

IMMUNE EQUILIBRIA INVOLVING ANTI-ANTIBODY

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ABSTRACT

IMMUNE EQUILIBRIA INVOLVING ANTI-ANTIBODY

Sheep red blood cells sensitized with rabbit-produced amboceptor unite with guinea pig-produced anti-rabbit globulin (anti-antibody), thus becoming less susceptible to complementary lysis. An equilibrium is established between the processes of union of anti-antibody with and dissociation from sensitized cells. The present work involves the effect on this equilibrium of four materials which influence antibody production. The study was of interest because of certain theoretical considerations regarding antibody production.

Three techniques were attempted, two of which were found to be inadequate and discarded. These were the measurement of anti-antibody before and after absorption by sensitized cells and titration of anti-antibody in the presence and absence of additives which were thought on theoretical grounds capable of shifting the equilibrium in either direction. The third technique involved a study of the dynamics of complementary lysis in the presence of additives. The additives used were endotoxin, pertussis vaccine, hydrocortisone succinate, cortisone acetate and Freund's incomplete adjuvant.

Of the additives, only hydrocortisone succinate appeared to influence lysis, causing a decrease. However,

this effect appeared to be attributable to an anticomplementary effect of the steroid rather than an effect on the equilibrium.

An attempt was made to produce homologous anti-antibody.

Author - Joseph Mazur

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INTRODUCTION

INTRODUCTION

This study arose out of certain theoretical considerations concerning antibody production.

Romeyn and Onysko (1964) produced a substance which behaved as an antibody against a cytolytic antibody and termed the substance anti-antibody (synonymous with anti-sensitizer). They found that this anti-antibody, produced by the injection of guinea pigs with normal rabbit serum, inhibited complementary lysis of sheep red blood cells sensitized with rabbit produced hemolysin.

Quantitative relationships among the components of the immune system were further studied. Antisensitizer could be absorbed on sensitized cells and promptly eluted from these cells on the addition of saline. The degree of inhibition of lysis was found to depend on the relative amounts of complement and antisensitizer, rather than the absolute amounts. Further, prolongation of incubation time resulted in a decrease in lysis inhibition by antisensitizer, ultimately reaching a level obtained in the absence of antisensitizer, provided complement was not non-specifically destroyed. These observations were taken as evidence for the existence of a dynamic equilibrium, the antisensitizer competing with complement for the same or closely related receptor sites

on the sensitized cells and in a continuous state of association and dissociation with these cells.

The theory referred to above proposes that the fundamental stimulus to antibody production is the action of cytolytic antibody on a potentially antibody-producing cell. The antibody which such a stimulated cell produces against the cytolytic antibody then serves to protect the cell from lysis by complement. In the case of the introduction of a foreign antigen, it is considered that the configuration of the combined cytolytic antibody is modified by the foreign antigen. The sensitized cell then proceeds to produce an antibody which is directed against the foreign antigen, and which again protects the cell from lysis by complement. Antibody against a foreign antigen is thus viewed as an "abnormal" anti-antibody.

These theoretical considerations can be conveniently expressed by the use of symbols: L represents the potentially antibody-producing cell; LBg is the cytolytic antibody, an antibody (B) directed against the cell L and composed of gamma globulin g. Anti-antibody is correspondingly represented by gBg. The antibody-producing cell, protected from lysis, then becomes L.LBg.gBg. If F represents a foreign antigen (possibly altered by macrophage activity), the sensitized cell after absorbing F becomes L.LBgF and the "abnormal" anti-antibody subsequently formed is FBg, the antibody-producing

cell becoming L.LBgP.FBg. (The abnormal anti-antibody might also be written FgBg.)

From these considerations, antibody production would be governed by the supply of free, sensitized cells, cells with the cytolytic antibody attached (L.LBg). This, in turn, would be governed by the same sort of dynamic equilibrium noted in the system involving sensitized sheep cells, the equilibrium being written as follows:



If this equilibrium were shifted in the direction of either a greater or lesser supply of free, sensitized cells, antibody production would be correspondingly greater or less. It was the object of this study to determine whether certain substances would shift the equilibrium in either direction.

It is known that certain substances may have an effect on the immune response in animals. Kind and Johnson (1959) showed that injection of rabbits with endotoxin plus protein antigens produced a marked increase in antibody formation. Injection of endotoxin prior to antigen had no effect while injection two to three days after antigen still enhanced the antibody response. Further, Ward and Johnson (1959) showed that bacterial endotoxins incited antibody formation that had been previously inhibited by cortisone treatment.

Levine and Wenk (1965) showed that vaccination of mice and rats with B. pertussis enhanced the antigenic effect

of central nervous system emulsion in adjuvant in producing experimental encephalomyelitis. Further, injection of pertussis vaccine mixed with nervous tissue in the absence of adjuvant still resulted in encephalomyelitis. These two substances, endotoxin and pertussis vaccine, thus appear to act as does Freund's adjuvant in enhancing the immune response.

The anti-inflammatory effect of hydrocortisone is well known. This steroid has been shown to reduce the antibody response in such species as mouse and rabbit while not affecting the response in other species such as human. Raffel (1961) describes how this species difference has been related to the differential production of the hormone by the various species. Hydrocortisone administration has the most pronounced effect in the species that produce little of the hormone, the rat, mouse and rabbit; on the other hand, administration of hydrocortisone has little effect in the species in which the hormone is normally produced, man and monkey.

It was of interest, therefore, to determine whether the substances which affect the immune response in a given way would correspondingly affect the equilibrium of the immune system involving anti-antibody: an increase in the amount of free, sensitized cells corresponding to increased antibody production, as indicated in the theoretical considerations.

