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

III. The immunizing potencies of acetone-killed vaccines prepared from *in vivo*- and *in vitro*-grown bacteria and the immunizing potency of substances isolated from infected organs

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In previous experiments of Olitzki & Godinger (1963) it was demonstrated that *Salmonella typhi* Ty 2 grown *in vivo* was more virulent for mice than its corresponding culture grown *in vitro*. These authors also showed that extracts of infected organs acted as infection-promoting substances. *In vitro* experiments of Olitzki & Kaplan (1963) proved that the majority of organ extracts of mice infected with strain Ty 2 inhibited markedly the bactericidal action of serum on *S. typhi*. The experiments described below were carried out in order to examine whether the *in vivo*-grown *S. typhi* strain Ty 2, employed for the preparation of an acetone-killed vaccine, possesses a higher immunizing potency than its corresponding culture grown *in vitro*. Experiments were also carried out in order to show whether organ extracts of infected animals contained immunizing antigens similar to those of the intact bacteria isolated from the infected organs.

METHODS

The immunization and challenge procedures varied according to the conditions of the individual experiment. The details will be described below. *S. typhi*, strain Ty 2, was used in all experiments for the preparation of the vaccines and for the challenge dose as well. In all experiments white mice of both sexes, 3–4 weeks old at the start of the experiment, and weighing on an average 20 g. were used. The interval between the immunizing injection and the challenge dose was 14 days. Both injections were given intraperitoneally. The total volumes of the immunizing material and the challenge dose was in all experiments 0·5 ml. The challenge dose contained in the majority of the experiments 2×10^8 bacteria with the exception of one experiment, presented in Table 2, where $2{\cdot}5 \times 10^8$ were given.

In the first group of experiments we compared the immunizing power of strain Ty 2 subcultured 20 times on trypticase-agar (Difco) and harvested after an incubation of 20 hr. at 37° C. with the immunizing power of bacteria of the same parent strain after ten intraperitoneal passages *in vivo* in mice or guinea-pigs and harvested from the peritoneal fluid 18 hr. after the onset of the infection. The infecting dose was 2×10^8 bacteria. The *in vivo*-grown bacteria were carefully freed

from all cell debris by repeated slow centrifugation. Then the bacteria were spun down by centrifugation at 10,000 rev./min. and the living bacterial count of both vaccines was determined by plating, before drying them in acetone. Before each experiment the O-agglutinability of both the *in vitro*- and the *in vivo*-grown bacteria was tested by an anti-O 901 immune serum. The titre for the strain O 901 was 1/10,000, for Ty 2 grown *in vitro* 1/80, and for Ty 2 grown *in vivo* 1/40. From the dried material suspensions were prepared which contained, as calculated from the living count, 6×10^9 bacteria/ml. Groups of mice received intraperitoneally 0·5 ml. of 5-fold dilutions starting with dilution 1/80 which contained $3{\cdot}75 \times 10^7$ micro-organisms/0·5 ml. After an interval of 14 days the mice were challenged with 2×10^8 bacteria of strain Ty 2 grown *in vitro*.

In a second group of experiments we examined the immunizing power of extracts of infected organs. The technique of the preparation of organ extracts was the same as that described by Olitzki & Godinger (1963). The interval between administration of the vaccinating material and the challenge dose was again 14 days. The way of injection and the challenge dose were the same as in the previous experiment with intact bacteria. In the first experiment, in which we worked with untreated extracts, we had to exclude the possibility that residual living bacteria could exert some immunizing effect. For this purpose, the survivors of primary infections with different quantities of living bacteria, suspended in saline or in organ extracts, were challenged in the same way as those treated with organ extracts. However, later it became evident that organ extracts heated at 50° C. for 1 hr. which contained only dead bacteria and also those filtered through Seitz filter SS 1 and freed from living and dead bacteria as well exerted marked immunizing effects. Thus, the participation of residual living or dead bacteria could be completely excluded.

