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Studies on Immunity and Pathogenesis of Salmonellosis

II. ANTIBODY PRODUCTION AND ACCUMULATION OF BACTERIAL POLYSACCHARIDE IN THE TISSUES OF CHICKENS INFECTED WITH *SALMONELLA GALLINARUM*

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Summary. The bacterial agglutination, haemagglutination and antiglobulin haemagglutination tests have been used to detect antibody production during the development of *Salmonella gallinarum* infection in chickens. The latter test has detected serum antibodies as early as 1 day after oral infection in some cases of acute experimental disease, and antibodies were detected in the sera of all birds at the time of death.

The accumulation of bacterial polysaccharide in the tissues of infected birds has been detected by a haemagglutination inhibition test. High but variable concentrations occurred in different organs of chickens which died from the disease.

The presence of bacterial antibody and bacterial polysaccharide in the tissues of infected birds at death is discussed in relation to the pathogenesis of this disease. It is postulated that an antigen–antibody reaction, developing as an anaphylactic type of hypersensitivity, may be closely associated with the production of symptoms and death of chickens infected with *Salm. gallinarum*.

INTRODUCTION

The experimental approach to the pathogenesis of enteric infections has been directed mainly towards the response of the host to preparations of bacterial polysaccharide given under various defined conditions. One aspect of this problem is the possible relationship which specific antibodies may have to the development of clinical infection with Gram-negative organisms. Thomlinson and Buxton (1963) have shown the close association between antigen–antibody reactions and the pathogensis of *Escherichia coli* infections in pigs. During the development of *Salm. gallinarum* infection in chickens cytophilic antibodies are produced (Buxton and Allan, 1963), and these antibodies can be detected in the serum by an antiglobulin haemagglutination test. The purpose of the following experiments was to observe the production of these antibodies and the accumulation of bacterial polysaccharide in the organs during the development of infection.

MATERIALS AND METHODS

Methods for infecting chickens with *Salm. gallinarum*, for carrying out the haemagglutination and antiglobulin haemagglutination tests and for preparing *Salm. gallinarum* polysaccharide have been described by Buxton and Allan (1963).

Bacterial Agglutination Tests

The antigen consisted of *Salm. gallinarum* which had been killed in ethanol and

resuspended in saline to give an opacity of approximately 800 million organisms per ml. The method for carrying out the tests was similar to that used by Buxton and Thomlinson (1961).

Extraction of Salm. gallinarum *Polysaccharide from Tissues*

Two samples of each tissue were removed from the carcase. One sample was used for estimating the dry weight of the tissue and an additional 2 g. of tissue were homogenized in 2 ml. of physiological saline and the bacterial polysaccharide extracted by the method described by Buxton and Allan (1963).

Modification of Erythrocytes with Salm. gallinarum *Polysaccharide*

Five ml. of a 1·0 per cent suspension of thrice-washed chicken erythrocytes was incubated with 0·20 ml. of polysaccharide solution at 37° for 1 hour and mixed repeatedly. The cells were then washed three times and finally resuspended to give a 0·75 per cent suspension in normal saline.

Haemagglutination Inhibition Test

The technique used was a modification of that reported by Crumpton, Davies and Hutchison (1958). Pooled antiserum to *Salm. gallinarum* from two infected chickens was diluted 1/10 in saline, inactivated at 56° for 30 minutes and titrated against modified chicken erythrocytes. The titre of the serum was 1/40 and it was stored at −20° until required. To standardize the results the titration was repeated before each series of tests.

To one volume of doubling dilutions of the extract was added an equal volume of the 1/10 dilution of antiserum. The mixtures were incubated in a water bath for 30 minutes at 37°, and one volume of a 0·5 per cent suspension of sensitized erythrocytes was then added to each tube. Incubation was continued for 30 minutes before reading the haemagglutinin titre.

Estimation of the Bacterial Polysaccharide Content of Tissues

The amount of *Salm. gallinarum* polysaccharide in tissue extracts was calculated by a modification of the method used by Ravin, Rowley, Jenkins and Fine (1960). Doubling dilutions of a known amount of *Salm. gallinarum* polysaccharide extract were tested against the antiserum and the smallest amount to give complete inhibition was determined. The polysaccharide content of the tissue extracts was then estimated as the product of the highest dilution of tissue extract and the minimum amount of *Salm. gallinarum* polysaccharide which produced complete inhibition of the haemagglutination test. These results were expressed as μg. polysaccharide per g. dry weight of tissue.