The experimental model used in these studies consisted

of sheep erythrocytes (E), rabbit-produced sensitizer (R₂g)
and guinea pig-produced antisensitizer (g₂g).

CHAPTER I

REVIEW OF THE LITERATURE

CHAPTER I

REVIEW OF THE LITERATURE

There is little in the literature concerning immune lysis and antisensitizer as a system in equilibrium. Evidence for the existence of a dynamic equilibrium has been considered. The effects of endotoxin and pertussis vaccine have also been considered. The effect of corticosteroids on antibody formation has been comprehensively reviewed by McMaster and Franzel (1961) and on immunity in general by Kaas and Finland (1953) and will not be considered here. Literature will be reviewed from 1950 to 1966 on the subject of the effect of hydrocortisone on immune lysis in vitro.

The Effect of Hydrocortisone on Immune Lysis in vitro

Some workers have reported that hydrocortisone has no effect on immune lysis. Simonsen (1950) titrated complement in vitro in the presence of the acetate form of the steroid and found no difference in titre although in in vivo studies, he found that the complement titre dropped after injection of the hormone. Irvine (1960) cultured thyroid cells and found that the addition of the steroid in succinate form did not affect the action of cytotoxic sera in damaging the cells.

Weiss and Dingle (1964) found using rat dermal

fibroblasts in culture that cell death resulted when an unheated antilyosomal serum was added to the culture in the presence of hydrocortisone succinate, although the lysosomes appeared stabilized.

The majority of studies in this field indicate that hydrocortisone has an inhibitory effect on complementary lysis. Fell and Weiss (1965) observed foetal mouse bone cultures in the presence of an unheated antiserum and hydrocortisone and found that the steroid abolished necrosis and preserved the integrity of cells as compared to control cultures without hydrocortisone.

An inhibitory effect on the immune lysis of red blood cells has also been noted. Jennings and Taylor (1964) reported an inhibitory effect when titrating complement. In 1966, Jennings reported a detailed study of the hydrocortisone effect. He provided what he considered evidence that hydrocortisone forms an unstable association with the reactants of the immune system which blocks the combination of complement with the sensitized cells. Pre-incubation of hydrocortisone and complement did not cause a greater reduction in lysis and Jennings concluded that the steroid was not directly anticomplementary, that is, did not interact with free complement. Jennings used higher steroid concentrations than previous workers and thus suggests an explanation for their negative results.

Cewurz, Wernick, Quie and Good (1965) came to a different conclusion, however. In their studies, hydrocortisone succinate and complement were pre-incubated before adding to sensitized red cells and it was found that as pre-incubation time was increased, there was a proportionate increase in inhibition of lysis in their system. These workers concluded that the action of hydrocortisone was on the fluid phase of the sera supplying the complement and not on the cell membrane. Both mechanisms may operate, however, since Jennings also found that hydrocortisone inhibited non-immune lysis such as that by bacterial lysins and ultraviolet radiation.

MADEIRA AND METHODS

CHAPTER II

CHAPTER II

MATERIALS AND METHODS

General

All sera were inactivated at 58°C for thirty minutes when used for the first time and subsequently at 58°C for ten minutes before each use.

Optical densities were measured using a Bausch and Lomb Spectronic Twenty at a wavelength of 540 $m\mu$ unless otherwise specified.

Sheep Cells

Sheep's blood was collected in a Baxter Transfuso Vac¹ bottle containing 2.30 gm. per cent Dextrose and 1.70 gm. per cent Sodium Citrate. It was stored in the refrigerator at least four days before use. When used, the cells were washed three times with modified Dulbecco Phosphate Buffered Saline (DBS)² in a 15.0 ml. centrifuge tube at a centrifuge speed of 2000 R.P.M. for five minutes each time. From the sediment of packed red cells remaining after the final washing, a suspension was made in DBS such that 1.0 ml. plus 9.0 ml. distilled water gave an optical density of 0.225 at a

¹Baxter Laboratories of Canada Ltd., Alliston, Ontario.

²see Appendix.

wavelength of 550 μ . This suspension will be referred to as "0.225 cells" and is approximately a one per cent suspension. Cells were prepared freshly each day and refrigerated until use.

Sensitizer

Rabbit anti-sheep cell hemolysin was obtained from Markham Laboratories, Chicago 20, Illinois.

Titration of Sensitizer

Sensitizer was diluted in DBS in 20.0 ml. volumes in 50.0 ml. centrifuge tubes to which were added equal volumes of 0.225 cells. After incubation for twenty minutes at 37°C with intermittent shaking, the tubes were centrifuged at 5°C for five minutes at 2000 R.P.M. and the supernatants discarded. DBS was added to the packed cells without resuspending and centrifugation was repeated. This procedure will be termed "washing briefly once". The supernatants were discarded and the cells resuspended to their original concentration by the addition of 19.8 ml. DBS to each tube, using a Vortex Junior Mixer to resuspend. From each tube 1.0 ml. was then transferred to a corresponding series of 16 x 100 mm. serology tubes and 2.0 ml. DBS and 1.0 ml. of 1:30 guinea pig complement was added to each tube. The tubes were incubated at 37°C for twenty minutes with intermittent shaking and centrifuged at 5°C for five minutes at 2000 R.P.M.