RESULTS

Immunization experiments with vaccines prepared from in vitro- *and* in vivo-*grown* S. typhi *strain* Ty 2

The results of two vaccination experiments are summarized in Table 1. Although the non-protective dilutions of the vaccines were not reached, Table 1 shows that $1{\cdot}5 \times 10^6$ *in vitro*-grown bacteria were able to protect only 68 % of the animals, while the same number of *in vivo*-grown bacteria afforded complete protection.

We therefore extended our experiments and examined the immunizing power of bacteria grown in the abdominal cavity and in the spleen of mice and guinea-pigs as well. The acetone-dried bacteria taken from these environments proved to be non-toxic, when injected in quantities of $7{\cdot}5 \times 10^7$ or less. No deaths were observed within an observation time of 14 days after the vaccination had taken place. The results of these experiments are summarized in Table 2. Although the challenge dose was somewhat higher than in the previous experiments ($2{\cdot}5 \times 10^8$ bacteria grown *in vitro*) the differences in the potencies of the vaccines were similar to those presented in Table 1. $1{\cdot}5 \times 10^6$ bacteria grown in the mouse abdomen and killed by acetone were able to protect 16 out of 20 mice (= 80 %), while the same

quantity of bacteria grown *in vitro* or *in vivo* in the spleens of mice or in guinea-pigs were unable to protect.

Table 1. *Vaccination of mice with* in vitro- *and* in vivo-*grown* S. typhi, *strain* Ty 2 *acetone dried and resuspended in saline*

(Challenge dose 2×10^8 bacteria.)

Expt.	Vaccine prepared from strain Ty 2 grown	Bacterial count in the vaccine	Survivors after challenge in groups of 10	Survivors after challenge in groups of 15
1	*In vitro*	$3{\cdot}75 \times 10^7$	10	—
		$7{\cdot}50 \times 10^6$	10	—
		$1{\cdot}50 \times 10^6$	6	—
	In vivo	$3{\cdot}75 \times 10^7$	10	—
		$7{\cdot}50 \times 10^6$	10	—
		$1{\cdot}50 \times 10^6$	10	—
	Controls non-vaccinated		0	—
2	*In vitro*	$7{\cdot}50 \times 10^6$	—	15
		$1{\cdot}50 \times 10^6$	—	11
		$3{\cdot}00 \times 10^5$	—	10
	In vivo	$7{\cdot}50 \times 10^6$	—	15
		$1{\cdot}50 \times 10^6$	—	15
		$3{\cdot}00 \times 10^5$	—	13
	Controls non-vaccinated	—	—	0

Table 2. *Vaccination of mice with* in vitro- *and* in vivo-*grown* S. typhi

(Challenge dose $2{\cdot}5 \times 10^8$ bacteria grown *in vitro*.)

Vaccine from strain Ty 2 grown	No. of survivors in groups of 20: Vaccinated with acetone-killed micro-organisms $3{\cdot}75 \times 10^7$	$7{\cdot}5 \times 10^6$	$1{\cdot}5 \times 10^6$	$3{\cdot}0 \times 10^5$	Non-vaccinated
On trypticase agar	12	1	2	0	—
In mouse spleen	12	3	0	0	—
In mouse abdomen	18	16	16	4	—
In guinea-pig spleen	17	6	1	0	—
In guinea-pig abdomen	19	16	0	0	—
Control	—	—	—	—	0

Furthermore, Table 2 shows that there was also a marked effect exerted by bacteria grown in the abdominal cavity of guinea-pigs. $7{\cdot}5 \times 10^6$ bacteria grown in abdominal cavities of guinea-pigs and mice as well protected 16 out of 20 mice (= 80 %), while the protective effect of $7{\cdot}5 \times 10^6$ *in vitro*-grown bacteria was almost zero and that of the spleen-grown bacteria not higher than 30 %.