RESULTS

ANTIBODY PRODUCTION BY CHICKENS INFECTED EXPERIMENTALLY WITH *Salm. gallinarum*

A number of experiments were made to observe the earliest time after oral infection with *Salm. gallinarum* at which humoral antibodies could be detected. In all cases it was found that the antiglobulin haemagglutination test detected antibodies earlier and to a higher titre than the haemagglutination test or bacterial agglutination test. It has been possible to identify humoral antibodies in infected chickens before death, irrespective of the severity of the infection. The following experiment illustrates the typical results recorded from these investigations.

Six Rhode Island Red cockerels, aged 1 year, were infected orally with *Salm. gallinanum.* Thereafter, serum samples from these birds were examined at frequent intervals for antibodies by the bacterial agglutination test, haemagglutination and antiglobulin haemagglutination tests. Four of the birds died on the 11th or 12th day, and their antibody titres are shown in Fig. 1.

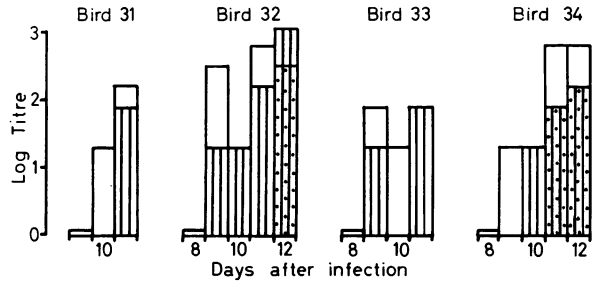

FIG. 1. Typical antibody responses of four chickens which died on the 11th or 12th day after experimental oral infection with *Salm. gallinarum.* □, antiglobulin haemagglutinin titre; ▥, haemagglutinin titre; ⊡, bacterial agglutinin titre.

Antibodies were detected from 1 to 3 days before death. Antibodies demonstrable by the antiglobulin haemagglutination test were particularly evident during the initial stages of infection. In two birds that survived the disease antibody was also first detected by the antiglobulin haemagglutination test. The other notable feature which frequently occurred in birds surviving infection was the extremely high antibody titre demonstrated by the antiglobulin haemagglutination test at about the 14th day. This was maintained only for 24–36 hours before declining to a level approximating to that of a day or two earlier (Fig. 2).

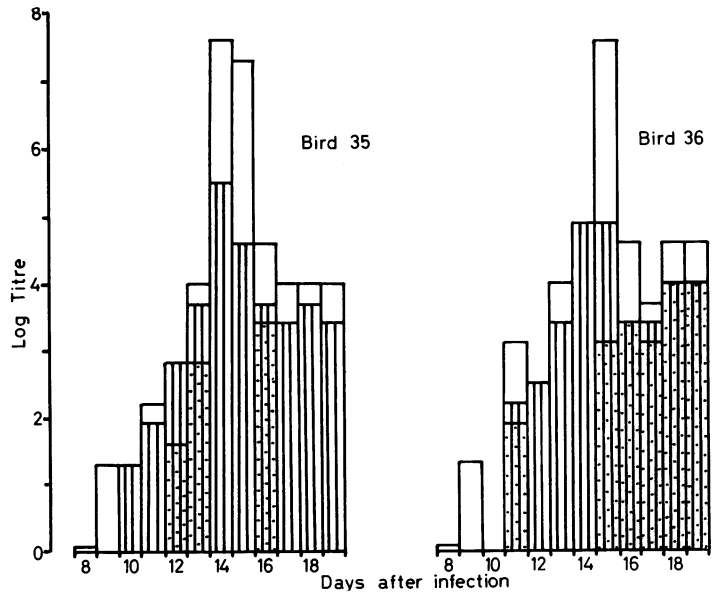

FIG. 2. Typical antibody responses of two chickens which survived experimental oral infection with *Salm. gallinarum.* Key as in Fig. 1.

THE ACCUMULATION OF BACTERIAL POLYSACCHARIDE IN TISSUES OF CHICKENS INFECTED WITH *Salm. gallinarum*

Previous experiments had shown that during the rapid development of a generalized and fatal infection, humoral antibodies were detectable in the sera for several hours before death. The following experiments were made to observe the distribution and concentration of polysaccharide in the tissues of infected birds, particularly at the time when antibodies become detectable in the serum.