Optical densities of the supernatants were measured against a blank of 1.0 ml. of 1:30 complement plus 3.0 ml. DBS. The percentage lysis was determined using cells lysed in the presence of excess sensitizer as one hundred per cent lysis.

$$\text{Percentage lysis} = \frac{\text{Optical density of tube}}{\text{Optical density at 100 per cent lysis}} \times 100$$

The readings were plotted on logarithmic-probability paper with sensitizer dilutions as abscissae and percentage lysis as ordinates. The best straight line was drawn and the dilution giving fifty per cent lysis was determined. Four fifty per cent hemolytic doses were used throughout this work in routine sensitization, the dilution being 1:40,000. Control tubes included one with no complement, one with no sensitizer and one with neither sensitizer nor complement.

Sensitization of Cells

Twenty ml. of 0.225 cells were added to 20.0 ml. of 1:40,000 sensitizer in 50 ml. centrifuge tubes which were then incubated at 37°C for twenty minutes. The cells were washed briefly once and resuspended in 19.8 ml. DBS and pooled after mixing. The cells were refrigerated or kept in an ice bath.

Complement

The source of complement was dehydrated guinea pig serum¹

¹Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

It was reconstituted to the requisite volume with distilled water before use and kept in an ice bath.

Titration of Complement

Complement was titrated each day before use. A dilution of 1:100 was made in DBS. A row of ten tubes was set up and 1.0 ml. of 1:100 complement was added to the first tube, 0.9 ml. to the second tube, 0.8 ml. to the third tube, and so on to the tenth tube which received 0.1 ml. complement. DBS was then added, 0.1 ml. to the second tube, 0.2 ml. to the third tube, 0.3 ml. to the fourth tube and so on to bring the volume to 1.0 ml. The dilutions were made in duplicate and kept in an ice bath. Next, 2.0 ml. DBS and 1.0 ml. sensitized cells were added. Controls included only sensitized cells and DBS, only unsensitized cells and DBS and unsensitized cells, complement and DBS. The tubes were incubated at 37°C for twenty minutes with intermittent shaking and centrifuged at 5°C for five minutes at 2000 R.P.M. Optical densities of the supernatants were measured against a blank of DBS. Percentage lysis was then determined using the density of the supernatant from 1.0 ml. sensitized cells lysed with 3.0 ml. distilled water as one hundred per cent lysis.

Results were plotted on probability paper with volumes of undiluted complement as abscissae (arithmetic scale) and percentage lysis as ordinates (probability scale). The

The best straight lines were drawn and the average of the volumes of complement giving fifty per cent lysis was taken as one fifty per cent hemolytic dose (HD_{50}). Four HD_{50} was used in titrations of antisensitizer.

Antisensitizer

Antisensitizer was prepared in guinea pigs by the intraperitoneal injection of 2.0 ml. amounts of eighty per cent guinea pig red cells sensitized with rabbit-produced amboceptor. The animals were injected in two courses of six injections during a three week interval with a two week rest period between courses. After the final injection, the animals were bled on the fifth and sixth days by cardiac puncture and the serum stored at -20°C .

Titration of Antisensitizer

The sera were titrated by a method involving inhibition of complementary lysis of sensitized red cells. Serial doubling dilutions of serum were made in 2.0 ml. volumes of PBS, 1.0 ml. of each dilution being transferred to a corresponding tube in a second row. To each tube in the first row was added 1.0 ml. sensitized cells and a similar volume of unsensitized cells was added to the second row of tubes. The tubes were incubated at 37°C for twenty minutes with intermittent shaking and removed to an ice bath where 2.0 ml. PBS containing $\frac{1}{4}$ HD_{50} complement was added. The tubes were again

incubated at 37°C for twenty minutes with intermittent shaking. The tubes were then centrifuged at 5°C for five minutes at 2000 R.P.M. Optical densities of the supernatants from the first row of tubes were measured using corresponding tubes in the second row as blanks. This allowed for any colour in the supernatants due to the presence of the serum being titrated. Using one hundred per cent lysis as the optical density of the supernatant of a tube containing 1.0 ml. sensitized cells, 1.0 ml. DBS and 2.0 ml. complement (4 ED₅₀) incubated with the other tubes, the percentage lysis was determined. The percentage inhibition of lysis in any tube is the percentage lysis subtracted from one hundred. The last tube showing more than seven and one half per cent inhibition of lysis was taken as the end point in a titration, and titres were expressed as initial dilutions. Results were plotted with dilutions of antisensitizer as abscissae and percentage lysis as ordinates, both on an arithmetic scale.

Endotoxin

The endotoxin used was Lipopolysaccharide W.E.Coli O111:B₄, obtained from Difco Laboratories, Detroit, Michigan.

Pertussis Vaccine

B. pertussis vaccine containing 15 billion killed organisms per ml. was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

Hydrocortisone

Cortone (Cortisone Acetate) was obtained from Merck, Sharp and Dohme of Canada, Ltd., Montreal. Solu Cortef (Hydrocortisone Sodium Succinate) was obtained from the Upjohn Company of Canada, Don Mills, Ontario.

The above four materials will be referred to as "additives".

Absorption of Antisensitizer by Sensitized Cells

For absorption of antisensitizer, a heavy suspension of cells, twenty-five per cent, was used. Cells were sensitized as usual in 50 ml. centrifuge tubes and centrifuged at 5°C for five minutes at 2000 R.P.M. The sedimented cells were then transferred to a 15.0 ml. graduated centrifuge tube and centrifuged at 5°C for ten minutes at 2000 R.P.M. The supernatants were discarded and DBS was added in a volume equal to that of the cell pellet giving a fifty per cent suspension after mixing. Unsensitized cells were prepared similarly.

To 1.0 ml. amounts of antisensitizer dilution, usually in quadruplicate, were added equal volumes of fifty per cent sensitized cells, and to four further tubes, unsensitized cells. The tubes were incubated at 37°C for twenty minutes followed by centrifugation at 5°C for five minutes at 2000 R.P.M. The supernatants were then removed and stored overnight at -20°C.

