, ii, 578.

BARBER, M. & WARREN, S. (1962). Control of cross-infection in a surgical ward. *Lancet*, ii, 374.

BASSETT, H. F. M., FERGUSON, W. G., HOFFMAN, E., WALTON, M., BLOWERS, R. & CONN, C. A. (1963). Sources of staphylococcal infection in surgical wound sepsis. *J. Hyg., Camb.*, 61, 83.

COLBECK, J. C., ROBERTSON, H. R., SUTHERLAND, W. H. & HARTLEY, F. C. (1959). The importance of endogenous staphylococcal infections in surgical patients. *Med. Serv. J. Can.* **15**, 326.

DAVIES, D. M. (1960). Staphylococcal infection in nurses. *Lancet*, i, 644.

ELEK, S. D. & CONEN, P. E. (1957). The virulence of *Staphylococcus pyogenes* for man. A study of the problems of wound infection. *Brit. J. exp. Path.* **38**, 573.

GARDNER, A. M. N., STAMP, M., BOWGEN, J. A. & MOORE, B. (1962). The infection control sister, a new member of the control of infection team in general hospitals. *Lancet*, ii, 710.

GILLESPIE, W. A. (1958). Hospital cross-infection. *Med. J. South-West*, **73**, 56.

GILLESPIE, W. A., ALDER, V. G., AYLIFFE, G. A. J., BRADBEER, J. W. & WYPKEMA, W. (1959). Staphylocococcal cross-infection in surgery. *Lancet*, ii, 781.

GILLESPIE, W. A., ALDER, V. G., AYLIFFE, G. A. J., POWELL, D. E. B. & WYPKEMA, W. (1961). Control of staphylococcal cross-infection in surgical wards. *Lancet*, i, 1299.

HARE, R. & COOKE, E. M. (1961). Self-contamination of patients with staphylococcal infections. *Brit. med. J.* ii, 333.

HENDERSON, R. J. & WILLIAMS, R. E. O. (1961). Nasal disinfection in prevention of post-operative staphylococcal infection of wounds. *Brit. med. J.* ii, 330.

HOWE, C. W. (1956). Prevention and control of postoperative wound infections owing to *Staphylococcus aureus*. *New Engl. J. med.* **255**, 787.

LIDWELL, O. M. (1961). Sepsis in surgical wounds. Multiple regression analysis applied to records of post-operative hospital sepsis. *J. Hyg., Camb.*, **59**, 259.

MCNEILL, I. F., PORTER, I. A. & GREEN, C. A. (1961). Staphylococcal infection in a surgical ward. *Brit. med. J.* ii, 798.

MOORE, B. (1960). A new screen test and selective medium for the rapid detection of epidemic strains of *Staph. aureus*. *Lancet*, ii, 453.

PUBLIC HEALTH LABORATORY SERVICE (1960). Incidence of surgical wound infection in England and Wales. *Lancet*, ii, 659.

ROUNTREE, P. M. & BARBOUR, R. G. H. (1951). Nasal carrier rates of *Staphylococcus pygenes* in hospital nurses. *J. Path. Bact.* **63**, 313.

ROUNTREE, P. M. & BEARD, M. A. (1962). Observations on the distribution of *Staphylococcus aureus* in the atmosphere of a surgical ward. *J. Hyg., Camb.*, **60**, 387.

ROUNTREE, P. M., HARRINGTON, M., LOEWENTHAL, J. & GYE, R. (1960). Staphylococcal wound infection in a surgical unit. *Lancet*, ii, 1.

RUBBO, S. D., STRATFORD, B. C. & DIXSON, S. (1962). Spread of a marker organism in a hospital ward. *Brit. med. J.* ii, 282.

SANDYS, G. H. (1960). A new method of preventing swarming of *Proteus* sp. with a description of a new medium suitable for use in routine laboratory practice. *J. med. Lab. Technol.* **17**, 224.

SHOOTER, R. A., SMITH, M. A., GRIFFITHS, J. D., BROWN, M. E. A., WILLIAMS, R. E. O., RIPPON, J. E. & JEVONS, M. P. (1958). Spread of staphylococci in a surgical ward. *Brit. med. J.* i, 607.

STOKES, E. J. & MILNE, S. E. (1962). Effect of Naseptin cream prophylaxis on staphylococcal infection in adult surgical wards and infant nurseries. *J. Hyg., Camb.*, **60**, 209.

THOMAS, M. (1961). The sticky film method of detecting skin staphylococci. *Mon. Bull. Minist. Hlth Lab. Serv.* **20**, 37.

WEINSTEIN, H. J. (1959). The relation between the nasal staphylococcal carrier state and the incidence of postoperative complications. *New. Engl. J. Med.* **260**, 1303.

WILLIAMS, J. R. B., TALBOT, E. C. S. & MAUGHAN, E. (1959). Hospital outbreak of cross-infection due to *Staphylococcus pyogenes* phage type 80. *Brit. med. J.* i, 1374.

WILLIAMS, R. E. O. (1947). Cited by S. D. Elek (1959) in Staphylococcus pyogenes *and its Relation to Disease*, p. 154. Edinburgh and London: E. & S. Livingstone Ltd.

WILLIAMS, R. E. O., BLOWERS, R., GARROD, L. P. & SHOOTER, R. A. (1960). *Hospital Infection—Causes and Prevention*. London: Lloyd-Luke.

WILLIAMS, R. E. O., JEVONS, M. P., SHOOTER, R. A., HUNTER, C. J. W., GIRLING, J. A., GRIFFITHS, J. D. & TAYLOR, G. W. (1959). Nasal staphylococci and sepsis in hospital patients. *Brit. med. J.* ii, 658.

WILLIAMS, R. E. O. & MILES, A. A. (1949). *Infection and Sepsis in Industrial Wounds of the Hand. Spec. Rep. Series M.R.C.* No. 266. London: H.M.S.O.

WILLIAMS, R. E. O., NOBLE, W. C., JEVONS, M. P., LIDWELL, O. M., SHOOTER, R. A., WHITE, R. G., THOM, B. T. & TAYLOR, G. W. (1962). Isolation for the control of staphylococcal infection in surgical wards. *Brit. med. J.* ii, 275.

WILLIAMS, R. E. O. & RIPPON, J. (1952). Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg., Camb.*, **50**, 320.

J. Hyg., Camb. (1963), **61**, 115
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Measles in Nigerian children

A study of the disease in West Africa, and its manifestations in England and other countries during different epochs

BY D. MORLEY, M.D., D.C.H.,*
MARGARET WOODLAND, S.R.N., S.C.M.,
Child Health Unit of the West African Council for Medical Research, Wesley Guild Hospital, Ilesha, Nigeria

AND

W. J. MARTIN, Ph.D., D.Sc.,
Medical Research Council, Statistical Research Unit, London School of Hygiene and Tropical Medicine

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'The measles which are of a deep red and violet colour are of a bad and fatal kind.'
Rhazes, A.D. 850

During the years 1956–61 an investigation into morbidity and mortality among children under 5 was undertaken in the village of Imesi, Western Nigeria, and at the nearby Wesley Guild Hospital in Ilesha. One of its results was to emphasize the contribution to morbidity and mortality made by the infective diseases of childhood. Of the acute infective fevers, measles, with its complications, was found to be outstanding as a cause of death.

In this paper an attempt will be made to describe some of the epidemiological and clinical characteristics of measles in West Africa today; they are quite unlike present experience of the disease in England. It will be shown that the disease in West Africa seems to resemble closely measles as seen in England prior to the 20th century. In the description of measles in West Africa, special attention will be given to its effect on the child's nutrition, and it will be shown that it is often responsible for the development of kwashiorkor.

BACKGROUND TO THE STUDY

Ilesha lies 74 miles north-east of Ibadan, in the rain forest area of Southern Nigeria. The Wesley Guild Hospital serves a population of 75,000 in the township, and draws many patients from the surrounding villages, where a further 75,000 live. Yorubas predominate, the majority being farmers producing yams, maize and cassava, with cocoa as the cash crop.

Within recent years the hospital has rapidly expanded, and there has been an explosive increase in the number of child out-patients. Simultaneously, the

* Now in the Department of Human Nutrition in the London School of Hygiene and Tropical Medicine.

paediatric services have been improved, and research in the paediatric field undertaken. But in spite of these developments, the hospital has difficulty in meeting the needs of the people.

No attempt has been made, in organizing the hospital services, to separate curative and preventive paediatrics. All children under 5 attend one clinic, at which there is a daily attendance of about 400 children on 6 days a week. The children are all seen and treated by locally trained nurses who have been given additional training in malaria, malnutrition, whooping cough, smallpox and tuberculosis, and in how to treat, in their early stages, the acute respiratory and diarrhoeal illnesses which are so common among Nigerian children. Only children with complex conditions and those who are seriously ill are referred to the paediatrician.

LONGITUDINAL STUDY IN IMESI

Imesi is a village 95 miles from Ibadan, in Western Nigeria. Situated nearly 1000 ft. above sea level, it lies on a small plateau in the hills, 25 miles north-east of Ilesha. In November 1956 it was chosen as the site for a longitudinal study of children, which began during their mothers' pregnancy. The investigation, centred in a dispensary, was undertaken by a paediatrician (D. M.) and a nursing sister (M. W.); the latter has been permanently resident in the village throughout, except for periods of furlough, when a relief nursing sister took over. In addition to carrying out the research, these workers also offered medical services to those of the population of 5000 who desired them. The study was designed to trace the growth and development of a group of 405 African village children from birth to 5 years. In this paper, records of the first three years are used.

THE INCIDENCE AND SEVERITY OF MEASLES

Measles occurs every year in this part of Nigeria, but the outbreak is more severe in alternate years. The epidemic begins in the middle of the dry season, and declines with the onset of the wet season (Morley, 1962). During the dry season there is little activity on the farms and the people celebrate various festivals. At these festivals, which are spent in dancing and feasting, the child is carried on the mother's back, and excellent opportunities are presented for the spread of droplet infections. The decline of the epidemic is associated with the coming of the rains, when the people spend much of their time on the farms, planting the next season's crops. The rise and fall of the measles epidemic may be associated with these movements of population.

Of the 405 children in the Imesi village study, 82 were stillborn or died from causes unrelated to measles, so that full records of only 323 children are available; of them, 222 had measles during the study period, and among these children with measles there were 15 deaths in which the disease played a major part. Clinical data relating to 12 of these fatal cases are available, and are used in the descriptions of bronchopneumonia, diarrhoea and kwashiorkor given in a later section.

Similarly in the hospital at Ilesha measles was a frequent and severe disease among the child patients. During 1961, 2030 cases of measles were seen in the children's clinics. In the 3-year period ending June 1961, 1283 children with

measles were admitted to the children's ward; of these 1283 children, 325 died, a mortality of 25·3 %.

Gans, Macnamara, Morley, Thomson & Watt (1961) have estimated an overall case mortality of 5 % in Nigeria. The following figures, though limited, suggest a high mortality throughout West Africa.

Vallée de Sénégal. In 1956–7, Cantrelle, Etifier & Masse, (1960) reported from this area that measles accounted for 24 % of all deaths in children under the age of 15, and diarrhoea complicating measles, a further 13 %. Senecal, Aubry & Falade (1962) considered measles to be the most killing disease of the pre-school child.

Gambia. In an outbreak in the village of Keneba, the first for 12 years, 18 % of the children under 5 died (McGregor, 1962).

Sierra Leone. Avery (1962) analysed 573 admissions for measles to the Nixon Memorial Hospital, Segbwema, between 1954–61, and reported a 17 % mortality.

Haute Volta. In 1960 measles accounted for 50 % of all deaths in the age group 1–4 years (Boué, 1962).

Ghana. Murphy (1962) reported a 15 % mortality among 157 children admitted with measles to an Accra hospital. Of 330 children admitted with kwashiorkor, 62 had measles in the preceding 6 months. Field studies in Ghana suggest a mortality in excess of 3 %.

Lagos, Nigeria. Gans (1961) recorded that during 1959, 185 children with measles were admitted to the Lagos General Hospital; of these, 42 died, a mortality of 22·7 %.

Congo. McFie & Yarom (1962) recorded 7 deaths in a group of 36 children who developed measles.

Statements to the effect that measles, as an endemic disease, is less prevalent in the tropics than in the temperate zone, and less serious among negro than white populations, are still to be found in some text-books. Some contribution to this belief may have been made by the fact that African parents have tended to regard an attack of measles as a 'normal' and inevitable occurrence in the life of their children and hence did not consider it worthwhile to bring them to hospital when they were suffering from it. This in turn meant that doctors in Africa gained little experience of the disease and hence erroneous ideas about its prevalence. In this connexion, a distinction must of course be made between endemic measles and the severe epidemics which have sometimes followed the introduction of the disease into isolated communities, an account of which will be found in a review by Brincker (1938). These are unlikely to recur, since modern developments in transport have eliminated isolation and no community can now remain free from measles for a long period and hence become susceptible to epidemics.

MORTALITY FROM MEASLES IN THE PAST

Creighton (1894) gives many accounts of epidemics with a high mortality, the highest of which seems to have been recorded by Gannelon (1892) in the Hospice des Enfants Assistés in Paris. In this orphanage, between the years 1867–72, there were 612 deaths among 1256 children who developed measles. Glasgow provides

an example of high mortality in the general population; in the years 1807–12, measles accounted for 10·76 % of all deaths in that city. Over the next 100 years the mortality declined, but still remained high among young children, as is shown in Table 1.

Table 1. *Comparative mortality from measles in Great Britain and West Africa*

Age (years)	Glasgow, 1908		England and Wales, 1960	
	Notifications per 100,000 population	% mortality	Notifications per 100,000 population	% mortality
Under 1	7337	11·7	777	0·104
1–	14177	14·2	2126	0·045
2–	17422	4·2	2740	0·046
3–	16643	1·7	3041	0·010
4–5	16560	1·2	3242	0·005
Under 5	14215	5·8	2356	0·030

Age (years)	London fever hospitals, 1911–14		Ilesha, W. Nigeria, July 1958–61	
	Hospital admissions	Case fatality (%)	Hospital admissions	Case fatality (%)
Under 1	1080	22·9	276	19·9
1–	2728	21·3	521	29·9
2–	2125	11·8	217	26·7
3–	1874	7·1	138	21·7
4–5	1470	5·2	72	23·6
Under 5	9277	13·9	1224	25·8

Table 1 gives comparative data on mortality from measles in infants and young children in Glasgow in 1908 and England and Wales in 1960, the former taken from the Annual Report of the Registrar-General for Scotland, 1908, and Chalmers' *The Health of Glasgow*, 1818–1925, the latter from the Annual Report of the Registrar-General for England and Wales, 1960. Case fatality in London fever hospitals 1911–14, as derived from the Reports of the Local Government Board, (1918) is compared with that in the Ilesha hospital, 1958–61. The latter comparison raises a point of interest, namely that the fatality in the Ilesha children in the age group 3–5 years was higher than that in London children 50 years ago, perhaps because only serious cases were admitted to the Ilesha hospital, and because malnutrition contributes to measles mortality in African children.