If we summarize the results of this experiment we find for the four doses of the intraperitoneally grown bacteria (from guinea-pigs or mice) the following percentages of survivors: 92·5, 80·0, 40·0 and 10·0. The corresponding percentages of

all the other groups are: 68·3, 16·7, 5·0 and zero. The average ED 50 of the first group of vaccines was according to this result about 3×10^6 and of the second $1{\cdot}7 \times 10^7$.

In the following experiment we investigated the immunizing potency of the *in vivo*- and *in vitro*-grown strains against challenge doses of both the *in vivo*- and the *in vitro*-grown strains. The *in vitro*-grown bacteria used for vaccine preparation and challenge doses were subcultured 20 times on trypticase agar, the *in vivo*-grown bacteria used for both purposes were subcultured 10 times in the peritoneal cavities of mice. The results of this experiment are summarized in Table 3.

Table 3. *The immunizing potency of* in vitro- *and* in vivo-*grown* S. typhi *strain* Ty 2 *against* in vitro- *and* in vivo-*grown challenge doses*

(All *in vivo*-grown bacteria were harvested from the abdominal cavities of mice. Challenge dose 2×10^8.)

Immunizing dose	No. of survivors in groups of 10 mice after vaccination with vaccine grown: *In vitro* and challenge with bacteria grown — *In vitro*	*In vitro* and challenge with bacteria grown — *In vivo*	*In vivo* and challenge with bacteria grown — *In vitro*	*In vivo* and challenge with bacteria grown — *In vivo*
$1{\cdot}5 \times 10^8$	10	5	10	10
$3{\cdot}0 \times 10^7$	6	1	10	9
$6{\cdot}0 \times 10^6$	1	0	9	5
$1{\cdot}2 \times 10^6$	0	0	7	2
$2{\cdot}4 \times 10^5$	0	0	5	2
$4{\cdot}8 \times 10^4$	0	0	2	0
$9{\cdot}6 \times 10^3$	0	0	0	0
Control non-vaccinated	0	0	—	—

In order to understand the results of this experiment, we have to take into consideration that 2×10^8 bacteria grown *in vitro* represented somewhat more than one lethal dose, while the lethal dose of the *in vivo*-grown strain was $1{\cdot}6 \times 10^6$. The challenge dose, 2×10^8, of the *in vivo*-grown strain represented, therefore, more than 100 lethal doses. The table shows that the *in vitro*-grown strain yielded a relatively weak vaccine. About 3×10^7 micro-organisms were required to afford a 50 % protection against the *in vitro*-grown and $1{\cdot}5 \times 10^8$ to afford a 50 % protection against the *in vivo*-grown challenge organisms. The corresponding quantities of the *in vivo*-grown bacteria were much lower: $2{\cdot}4 \times 10^5$ and $6{\cdot}0 \times 10^6$. Therefore, if we gave the *in vitro*-grown micro-organisms as challenge, the *in vivo*-grown vaccine was about 100 times more effective than the *in vitro*-grown vaccine. However, if we gave the *in vivo*-grown challenge the *in vivo*-grown vaccine was only 25 times more effective than the *in vitro*-grown vaccine.

We also compared the effect of the potency of the bacteria grown in the peritoneal cavity with those grown in the spleen against both types of challenge doses, *in vitro* and *in vivo*. The results are summarized in Table 4.

Table 4 shows that the intraperitoneally grown bacteria immunize better than

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the spleen-grown ones. With the intraperitoneally grown vaccine, $1{\cdot}2 \times 10^6$ organisms gave 100 % protection against the *in vitro*-grown challenge, but $6{\cdot}0 \times 10^6$ organisms were required for protection against the *in vivo*-grown challenge. The respective 100 % protecting doses of the spleen-grown vaccine were $3{\cdot}0 \times 10^7$ and $1{\cdot}5 \times 10^8$. These results, together with those presented in Table 3, indicated that the vaccinating potency of spleen-grown bacteria was lower than that of the intraperitoneally grown bacteria. However, the potencies of both vaccines were higher than those of the *in vitro*-grown micro-organisms.