On the following day, antisensitizer titrations were done. The titration results were plotted with the amounts of undiluted antisensitizer as abscissae and the percentage inhibition of lysis ("protection") as ordinates. The best straight line was drawn and the amount of antisensitizer giving fifty per cent protection from lysis was taken as one HPD_{50} . The total number of HPD_{50} in 1.0 ml. of the supernatants was then calculated. The HPD_{50} value of the supernatant from unsensitized cells was taken as the value without specific absorption and the HPD_{50} value of the supernatant from sensitized cells the value after specific absorption by sensitized cells.

Dynamic Studies of Complementary Lysis

The dynamic studies provided a more sensitive means of observing complementary lysis than the amount of lysis after the usual twenty minute incubation period. In this method, 1.0 ml. of antisensitizer dilution was added to two rows of tubes followed by the addition of 1.0 ml. sensitized cells. These were incubated at 37°C for twenty minutes and removed to an ice bath where 1.0 ml. amounts of DBS to the first row and additive to the second row were added, followed by the addition of 1.0 ml. of an appropriate complement dilution to each tube. The tubes were incubated at 37°C and duplicate tubes from each row removed to an ice bath at given time intervals, usually five minutes. After forty-five minutes,

the tubes were centrifuged at 5°C for five minutes at 2000 R.P.M. The optical densities of the supernatants were measured using a tube removed to the ice bath at zero time as blank. The percentage lysis in each tube was determined using as one hundred per cent lysis a tube containing 2.0 ml. DBS, 1.0 ml. sensitized red cells and 1.0 ml. complement incubated for forty-five minutes. A curve was drawn with times of incubation as abscissae and percentage lysis as ordinates.

Production of Homologous Antisensitizer

The source of the Freund's adjuvant formula was Crowle (1961). Approximately 100 mg. of heat-killed tubercle bacilli were emulsified in 5.0 ml. of one per cent guinea pig gamma globulin¹ dissolved in DBS. To this was added 3.75 ml. Bayol F and 1.25 ml. Arlacel A, the mixture being emulsified with a ten ml. syringe.

The immunization schedule consisted of three subcutaneous injections of 0.5 ml. emulsion per guinea pig at weekly intervals. The animals were bled on the fifth and sixth days following the last injection, the serum separated and stored at -20°C.

¹Hyland Laboratories, Los Angeles, California.

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

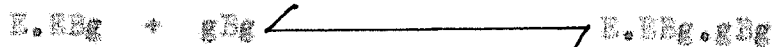
CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

Three techniques were attempted in the study of the equilibrium. The first two were found inadequate and discarded. These were absorption of antisensitizer by sensitized cells followed by titration of the supernatant to determine the amount of free antisensitizer remaining, and titration of antisensitizer in the presence and absence of additive. The third technique involved a study of the dynamics of complementary lysis in the presence of antisensitizer and additive. Experiments were performed in the presence of the additives endotoxin, pertussis vaccine, hydrocortisone succinate, cortisone acetate and Freund's adjuvant.

I. ABSORPTION OF ANTISENSITIZER BY SENSITIZED CELLS

The experimental equilibrium is as follows:



In the absorption system, $E.SB_g$ represents a large dose of sensitized cells, the absorbing cells; gB_g is the free antisensitizer which is titrated before and after absorption. The remainder of the equation, the antisensitizer bound to the sensitized cell, can be calculated from the known free antisensitizer before and after absorption. The amount

of free antisensitizer after absorption, determined by titration of the supernatant, represents the amount at equilibrium. If the presence of an additive changes the equilibrium, the amount of free antisensitizer would be changed, decreasing in the case of a shift to the right and increasing in the case of a shift to the left in the above equation. Such shifts would also indicate a change in the amount of free, sensitized cells.

Procedure

Dilutions of antisensitizer were absorbed¹ both in the presence and absence of cortisone acetate.

Results

The results, with antisensitizer expressed in NPD₅₀ per ml., are shown in Table I. There was no significant difference in the amount of free antisensitizer after absorption in the presence and absence of cortisone acetate and thus no apparent effect of the steroid on the equilibrium of the system. The decrease in free antisensitizer after incubation showed that absorption had taken place.

This technique was considered too insensitive to detect small changes in the equilibrium because of the heavy suspension of red cells necessary to obtain measurable absorption. There was also difficulty in obtaining similar results

¹See Materials and Methods

in duplicate samples. Accordingly, this technique was discarded at this point.

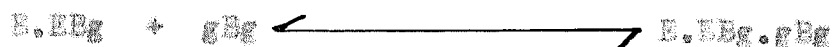
TABLE I

ABSORPTION OF ANTISENSITIZER WITH SENSITIZED RED CELLS
IN THE PRESENCE AND ABSENCE OF
CORTISONE ACETATE

Antisensitizer Dilution (Serum #235)	Cortisone Acetate (μ g./ml.)	Free antisensitizer without absorption (HPD_{50} /ml.)	Free antisensitizer after absorption (HPD_{50} /ml.)
1:68	0	35,35,35,35	11,13,18,13
	25	35,35,35	10,11,12
	50	35,35,35,35	8,12,11,12
1:75	0	31,31	10,10,10,9
	333.3	31,31	9,9

II. TITRATION OF ANTISENSITIZER IN PRESENCE OF ADDITIVES

The equilibrium again is written as follows:



If the presence of additive caused a shift in the equilibrium to the left, the titration curve would show a greater amount of lysis because of the increase in free, sensitized cells susceptible to lysis by complement. If the titration curve showed less lysis in the presence of additive

this would indicate a shift to the right, where antisensitizer is bound to the sensitized cell and protects it from lysis by complement.