THE CLINICAL PICTURE OF MEASLES IN WEST AFRICA

Certain aspects of the clinical picture in West African children are of special importance. Bronchopneumonia, laryngitis and diarrhoea are common and serious accompaniments of the disease; darkening of the rash followed by extensive desquamation is frequently seen; the effect of the disease on the state of nutrition is severe.

The clinical accounts which follow are confined largely to bronchopneumonia,

diarrhoea, malnutrition and skin changes. Encephalitis, cancrum oris, conjunctivitis, and keratitis leading to blindness, which are not uncommon accompaniments of the disease in West Africa, will not be considered in the paper.

The term 'complication' is avoided, because it is impossible to distinguish between clinical signs which are part of the disease and signs superimposed on it.

Measles bronchopneumonia

In the village study, as far as possible, day to day examinations of children with measles were made. Dyspnoea and crepitations audible on auscultation of the chest were often observed in the early stages before secondary bacterial infection was likely to have occurred. There were a number of instances of clear-cut bronchopneumonia, of which the following are examples:

No. 288, *N.D.* (*F*) *aged* 13 *months*, developed measles on 9 March 1959. As the rash appeared, she had some diarrhoea and vomiting, and was treated with sulphadimidine. Ten days later she was brought back to the dispensary, looking very miserable, with a running nose, desquamation of the skin, and dyspnoea. Crepitations were present at both lung bases. She was treated with penicillin, to which was added streptomycin the following day. With this treatment, she made a good recovery during the next 6 days. In this child the skin changes and the bronchopneumonia developed simultaneously; this association was seen many times, and will be referred to again later.

Among the village children there were four deaths associated with measles bronchopneumonia. All four children showed some degree of marasmus. Recent whooping cough, anaemia and convulsions respectively, were present in three. The fourth is perhaps typical of this type of case, and is described below:

No. 61, *M.J.* (*F*) *aged* 12 *months.* M.J. had gained weight fairly satisfactorily until the 4th month, but from then until the 9th month she had not gained weight well. From the 9th months until her death, her mother received a skimmed milk supplement to give to her. She was seen on 3, 4 and 5 January 1958, with fever, misery and cough; she was 1 year old, and weighed only 13 lb. The rash of measles did not become obvious until 11 January. During the next 3 days the child became very ill and was treated at first with sulphadimidine and penicillin, and later with chloramphenicol. On the 5th day following the appearance of the rash, the skin started to desquamate in large plaques. Cough and misery became worse, and the child steadily lost weight, losing 1 lb. in 9 days. She died 9 days after the appearance of the rash.

In the hospital at Ilesha, severe bronchopneumonia exceeded other manifestations of measles as a reason for admission (Table 2). The children in Ilesha were not under such close observation as those in the village of Imesi, and their condition was usually serious on admission.

Of the 1283 children admitted with measles to the Ilesha hospital, 604 had bronchopneumonia as the most prominent condition: of these 169 (28 %) died. The children aged 6–12 months showed a lower mortality (23 %) than those in the 2nd year of life; 276 in this age group were admitted, with a mortality of 32 %. This difference may well have been due to selective admission, but it was our impression

that measles was more severe in the 2nd year than in the latter half of the 1st year, when breast milk was more abundant, and the state of nutrition of the children more satisfactory. Furthermore, in a proportion of the younger children the disease may have been mitigated by traces of maternal antibody.

Table 2. *Measles bronchopneumonia, admissions to Ilesha Hospital, W. Nigeria, July* 1958–*June* 1961

	−6 months	6 months–	1 year–	2 years–	3 years–	4 years–	5 years–	6 years–	9–12 years
Affected	5	139	276	80	54	23	10	15	2
Died	0	32	89	27	10	7	1	3	0

Three centuries ago, Thomas Sydenham (1674) had little doubt as to the severity of measles bronchopneumonia. In describing its symptoms he says they '... are so fatal, that they do more to fill Charon's boat than the smallpox itself'. Gregory (1843) records that in 1839, a peak year, measles was responsible for 10,937 deaths in England and Wales, a third of all deaths due to epidemic diseases in that year. In the same book he suggests that as many as nine-tenths of the deaths from measles may have been due to pneumonia. Trousseau (1869) observed an epidemic at the Necker hospital in Paris in 1845, in which 22 out of 24 children with measles died from pneumonia. In England today, however, bronchopneumonia is no longer a frequent cause of death following measles. Watson (1956) reported from the results of a study of 993 cases of measles as seen in general practice, that chest conditions of any kind occurred in only 30 previously healthy children, while ear conditions occurred in 51. It appears that mortality from measles bronchopneumonia in England was declining before the 'thirties' of this century, that is, before the introduction of sulphonamide and antibiotics. Experience in Nigeria shows that they are valuable in the treatment of measles bronchopneumonia and will prevent some deaths from this cause. But they can have been only partly responsible for the decline in the incidence of measles bronchopneumonia, and the mortality associated with it, in England during recent decades.

Laryngitis

Among the Imesi village children, laryngitis associated with measles developed a number of times. None of these children required admission to hospital. Among the 1283 children admitted to Ilesha Hospital with measles, laryngitis, severe enough to cause concern, was present in 32 (2·3 %). Among these 32, there were 8 deaths, 2·5 % of all the measles deaths. Laryngitis was particularly common and serious between the 1st and 3rd birthdays; 21 of the children admitted for laryngitis, and all the fatal cases, belonged to this age group.

Balme (1904) found laryngitis to be present in 8 % of 227 children with measles seen at a clinic for the London poor in 1904. Holt (1911) stated, in the sixth edition of his *Diseases of Children*, that severe catarrhal laryngitis occurred in about 10 % of children with measles, and might give rise to signs closely resembling those of membranous laryngitis. According to a Report of the Local Government Boards

(1918), there were 248 deaths due to laryngitis associated with measles in 1911, 1·9% of all deaths from measles in that year. Thursfield (1914) recorded that tracheotomy was usually fatal in this condition. At Ilesha, this operation was performed on six children, of whom four died.

Diarrhoea associated with measles

Both among the village children and among those admitted to the children's ward at Ilesha, diarrhoea came second in importance only to bronchopneumonia as a feature of measles. The Ilesha people themselves know that diarrhoea is a dangerous occurrence, and fear it; sometimes the parents of children with measles specifically ask how it can be prevented. Diarrhoea occurs in many of the acute diseases of childhood, and its frequency in relation to these was investigated in the village study. In a group of 259 children who were permanently in the village, and observed for 3 years, 1804 attacks of diarrhoea were recorded, of which 179 were associated with acute infections. The frequency of the occurrence of diarrhoea in different infections is shown in Table 3.

Table 3. *Acute infections that were associated with diarrhoea in Imesi village children*

Condition	No. of attacks
Measles	109
Whooping cough	26
Malaria	10
Ear infections	8
Respiratory	7
Other	19
	179

Table 4. *Time relationship between* 205 *attacks of diarrhoea and appearance of rash in* 142 *children*

7–14 days before rash	1–6 days before rash	0–6 days after rash	7–14 days after rash	Later
11	39	50	23	82

Table 3 shows that in this community, diarrhoea is more commonly associated with measles than with the other diseases listed. The time relationship between attacks of diarrhoea and the appearance of the rash is shown in Table 4.

Diarrhoea may occur in the prodromal period or together with the rash. The children observed in Imesi were susceptible to diarrhoea for a long period after the measles itself had subsided, as is shown in Table 4. The diarrhoea may continue or reappear over a period of many weeks.

In five children diarrhoea following measles was a major factor in the child's death. The following history is typical of these children:

No. 150, *A.S.* (*M*) *aged* 35 *months.* The child had been brought regularly to the clinic by his mother. Weight gains had been satisfactory, the weight keeping 1–4 lb. above the mean weight for children in this area. When the child was seen on

2 August 1960, the mother gave a history of measles beginning a few days previously. This was confirmed by the condition of the child's skin, which showed peeling. He was severely anaemic, and ova of *Ancylostoma duodenale* were found in his stool. On 6 August he had a discharging ear, and on the 9th he had signs of bronchopneumonia, for which he was treated. On 13 August he was brought with a most profuse diarrhoea. As the child was being examined on his mother's knee, there was a continual dripping of blood-stained stools into a bowl below. His weight had fallen from 29 lb. 4 oz. to 22 lb. 8 oz. during the month since he had contracted measles, a loss of 24 %. He was admitted to hospital for treatment with parenteral fluid, but died in a convulsion the following day. Death was considered to be due to measles associated with diarrhoea, bronchopneumonia, and anaemia.

Of the 1283 children admitted to Ilesha hospital for measles, 355 (28 %) had diarrhoea and dehydration as one of the major signs. The age of these children, and the number of deaths, is shown in Table 5.

Table 5. *The age distribution and mortality in* 355 *children with measles accompanied by diarrhoea*

	−1 year	1 year–	2 years–	3 years–	4 years–	5 years–	6 years–	9 years +
Affected	78	131	66	43	21	7	9	–
Died	12	42	24	13	7	2	3	–

From Table 5 it will be seen that children of all ages are prone to diarrhoea, and the mortality from this cause is high in all age groups. Of the children admitted with diarrhoea, 196 (55 %) required parenteral fluid therapy.

McGregor (1962) observed that after a severe outbreak of measles in the village of Keneba, in the Gambia, many of the children had diarrhoea, and that the stools were commonly blood-stained. At Imesi, diffuse diarrhoea was sometimes seen in association with measles, but the most common sign was the passing of mucus in the stool, with tenesmus. Blood was noticed in the stools of 13 % of these children.

An early account of measles in West Africa was given by Daniell in 1852. He believed that dysentery and diarrhoea were then more serious and fatal than pneumonia among the African children. Diarrhoea occurring in the course of measles is now seldom mentioned in text-book descriptions of the disease, and is rarely seen in England or the U.S.A. Early in the 20th century it was still important; in Balme's account (1904) of an epidemic seen at a clinic for the London poor in 1904, it is recorded that out of 227 cases, 64 (28 %) had diarrhoea. D. Williams, (1906), states that 'Diarrhoea of foul-smelling slimy stools very commonly occurs during the eruptive stages, either at the onset of the rash or as it fades'. He later adds: 'Diarrhoea of a dysenteric character, due apparently to diffuse catarrhal colitis, sometimes occurs, and is very apt to continue as a chronic condition for weeks or months'. In certain earlier epidemics, diarrhoea seems to have been particularly severe. Creighton (1894), writing of the epidemics in 1807 and 1808 in Glasgow and Edinburgh, records that '...there

were troublesome symptoms in almost every case—a violent pain in the belly, frequently accompanied with diarrhoea (and even with vomiting), and with the dysenteric symptoms of tenesmus and mucus in the stools. This bowel complaint usually lasted three or four days and wasted the patients remarkably.' This picture of diarrhoea closely resembles that seen in the African children, among whom the passage of mucus and tenesmus were particularly common. Many of the African children showed severe wasting associated with the diarrhoea, an observation which will be considered later under 'Weight loss'. In the village, the diarrhoea most commonly appeared after the rash, during or after the period of desquamation (Table 4). Thursfield (1914), in England, recorded that fatal diarrhoea usually occurred in the convalescent stages, and that in the eruptive stage it was usually harmless.

Loss of weight, wasting and kwashiorkor

The considerable loss of weight which follows measles was an important observation in the longitudinal child study in Imesi village. It was found that measles, whooping cough, cessation of breast feeding, and the birth of a sibling, were the most common events associated with a striking loss of weight. The weight changes occurring after measles in 220 children in Imesi were analysed and expressed as a percentage of the child's weight immediately prior to the measles infection. The results are shown in Table 6.

Table 6. *Percentage of former weight lost during measles by 220 Imesi village children*

	None	Under 5 %	5 %–	10 %–	15 %–	20 % and over
No. of children	24	71	72	34	16	3
Percentage	11	32	32·7	15·5	7·3	1·5

Table 7. *Time taken to regain former weight after measles, Imesi village children*

	0–4 weeks	5–8 weeks	9–12 weeks	Over 12 weeks
No. of children	77	70	16	29
	(40 %)	(36·5 %)	(8·3 %)	(15·1 %)

Mean period = 7·2 ± 0·45 weeks

Table 6 shows that almost one in four of the children lost more than 10 % of their former weight as a result of this infection. In Table 7 the duration of weight loss is recorded for 192 children who attended regularly and who were weighed for a period after the disease.

The height of the children included in the study was measured every 6 months. The height records of 38 children who had taken 8 weeks or more to recover their weight after measles were analysed. No significant change in the rate of growth, as judged by height, could be demonstrated among these children.

In West Africa, as elsewhere, there is a tendency to restrict the child's diet during measles, accentuating nitrogen depletion. The foods that are given are mainly of

the starchy type. Nine of the 222 children in the village study developed kwashiorkor after measles, and 4 of these died. All but one of the 9 had lost more than 10 % of their weight before the onset of oedema. Kwashiorkor is occasionally seen in children who for some reason lose weight suddenly and dramatically after having previously shown satisfactory weight gains; more commonly, however, it appears after a prolonged period of failure to gain weight. This happened in the case of the four children who died. The following case history is fairly typical of these:

No. 375, *F.A.* (*F*) *aged* 19 *months.* On 25 January 1960 this child was brought to the dispensary for a routine examination, and may, on that occasion, have been in contact with a child incubating measles. A typical measles rash appeared on 8 February. During the next 8 days F.A. did not appear ill, but showed a steady loss of weight. A marked desquamation of the skin was noticed, and her mother said she had lost her voice. Two weeks after the appearance of the rash, the child was brought to the clinic, oedematous and seriously ill; she died the same evening. Her weight had been almost stationary at about 19 lb. for 7 months prior to the measles, and she lost 3 lb. 3 oz. (16·5 %) of this weight during the course of the illness. Death was attributed to acute kwashiorkor, probably accompanied by an underlying bacterial infection following measles.

HOSPITAL EXPERIENCE OF KWASHIORKOR FOLLOWING MEASLES AT ILESHA

The relationship between measles and kwashiorkor is illustrated by the figures in Table 8.

It will be seen from Table 8 that the age distributions of admissions for, and deaths from, measles and kwashiorkor respectively, are very similar, except in the

Table 8. *Age distribution of children with measles and kwashiorkor respectively admitted to Ilesha Hospital, July* 1958–*June* 1961

(Figures in brackets refer to the number of deaths.)

Age	Children with measles		Children with kwashiorkor		Children with recent measles and kwashiorkor	
Under 1	276	(55)	29	(8)	12	(3)
1–	521	(156)	180	(31)	52	(10)
2–	217	(58)	320	(34)	58	(6)
3–	138	(30)	163	(31)	30	(9)
4–	72	(17)	65	(10)	11	(2)
5–	23	(3)	29	(4)	4	(–)
6–	33	(6)	32	(6)	1	(1)
9–12	3	(–)	7	(2)	–	–
Total	1283	(325)	825	(126)	168	(31)

1st year, and that a considerable number of cases of kwashiorkor occurred in children who had recently had measles. Kwashiorkor developed most frequently as a sequela of measles between the 2nd and 4th birthdays. Since more than half

the children with measles seen at Ilesha are under the age of 2 years (Morley, 1962), it is apparent that measles affects the child's nutrition more severely between 2 and 4 years than in the first 2 years of life, when the majority of the children are still getting some breast milk.