Table 4. *The immunizing potency of two* in vivo*-grown and acetone-killed* S. typhi *vaccines, one grown in the spleen and the other in the abdominal cavity, against challenge by* in vitro *and* in vivo *intra-abdominally grown bacteria derived from the same parent strain*

(Challenge dose 2×10^8.)

Bacterial count of the vaccine	No. of survivors in groups of 10 mice after vaccination with *S. typhi*, strain Ty 2, grown in			
	The spleen and challenged by bacteria grown		The abdominal cavity and challenged by bacteria grown	
	In vitro	*In vivo*	*In vitro*	*In vivo*
$1{\cdot}5 \times 10^8$	10	10	10	10
$3{\cdot}0 \times 10^7$	10	8	10	10
$6{\cdot}0 \times 10^6$	3	0	10	10
$1{\cdot}2 \times 10^6$	1	0	10	6
$2{\cdot}4 \times 10^5$	1	0	7	0
$4{\cdot}8 \times 10^4$	0	0	0	0
Controls non-vaccinated	0	0	—	—

Immunization experiments with extracts from infected organs

In order to determine whether extracts of infected organs possessed any immunizing potency, we had to exclude the possibility that residual living or dead bacteria, remaining in the extracts after centrifugation, could exert some immunizing effect. Table 5 demonstrates immunity resulting from a primary infection with different quantities of living bacteria suspended in saline or in organ extracts. The animals which received higher inocula of strain Ty 2 died. When the survivors were challenged 14 days later with 2×10^8 bacteria of strain Ty 2 then the majority of those animals survived which had received 2×10^5 or more living bacteria suspended in saline, 2×10^2 or more bacteria suspended in liver extract and 2×10^4 or more bacteria suspended in spleen extract.

The results of the experiment presented in Table 6 show that extracts of infected liver, and spleen and the peritoneal fluid of infected animals exerted some immunizing effect. However, the control experiment carried out simultaneously and presented in Table 7 showed that organ extracts from non-infected animals also exerted a certain immunizing effect, when living bacteria were added to them in such quantities which, when suspended in saline, were unable to immunize. The

minimal quantity of living bacteria able to immunize when injected intra-abdominally together with liver extracts was 10^2 and together with spleen extracts was 10^4. Such a bacterial inoculum when injected together with normal organ extracts was apparently able to multiply *in vivo* and to reach such a high antigen concentration that a solid immunity was established. The control mixtures which contained normal extracts heated at 50° C. or exposed to chloramphenicol together with the added bacteria exerted also some immunizing effects, but the sterility controls showed that they still contained some living bacteria.

Table 5. *The immunity of the survivors from a preceding intraperitoneal infection with* in vitro-*grown* S. typhi *strain* Ty 2 *after a second infection with* 2×10^8 *bacteria of the same strain grown* in vitro

No. of infecting bacteria	Survivors after a second injection in groups surviving after a first injection of bacteria				
		With extract prepared from			
		Liver		Spleen	
	Without extracts	Normal	Infected	Normal	Infected
2×10^8	0/0	—	—	0/0	0/0
2×10^7	1/1	—	—	1/1	1/1
2×10^6	2/3	0/0	0/0	3/3	3/3
2×10^5	4/4	1/1	1/1	5/5	5/5
2×10^4	0/5	2/2	1/1	3/5	5/5
2×10^3	0/5	5/5	3/3	1/5	4/5
2×10^2	—	5/5	5/5	1/5	3/5
Control untreated	0/10	—	—	—	—

The peritoneal fluid behaved differently. When it was taken from infected mice, and the bacteria removed by high-speed centrifugation, it exerted a marked immunizing effect. When it was taken from normal mice and bacteria were added in concentrations up to $10^4/0{\cdot}5$ ml. no immunizing effects were observed. This result indicated that residual bacteria present in the peritoneal fluid after centrifugation were not identical with the immunizing agent involved.