Procedure

In order to rule out any effect of additive on the complement rather than on the equilibrium, complement was titrated in the presence of additive by incorporating additive in the DBS. All concentrations of additive quoted are final concentrations.

Results

Table II gives the results of complement titrations in the presence and absence of cortisone acetate. There was an apparent decrease in lysis as shown by the increased ED_{50} values in the presence of the steroid.

Table III gives the results of complement titrations in the presence of pertussis vaccine. The concentration of vaccine used in vivo to enhance the production of encephalomyelitis was a 1:5 dilution.

Table IV shows the results of titrations of complement in the presence and absence of endotoxin. There was no significant change in lysis in the presence of the endotoxin. The concentration of endotoxin used in vivo to enhance antibody production was 10 μ g. per ml.

Table V shows the results of complement titrations in

the presence and absence of hydrocortisone succinate. There was an apparent, but not consistent, decrease in lysis in the titrations.

TABLE II

TITRATION OF COMPLEMENT IN THE PRESENCE
AND ABSENCE OF CORTISONE ACETATE

Cortisone Acetate ($\mu\text{g./ml.}$)	Complement ND_{50} (ml.)
0	0.0036
100	.0036
0	.0034
20	.0045
1000	.0052
0	.0045
1000	.0058
0	.0040
500	.0049

TABLE III
 TITRATION OF COMPLEMENT IN THE PRESENCE
 AND ABSENCE OF PERTUSSIS VACCINE

<u>H. pertussis</u> dilution	Complement ND ₅₀ (ml.)
0	0.0047
1:92	.0047
0	.0046
1:64	.0050
0	.0048
1:64	.0048
0	.0054
1:53	.0052
0	.0054
1:53	.0054

TABLE IV
 TITRATION OF COMPLEMENT IN THE PRESENCE
 AND ABSENCE OF ENDOTOXIN

Endotoxin ($\mu\text{g./ml.}$)	Complement HD_{50} (ml.)
0	0.0050
1.0	.0054
100	.0054
0	.0074
0.5	.0080
50	.0078
0	.0067
6.25	.0063
.00625	.0064

TABLE V
 TITRATION OF COMPLEMENT IN THE PRESENCE AND
 ABSENCE OF HYDROCORTISONE SUCCINATE

Hydrocortisone Succinate (μ M)	Complement HD_{50} (ml.)
0	0.0034
100	.0046
0	.0048
1.0	.0056
100	.0056
0	.0050
0.01	.0054
.1	.0058
1.0	.0058
10	.0060
0	.0042
0.001	.0042
.01	.0042
.1	.0042
0	.0052
0.001	.0052
.01	.0052
.1	.0052

From the above results of complement titrations, it was seen that in some cases there was an apparent interaction of additive with complement. This would necessitate increasing the amount of complement in the antisensitizer titrations to obtain the corresponding 4 HD_{50} dilution used. Thus any subsequent alteration in lysis might be attributed to the higher complement level in the presence of additive rather than an effect of additive on the equilibrium. Accordingly, this technique was abandoned at this point.

III. DYNAMIC STUDIES OF COMPLEMENTARY LYSIS

It was thought at this point that the rate of lysis might provide a more sensitive measure of any difference in lytic behavior in the presence of additive than merely the amount of lysis after a given incubation period. It was considered that the amount of lysis might be the same after incubation whether the additive was present or not, even though the amount of lysis was different earlier during the incubation period. Accordingly, dynamic studies were devised and adapted to the equilibrium system.

The equilibrium is written as follows:



Again, any equilibrium shift in the presence of additive would be indicated by a greater or lesser lysis, the greater lysis occurring in a shift toward the free, sensitized cells ($E.BB_g$)

and the lesser lysis in a shift toward the cells with anti-sensitizer attached (E. 52g.gHg).

Procedure for dynamics of lysis with hydrocortisone

Cortisone acetate was no longer used because of its small solubility and the more soluble form, hydrocortisone succinate, was used instead. The hydrocortisone concentration was chosen to correspond to the highest level which was shown by Gewurz et al. (1965) not to give an anticomplementary effect. In terms of final concentration, this was 0.32 mg. per ml.

A forty-five minute incubation period was chosen and from preliminary experiments the concentrations of antisensitizer and complement were obtained which gave a suitable range of lysis during this incubation period. These concentrations were 1:500 antisensitizer and 6 HD₅₀ complement. The same antisensitizer serum, #660, was used throughout the majority of the dynamic studies.

Results

Figure 1 shows the lysis curve at 1:500 antisensitizer and 0.32 mg. per ml. hydrocortisone using 6 HD₅₀ complement over the forty-five minute incubation period. There was a reduction in lysis in the presence of the steroid up to a maximum of approximately ten per cent. This result was confirmed in ten other experiments. The reduction of lysis after