The importance of infection in causing protein malnutrition has recently received emphasis from Platt (1957), and from Scrimshaw, Taylor & Gordon (1959) and Scrimshaw (1961). Investigations by Scrimshaw (1962) have shown that measles is associated with a marked negative nitrogen balance.

Gans (1961) recorded that kwashiorkor appeared after measles in a considerable number of children admitted to Lagos Hospital with measles. In Ilesha, measles was the most common of the infective illnesses of childhood which occurred, according to the histories given by the mothers, in the weeks preceding the onset of kwashiorkor.

Skin changes in kwashiorkor following measles

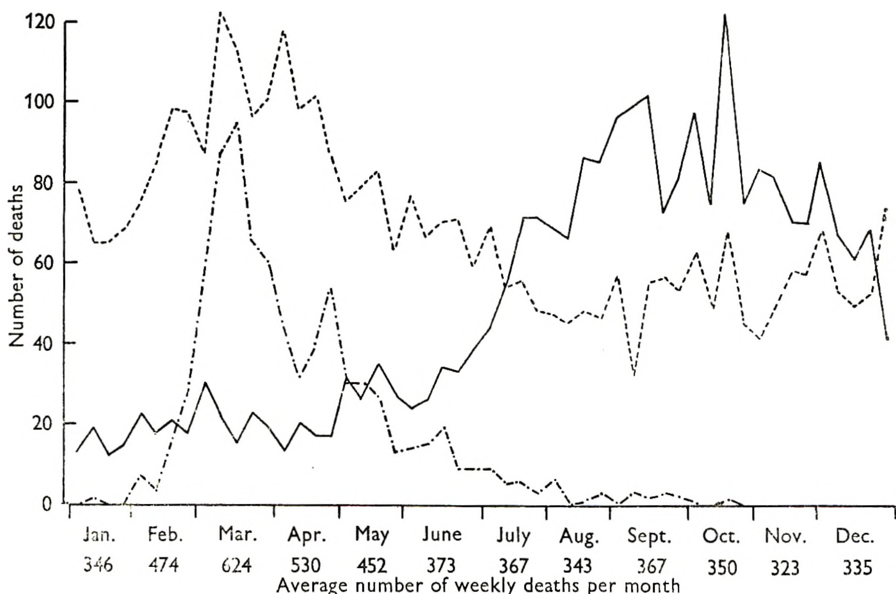
In children with kwashiorkor developing shortly after measles, the skin changes may be different from those seen in kwashiorkor due directly to dietary deficiency or precipitated by other illnesses. They are more widely distributed, and are frequently present on the face. Cracks may appear round the mouth. Paterson & Lightwood (1956) give a good illustration of the appearance of a child with acute kwashiorkor after measles.

Sore mouth and stomatitis

The African mother is accustomed to prolonged breast feeding and is therefore alert to the occurrence of a sore mouth in her child. That a sore mouth may interfere with breast feeding and thus be among the causes of weight loss, was not appreciated in the earlier stages of the village study. Many mothers said that their child had a sore mouth, but only a few volunteered the information that he would not suck as a result. Later, a series of mothers whose children had been admitted to hospital with severe measles were asked whether their children had stopped sucking. Twenty-six out of 160 said that their children had gone off the breast for a period of more than 1 day. In a few cases, breast feeding had ceased for long periods, or altogether. One mother in the village group developed a breast abscess after her child had measles, and this also occurred in a number of mothers of children with measles attending the Ilesha hospital. The breast abscesses were almost certainly related to the sore mouth. The succession of events observed was: failure to suck owing to the sore mouth, engorgement of the breast, damage to the nipple from attempts to feed, infection of the damaged nipple from the infected mouth, spread of infection from the cracked nipple to the breast, and finally a breast abscess. A breast abscess naturally interferes with breast feeding and may seriously upset the child's nutrition. The importance of a sore mouth was well recognized in the past, for example, Goodhart & Still (1921) recorded that 'the tongue and mouth become dry and ulcerated or covered with sores, and rapid emaciation takes place...'. This resembles the picture often seen in West Africa, where the sore mouth caused by measles may seriously upset the nutrition of the infant.

Marasmus following measles in England before 1920

The wasting that may occur after measles is mentioned by early writers. Creighton (1894) produced evidence that the loss of weight and emaciation associated with the disease might be an important cause of mortality. He wrote: 'We shall not correctly understand the part played by measles among the infective maladies of children unless we keep that grand character of it in mind—that its effect upon the mortality of infancy and childhood are only in part expressed by the deaths actually appearing under its name.' He drew support for this statement from 17th-century London Bills of Mortality, based on the records of women 'searchers' appointed by each parish. The records for 'consumption', 'smallpox' and 'measles' in the Bills of Mortality for the year 1674, taken from Creighton's book, are set out



Text-fig. 1. Weekly deaths from measles, smallpox and consumption in London in 1674. —·—·—, Measles; ——, smallpox; - - - -, consumption (i.e. wasting, see text).

in Text-fig. 1. Creighton's argument turns on the meaning of the word 'consumption' at the time. He quotes a Dr Fothergill, who in 1751 commented that this word had a broad meaning for the 'searchers', saying that '...if the body is emaciated, which may happen even from an acute fever, it is enough for them to place it to the account of Consumption...'.

The epidemic of measles reached its peak in the week ending 17 March when 95 cases were recorded. In that week there was a total of 695 deaths, 300 above the weekly average of that season. This increase was due in part to 'consumption'. Deaths from 'teeth' and 'convulsions' also reached a peak for the year in the same season. While mortality was always higher in the spring, Creighton was confident that the exceptionally high mortality in this particular year was due to measles.

Later in the same year there was an outbreak of smallpox. Although many more

deaths were attributed to smallpox than to measles, the total number of deaths remained lower, and, in particular, there was little or no increase in the number of deaths from 'consumption'. 'Consumption' or emaciation was doubtless due to several causes, but the fact that its incidence seems to have been much increased by the measles epidemic is significant. By 1733, age at death was also recorded in the Bills of Mortality. In that year there was a serious epidemic of measles, again associated with an increase in the number of deaths from 'consumption'. Creighton was able to show that this increase in mortality from 'consumption' was mainly among infants.

At the beginning of the present century, wasting and marasmus were still associated with measles in Northern Europe. Meunier (1898), in France, described loss of weight in the pre-eruptive stage, a sign which has been used in the differential diagnosis. The occurrence of marasmus in the later stages of the disease was also recognized.

In the village study, diarrhoea with mucus and tenesmus was among the causes of weight loss in children with measles. The time taken for children to regain their former weight after measles is shown in Table 7. If these children are separated into those with and those without diarrhoea, the mean period taken for the former to regain previous weight was $8{\cdot}06 \pm 0{\cdot}31$ weeks, as compared with $4{\cdot}49 \pm 0{\cdot}39$ weeks for the latter. Creighton also noticed an association between diarrhoea and weight loss, and wasting in patients suffering from measles accompanied by diarrhoea is referred to in the older literature.

It is possible that what might today be called a 'kwashiorkor-like' syndrome sometimes occurred in children after measles in earlier times in England. Creighton, in describing an epidemic in 1807, records: 'Numbers who recovered from the measles were afterwards affected with debility, cough, emaciation and oedematous swellings of the face and extremities, which proved very difficult to remove.'

The rash and subsequent changes in the skin

The unusual skin changes noticed in the Imesi and Ilesha children suggested a reason for the differences between measles in Africa and England today. In the early stages of measles in the African child, Koplik spots are commonly recognized, and the first appearance of the rash is similar to that seen in English children, when due allowance is made for the darker skin. In a proportion of the children, however, the rash became confluent. In some, it darkened to a deep red colour, and even progressed to a violet or purple hue, a change not now seen in English children. Two to four days after the appearance of the rash, desquamation began. Some degree of desquamation developed in every child in this area who had measles, the degree of desquamation being apparently related to the extent that the rash darkened in colour. In children in whom the rash acquired a purple hue, large scales of skin were likely to separate (Pl. 1).

The desquamation in the African child was more apparent for two reasons: first, the scales are white and hence show up against the dark skin, and secondly, as mentioned below, the parents believe that a child should not be washed during the course of the illness, and washing might clear away an accumulation of the

scales. After the desquamation there was a variable and patchy depigmentation which lasted for some weeks.

A number of references to similar skin changes can be found in the older English literature. Willan (1821), who has been called 'the father of English dermatology', refers to the darkening of the rash in a child who had previously suffered from, and had presumably been weakened by, whooping cough and who then developed measles. He says: '...the rash was succeeded by numerous livid spots, diffused over nearly the whole body, and resembling those of the Purpura....' Drinkwater (1885) described a severe measles epidemic in Sunderland during a period of industrial depression and semi-starvation. There was an 8% mortality among the children with measles under his care. In half the children the rash was unusually dark, darker than he had noticed in previous years. Moore (1892), in his *Textbook of the Eruptive and Continued Fevers*, refers to a 'stage of desquamation', and states that it starts at the 8th day and ends at about the 18th day after the onset of the catarrhal stage. The branny scales, he says, may not be seen as they adhere to the clothes. Another account of severe desquamation after measles is given by Edlin (1802) in a description of an epidemic in the winter of 1801–2: 'Another peculiarity in this epidemic was that the cuticle in many children did not separate after the disappearance of the eruption, and in several others that I particularly noticed, it came off in large flakes instead of branny scales; and the appearance of the rash in others assumed so striking a resemblance to the scarlet fever that, had it not been for the violent cough and other measly symptoms, many such cases occurring singly might, upon a superficial view, have been considered and treated as that disorder.'

In the 19th century excessive exfoliation of the skin following measles is likely to have been credited to scarlatina. The same possibility was considered in the earlier stages of the African studies, but was excluded on clinical grounds. The course of the disease was typically that of measles, and streptococcal disease, with its complications of nephritis and acute rheumatism, seems to be rare in this part of Africa.

Whether the type of measles seen in Nigeria, in which there is the darkening of the rash, is the same as what used to be called 'black measles', is difficult to say. Holt (1911) records that in 5% of his patients the rash was haemorrhagic; he acknowledged this as a bad, but by no means a fatal symptom. No satisfactory account of a number of cases of 'black measles' has been found, and the descriptions of individual cases vary considerably. The changes described in the rash among African children were not associated with gastro-intestinal or urinary haemorrhage, nor were they similar to thrombocytopenic purpura, which was occasionally encountered. Their appearance suggested an increased exudate of blood cells into the rash.

One final stage following desquamation was the development of multiple small boils; this was seen in 41 (18·5%) of the children in the village study. The boils occurred most commonly on the head and trunk and usually did not appear until 2 or more weeks after the appearance of the rash. Willan (1821) records that measles could be succeeded by '...glandular swellings, and eruptions of painful

inflamed pustules, some of them being nearly as large as boils'. In Balme's (1904) record of a measles epidemic, 12 (5·3 %) of the cases suffered from subsequent impetigo. Holt (1911) stated that boils and superficial sepsis of the skin were uncommon as complications of measles, and they are rarely seen in England or the U.S.A. today.

BELIEFS ABOUT MEASLES

Reed (1957) has described some of the beliefs about measles held in West Africa. One of the most important is a conviction that measles is inevitable in young children—justified since practically every child does get measles—which makes parents indifferent towards seeking medical treatment for children with the disease. Reed noted this attitude in Ibadan, Nigeria, and it was also evident in the village study in Imesi, where it contributed to the death of three children. Within recent years some change has taken place. Thus Whitbourne (1930), writing of experience in Lagos, recorded that there were only two deaths from measles in hospitalized children in 1928 and 1929. In 1959, however, 187 children with measles were admitted to the Lagos General Hospital, 44 of whom died (Gans, 1961).

Beliefs in Europe and America have been discussed by Radbill and Hamilton (1960), who described the old remedies for measles. Apathy towards seeking medical care for children with measles was also observed in past years. It receives some mention in the Report of the Local Government Board for 1918.

Another belief mentioned by Reed is that children with measles should never be washed. This was generally held by mothers in Ilesha; some would neither wash them nor allow them water to drink. It was also prevalent in England in the past, and is still remembered by older physicians; thus Balme (1904) records, on the basis of experience at a clinic for the London poor, that mothers were averse to wetting their children when they had measles. Failure to wash leads to an accumulation of desquamating skin, and may, in the past, have increased opportunities for the physicians to observe the desquamating stage of measles.

THE GENERAL CLINICAL PICTURE AND ITS VARIATIONS

A resumé of some of the main features of measles, as observed in West Africa today and reported in this paper, is given in Table 9. This is compared with the current picture in Great Britain, based largely on a Report of a Study Group of Physicians (Watson, 1956), and with the picture obtained from the works of earlier writers. As source material for what might be called the 'pre-1920' picture, the 6th edition of Holt's *Diseases of Infancy and Childhood* (1911) was found to be particularly useful; while Holt's account relates primarily to the U.S.A., it is in line with contemporary and earlier descriptions given by physicians in Great Britain.

DISCUSSION

The material presented in this paper suggests that measles as now seen in West Africa closely resembles the formidable disease of an earlier era in Great Britain. If this is true, then an opportunity presents itself of studying the disease as it once

existed in Great Britain and other countries in Northern Europe and America. Such studies, which will enhance our knowledge of the disease, should be made without delay. Once an effective vaccine becomes available, it must be widely used and it will no longer be possible to observe the uncontrolled disease in communities in which its manifestations are severe.

It is proposed to discuss possible reasons for these resemblances and differences. In doing this, the virus, the environment and the host will be considered.

Table 9. *Features of measles in West Africa today, and in Great Britain today and before about* 1920

	West Africa (today)	Great Britain (today)	Great Britain (before 1920)
Peak age incidence	17 months (Ilesha)	4 years	Under 3 years
Bronchopneumonia	Common and severe	Uncommon	Common and severe
Laryngitis	Common	Uncommon	Common
Diarrhoea	Frequent	Almost unknown	Frequent
Darkening of rash	Common	Unknown	Common
Desquamation	Common, may be severe	Practically unnoticed	Common
Effect on child's nutrition	Severe	Transient	Probably severe
Otitis media	?Uncommon	Common	Uncommon
Mortality	1:20	1:5000	1:20 (Glasgow, 1908)

The virus. At present it is impossible to exclude altogether the possibility that the strain of measles virus which causes the disease in Nigeria and elsewhere in the tropics is more virulent than the strain occurring in England. Repeated isolation of the former and appropriate laboratory studies will be needed to reach a final conclusion on this point. However, it would seem that any virulent virus would be brought from time to time to England and other countries from the tropics, particularly in these days of 'overnight' air transport. There is no evidence that this has happened.