We continued, therefore, the immunization experiments with infected peritoneal fluids and spleens of guinea-pigs which yielded greater quantities of immunizing material. When these products were taken from normal animals and injected together with small quantities of living bacteria they did not show any immunizing effect.

The spleen of each guinea-pig was suspended in 5 ml. of saline and the peritoneal fluid taken up in 10 ml. saline. The number of bacteria before heating at 50° C. for 60 min. was in the spleen extract 2×10^4/ml. and in the peritoneal fluid 10^3/ml.

The experiment presented in Table 8 shows that the peritoneal fluid of guinea-pigs possesses a powerful immunizing potency which could not be ascribed to residual bacteria, since it retained its immunizing power after it was freed from bacteria by filtration through Seitz filter SS 1.

We tried therefore to isolate this agent by precipitation with 25, 40 and 66·5 % of ethanol. The substances precipitated after each addition of ethanol were dried and then taken up in saline at a concentration of 20 mg./ml. When varying quantities of substances were injected into mice and the mice challenged with 2×10^8 bacteria of strain Ty 2 then the results were obtained, which are presented in Table 9.

Table 6. *Immunization experiment with extracts from infected organs*

(Total volume 0·5 ml. Challenge dose 2×10^8 micro-organisms of strain Ty 2 grown *in vitro*.)

Antigen			Living bacteria present in the vaccine	Survivors in groups of 10 mice after injection of the	
Preparations	Origin	Dilution		Immunizing antigen	Challenge dose
Disintegration and centrifugation	Liver	Undiluted	15000	4	4
		1/2	7500	9	5
		1/5	3000	10	2
	Spleen	Undiluted	3000	10	10
		1/2	1500	10	1
		1/5	600	10	1
	Peritoneal fluid	Undiluted	14000	10	10
		1/2	7000	10	0
		1/5	2800	10	0
As above then kept for 1 hr. at 50° C.	Liver	Undiluted	0	10	10
		1/2	0	10	5
		1/5	0	10	3
	Spleen	Undiluted	0	10	10
		1/2	0	10	3
		1/5	0	10	0
	Peritoneal fluid	Undiluted	0	10	10
		1/2	0	10	2
		1/5	0	10	0
As above then kept for 18 hr. at 37° C. with 1 mg. chloramphenicol/ml.	Liver	Undiluted	1000	10	10
		1/2	100	10	4
		1/5	40	10	1
	Spleen	Undiluted	50	10	10
		1/2	25	10	4
		1/5	10	10	0
	Peritoneal fluid	Undiluted	0	10	10
		1/2	0	10	0
		1/5	0	10	0
Controls, untreated					0

As control for this experiment we used the original infected peritoneal fluid which allowed, in groups of five mice, in the dilutions 1/10, 1/20 and 1/40, the respective survival of 5, 3 and 1 mice, while in the untreated group no mice survived.

Table 9 shows that the fraction precipitated by 40 % ethanol was the most active one and 0·25 mg. still prevented the death of three out of five mice. The fraction precipitated with 66 % ethanol was almost inactive and protected only when injected in

quantities of 4·0–20·0 mg. The supernatant fluid which remained after the ethanol precipitation and evaporation of the ethanol was inactive and all mice in groups of five died after treatment with 1·0 and 0·5 ml. of the supernatant fluid and infection with 2×10^8 *in vivo*-grown bacteria of strain Ty 2. Also the protein precipitated from this fraction by saturation with ammonium sulphate did not exhibit any immunogenic properties. All fractions proved to be non-toxic and no deaths occurred after the injection of 10·0 and 20·0 mg of any fraction.

Table 7. *Control experiments for the experiment shown in Table* 6

(Immunization of mice with extracts of non-infected organs with addition of living or killed bacteria. Organ extracts undiluted. Total volume 0·5 ml. Challenge dose: 2×10^8 bacteria of strain Ty 2 grown *in vitro*.)