The purpose of the first experiment was to estimate the sensitivity of the method for detecting polysaccharide in various tissues. Twenty-two adult chickens were infected orally. Portions of liver, spleen, lung, kidney, duodenum, ileum and caecum were removed as soon as possible after death and stored at −20°. Those birds which survived acute infection were killed at varying times after recovery and samples of their tissues were

TABLE 1

THE CONCENTRATIONS OF BACTERIAL POLYSACCHARIDE IN THE TISSUES OF CHICKENS AFTER ORAL INFECTION WITH *Salm. gallinarum*

Bird No.	*Time of death after infection (days)*	*Bacterial polysaccaride per g. dry weight of tissue* (μg.)						
		Liver	*Spleen*	*Lung*	*Kidney*	*Duodenum*	*Ileum*	*Caecum*
1	7 D	53·5	260·0	248·6	204·8	337·9	151·1	88·4
2	7 D	155·6	111·2	N.T.	15·1	85·8	12·3	12·1
3	8 D	436·3	249·0	131·2	30·8	1030·4	1092·9	561·4
4	9 D	54·6	108·0	29·2	353·4	205·8	1279·3	0
5	9 D	330·4	337·8	180·7	214·5	229·1	224·8	14·2
6	10 D	45·8	30·8	0	16·3	8·3	0	0
7	10 D	30·6	7·3	8·4	0	0	0	15·9
8	11 D	337·8	1168·9	515·7	993·5	1013·2	518·9	366·2
9	11 D	335·7	908·7	680·9	169·9	173·0	355·6	36·0
10	11 D	1020·9	1017·3	2062·9	453·1	517·9	99·9	504·5
11	12 D	231·4	42·9	9·4	15·4	32·2	15·1	0
12	13 D	177·6	390·2	255·4	61·2	17·7	N.T.	7·9
13	16 D	177·6	330·0	221·3	39·9	16·7	N.T.	7·3
14	21 K	0	0	0	0	0	0	0
15	21 K	0	0	0	72·5	0	0	0
16	23 K	0	0	0	0	0	0	0
17	23 K	0	0	0	0	0	0	0
18	26 K	0	0	0	0	0	0	0
19	26 K	20·1	0	0	0	0	0	0
20	27 K	19·2	0	0	0	0	0	0
21	27 K	0	0	0	0	0	0	0
22	27 K	0	0	0	0	0	0	0

N.T = not tested; D = died; K = killed.

similarly removed and stored. The results showed that high concentrations of polysaccharide could be readily detected in many samples derived from fatally infected chickens (Table 1). In contrast, the quantities observed in the nine surviving chickens were small or undetectable.

The organs from birds 8, 9 and 10, which died 11 days after infection, contained particularly high concentrations of polysaccharide and it was significant that in these instances tissues were not removed from the carcases until some hours after death. It seemed probable that this resulted from continued bacterial multiplication in the tissues *post mortem* while the carcase was cooling. Tissues from birds 6 and 7 which were removed at the time of death and immediately frozen contained very much less polysaccharide.

THE EFFECT OF INCUBATION ON THE CONCENTRATIONS OF BACTERIAL POLYSACCHARIDE DETECTED IN TISSUES FROM INFECTED CHICKENS

The following tests were made to observe the extent to which the delayed removal of organs from infected carcases could alter the concentration of bacterial polysaccharide found in the tissues.

Samples of liver, kidney and ileum were removed from five infected chickens. Birds 86 and 100 were killed after 5 days, birds 93 and 873 were killed a day later, and bird 44 died on the 7th day. The samples were divided into four or five portions. One portion was frozen and the others incubated at 37° for 1, 2½, 4 and 5 hours, respectively, before being stored at −20°.

TABLE 2

THE EFFECT OF INCUBATION AT 37° ON THE CONCENTRATIONS OF BACTERIAL POLYSACCHARIDE DEMONSTRABLE IN THE TISSUES OF CHICKENS INFECTED ORALLY WITH *Salm. gallinarum*