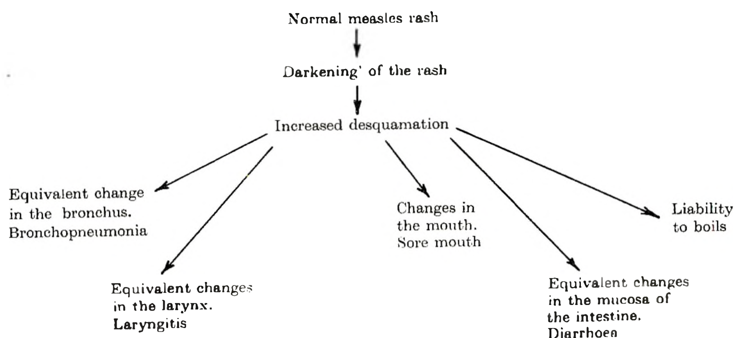
The environment. The child in the tropics today lives in an environment in which chances of secondary infection are high, and the same was true of the English child in the past. Today the chances of secondary infection in the English child are greatly reduced. This difference in the environment may in part account for the differences in liability to secondary infection and the mortality associated with it. Another important environmental difference, considered in this paper, is in feeding practices and state of nutrition.

The host. Catastrophic epidemics of measles have occurred in populations previously unexposed to the disease, as in Fiji and the Faroes. There is no indication of such lack of immunity, associated with sudden and lethal epidemics, in West Africa, where the disease has probably been endemic for a long time. In the measles epidemic in Accra in 1852 described by Daniell and mentioned earlier, it was the small children who were infected and epidemics were known to be frequent. The present low mortality from measles in England can scarcely be attributed to an acquired immunity, since the decline in mortality has taken place over a period of

50 years or so, after centuries during which the disease seems to have remained severe, with only minor variations in its severity.

Racial factors can probably be excluded. In the U.S.A. the mortality rates from measles in the negro and white population between 1921 and 1940 were similar, and declined at the same rate (*Vital Statistics Rates in the U.S.* 1900–1940).

A high mortality from measles seems to be related to the severity of the rash, and the subsequent desquamation. To repeat the quotation from Rhazes: 'The measles which are of a deep red and violet colour are of a bad and fatal kind'. Darkening of the rash is often seen in African children today, and seems to have been common in England in the past. Copland (1858) comments: 'The more prominent and copious the eruption, the more marked the desquamation becomes'. He also states that the red rash occurred more intensely in the ill-fed, and was associated with croup and pneumonia. It seems likely that the deep red rash and the desquamation are accompanied by equivalent changes in other epithelial surfaces of the body, which give rise to bronchopneumonia, laryngitis and diarrhoea. Gunn (1955), in his account of the morbid anatomy of measles, says: 'The essential lesions consist in a catarrhal inflammation of the respiratory and alimentary tracts. The initial inflammation of the epithelial cells is rapidly followed by fatty degeneration and by exfoliation of dead cells.' In describing the changes in the gastro-intestinal tract, he says: 'In all but the mildest attacks the whole length of the gastro-intestinal tract shows an inflammatory catarrh: complete resolution is the rule, but widespread denudation of epithelium may end in mucosal atrophy.' These pathological findings are in line with experience in West Africa, where broncho-pneumonia, laryngitis and chronic or relapsing diarrhoea are often encountered as complications or sequelae, tending to coincide with desquamation; they are also in line with the description or measles given by physicians in England in the past. The sequence and associations of epithelial changes in the African child may perhaps be summarized as follows:



It seems that the danger of an attack of measles depends largely on the severity and extent of the epithelial lesions. The fact may provide a clue to differences in the epidemiological picture. Conceivably, a poor state of nutrition may make the epithelial surfaces more vulnerable.

SUMMARY

1. An investigation of measles was made in Nigeria as part of a longitudinal study of 405 village children, supplemented by observations on 1,283 children admitted to a Mission Hospital with the disease.

2. Measles was found to be the most serious of the acute infectious diseases of Nigerian children. During the Imesi village study measles played a major part in the death of 15, or 7 % of the 222 children who were seen with measles. The overall case mortality in Nigeria is believed to be in the region of 5 %. For children admitted to hospital with the complications of measles, a mortality in excess of 20 % is not infrequent. This contrasts with the present situation in Northern Europe and America, where measles is of relatively minor public health importance.

3. Bronchopneumonia was present in nearly half the children needing admission to hospital. Of those with bronchopneumonia, 28 % died.

4. Diarrhoea occurred in all stages of the disease, but was most common and severe during and following the period of desquamation. Treatment by parenteral fluid to combat dehydration was required in 55 % of children admitted with this complication.

5. Extensive loss of weight was associated with the disease in the majority of children. In the village study nearly one child in four lost 10 % of his former weight. The mean time taken to recover former weight was 7·2 weeks.

6. Children frequently developed marasmus and kwashiorkor after an attack of measles.

7. Striking appearances were observed in the rash and subsequently in the skin. In some children the rash darkened in colour in the manner described by Rhazes and other early writers. Extensive desquamation often appeared after the rash. The desquamation was most severe when the rash darkened.

8. The picture of measles in this study resembles descriptions of the disease to be found in the older literature. Accounts of the incidence and severity of bronchopneumonia, laryngitis, diarrhoea and weight loss before 1920 are presented to illustrate this similarity.

9. Possible reasons for differences in the frequency and severity of measles in different places and epochs are discussed. Differences in strains of the virus and host immunity seem unlikely factors, but liability to secondary infections may be of some importance. It is concluded that the severity of the disease is related to the manifestations of the rash. A dark rash, followed by profuse desquamation, is associated with equivalent changes in the larynx, bronchus, and intestines, which are likely to be responsible for the occurrence of bronchopneumonia, laryngitis and diarrhoea. Defective nutrition is a possible cause of the 'vulnerability' of the epithelium.

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REFERENCES

Annual Report of the Registrar-General for Scotland, 1908, p. 284.

Avery, T. (1962). Private communication.

Balme, H. (1904). The signs and symptoms of measles in relation to diagnosis and prognosis, *Practitioner*, ii, 504.

Boué, A. (1962). Private communication.

Brincker, J. A. H. (1938). A historical, epidemiological and aetiological study of measles, *Proc. R. Soc. Med.* **31**, 807.

Chalmers, A. K. (1930). *The Health of Glasgow*, 1818–1925, p. 340. Glasgow: Bell and Bain.

Cantrelle, P. A., Etifier, J. & Masse, N. (1960). *Journées Africaines de Pédiatrie*, p. 66. Paris: Centre International de l'Enfance.

Copland, J. (1858). *Dictionary of Practical Medicine*, vol. 2, p. 814. London: Longman, Brown, Green, Longmans and Roberts.

Creighton, C. (1894). *A History of Epidemics in Britain*, vol. 2, pp. 638, 651, 656. Cambridge University Press.

Daniell, W. F. (1852). On the epidemic rubeola of Accra, West Coast of Africa, *Dublin Quart. J. med. Sci.* **14**, 25.

Drinkwater, H. (1885). *Remarks upon the Epidemic of Measles prevalent in Sunderland*, p. 45. Edinburgh: James Thin.

Edlin (1802). Observations on measles, *Med. phys. J.* **8**, 28.

Fothergill (1751). *Observations of the Weather and Diseases.* Quoted in *Miscellaneous Works of the late R. Willan*, p. 196. London: Ashby Smith. 1821.

Gannelon, C. (1892). *La Rougeole à l'Hospice des Enfants Assistés.* Paris: G. Steinheil.

Gans, B. (1961). Paediatric problems in Lagos, *W. Afr. Med. J.* **10**, 33.

Gans, B., Macnamara, F. N., Morley, D. C., Thomson, S. W. & Watt, A. (1961). Some observations on the epidemiology of measles in West Africa, *W. Afr. Med. J.* **10**, 253.

Goodhart, J. F. & Still, G. F. (1921). *The Diseases of Children*, 11th ed., p. 223. London: Churchill.

Gregory, G. (1843). *Lectures on the Eruptive Fevers*, p. 111. London: Henry Renshaw.

Gunn, W. (1955). Sections on measles in *The British Encyclopaedia of Medical Practice* (2nd ed.) 8, 407. London: Butterworth and Co.

Holt, L. E. (1911). *The Diseases of Infancy and Childhood*; 6th ed., pp. 932, 936. New York: D. Appleton & Co.

McFie, J. & Yarom, R. (1962). Child health in Luluabourg, Congo (with a note on the age-distribution of kwashiorkor). *J. Trop. Paediat.* **7**, 123.

McGregor, I. (1962). Private communication.

Meunier, H. (1898). Sur un symptôme nouveau de la période précontagieuse de la rougeole et sur sa valeur prophylactique. *Gaz. Hebd. Méd. Chir.* **3**, 1057.

Moore, J. W. (1892). *Textbook of the Eruptive and Continued Fevers*, p. 140, Dublin: Fannin and Co.

Morley, D. C. & MacWilliam, K. M. (1961). Measles in a Nigerian Community. *W. Afr. Med. J.* **10**, 246.

Morley, D. C. (1962). Measles in Nigeria. *Amer. J. Dis. Child.* **103**, 230.

Murphy, E. (1962). Personal communication.

Paterson, D. & Lightwood, R. (1956). *Sick Children*, 7th ed., p. 516. London: Cassell.

Platt, B. S. (1957). Protein malnutrition and infection. *Amer. J. Trop. Med.* **6**, 773.

Radbill, S. X. & Hamilton, G. R. (1960). Measles in fact and fancy. *Bull. Hist. Med.* **34**, 430.

Reed, F. S. (1957). Measles and folklore. *W. Afr. Med. J.* **6**, 39.

Registrar-General's Statistical Review of England and Wales, 1960, Part I, Medical, p. 182.

Reports of the Local Government Board, Public Health and Medical Subjects (1918). New Series, no. 115, p. 7. London: H.M.S.O.

RHAZES, (A.D. 850). *A Treatise on the Smallpox and Measles.* Divisio Morborum, Cap. 149, Syd. Soc., London, 1848.

SCRIMSHAW, N. S., TAYLOR, C. E. & GORDON, J. E. (1959). Interactions of nutrition and infection, *Amer. J. med. Sci.* **237**, 367.

SCRIMSHAW, N. S. (1961). Nutrition and infection in *Recent Advances in Human Nutrition*, by Brock, J. F., p. 375. London: Churchill.

SCRIMSHAW, N. S. (1962). Private communication.

SENECAL, J., AUBRY, L. & FALADE, S. (1962). Infectious diseases in the child of pre-school age in Senegal. *W. Afr. Med. J.* **11**, 93.

SYDENHAM, T. (1674). *The Works of Thomas Sydenham*, vol. 1, p. 185, Syd. Soc., London, 1848.

THURSFIELD, H. (1914). Causes of death in measles in 42*nd Annual Report of the Local Government Board*, 1912–13, Supplement containing the Report of the Medical Officer, appendix B, no. 2, p. 370. London: His Majesty's Stationery Office.

TROUSSEAU, A. (1869). *Lectures on Clinical Medicine*, vol. 2, p. 228. New Sydenham Society.

Vital Statistics Rates in the U.S., 1900–1940 (1947). P. 284. Washington: U.S. Govt. Printing Office.

WATSON, G. I. (1956). The complications of measles, Supplement to Research Newsletter, No. **11**, of the College of General Practitioners.

WHITBOURNE, D. (1930). Notes on the infant mortality of the colony of Lagos, Nigeria. *W. Afr. Med. J.* **4**, 3.

WILLAN, R. (1821). In *Miscellaneous Works of the Late R. Willan*, pp. 139, 288, Ashby Smith (Ed.), Cadell, Strand, London.

WILLIAMS, D. (1906). *Allbutt and Rolleston's A System of Medicine*, vol. 2, part 1, pp. 390, 394. London: MacMillan and Co.

DESCRIPTION OF PLATE

Measles in an 8 months old child. The rash has blackened and areas of skin are desquamating.

J. Hyg., Camb. (1963), **61**, 135
Printed in Great Britain

The clinical reaction of Nigerian children to measles vaccine with and without gamma globulin

By D. MORLEY, M.D.*
Child Health Unit, West African Council for Medical Research, Wesley Guild Hospital, Ilesha, W. Nigeria,

S. L. KATZ, M.D.
Department of Paediatrics, Harvard Medical School, Children's Hospital Medical Centre, Boston

AND S. KRUGMAN, M.D.
Professor of Paediatrics, New York University School of Medicine

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In earlier papers (Morley, 1962; Morley, Woodland & Martin, 1963) the manifestations of measles in Nigerian children were described and it was shown that measles in these children was outstanding among the acute infectious diseases as a cause of death. The prevalence and seriousness of the disease prompted a trial of live attenuated measles vaccine in which advantage was taken of the experience with measles vaccine gained in the U.S.A. by two of the authors (S.L.K. and S.K.). In this paper the clinical reaction of 500 children to the attenuated vaccine will be reported. Among the purposes of the trial was to ascertain whether the reaction of the African child, who is often malnourished, differs from that of the American child.

A more limited trial of measles vaccine in Ibadan, Nigeria, has been reported by Collard and his associates (1961).

RESULTS

The trial was made in the village of Imesi, and among out-patients attending the Ilesha Hospital. It was divided into three stages as follows:

Stage 1. *Preliminary observations on the effect of vaccine*†

Twenty-six children were given Enders 'B' liquid measles vaccine (Merck, U.S.A. Lot no. 15) which was flown to Nigeria in solid carbon dioxide. A similar control group were given pertussis/tetanus vaccine.

* Dr Morley is now in the Department of Human Nutrition of the London School of Hygiene and Tropical Medicine.

† The vaccines and gamma globulin were supplied by Dr Maurice Hilleman, Director, Merck Institute for Therapeutic Research.

Stage 2. Effect of dry vaccine alone

Lyophilized vaccine (Merck, Lot no. 22) was given to 120 children; this was expected to give them permanent immunity to measles. A similar control group of children was given an injection of gamma globulin together with some inert material; this was expected to give them a temporary immunity from the severe effects of measles.

Stage 3. Effect of dry vaccine modified by gamma globulin

Dry vaccine (Lot no. 22) was given to over 800 children with a simultaneous injection of gamma globulin. A control group received gamma globulin plus inert material. This stage was similar to stage 2 except that an attempt was made to modify the reaction to the vaccine by the use of gamma globulin. The observations on the reaction to the inoculation were confined to 378 vaccinated children, and 367 children in the control group.

The inoculations in stage 1 were given in the village of Imesi where a 'longitudinal' health study of a group of children is in progress. The children were seen twice daily at the local clinic from the 5th to 14th day after inoculation. The co-operation of the mothers was good and a remarkable attendance of 100% was achieved. Blood specimens were taken immediately before inoculation and again 3 weeks later. It was found, however, that because of local beliefs the parents disliked having blood taken from their children. Because of this attitude, and also because of shortage of staff, specimens of blood were not taken in the later two stages of the trial.

The preliminary first stage was undertaken in November 1960. Measles was not present in Imesi at that time. The second and third stages were undertaken from February 1961 onwards in Ilesha, when measles was becoming increasingly common. A number of children receiving gamma globulin had a mild attenuated attack but were spared serious or fatal illness. The occurrence of mild measles in these children made the interpretation of the results more difficult.

The children given the inoculations were between the ages of 6 months and 2 years. As the last epidemic had subsided only 6 months previously, the records of all the inoculated children, particularly those over a year old, had to be examined, and the mothers questioned, to discover whether measles had occurred previously.

Age and weight of vaccinated and control children

The mean age and weight of the boys and girls in the vaccinated and control groups are shown in Table 1. It will be seen that there was no statistical difference between the two groups in respect of age and weight.