Antigens			Survivors in groups of 5 mice	
Origin of antigen	Bacteria added (strain Ty 2)	Further treatment	After vaccination	After challenge dose
Normal liver	None	None	10	2
	10^2		10	8
	10^3		8	8
Normal spleen	None	None	10	0
	10^2		10	0
	10^3		10	0
	10^4		10	4
Normal peritoneal fluid	None	None	10	0
	10^2		10	0
	10^3		10	0
	10^4		10	0
Normal liver	None	1 hr., 50° C.	10	0
	10^2		10	2
	10^3		10	6
	10^4		10	8
Normal liver	None	Chloramphenicol,	10	0
	10^2	1 mg./ml., 18 hr.,	10	2
	10^3	37° C.	10	8
	10^4		10	8
Extract not added	10^2		10	0
	10^3		10	0
	10^4		10	0
Controls untreated	—	—	—	0

Differences in the chemical and physical properties of in vivo- *and* in vitro-*grown bacteria*

The *in vivo*-grown bacteria differ from the *in vitro*-grown ones in their optical density, weight, nitrogen and protein content as summarized in Table 10.

All these data were obtained after careful removal of cell debris until microscopical examination did not reveal any stainable structures beside bacteria. However, these differences, mainly those between the *in vitro*-grown bacteria and those grown in the abdominal cavity, were not sufficiently great to explain the

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Table 8. *Immunization experiment with extracts of spleen and peritoneal fluid of infected and non-infected guinea-pigs, heated at* 50° C.

(Total volume 0·5 ml. Challenge dose 2×10^8 bacteria of strain Ty 2.)

Antigen from guinea-pig	Dilution	Living bacteria present in vaccine before heating	Survivors in groups of 5 mice after injection of strain Ty 2 grown	
			In vitro	*In vivo*
Infected spleen	Undiluted	1000	5	3
	1/4	250	5	0
	1/16	60	2	0
Non-infected spleen	Undiluted	0	0	0
	1/4	0	0	0
	1/16	0	0	0
Infected peritoneal fluid	Undiluted	50	5	5
	1/4	12	5	5
	1/16	3	5	5
Non-infected peritoneal fluid	Undiluted	0	1	0
	1/4	0	0	0
	1/16	0	0	0
Infected peritoneal fluid passed through Seitz filter	1/7·5	0	5	—
	1/15	0	4	—
	1/30	0	2	—
Controls non-vaccinated	—	—	0	0

Table 9. *The immunizing properties of three fractions precipitated from the peritoneal fluid of infected guinea-pigs by addition of* 25, 40 *and* 66 % *ethanol, compared with those of the original peritoneal fluid*

Injected dilutions of the original peritoneal fluid (volume 0·5 ml.)	Injected quantity of each fraction (mg.)	No. of survivors in groups of 5 mice infected with 2×10^8 *in vitro*-grown bacteria after immunization with			
		Fractions precipitated with ethanol			Original infected peritoneal fluid heated at 50° C., 60 min.
		25 %	40 %	66 %	
—	2·0	4	5	1	—
—	1·0	5	5	0	—
—	0·5	4	4	0	—
—	0·25	0	3	0	—
1/10	—	—	—	—	5
1/20	—	—	—	—	3
1/40	—	—	—	—	1

differences in immunological activity. The differences in the optical density were 2·6-fold, the differences in the weight 4-fold and the differences in the nitrogen content 1·5-fold, while the differences in the immunizing potencies were 25-fold against the *in vivo* challenge and 100-fold against the *in vitro* challenge as shown in Table 3. The morphological differences between the *in vivo*- and *in vitro*-grown

bacteria were as follows: the average length of the *in vivo*-grown bacteria, taken from the peritoneal fluid 18 hr. after the onset of infection, was 1·50 μ, 91·8 % of them varying in their length between 0·5 and 2·0 μ and only 8·2 % representing elongated forms varying from 2·5 to 6·0 μ. The average length of the *in vitro*-grown bacteria from an 18 hr. agar culture was 1·74 μ, only 81·1 % of them varied in their length between 0·5 and 2·0 μ, and 18·9 % represented elongated forms varying from 2·5 to 7·0 μ. According to this finding a higher weight of the *in vitro*-grown bacteria should be expected, while in reality the *in vivo*-grown bacteria weighed more. This phenomenon may be explained by the temporary absorption of host-substances during the *in vivo* growth, which was described in the previous communication of Olitzki & Godinger (1963).