Time tissue incubated at 37° before extraction (hours)	*Bacterial polysaccharide per g. dry weight of tissue* (μg.)														
	Bird 86			*Bird* 100			*Bird* 93			*Bird* 873			*Bird* 44		
	Liver	*Kidney*	*Ileum*	*Liver*	*Kidney*	*Ileum*	*Liver*	*Kidney*	*Ileum*	*Liver*	*Kidney*	*Ileum*	*Liver*	*Kidney*	*Ileum*
0	72·9	0	15·5	8·5	0	0	15·2	0	0	0	0	0	18·9	0	0
1	0	5940·6	0	14·6	0	49·9	50·1	0	0	0	0	0	158·8	125·4	100·7
2½	0	N.T.	17·8	20·1	0	0	91·5	57·6	14·7	70·2	0	2144·8	N.T.	188·9	124·3
4	0	249·6	0	70·1	131·4	0	N.T.	N.T.	N.T.	73·6	0	84·4	225·0	711·2	158·8
5	0	N.T.	N.T.	0	N.T.	N.T.	63·3	53·1	16·0	N.T.	N.T.	N.T.	2506·3	N.T.	N.T.

Birds 86 and 100 were killed on the 5th day after infection and birds 93 and 873 on the 6th day after infection. Bird 44 died on the 7th day.
N.T. = not tested.

The results of this experiment (Table 2) show some interesting features. During incubation at 37° there was a definite increase in the amount of demonstrable polysaccharide and it is clear that if an accurate assessment is to be made the samples must be removed and frozen immediately after death. In nine of the fifteen samples polysaccharide was detected only after the tissue had been incubated at 37° for 1 or more hours.

These results were limited by the sensitivity of the method employed and also by the probability that bacteria may not be evenly distributed throughout an organ. Focal areas of infection may account for the variable results recorded from the ileum of birds 100 and 86, from the liver and kidney of bird 86 and from the ileum of bird 873.

A COMPARISON BETWEEN THE SIMULTANEOUS ACCUMULATION OF BACTERIAL POLYSACCHARIDE IN TISSUES AND THE DEVELOPMENT OF HUMORAL ANTIBODIES IN CHICKENS INFECTED WITH *Salm. gallinarum*

In different groups of infected birds bacterial polysaccharide becomes variously deposited in the tissues and humoral antibodies are demonstrable before death occurs. To obtain further evidence on the possible significance of these two factors they were assessed together in a group of twelve R.I.R. chickens. After oral infection, serum samples were

examined for antibodies at frequent intervals (Fig. 3). From the 4th day onwards, the tissues from at least two birds were examined each day for the presence of bacterial polysaccharide. All the samples from birds that were killed or that died were removed at the time of death and immediately frozen.