Inoculation and follow-up techniques

On the day the child was registered, an injection of gamma globulin (0·02 ml. per pound of body weight containing 40 measles antibody units per ml.) was given into the left buttock, and either 0·50 ml. of the vaccine or 0·50 ml. of inert material into the right buttock. A system of random numbers was used, but it was arranged

that all children attending on 1 day should receive the same inoculation to make recording errors less likely. The mothers were told and understood that only half of the children would be fully protected. The above relates to stage 3. The procedure followed in stage 2 was similar, except that no gamma globulin was given to the children receiving vaccine.

Table 1. *Age and weight of children in stage* 2 *and* 3 *of the measles vaccine trial*

	Age (months)		Weight (lb.)	
	Vaccine	Control	Vaccine	Control
Boys	13·50 ± 0·32	13·92 ± 0·31	18·30 ± 0·22	18·67 ± 0·19
Girls	12·91 ± 0·28	13·15 ± 0·30	17·03 ± 0·20	17·47 ± 0·18

The children should have been seen on the 8th, 10th and 12th day after inoculation. This was in fact done in the third stage. In the second stage, however, owing to a misunderstanding, the children were brought back on the 7th, 9th and 11th day. In this stage they were brought to the hospital at 4 p.m. (16.00 hours), but an afternoon visit was found to be difficult, and in the third stage they were seen at 7 a.m. (07.00 hours). Because of these differences, together with the fact that the third stage took place when there was more infection in the community, the second and third stages cannot be directly compared.

Only a very simple follow-up examination for children in stage 2 and 3 was possible. Rectal temperatures were taken by a nurse, and the children were then briefly seen in bright daylight by the paediatrician. At the same time the mother was asked if the child was well, and any complaints were recorded. The paediatrician did not know whether the children were in the vaccine or control group.

Sterile disposable syringes were used. One of the children developed a small injection abscess that required incision. One mother also said that the site of injection was painful.

Results of the first stage

Serological investigations undertaken in Dr Enders' laboratories in Boston revealed the following: of the 26 children, 7 showed evidence of previous measles, with a low titre of complement fixing antibodies in the first specimen taken and no rise in the second. In the remaining children there was a strong response to vaccination, with titres in the second specimen equal to or greater than 1:512. This is a higher titre than usually observed among American children, using an identical technique in the same laboratory.

The clinical reactions are set out in Table 2. There was a high incidence of minor reactions in the vaccinated group, but reactions were also high in the children who were already immune, and also in the control group. Over half the inoculated children had a rash and diarrhoea: in many children the distinction between a vaccine and a 'sweat' rash was difficult, and was not attempted. None of the children was, however, seriously upset, and their reaction resembled that seen by two of us (S.L.K. and S.K.) in American children receiving measles vaccine. On the basis of the results, it was considered safe to proceed with the next stages.

Results of the second and third stages

In stage 2 the reaction of children given vaccine was compared with that of children given gamma globulin and inert material. In stage 3 the comparison was between children given vaccine and gamma globulin together, and children given gamma globulin and inert material together. The results were transferred to punch cards and mechanically analysed.

Table 2. *Clinical reactions of children receiving live attenuated measles vaccine, stage 1 of the trial*

	Children receiving vaccine		Controls receiving pertussis/tetanus
	Non-immunes (19)	Immunes (7)	(26)
Fever (101° F.–105° F. 38·3° C–40·6° C.)	19	4	11
Catarrh	14	6	10
Loose stools	11	3	7
Restlessness	6	3	5
Refused solid food	15	3	13
Refused breast	0	0	0
Rash	10	4	5

Fever

The incidence of fever is shown diagramatically in Fig. 1. It will be seen that 38 % of the African children given the vaccine alone developed temperatures between 101° F. (38·3° C.) and 102° F. (38·9° C.), and 19 % temperatures of 103° F. (39·4° C.) and over. Less fever occurred in the group given vaccine plus gamma globulin, the corresponding percentages being 20 and 6. Data relating to American children are included in Fig. 1 for purposes of comparison. Less fever was recorded among the African than the American children. The difference is, however, probably due to the fact that the American children had their temperatures taken twice daily for 18 days, while the African children were available on only 3 days for temperature recordings. In a few instances, the temperatures of the African children were taken more often, because their mothers considered them unwell and brought them specially to the clinic. In general African mothers do not regard fever in their children as anything very unusual, and few showed concern about the febrile reaction produced by the vaccine.

In the Nigerian community in which the trials were made, there is always a proportion of children with marasmus, or in danger of developing kwashiorkor. It was felt possible that such children might react differently to measles vaccine than children in a better stage of nutrition. To test this possibility, the temperature records were separated by sex and age in months and then divided by weight into percentiles. The temperatures in the percentile groups are shown in Fig. 2. The children of below the local average weight in the left hand column (10 and 25 percentile groups) fared no worse than those with a relatively good weight in the 90 and 75 percentile groups.

The incidence of fever following vaccination is of considerable importance, since high fever from any cause is likely to cause convulsions, a serious event which tends to disturb both the parent and the doctor more than most other illnesses. In the present trials no children with convulsions were seen, but eight mothers gave

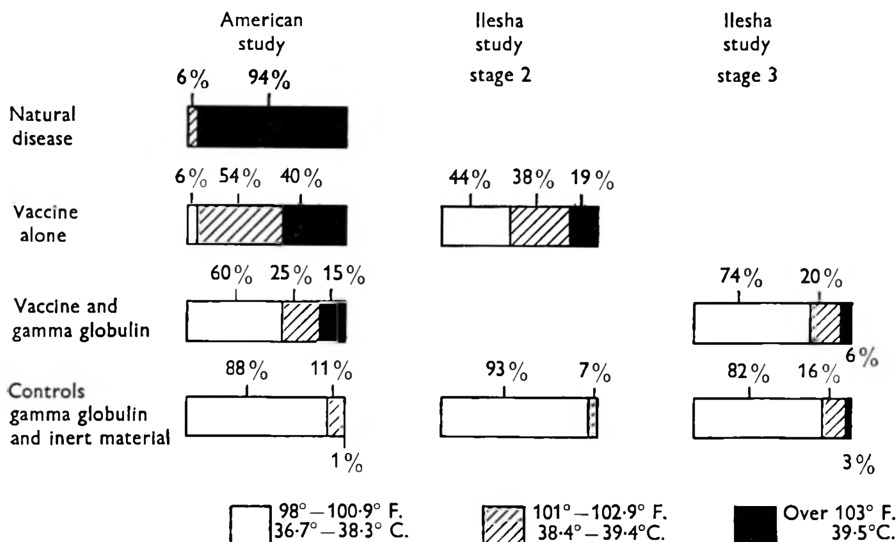

Fig. 1. Immunization with live attenuated measles virus vaccine. Incidence of fever in American and African children. The incidence of fever following measles virus vaccination is similar in American and Nigerian children. The American children (Krugman, Giles, Milton Jacobs & Friedman, 1962) had their temperatures recorded more frequently which may account for a slightly higher recorded incidence of fever.

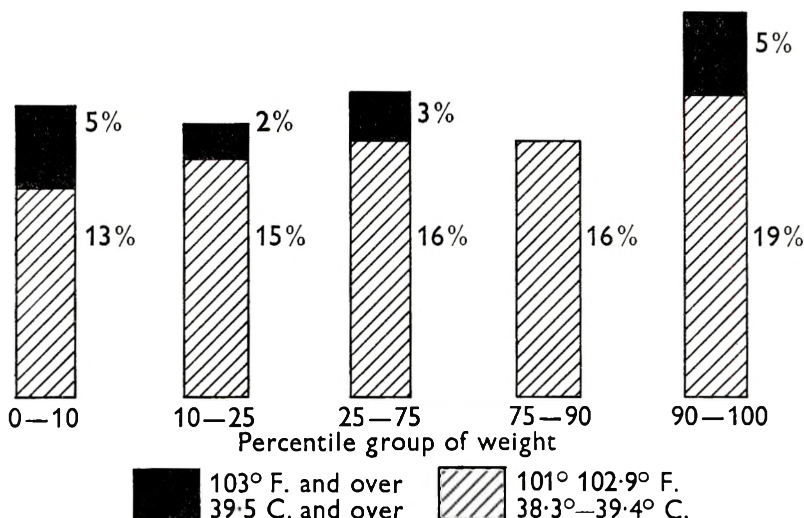

Fig. 2. 441 Nigerian children, standardized by age and sex, receiving measles vaccine. Percentage distribution of fever (over 100·9° F., 38·3° C.) by weight percentile. In this figure is set out the incidence of febrile reaction in each weight group. There is no evidence that children in the low percentile group, who are below average weight for this community have more fever than children of above average weight.

suggestive histories; these related to five children given vaccine and three given gamma globulin and inert material. There was nothing to suggest that the vaccine had any specific effect in producing convulsions.

Cough

Respiratory infections were prevalent among the children in the community at the time when the trials were being made. In stage 2, complaints of cough in their children were made by 47 and 39 mothers in the two groups respectively, while in stage 3 the incidence of cough so reported was also approximately the same in both groups.

Diarrhoea

In a previous paper one of the authors (Morley, 1962) reported that diarrhoea is frequently associated with the acute stages of measles and that the child is apparently more susceptible to diarrhoea in the ensuing months. In the trials diarrhoea was more frequent among the children who received vaccine alone; 31 % of the mothers complained of abnormally watery and frequent stools in their children, and among the controls only 19 %. In no case was the diarrhoea severe. Among the 356 children who had vaccine and gamma globulin and were followed up, 22 % had diarrhoea, and of 335 controls, 21 %. Possibly diarrhoea is part of the reaction to measles vaccine that is suppressed by gamma globulin.

Rash

Among the vaccinated children in stage 2 who did not have gamma globulin the rash was recognized in 25 %. American workers have observed rash in 50–60 % of white children given the vaccine without gamma globulin. This difference may be due to the fact that the African children were seen only on alternate days, and the measles vaccine rash may appear for only a brief period and is not easily detected on a dark skin. The incidence of rash in stage 3 amongst the children given vaccine and gamma globulin was similar to that in the control group.

Comparison of the incidence of cough, diarrhoea and rash in the various weight groups showed no higher incidence among underweight children in the 10 and 25 percentile groups than in children of greater weight in the 90 and 75 percentile groups.

Treatment of conditions appearing during the trials

Coughs were treated with a simple cough mixture. For fever, chloroquin was given as a routine, and an aspirin mixture in many cases. Children who apparently had true measles were given sulphonamide, with penicillin if there were respiratory signs. Diarrhoea was treated with a routine mixture containing sodium and potassium chloride, which the mothers were instructed to add to the child's drinking water. The two children with rectal temperatures over 106° F. were both admitted to the ward for a few hours. Neither seemed to be ill, and after tepid sponging and observation the mothers were allowed to take them home. Both were well when seen on the following day.

DISCUSSION

The trials described here are open to criticism on the following grounds: the children were routinely seen only on 3 of the days on which they were likely to have reactions: the examination they were given could not be more than cursory; 40–50 children had to be seen each morning in addition to the routine work of a heavy paediatric unit. However, they were seen in a very good light, fully stripped, on their mother's knees. The authors are confident that any major abnormality would have been noticed.

SUMMARY

1. The severity of measles in infants and young children in Ilesha in Nigeria suggested this as a suitable centre for a trial of measles vaccine.

2. The trial was divided into three stages. In the first the vaccine was given to a group of 26 children who could be kept under close observation and were seen twice daily. In the second the vaccine was given to 120 children, a similar group receiving gamma globulin and an inert material. In the third stage the effect of vaccine and gamma globulin given as separate injections to 378 children, was compared with that of gamma globulin and inert material given to another control group. The second and third stages were run on the 'double blind' principle.

3. The children receiving vaccine alone showed fairly severe reactions but none were disabled by them; for example, 19 % had fever over 103° F. The reactions were similar to those observed in American children, with the one exception that diarrhoea was seen amongst African children receiving vaccine without gamma globulin. Those receiving vaccine and gamma globulin had only minor reactions. In the vaccinated children there was a notable absence of the severe complications seen with the natural disease in Nigeria.

4. Children who were well below the average weight for their age and sex in this community did not show more severe reactions than those above the average weight.

We wish to thank Mrs J. D. Harris and Mr W. E. Bird of the Machine Accountancy Department of Burroughs Wellcome for assistance in the mechanical sorting of the records, and Dr W. J. Martin, Ph.D., D.Sc., of the Medical Research Council's Statistical Unit for statistical help.

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REFERENCES

Collard, P., Hendrickse, R. G., Montefiore, D., Sherman, P., Van der Wall, H. M., Morley, D., Goffe, A. P., Laurence, G. D., & Pollock, T. M. (1961). Vaccination against measles. Part II. *Brit. med. J.* ii, 1246.

Krugman, S., Giles, J. P., Milton Jacobs, A. & Friedman, H. (1962). Studies with live attenuated measles-virus vaccine. *Amer. J. Dis. Child.* **103**, 353.

Morley, D. C. (1962). Measles in Nigeria. *Amer. J. Dis. Child.* **103**, 230.

Morley, D. C., Woodland, M. & Martin, W. J. (1963). Measles in Nigerian children, *J. Hyg., Camb.*, **61**, 115.

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Efficacy of measles vaccine

By J. HARTFIELD, M.B., D.A., D.Obst.R.C.O.G.
and D. MORLEY, M.D., D.C.H.*
Child Health Research Unit of the West African Council for Medical Research, Wesley Guild Hospital, Ilesha, W. Nigeria

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In previous papers the incidence and severity of measles (Morley, 1962; Morley, Woodland & Martin, 1963) and the clinical reactions of Nigerian children to Enders 'B' live attenuated vaccine (Morley, Katz & Krugman, 1963) in West Africa, have been described. In this paper the results of the use of this vaccine in preventing measles are recorded.

Vaccination trials were made on two groups of children. The first consisted of 53 children from the village of Imesi, where a longitudinal child health study has been undertaken, and the second of 2000 children attending the 'under fives' clinic at the Wesley Guild Hospital in Ilesha.

THE IMESI VILLAGE GROUP

These children were involved in the preliminary trial of measles vaccine described under stage 1 of the preceding paper. They were paired as far as possible by sex, age and weight. Twenty-six children received live liquid measles vaccine; the 27 controls were given pertussis/tetanus vaccine. This was not a blind study, since the investigators knew which children had received measles vaccine. The children were followed for 18 months; during this period there was one minor and one major outbreak of measles in the village. The incidence of measles in the groups is set out in Table 1.

Measles was not seen in any of the children in the vaccine group, as opposed to 19 in the control group. The three deaths, of which two were due to measles, were all in the control group.

THE ILESHA HOSPITAL GROUP

This group was drawn from the 'under fives' clinic at the Wesley Guild Hospital, Ilesha, and included all the children in Stages 2 and 3 described in the preceding paper (Morley, Katz & Krugman, 1963), and a further 1000 on whom observations on the severity of the vaccine reaction were not made. The children have been followed through a period varying from 6 to 20 months. All have been exposed in one epidemic of measles, and some in two.