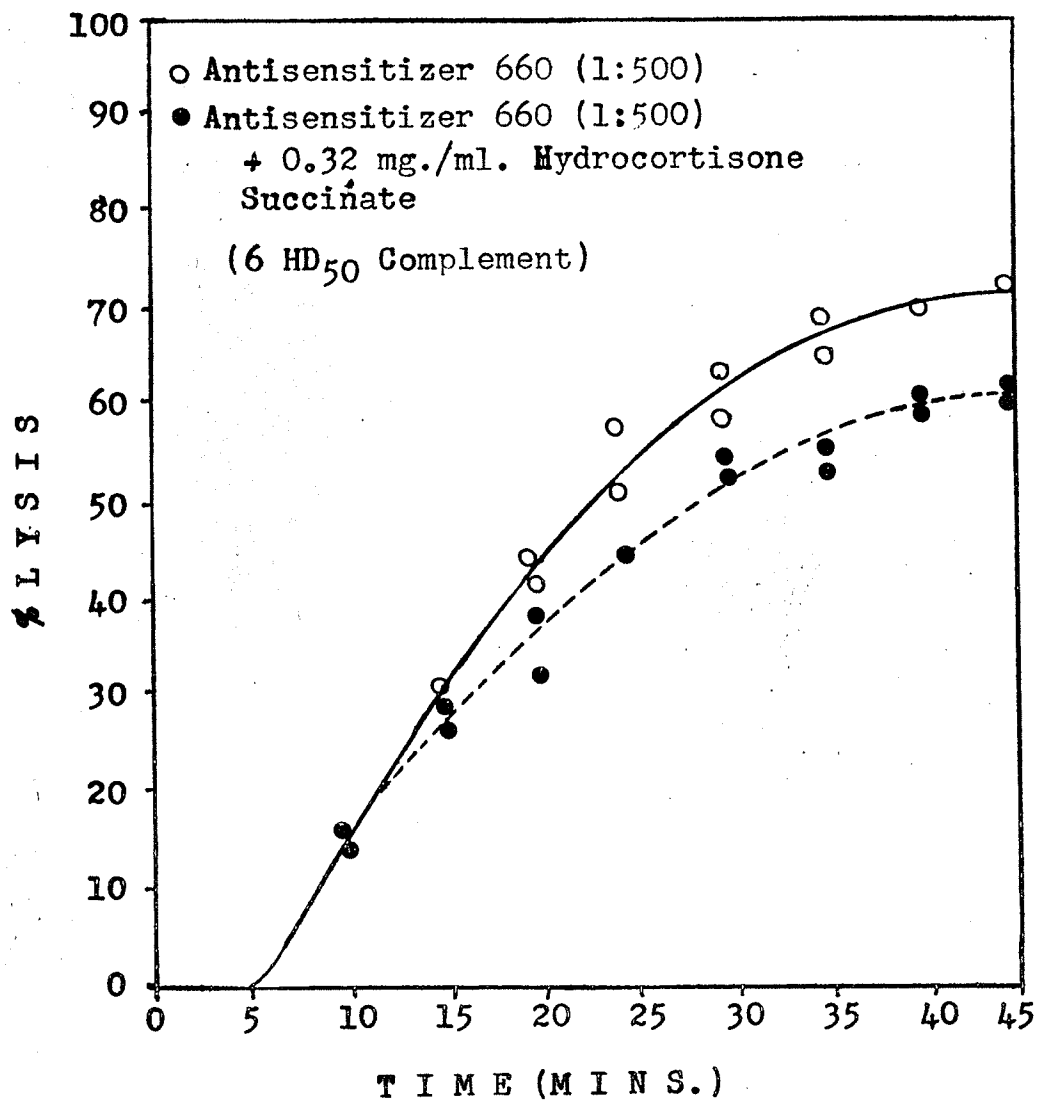


Fig. 1. Dynamics of complementary lysis in the presence of antisensitizer, with and without hydrocortisone succinate.

forty-five minutes of incubation in these experiments is tabulated in Table VI. The mean value is 8.3 per cent, the standard deviation 3.1 and the standard error 0.9.

TABLE VI

REDUCTION OF LYSIS BY HYDROCORTISONE SUCCINATE
IN THE PRESENCE OF ANTISENSITIZER

Experiment number	Reduction of lysis after 45 minutes incubation (%) (1:500 Antisensitizer 6 ED_{50} Complement)
1.	7
2.	8
3.	3
4.	10
5.	11
6.	7
7.	7
8.	12
9.	12
10.	3
11.	11

Procedure to test possibility of technical error

In order to rule out technical error, the experiment was repeated at lower concentrations of hydrocortisone, using 1/10 and 1/1000 the usual level (0.032 and 0.00032 mg. per ml., respectively).

Results

Figure 2 shows the curve of lysis at a hydrocortisone level of 0.032 mg. per ml. There was a decrease in the inhibitory effect. At 0.00032 mg. per ml. as shown in Figure 3, there was a complete abolition of the inhibitory effect of hydrocortisone on lysis, thus ruling out technical error.

Procedure to test effect of Solu Cortef vehicle

Since Solu Cortef also contains buffer salts and the preservatives methylparaben and propylparaben, an experiment was performed to ensure that the effect on lysis was due to the steroid and not these substances. The vehicle¹ containing the ingredients of Solu Cortef but lacking hydrocortisone succinate was diluted similarly to the Solu Cortef in previous experiments and dynamic studies were performed.

Results

Figure 4 shows that the vehicle had no effect on

¹supplied through the courtesy of Dr. Edward L. Masson, Upjohn Company of Canada, Don Mills, Ontario.