Table 10. *Some properties of* in vitro- *and* in vivo-*grown* S. typhi, *strain* Ty 2

	6×10^2 bacteria/ml. of strain Ty 2 grown		
		In vivo in mice	
Properties	*In vitro*	Spleen	Abdominal cavity
Optical density*	0·72	3·76	1·88
Dry weight (mg.)	0·66	11·00	2·66
Nitrogen content (μg.)	16·0	130·0	24·4
Protein content (mg.)	0·10	0·81	0·15

* Wavelength, 5500 Å.

DISCUSSION

In the previous investigations of Olitzki & Godinger (1963) on *in vivo*- and *in vitro*-grown *S. typhi* it was shown that normal liver extract was able to enhance markedly the lethal effect of these pathogenic bacteria, when injected intraperitoneally into mice. In the above-described experiments we could determine that sublethal minimal amounts of *S. typhi* suspended in saline did not immunize, while the same quantities, about 10^2–10^3 bacteria, injected together with normal liver extract produced an immunizing effect. Also in an experiment reported by Olitzki & Kaplan (1963) it was demonstrated that at a 4 hr. incubation-time liver extract behaved differently from other organ extracts. While the majority of the organ extracts acted as growth-promoting substances *in vitro* and enhanced the bactericidal serum action on strain Ty 2, the normal liver extract acted mainly as an inhibitor of the bactericidal action. It has to be mentioned that this 2-fold effect, the promotion of growth in absence of serum and complement and the enhancement of bactericidal serum action in their presence, is not limited only to the majority of organ extracts. Michael & Braun (1959) described similar effects exerted by broth, glucose and certain amino acids on the susceptibility of *Shigella dysenteriae* and *Escherichia coli* to the bactericidal action of normal human serum.

Several properties of organ extracts from infected animals have already been demonstrated by previous experiments, namely the accumulation of infection-promoting substances and precipitinogens by Olitzki & Godinger (1963), and of

inhibitors of the bactericidal serum action by Olitzki & Kaplan (1963). The experiments described in this paper revealed an additional activity of the infected organ extracts: their immunogenic action. As in the previous communication, no indication has been found that these immunogenic substances are toxic. Even the most concentrated preparations of the immunogenic substances obtained by ethanol precipitation proved to be non-toxic.

There remains still to be investigated the relationship between these immunogenic substances and the enhanced immunogenicity of the *in vivo*-grown bacteria. The production of great quantities of *in vitro*-grown bacteria in the peritoneal cavity of the guinea-pig and their immunogenicity make possible a continuation of this investigation on the above lines.

SUMMARY

1. *Salmonella typhi*, strain Ty 2, grown *in vivo* and employed as acetone-dried vaccine possessed a higher immunizing potency than the descendants of the same parent strain grown *in vitro* and employed as vaccine.

2. When 2×10^8 *in vitro*-grown bacteria were employed as challenge, the immunizing effects of both types of vaccine were more marked than after administration of 2×10^8 *in vivo*-grown bacteria as challenge.

3. The higher potency of the *in vivo*-grown vaccine was apparent in all experiments, whether the challenge strain was grown *in vivo* or *in vitro*.

4. Immunogenic substances were isolated from infected organs of mice and guinea-pigs, and an immunogenic substance from the peritoneal fluid of the infected guinea-pigs was concentrated by precipitation with ethanol.

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