Vaccination was offered to children between the age of 6 months and 2 years. The mothers were warned that it would be effective in only half the children, but in spite of this over 2000 mothers brought their children for vaccination within

* Present address: Department of Human Nutrition, London School of Hygiene and Tropical Medicine, Keppel St, Gower St, London, W.C. 1.

14 months. By a system of random numbers, these children were divided into two equal groups. One group received measles vaccine, the first 120 without gamma globulin, and the remainder with gamma globulin at a dosage of 0·02 ml., equivalent to 40 units of measles antibody, per pound of body weight. It has been found that this dosage of gamma globulin represents approximately the amount needed to suppress post-vaccination reactions.

Table 1. *Incidence of measles in children receiving measles vaccine and in the control group*

	Number in group	Clinical measles	Deaths: Measles	Deaths: Other causes	Number observed for 18 months	Number that left the village
Vaccine group (given liquid measles (vaccine)	26	0	0	0	23	3
Control group (Given pertussis/tetanus vaccine)	27	19	2	1	22	2

The other group was a control one, receiving gamma globulin and inert material identical with that used in the culture of the virus; the children in this group only received temporary protection from the severe effects of measles.

In the follow-up period all children with 'measles-like' illness were seen, as far as possible, by one of us. The diagnosis of measles was at times difficult. Illnesses resembling measles were frequently seen; facilities for serological confirmation of the diagnosis were not available.

At the end of the trial period at the Ilesha Hospital 1962 record cards were available. Among these 272 showed a record of an illness suggestive of measles. Eighty of these illnesses were among the 991 children who had received vaccine, and 192 among the control 971 children. Successful vaccination against measles is believed to produce a life-long immunity, and there was no reason to believe that the vaccination was unsuccessful in a large proportion of the Ilesha children. It seems probable that the occurrence of so much 'measles-like' illness in the vaccinated children was a reflexion of the difficulty in making a firm diagnosis of measles in the African child at one visit. The problem of diagnosis also arose in the longitudinal study described in a preceding paper. The children in that study were, however, seen on many occasions during the illness, and there was less difficulty in deciding which illness was likely to be true measles. In the present study the follow-up had to be made in a crowded and over-worked clinic. Many of the children were seen only once and in the later stages of illness, and it was impossible to study each case individually and follow the illness through.

Among the children in this trial there were 17 known deaths. Six were due to causes other than measles, 5 of which were in the vaccinated group and 1 in the control group. Eleven deaths occurred during or immediately following measles. All of these were in the control group.

WEIGHT-GAIN FOLLOWING MEASLES VACCINATION

In a previous paper (Morley, Woodland & Martin, 1963) measles was shown to be responsible for a severe weight loss in a group of Nigerian village children. One-quarter of the children surveyed lost 10 % or more of their weight. In 15 % the weight lost was not recovered for more than 3 months. These findings were in a group of children under 3 years old, at a time when the normal child gains weight steadily. As a result of this experience an attempt was made to see whether the incidence of measles in the control group affected their weight-gain in the months following vaccination as compared with the group that were vaccinated and presumably were protected against measles. Mothers were encouraged to bring their children to be reweighed at the end of the trial. As the children had been followed for periods that varied between 6 and 20 months the average weight-gain per month for each child was estimated. The mean monthly weight in 283 vaccinated and 273 control children subdivided into age groups are set out in Table 2.

Table 2. *Mean weight-gain per month in children following measles vaccination compared with a non-vaccinated group*

Age in months	Vaccinated: Number	Vaccinated: Mean gain (oz)	Controls: Number	Controls: Mean gain (oz.)	Difference (vaccinated − control)
6–8	69	8·82	78	7·88	0·94 ± 0·62
9–11	90	7·89	82	7·25	0·64 ± 0·47
12–14	71	7·81	54	6·47	1·34 ± 0·70
15–17	36	7·42	33	6·77	0·65 ± 0·97
18–20	17	7·76	26	6·90	0·86 ± 1·2

The mean gain in the controls was lower than in the vaccinated. The differences for each age group are not statistically significant, but if the consistency of the five differences is tested the probability is significant ($P = 0{\cdot}006$). There is at least a suggestion that the mean gain in the control children was influenced by the occurrence of measles in some children in this group.

DISCUSSION

There is already good evidence that Enders 'B' vaccine will protect children against measles. This is based both on clinical observation, and laboratory studies of antibody titres (Krugman, Giles & Jacobs, 1960; McCrumb, Kness, Saunders, Snyder & Schleuderberg, 1961). The protection is known to last for two years and may be life-long (Krugman *et al.* 1960; Kempe, Ott, St Vincent & Maisel, 1960). When Enders 'B' vaccine alone was used, a serological conversion rate of 96·5 % was achieved. If gamma globulin is given simultaneously at a level of 40 units per pound of body weight, there is a slight fall in the conversion rate to 90·5 % (Krugman, Giles, Jacobs & Friedman, 1962).

The findings in this study further support the protective value of the vaccine. Information on the deaths of 11 children from measles was obtained; all 11 were

found to be in the control group. An attempt was also made to identify measles in the children in the out-patient clinic during 20 months after the start of the trial. As children were frequently only seen once and at any stage of the disease, they were broadly classified as having a 'measles-like' illness. The number with such an illness in the control group far exceeded that in the vaccinated group.

In this population measles is believed to be the most common infection to precipitate kwashiorkor (Gans, 1961; Morley, Woodland & Martin, 1963). In this trial the vaccinated children showed a better mean gain in weight subsequent to inoculation than the control children, many of whom are likely to have developed the disease. We believe that measles vaccination may well prove to be an important step in reducing the incidence of overt malnutrition in Nigeria.

Carrying out blind controlled trials in any community with a vaccine which may produce a severe reaction carries a considerable moral responsibility. The authors felt that, because of the difficulty of explaining the purpose of the trials to the people, these responsibilities are particularly heavy in an under-developed community. In justification of the trials, it may be pointed out that in Nigeria measles is a severe and often fatal disease held in awe by the people. It is the greatest single cause of child admission to the Wesley Guild Hospital, and, as stated in a previous paper, is a major hazard of Nigerian childhood with an overall mortality of around 5 %. Reactions to the measles vaccine, which might be considered too severe in a community where measles has a low morbidity and a negligible mortality, are less important when they are an alternative to a dangerous illness. Moreover, the authors are confident that the vaccine saved the lives of a few children, and that measles in a number of children in the control group in whom naturally acquired measles occurred, was attenuated by gamma globulin.

In a recent editorial in the *British Medical Journal* (11 November 1961) doubts were expressed whether there was sufficient evidence to justify extensive trials of measles vaccine. Since this article appeared, an International Conference on Measles has been held in Washington D.C., the results of which have been published in the A.M.A. *Journal of Diseases of Children*. The experience from several sources of measles vaccine presented at this conference should go a long way to establish the safety of the vaccine and justify larger scale trials. Using a combination of vaccine and gamma globulin, or a more attenuated vaccine such as that described by Schwarz (1962), the health services of West Africa should have at their disposal an effective method of preventing measles with all the hazards that this disease brings to West African children.

SUMMARY

1. Approximately 1000 Nigerian children between the age of 6 months and 2 years were given Enders 'B' measles vaccine, combined with gamma globulin in all except 120 children. A control group of similar numbers received gamma globulin alone plus inert material.

2. In a preliminary village study the 26 vaccinated children remained free of measles, 19 of the 27 control children developed measles and 2 of these children died.

3. In a blind study among children attending the large child out-patient clinic at the Ilesha hospital, a follow up was also attempted but was more difficult. In all, 272 cases of 'measles-like' illness were seen, 192 in control children, and 80 in the vaccinated group. The number in the vaccinated group is believed to be a reflexion of the difficulties under which this diagnosis was made, rather than a failure of the vaccine to immunize. Eleven children are known to have died from measles. All these 11 were in the control group who had not had vaccine.

4. The control children showed a smaller mean gain in weight than the vaccinated in the months following inoculation. The difference may have been due to poor weight-gain among the control children who developed measles.

This work was supported in part by the Health Research Council of the City of New York under contract U 1056 and by the office of the Surgeon General, Department of the U.S. Army, sponsored by the Commission on Viral Infection Armed Forces Epidemiological Board. We wish to thank Dr W. J. Martin, Ph.D., D.Sc. and Miss I. Allen of the Medical Research Council's Statistical Unit for Statistical help.

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REFERENCES

Gans, B. (1961). Paediatric problems in Lagos. *W. Afr. med. J.* **10**, 33.

Katz, S. L., Enders, J. F. & Holloway, A. (1960). Studies on an attenuated measles virus vaccine. II. *New England J. Med.* **263**, 159.

Kempe, C. H., Ott, E. W., St Vincent, L. & Maisel, J. C. (1960). Studies on an attenuated measles virus vaccine. III. *New England J. Med.* **263**, 161.

Krugman, S., Giles, J. P. & Jacobs, A. M. (1960). Studies on an attenuated measles virus vaccine. IV. *New England J. Med.* **263**, 174.

Krugman, S., Giles, J. P., Jacobs, A. M. & Friedman, A. (1962). Studies with live attenuated measles virus vaccine. *Amer. J. Dis. Child.* **103**, 353.

McCrumb, F. R., Jr., Kress, S., Saunders, E., Snyder, M. J. & Schleuderberg, A. E. (1961). Studies with live attenuated measles virus vaccine. *Amer. J. Dis. Child.* **101**, 689.

Morley, D. C. (1962). Measles in Nigeria. *Amer. J. Dis. Child.* **103**, 230.

Morley, D. C., Katz, S. L. & Krugman, S. (1963). The clinical reaction of Nigerian children to measles vaccine with and without gamma globulin. *J. Hyg., Camb.*, **61**, 135.

Morley, D. C., Woodland, M. & Martin, W. J. (1963). Measles in Nigerian children. *J. Hyg., Camb.*, **61**, 115.

Schwarz, A. J. F. (1962). Preliminary Tests of a highly attentuated measles vaccine. *Amer. J. Dis. Child.* **103**, 386.

J. Hyg., Camb. (1963), **61**, 149
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Electron microscopy of neurotropic African horse-sickness virus

By A. POLSON and D. DEEKS
C.S.I.R. and U.C.T. Virus Research Unit, Medical School, University of Cape Town, South Africa

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INTRODUCTION

African horse-sickness virus is an important member of the arthropod-borne viruses and the disease it causes is endemic on the African continent and in the Middle East. It affects all equines and the mortality rate in horses may be high. The virus was first attenuated by Alexander (1935) by intracerebral passage in mice and such attenuated strains form the basis of the polyvalent live virus vaccine which is widely used today.

Ultracentrifugation studies have shown that the infectivity is associated with components of two different sedimentation constants but that most of this activity is associated with particles having a sedimentation constant of 476 Svedberg units (Polson & Madsen, 1954).

In this communication electron micrographs of the virus are presented.

MATERIAL AND METHODS

Virus

The A 501 strain of horse-sickness virus was used in this work, and was kindly supplied by Dr R. A. Alexander, former director of Veterinary Services. The virus was injected intracerebrally into 3- to 6-day-old suckling mice and the brains harvested when the animals showed definite signs of involvement of the central nervous system 2–3 days after infection.

Thirty to sixty brains were emulsified in 0·066 M phosphate buffer of pH 7·0 for each experiment. Thirty suckling brains per 10 ml. buffer were used. After clarification of the emulsion in the model L Spinco ultracentrifuge at 10,000 rev./min. for 10 min., the supernatant fluid was stored at 21° C. before fractionation.

Virus and protein precipitant

Polyethylene glycol (p.e.g.) M.W. 6000 was found to be a suitable precipitating agent. A 32 % solution of polyethylene glycol in 0·066 M phosphate buffer pH 7·0 was prepared as stock virus precipitant (Polson, to be published).

Zone electrophoresis

The apparatus and methods for zone electrophoresis were similar to those used by Polson & Cramer (1958). Borate buffer of pH 8·6 and 40 % Analar sucrose

dissolved in borate buffer, the pH of which was finally adjusted to 8·6, were used in the zone electrophoresis column and in the formation of the sucrose concentration gradient.

Infectivity titrations

The titration of virus infectivity was carried out in mice 3–5 weeks old, using tenfold dilutions and six mice per dilution. The dose was 0·03 ml. and given by the intracerebral route.

Experimental

In preliminary tests on the most suitable concentration of polyethylene glycol for precipitation of horse-sickness virus, it was found that the maximum amount of virus was precipitated at a concentration of polymer between 3 and 4 %.

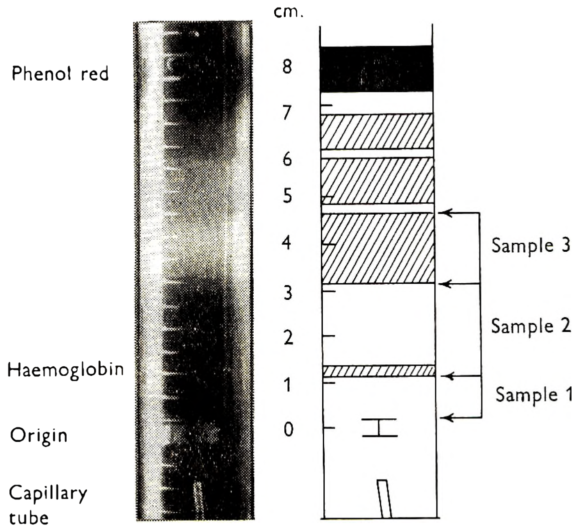


Fig. 1. Section of the zone electrophoresis column after completion of the electrophoresis run. On the left-hand side is a photograph of the column showing the different bands of opalescence and the position of the indicator substances, phenol red and haemoglobin. On the right is a diagrammatic presentation indicating the positions where the samples were taken for electron-microscopy relative to the opalescent bands (oblique lines).

To the virus suspension polyethylene glycol solution (32 %) was added to yield a final polymer concentration of 3 % and the mixture was kept in a waterbath at 21° C. A fairly heavy precipitate which formed was centrifuged off and discarded. The polyethylene glycol concentration was increased to 4 % by further addition of the 32 % solution, the reaction again being carried out at 21° C. The precipitate which formed was sedimented by centrifugation, dispersed in 10 ml. phosphate buffer and clarified at 10,000 rev./min. for 10 min. The virus in the S.N.F. was concentrated into a pellet by centrifugation at 30,000 rev./min. for 75 min. and re-dispersed in 2·2 ml. 35 % sucrose–borate–buffer mixture containing a trace of phenol red and rabbit haemoglobin. After clarification at 10,000 rev./min. for 10 min., the mixture was subjected to zone electrophoresis for 8 hr. By the end of this period the phenol red had migrated 8 cm. in the concentration gradient column.

In Text-fig. 1 the bands of opalescent material are clearly visible in a photograph of the gradient column after completion of the electrophoresis run. In Text-fig. 2 the diagram indicates the distribution of virus activity relative to the bands of opalescence. It is obvious that the virus activity is not confined to a single narrow zone of the gradient column, but that it is spread over a wide region. When comparing this diagram with Text-fig. 1, it is evident that a considerable portion of the virus activity is contained in the clear zone below the regions of the opalescence. Identical opalescent regions were also found in normal uninfected brain extracts treated in the same way as the infected material. These bands must therefore be regarded as due to normal brain components.