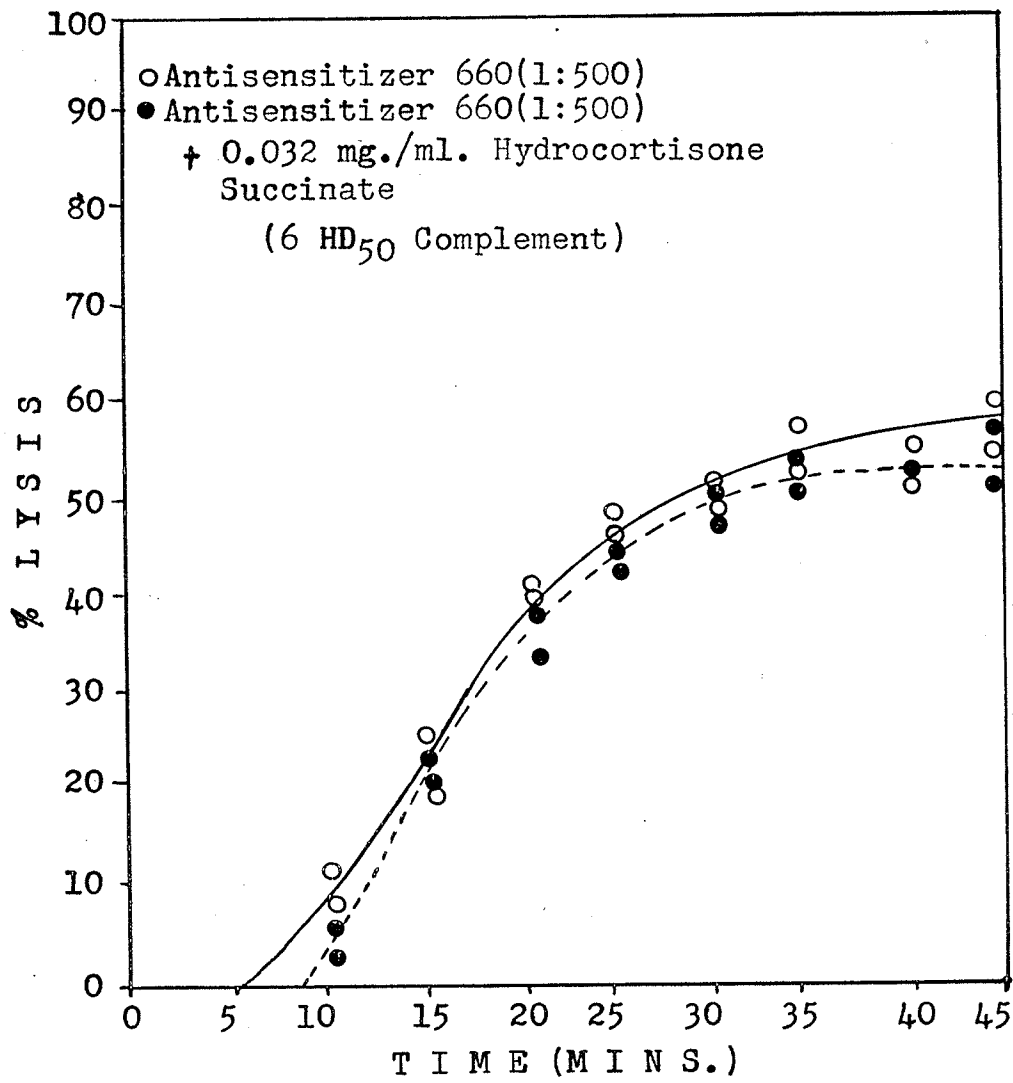


Fig. 2. Dynamics of complementary lysis in the presence of antisensitizer, with and without hydrocortisone succinate.

