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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

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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 & Olitzk 957) observed that in groups of non-immunized mice infected with *S. paratyphi* A . uantities corresponding to a half or a quarter of the LD_{100}, only those animals di 'n 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 2×10^8/0·5 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*

	Deaths in groups of 10 mice after intraperitoneal injection of micro-organisms grown					
	in vivo		*in vitro* No. subcultures			
No. micro-organisms	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

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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

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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 O 901. 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 O 901 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

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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

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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	By bacteria suspended in organ extracts — O 901	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	—	—	—	—

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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 micro-organisms 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.

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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.
Strain	Quantity	Condition	(mg./ml.)	0 min.	5 min.	10 min.	of protein
S. typhi (Ty 2)	$2{\cdot}0 \times 10^8$	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)	$4{\cdot}0 \times 10^{10}$	Acetone-dried	1·0	0·60	0·47	0·37	11·5
S. typhi (O 901)	$5{\cdot}0 \times 10^8$	Living	1·0	0·60	0·53	0·40	7·0
S. typhi (Ty 1)	$8{\cdot}0 \times 10^6$	Living	1·0	0·60	0·53	0·48	6·5
S. ballerup	$2{\cdot}5 \times 10^8$	Living	1·0	0·60	0·44	0·34	15·0
S. stanley	$1{\cdot}0 \times 10^7$	Living	1·0	0·60	0·52	0·43	13·0
S. hirschfeldii	$1{\cdot}0 \times 10^8$	Living	1·0	0·60	0·57	0·46	12·5
E. coli (O 111)	$1{\cdot}0 \times 10^7$	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

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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 unabsorbed
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

	Reciprocals of titres				
	Serum absorbed with:				
Immunoreaction with antigens	Ty 2 *in vitro*	Ty 2 *in vivo*	Normal spleen	Infected spleen	Serum unabsorbed
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

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.

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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.

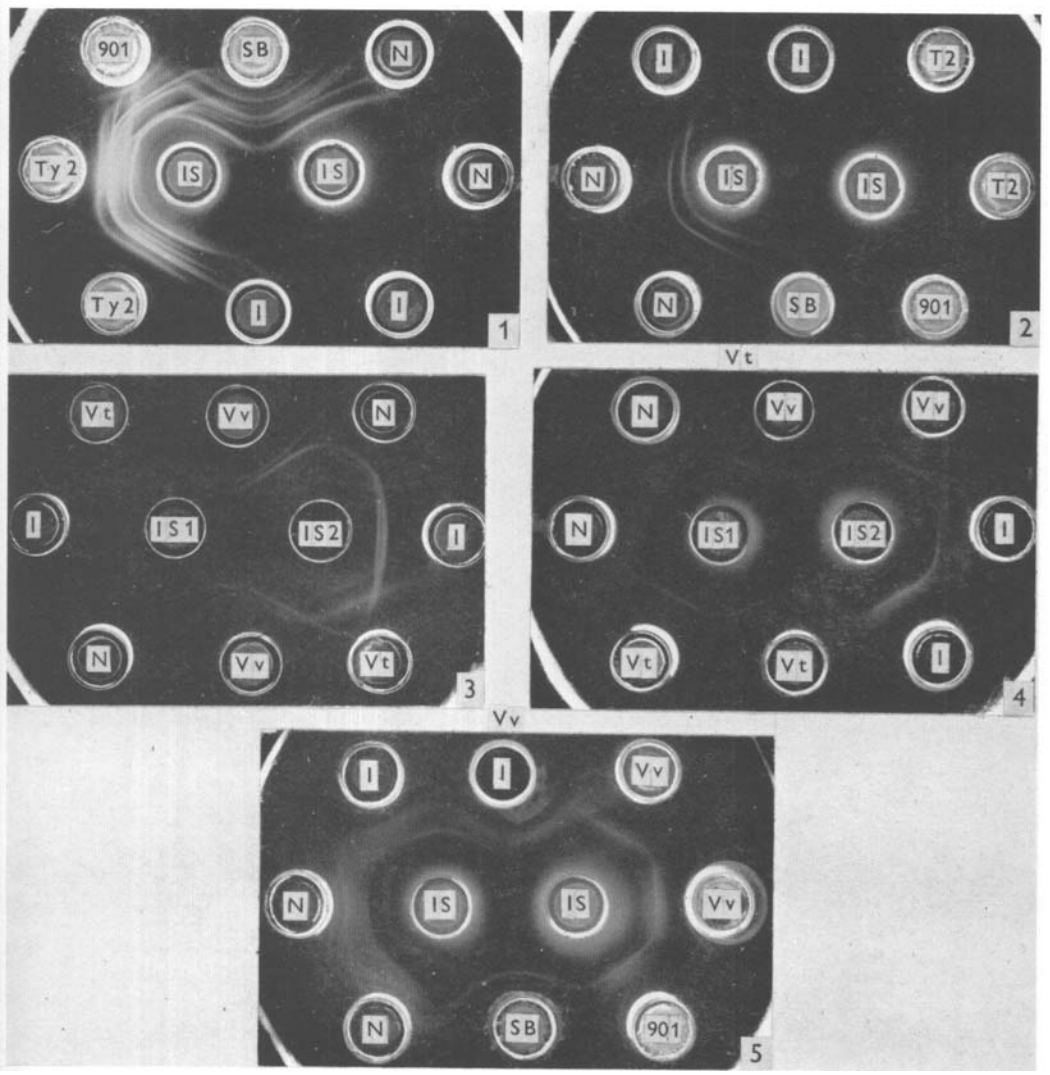

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Fig. 2. Reaction of an anti-mouse-spleen immune serum with the same antigens as used in the experiment demonstrated in fig. 1. IS, anti-mouse-spleen immune serum. The symbols of the antigens as in fig. 1.

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*.