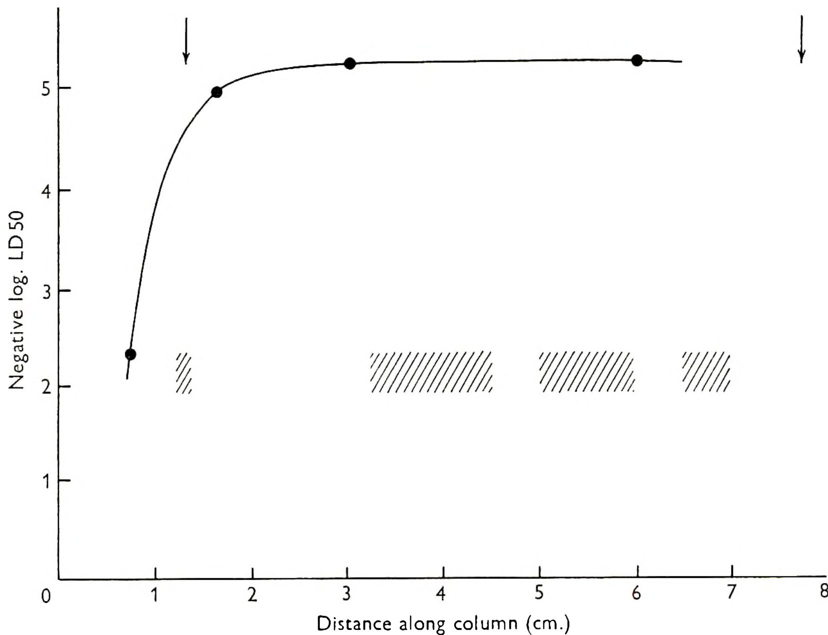


Fig. 2. Activity zone electrophoresis diagrams indicating the distribution of virus activity relative to the bands of opalescence (oblique lines). The arrows indicate the positions of the reference substances haemoglobin (left) and phenol red (right).

Each fraction taken from the column was freed of sugar by dilution with distilled water and ultracentrifugation. Two cycles of centrifugation at 33,000 rev./min. for 70 min. were adequate to reduce the concentration of the sugar to such a level that it did not interfere with electron microscopy. The final virus pellet was dispersed in 0·2 ml. of distilled water and negatively strained with an equal volume of neutral phosphotungstic acid. It was then placed on carbon films and examined in the Metropolitan Vickers EM 3A electron microscope.

RESULTS

Electron micrographs of the infected and the normal control material derived from fraction (2) (Text-fig. 1) taken at corresponding positions in the columns containing the infected and normal extracts respectively are shown in Pl. 1 (*a*)

and (*b*). Virus-like particles resembling those of *Herpes simplex* virus photographed by Horne, Brenner, Waterson & Wildy (1959) are present in Pl. 1 (*a*). These do not appear on the electron micrograph of the control material Pl. 1 (*b*). Both micrographs contain a large number of spherical bodies of varying diameter which are all smaller than the virus particles. These are regarded as normal brain constituents not removable by the present technique.

Judging from the size and shape alone, the virus particles appearing in the electron micrographs correspond with particles of sedimentation constant 476S reported for the main horse-sickness virus infective component (Polson & Madsen, 1954). It was not possible to identify virus particles with sedimentation constants lower than this in the electron micrographs on account of the presence of particles in the normal material.

Counting the number of subunits on the surface of the virus gave a figure of approximately 40. As the number of subunits visible on a negatively stained virus particle represents slightly fewer than half the actual number, it may be assumed that the particle of the A501 horse-sickness virus strain has 92 subunits on its surface by the classification of Horne & Wildy (1961). Little detail can be seen of the units, but they appear to be short rods which lie on the radii of a spherical body.

Using the polyethylene glycol precipitation technique in combination with ultracentrifugation and zone electrophoresis, successful electron micrographs of the virus have been obtained on three out of a total of five attempts. An essential in the purification procedure is to have as little delay as possible in the preparation of the electron microscope grid due to the instability of the virus in albumin-free media.

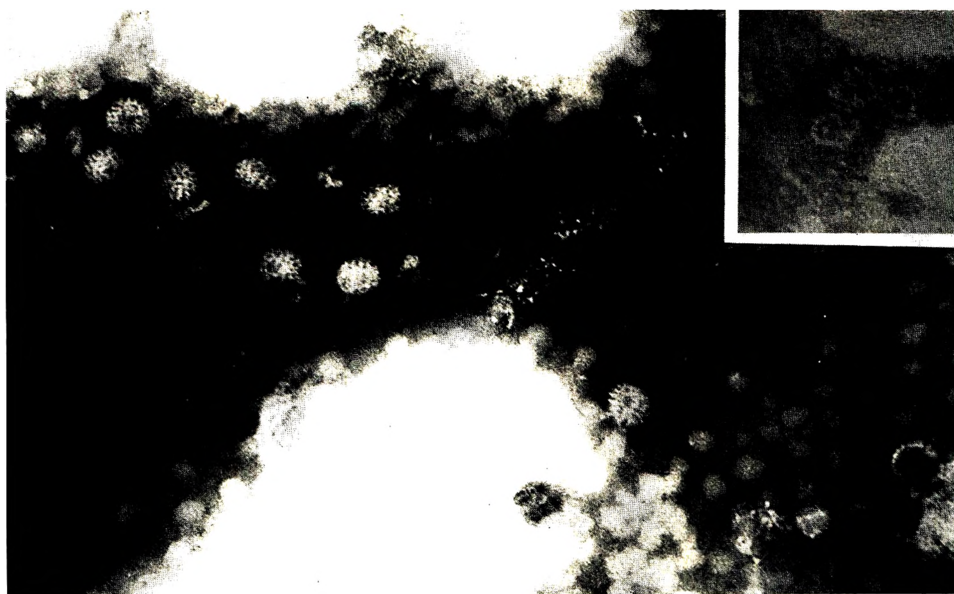
Electron micrographs of the virus have also been obtained from material collected from the fast-migrating opalescent zones (Pl. 2). The virus particles had the same appearance as those obtained from the slower-migrating clear zone of the column, but the background material was quite different.

Further attempts at purification of horse-sickness virus

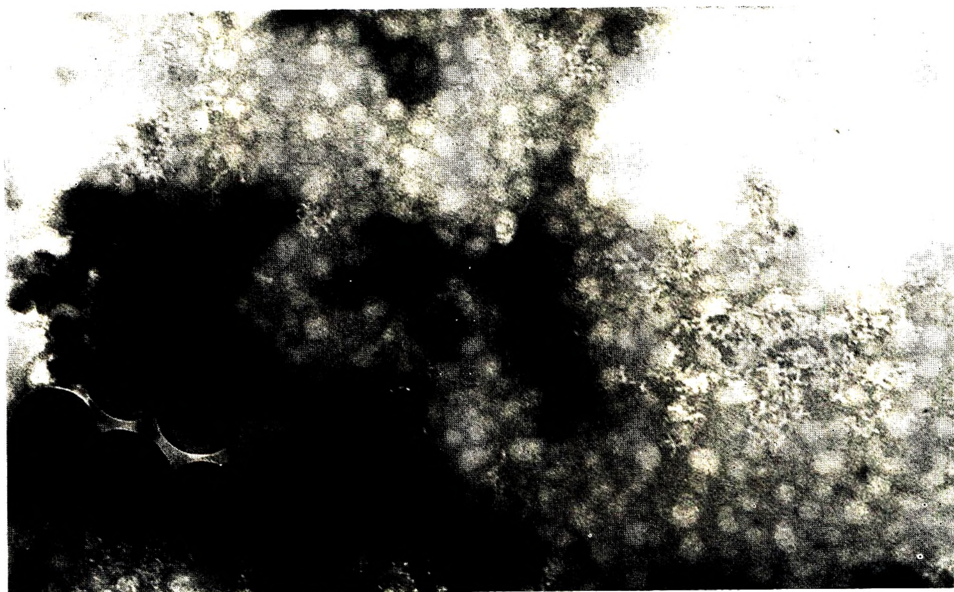
Various other methods coupled with zone electrophoresis have been tried for removal of extraneous proteins from infected brain extracts. These were treatments of the crude brain extracts with ether, chloroform and fluorocarbon, digestion of the associated proteins with trypsin and by precipitation of the virus with protamine sulphate. None of these methods was successful, as the virus was either inactivated or it was not recovered from the precipitate in any form suitable for electron-microscopy.

DISCUSSION AND SUMMARY

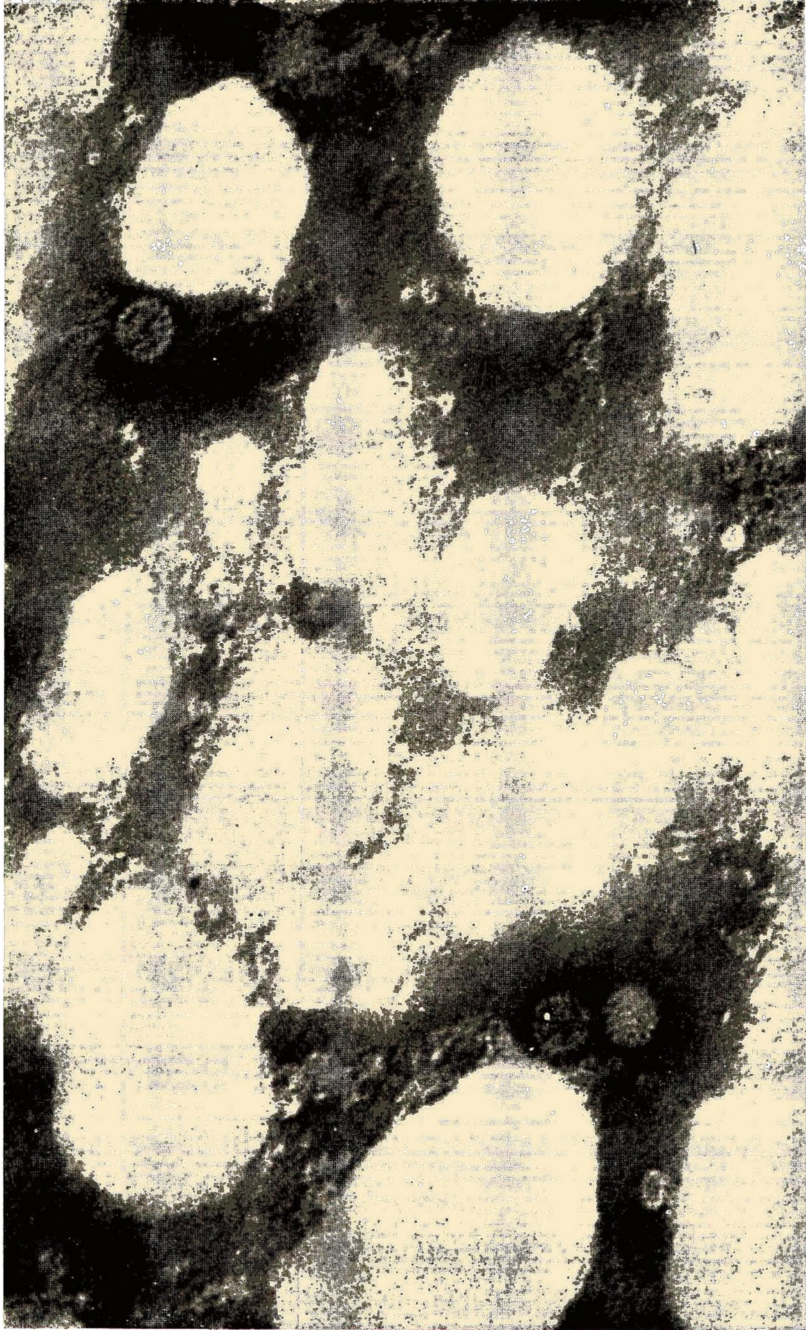
During attempts at purification of horse-sickness virus for electron-microscopy from infected mouse brain extracts, it was found that a combination of polyethylene glycol precipitation of the virus, ultracentrifugation and zone electrophoresis gave promising results. The final purified material could not be regarded as pure on account of the presence of considerable normal brain components, but



(*a*)



(*b*)



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it contained particles not present in normal brain treated identically, which could be recognized as virus particles. The virus particles appear to have diameters of 70–80 mμ and are estimated to have 92 rod-shaped subunits radiating from a spherical body.

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The electron micrographs were taken by Mr L. G. Fowle of the Department of Physics, University of Cape Town.

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REFERENCES

Alexander, R. A. (1935). *Onderstepoort J.* **4**, 349.
Polson, A. & Madsen, T. (1954). *Biochim. biophys. Acta*, **14**, 366.
Polson, A. & Cramer, R. (1958). *Biochim. biophys. Acta*, **29**, 187.
Horne, R. W., Brenner, S., Waterson, A. P. & Wildy, P. (1959). *J. mol. Biol.* **1**, 84.
Horne, R. W. & Wildy, P. (1961). *Virology*, **15**, 348.

EXPLANATION OF PLATES

Plate 1

(*a*) Infected material showing virus particles and a virus capsid lower right-hand corner. (*b*) Normal brain extracts subjected to same treatment as infected material. Note the occurrence of macromolecular material in both electron micrographs. The spherical bodies in lower left-hand corner of (*b*) are polystyrene latex particles of 138 mμ diameter. Inset in (*a*) probably virus capsids in a state of disruption at slightly lower magnification.

Plate 2

Electron micrograph of material collected in the region of heavy opalescence showing virus-like bodies. The typical macromolecular components observed in the previous micrographs are absent in this region of the gradient column.

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The distribution of adenovirus antibodies in normal children

BY C. W. POTTER AND W. I. H. SHEDDEN
From the Department of Bacteriology, Sheffield University and the Children's Hospital, Sheffield

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INTRODUCTION

Population surveys for the presence of adenovirus complement-fixing (C.F.) and neutralizing antibodies suggest that certain serotypes, particularly types 1 and 2, are endemic. Huebner, Rowe, Ward, Parrott & Betts (1954) estimated that, of children in Washington in the age group 6–11 months, over 50 % had antibodies against one or more serotypes and 30 % had antibody against type 1 or type 2. The percentages with neutralizing antibody against one or more viruses rose with age. Jordan, Badger & Dingle (1958) carried out a survey of 81 children in Cleveland over the first 5 years of life. This confirmed the endemic nature of adenovirus infection.

Whether or not a similar state of affairs exists in children of Great Britain, has never been determined. A study, similar to that of Huebner *et al.* (1954), is given here from Sheffield.

MATERIALS AND METHODS

Sera

Forty-two paired serum specimens of maternal and cord blood were supplied by Dr Bowley of the Sheffield Regional Blood Transfusion Centre, and in addition, 197 single serum specimens were collected over a 2-year period (November 1959 to November 1961) from Sheffield children coming to the Children's Hospital for examination prior to adoption or for treatment of burns, cleft palates and accidents. Specimens were not taken from children who had been previously in hospital or who had received previous blood transfusions or who were suffering from renal disease.

All serum specimens were stored at $-20°$ C.