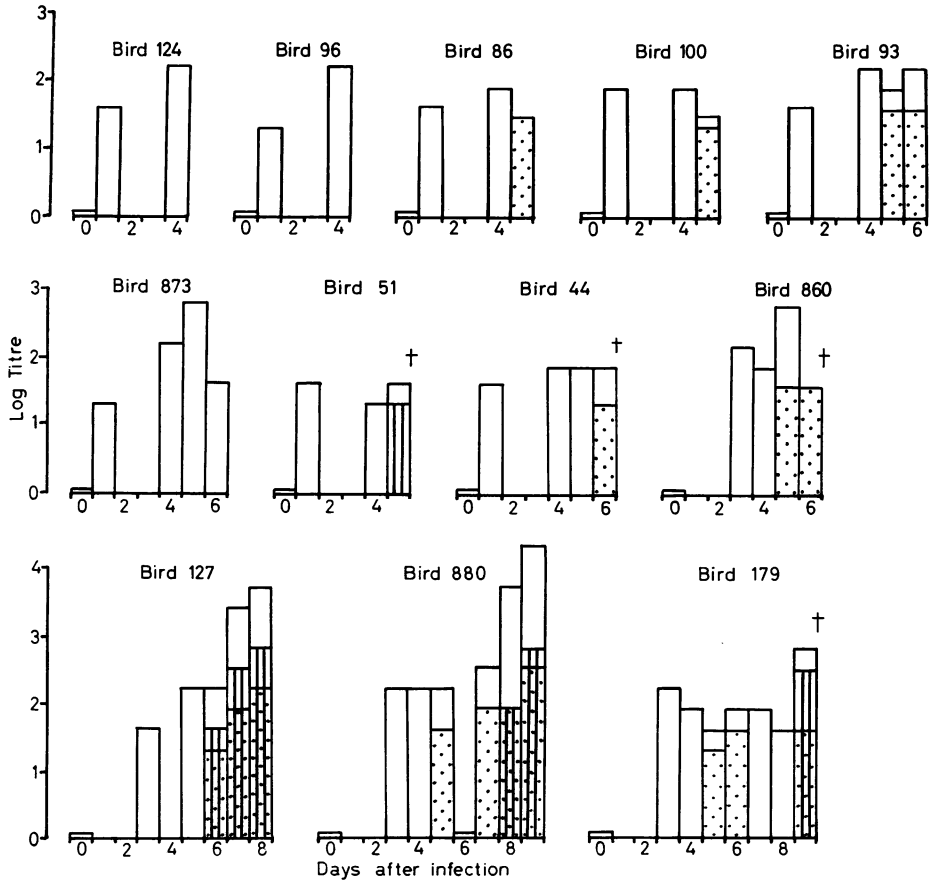

FIG. 3. Antibody responses of a group of twelve chickens which developed acute infection after oral dosage with *Salm. gallinarum*. Key as in Fig. 1. † Died.

The results showed that the concentration of bacterial polysaccharide increased from the 4th day onwards and was particularly high in those birds that died from the disease (Table 3). As in previous experiments there was considerable variation in the amounts found in different organs. An unusual feature was the rapidity with which these chickens developed humoral antibodies, which were demonstrable within a day or two after infection (Fig. 3). The disease was very acute and a number of deaths occurred as early as the 5th and 6th day. It was significant that even in these acute cases humoral antibodies were detectable in all cases before death.

The birds used in this experiment were from a different source to those used previously. Although they came from healthy stock and were known to be free from *Salm. gallinarum* infection, subsequent antibody production showed a typical secondary immunological

response. Preliminary bacteriological examination of these birds had shown that their intestinal tracts contained large Gram-positive bacilli possessing a common antigen with *Salm. gallinarum*. The possible significance of these organisms in relation to subsequent antibody production against *Salm. gallinarum* is the subject of further study.

TABLE 3

THE CONCENTRATIONS OF BACTERIAL POLYSACCHARIDE IN THE TISSUES OF CHICKENS DURING THE DEVELOPMENT OF ACUTE EXPERIMENTAL INFECTION WITH *Salm. gallinarum*

Bird No.	*Time of death after infection (days)*	*Bacterial polysaccharide per g. dry weight of tissue* (μg.)							
		Liver	*Spleen*	*Lung*	*Kidney*	*Heart*	*Duodenum*	*Ileum*	*Caecum*
124	4 K	0	0	0	12·1	0	0	0	0
96	4 K	7·1	0	0	0	0	0	0	0
86	5 K	72·9	25·8	45·5	0	0	24·4	15·5	0
100	5 K	8·5	21·0	0	0	0	6·9	0	0
93	6 K	15·2	7·5	6·9	0	0	0	0	0
873	6 K	0	0	92·5	0	0	0	0	200·1
51	6 D	64·5	356·1	88·2	0	25·0	23·9	0	0
44	7 D	18·9	172·2	7·6	0	187·8	0	0	6·9
860	7 D	220·2	181·9	44·0	0	40·3	0	0	0
127	8 K	21·8	0	0	0	0	21·4	14·2	0
880	9 K	127·0	0	24·1	173·5	0	695·1	825·1	14·7
179	9 D	272·6	174·2	0	0	0	0	0	0

K = killed; D = died.

DISCUSSION

The results, in general, showed that at the time of death there was a high concentration of *Salm. gallinarum* polysaccharide in the tissues of infected birds. In birds that had recovered from an acute infection the concentration was low or undetectable. No bacterial polysaccharide was found in healthy birds. These results are to be expected in a disease which develops as a severe generalized infection. However, there were wide differences in the concentrations of polysaccharide in various tissues from individual birds, and considerable variation in the concentration of polysaccharide in different birds at the time of death. Differences between individuals and between the various tissues were also apparent in the experiments of Ravin *et al.* (1960) who compared the absorption of *Escherichia coli* polysaccharide from the intestines of normal and shocked rabbits after receiving a standard oral dose of polysaccharide.

There are certain factors which may be concerned with the results obtained from experiments of this nature. The increase in bacterial polysaccharide in the tissues for some 2½ hours after death is the result of continued bacterial multiplication while the carcase is still warm. The subsequent fall in concentration may be the result of degradation by polysaccharidases or other enzymes. Variations of polysaccharide concentration may also arise from differences in the response of the reticulo-endothelial system of individuals and from the uneven distribution of bacteria in organs.