Viruses

Adenovirus serotypes 1–7 inclusive were obtained from Dr M. H. Hambling of the Central Public Health Laboratory, Colindale. Viruses were grown on HEp 2 tissue cultures in 4 oz. medical flat bottles. Tissue cultures were harvested, when full cytopathic effect (C.P.E.) was observed, by three cycles of freezing and thawing. The cell debris was removed by centrifugation at 3000 rpm for 20 min. and the virus suspensions collected and stored at $-20°$ C. Infectivity titrations were carried out on HEp 2 tissue cultures in $4 \times \frac{1}{2}$ in. test tubes using logarithmic virus dilutions. Infectivity titres were calculated using the method of Reed & Muench (1938) on a three-day titration.

Control antisera

Rabbit hyperimmune adenovirus antisera were prepared by inoculating 5·0 ml. of neat virus suspension intravenously into rabbits on alternate days for 10 days. Test serum specimens were taken 3 weeks after the first inoculation and neutralization titres against homologous and heterologous adenovirus serotypes calculated.

Neutralization tests

Doubling dilutions of each serum were tested. Virus–serum mixtures containing 0·1 ml. of serum dilution and 0·1 ml. of virus containing 10 CPD_{50} (Binn & Hilleman, 1960) were prepared. These were left at room temperature for 1 hr. and then 0·3 ml. of maintenance medium (2 % inactivated calf serum in Medium '199') was added. The total volume, 0·5 ml., was then transferred to a tube of semi-confluent HEp 2 tissue culture cells previously washed twice with phosphate-buffered saline (Dulbecco & Vogt, 1954). For each serum dilution, two or more tissue culture tubes were inoculated.

Tubes were incubated at rest at 37° C. Virus controls showed specific C.P.E. after 24 hr. incubation and complete degeneration after 3 days. Serum neutralization titres were read after 3 days incubation and are given as the highest serum dilution showing 50 % or less C.P.E.

Complement-fixation tests

Complement-fixation tests were performed by the method of Pereira (1956).

RESULTS

Neonatal passive immunity

Forty-two pairs of sera from maternal and cord blood were tested for neutralization titres against adenovirus type 1 and 3. Serum dilutions from 1/10 to 1/320 in doubling dilutions were used. Titration end-points are given as the highest serum dilution showing 50 % or less C.P.E. when challenged by standard virus dose in HEp 2 tissue cultures.

Neutralizing antibody for adenovirus type 1 was found at 1/10 dilution or higher in 22 (52·4 %) of maternal serum specimens and in 25 (59·5 %) of corresponding cord blood specimens. Antibody was found in three foetal serum specimens at 1/10, but not in the corresponding maternal specimens. The average maternal antibody titre was 1/40, and the average cord titre was 1/50. The relative foetal/maternal antibody concentration was therefore 1·25/1·0.

Neutralizing antibody for adenovirus type 3 was detected in 19 (45·2 %) of both maternal and cord specimens. In one pair antibody was detected at 1/10 dilution of maternal serum, but not in the corresponding cord serum, and in one other case the converse was true. The average maternal titre was 1/100 and the average cord titre 1/115. The relative foetal/maternal antibody concentration was thus 1·15/1·0.

Disappearance of neonatal passively acquired adenovirus antibodies

Fifty serum specimens from children 1 year old or younger were tested for the presence of neutralizing antibody against adenovirus types 1, 2, 3, 5, 6 and 7. Figure 1 shows the number of serum specimens giving adenovirus neutralization

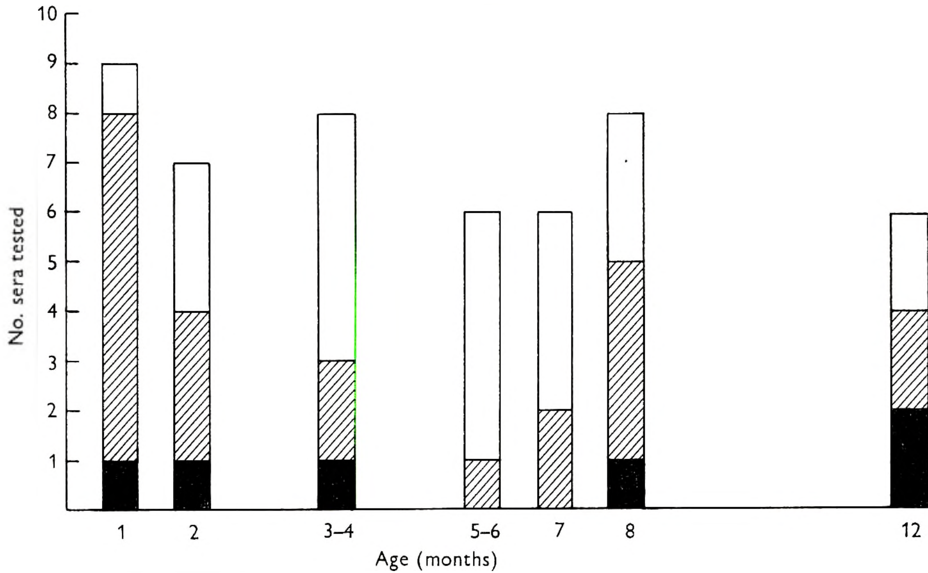

Fig. 1. Correlation of age with the presence of adenovirus neutralizing antibody. □, Number tested; ▧, number with neutralizing antibody; ■, number with antibody titres ⩾ 1:80.

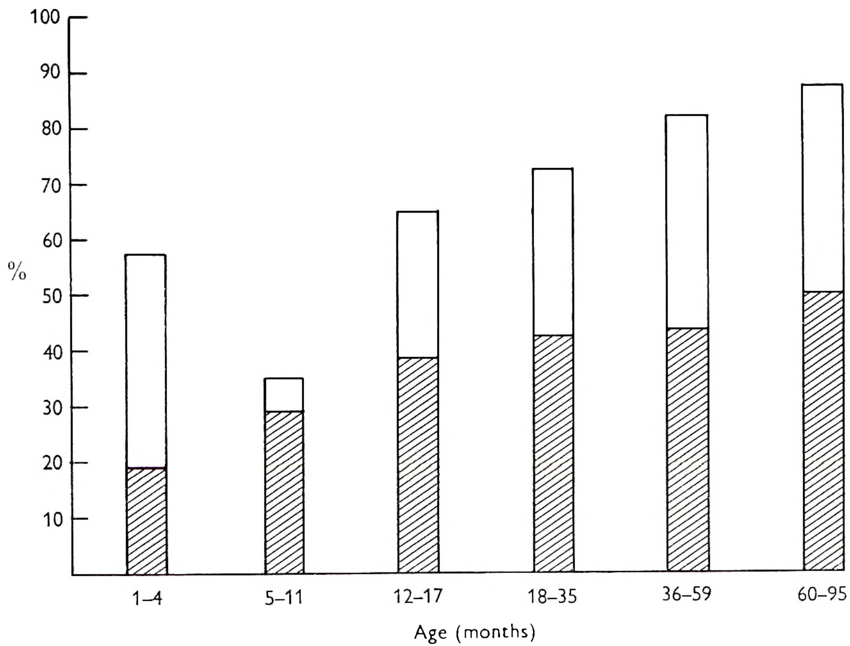

Fig. 2. Correlation of age with the presence of complement-fixing antibody and neutralizing antibody for adenovirus. □, Neutralizing antibody; ▧, complement-fixing antibody.

of any serotype at serum dilutions of 1/5 and 1/80. The neonatal level of passive immunity can be seen to have disappeared after 5 or 6 months of life.

Rate of development of active adenovirus antibodies

Figure 2 shows the percentage of children with demonstrable adenovirus C.F. antibody and neutralizing antibody against any of the adenovirus serotypes 1, 2, 3, 5, 6, or 7. At age 5–11 months, 35·5 % have adenovirus neutralizing antibody for one or more serotypes. The percentage rises with age until by 8 years

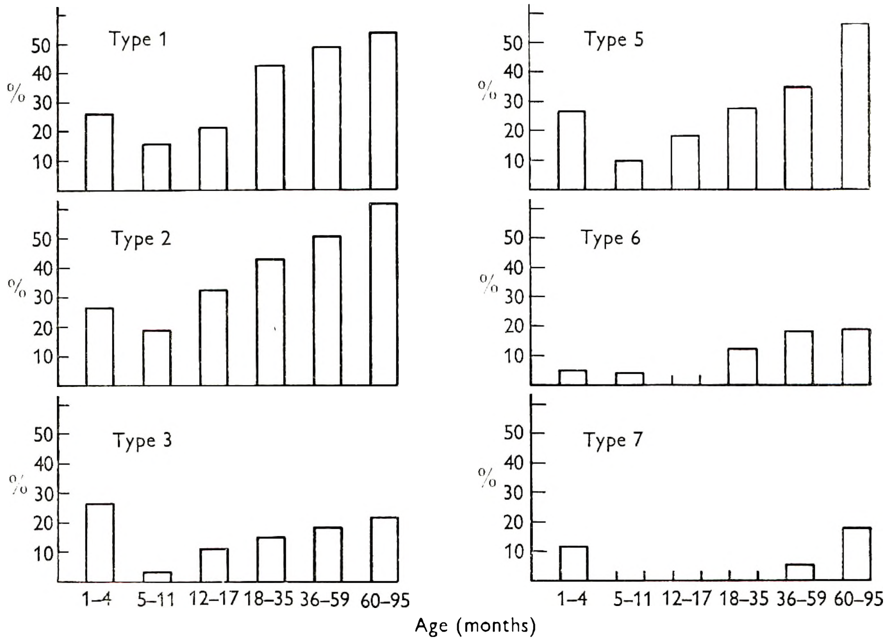

Fig. 3. Correlation of age with the presence of neutralizing antibodies for adenovirus.

Table 1. *Percentages of children with multiple adenovirus neutralizing antibodies*

Total number of adenovirus serotypes neutralized	Age (months)					
	1–4	5–11	12–17	18–35	36–59	60–95
0	42·4	64·5	35·4	27·6	18·2	12·5
1	10·3	19·4	46·4	29·8	27·2	9·4
2	23·1	16·1	17·8	27·6	33·3	43·8
3	11·4	—	—	4·4	6·1	18·7
4	—	—	—	10·6	9·1	6·2
5	—	—	—	—	6·1	9·4
6	3·8	—	—	—	—	—

87·5 % have neutralizing antibody against one or more types. At age 5–11 months, 29·3 % have demonstrable adenovirus C.F. antibody rising to 50 % by 8 years.

Figure 3 shows the percentages of children of various ages with demonstrable neutralizing antibodies against the six adenovirus serotypes. Neutralizing anti-

bodies against serotypes 1, 2 and 5 are much more widely distributed than against types 3, 6 and 7 in the group studied. At age 5–11 months, neutralizing antibody against type 1 is demonstrable in 16·1 % of children, rising to 46·9 % at age 8 years: for type 2 the proportion is 19·4 % at age 5–11 months, rising to 62·5 % at age 8 years. For type 5 the proportion is 9·7 % at age 5–11 months, rising to 56·2 % at age 8 years. Antibody against type 7 was relatively rare. None was detectable in children under 3 years of age.

Table 1 shows the percentage of children at various ages with antibodies against more than one adenovirus serotype.

The number having multiple antibodies is lowest at age 5 months. It increases steadily until, by age 5–8 years, one-third of the children have antibodies against three or more serotypes.

DISCUSSION

The passive acquisition of transplacental adenovirus neutralizing antibody was described by Jordan *et al.* (1958). The data presented in this paper show that the average titre in the new-born child is higher than that in the mother. The ratio of maternal to cord neutralizing antibody levels was approximately the same for adenovirus types 1 and 3.

Active neutralizing antibody for adenovirus serotypes begins to appear in serum specimens early in life and the percentage of children with antibodies against one or more virus types rises quickly with increasing age. Antibodies against adenovirus types 1, 2 and 5 are found more frequently than antibodies against types 3, 6 and 7. As the antibody response in young children is mainly homotypic (Jordan *et al.* 1958), heterotypic response being an antibody recall (Van der Veen & Prins, 1960), it is concluded that infection with adenovirus types 1, 2 and 5 is probably endemic in Sheffield children, just as it is in Cleveland children.

Type 7 antibodies, however, were not encountered in children under 3 years of age. This may be interpreted in two ways. Children over the age of 3 years might be particularly susceptible to infection by this serotype. On the other hand, there may have been no adenovirus type 7 infection in the past 3 years. The latter view is favoured since the presence of antibody in the older children coincided with the presence of this serotype in Sheffield in 1955, described by Tyrrell, Balducci & Zaiman (1956).

From a comparison of this study with two similar studies made in American populations (Huebner *et al.* 1954; Jordan *et al.* 1958) it is evident that adenovirus infection is endemic in all three communities. The Sheffield infection rate is probably higher than that in Cleveland, but lower than that in Washington. The magnitude of endemic infection must be related to the social circumstances of the population studied. Jordan *et al.* (1958) state that the Cleveland study was carried out in a population which 'both at home and school is subjected to the minimal crowding possible in contemporary metropolitan civilization'. This is not true for Sheffield. The social circumstances of the group studied by Huebner *et al.* (1954) are not known.

SUMMARY

The levels of neutralizing antibody against several adenovirus serotypes were determined on 42 paired specimens of maternal and cord serum and on 197 single serum specimens from children up to the age of 8 years.

The antibody distribution indicates that adenovirus infection is endemic in Sheffield just as it has been shown to be endemic in Cleveland and Washington.

We should like to thank Prof. C. P. Beattie for his advice and criticism, Dr C. C. Bowley of the Sheffield Regional Blood Transfusion Centre for paired maternal and cord sera, Dr M. H. Hambling for the provision of adenovirus stock strains and Mr Foster, medical artist, for preparation of the figures.

REFERENCES

Binn, L. N. & Hilleman, M. R. (1960). A guinea pig potency test for adenovirus vaccine. *J. Immunol.* **84**, 20.

Dulbecco, R. & Vogt, Marguerite (1954). Plaque formation and isolation of live lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167.

Huebner, R. J., Rowe, W. P., Ward, T. G., Parrott, R. H. & Betts, J. A. (1954). Adenoidal-pharyngeal-conjunctival agents. A newly recognized group of common viruses of the respiratory system. *New Engl. J. Med.* **251**, 1077.

Jordan, W. S., Badger, G. E. & Dingle, J. H. (1958). A study of illness in a group of Cleveland families. XV. Acquisition of type-specific adenovirus antibodies in the first five years of life—Implications for the use of adenovirus vaccine. *New Engl. J. Med.* **258**, 1041.

Pereira, H. G. (1956). Typing of adenoidal-pharyngeal-conjunctival (A.P.C.) viruses by complement-fixation. *J. Path. Bact.* **72**, 105.

Reed, L. J. & Muench, H. (1938). A simple method for estimating 50 per cent end-points. *Amer. J. Hyg.* **27**, 493.

Tyrrell, D. A. J., Balducci, D. & Zaiman, T. E. (1956). Acute infections of the respiratory tract and the adenoviruses. *Lancet*, ii, 1326.

Van der Veen, J. & Prins, A. (1960). Studies of the significance of the recall phenomenon in the antibody response to adenovirus vaccine and infection. *J. Immunol.* **84**, 562.