These results give some information on the rate and extent of accumulation of bacterial polysaccharide which can be expected to occur in the tissues of infected chickens. Noyes, McInturf and Blahuta (1959) have concluded that the lethal effect of *E. coli* endotoxin in mice could not be related to any particular pattern of distribution within the tissues. They

found, for example, that after intravenous inoculation, all the endotoxin was concentrated in the liver, whereas after intramuscular inoculation about 50 per cent remained at the site of injection. Further experiments on the metabolism of tissues in relation to bacterial polysaccharide and on the method of estimation are necessary before more precise results can be obtained. However, the apparent variation in the tissues of infected chickens would suggest that death occurs as the result of some other factor or factors interacting with polysaccharide *in vivo* rather than as a direct toxic action of this substance on tissue cells.

During the early stages of infection, antibodies to *Salm. gallinarum* can be demonstrated only by the antiglobulin haemagglutination technique and in some cases this was the only method which detected antibody at the time of death. In the tissues of fatally infected chickens the appearance of antibody coincides with the rapid accumulation of bacterial polysaccharide. The properties of this particular type of antibody must be taken into account when considering a possible relationship between antigen–antibody reactions and the pathogenesis of this disease. Buxton and Allan (1963) have shown that non-precipitating humoral antibodies produced by chickens infected with *Salm. gallinarum* and demonstrable by the antiglobulin haemagglutination test are cytophilic, and these antibodies have been detected on circulating leucocytes derived from infected chickens. In our experiments antibodies detected by the antiglobulin haemagglutination test showed a distinctive pattern compared to antibodies detected by the haemagglutination and bacterial agglutination tests. The antiglobulin haemagglutinating antibody appeared first, rose to a very high concentration for a few hours in those birds that survived acute infection, and then rapidly declined (Fig. 2). This characteristic form of early immune response can be accounted for on the assumption that large quantities of cytophilic antibody are released rapidly from the reticulo-endothelial system and some is adsorbed by tissue cells. It is notable that the high concentration of these antibodies in serum was demonstrable for only about 24–36 hours before declining to a level approximating to that of the haemagglutinins. Under experimental conditions, Humphrey and Mota (1959) have demonstrated *in vivo* a latent period of up to 24 hours before antibodies in rabbits become attached to mast cells. In infected chickens some antibody could also have combined with bacterial antigen as well as with tissue cells and this may induce a state of hypersensitivity (Uhr, Salvin and Pappenheimer, 1957). Experimentally, the inoculation into mice of an antigen–antibody mixture containing slight antigen excess causes an accelerated immune response. Moreover, this immune response takes place concurrently with the development of a specific anaphylactic hypersensitivity (Terres and Wolins, 1961).

If antiglobulin haemagglutinating antibodies are concerned in the development of an anaphylactic type of hypersensitivity in an acutely infected chicken, it is interesting to speculate on the combined effect of this developing hypersensitivity with the accumulation of bacterial polysaccharide in the tissues of fatally infected chickens. The ultimate development of an anaphylactic type of reaction would depend upon a variety of factors, including the rate of bacterial multiplication in the tissues, and the ability of the reticulo-endothelial system to cope with increasing quantities of bacterial polysaccharide. The rate of antibody production and its cytophilic quality are significant, and may be altered by the host's previous experiences with antigens from different sources that are related to those of *Salm. gallinarum*. These factors must combine to give an optimal antigen–antibody reaction if symptoms of an anaphylactic type of hypersensitivity are to develop, as has been shown by Makinodan, Wolfe, Goodman and Ruth (1952) who studied the relationship between circulating antibody to bovine albumin and anaphylactic shock in chickens. The

symptoms shown by chickens acutely infected with *Salm. gallinarum* are similar to those recorded by Makinodan *et al.* (1952) for moderately severe forms of anaphylactic shock and also to those resulting from the inoculation of histamine into chickens (Lecomte and Beaumariage, 1958). Experimentally, the injection of soluble antigen–antibody complexes into guinea-pigs can cause both an acute anaphylaxis and a delayed reaction lasting for as long as 24 hours before death (Germuth and McKinnon, 1957; Weigle, Cochrane and Dixon, 1960). The similarity of these reactions produced under different circumstances, together with the evidence that active production of cytophilic antibody occurs prior to death, even in acutely infected birds, suggest that further experiments may show more clearly the role of antigen–antibody reactions in the development of clinical disease and death of chickens infected with *Salm. gallinarum.*

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