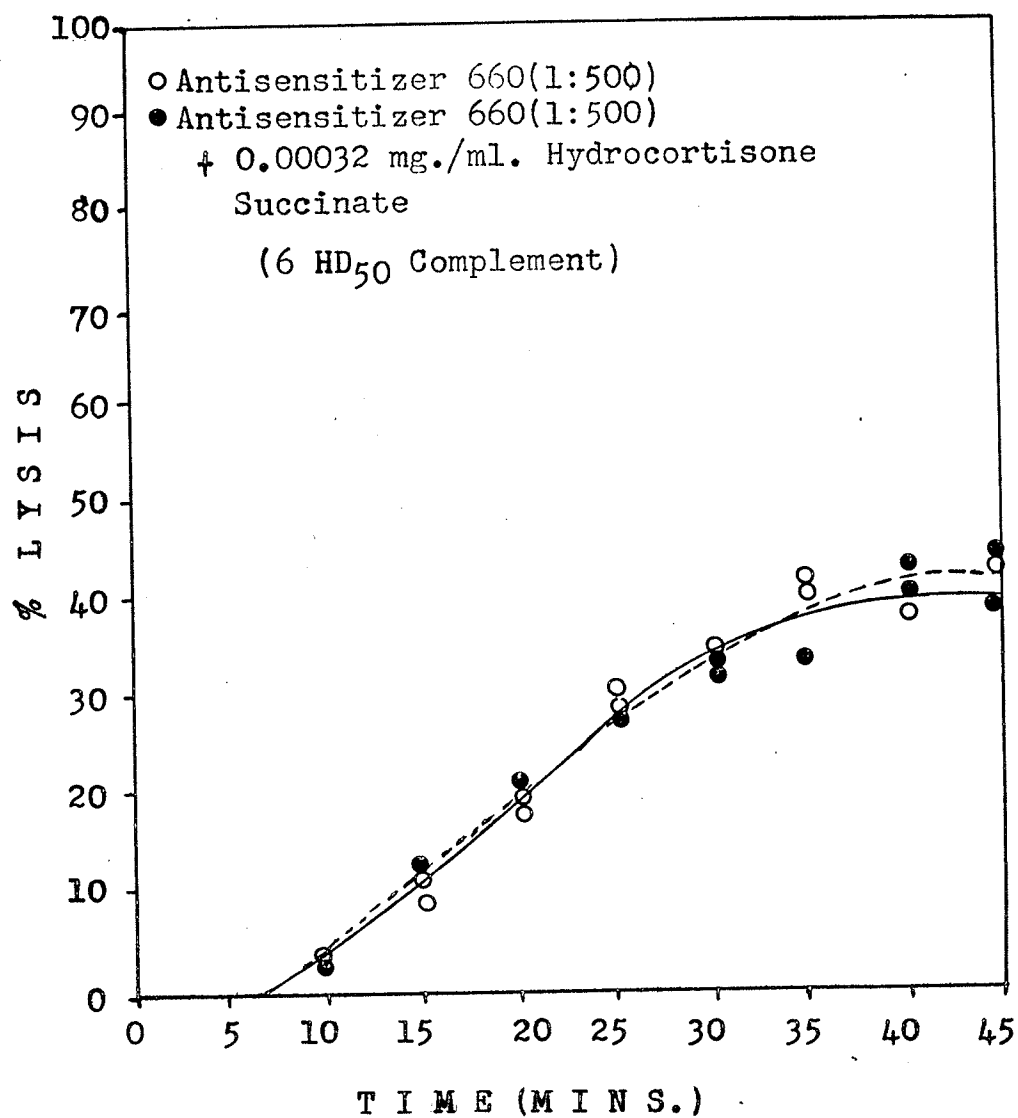


Fig. 3. Dynamics of complementary lysis in the presence of antisensitizer, with and without hydrocortisone succinate.

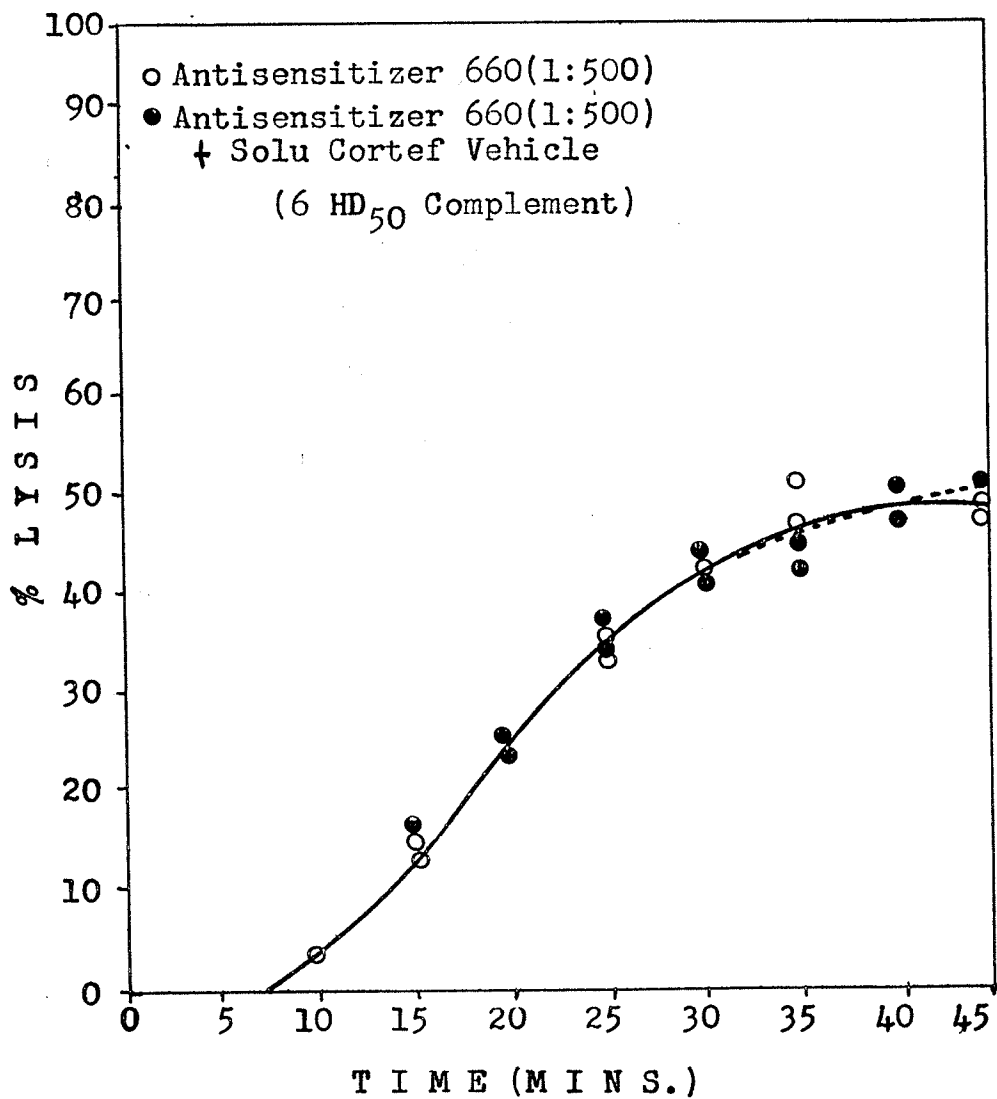


Fig. 4. Dynamics of complementary lysis in the presence of antisensitizer, with and without Solu Cortef vehicle.

complementary lysis and that the inhibitory effect observed with Solu Cortef was due to the hydrocortisone succinate. This experiment was repeated once with the same result.

Procedure to test effect of normal serum

To test the possibility that hydrocortisone was acting on the complement rather than shifting the equilibrium, normal guinea pig serum at a dilution of 1:500 was substituted for antisensitizer. The complement level was lowered to 3 HD_{50} in order to decrease the rate of lysis suitably.

Results

Figure 5 shows that in the presence of normal serum there was an inhibitory effect on lysis, the inhibition apparently due to some sort of anticomplementary action of the steroid. This result was confirmed in four other experiments while two experiments showed no difference in lysis using various concentrations of normal serum. The results are tabulated in Table VII. The mean reduction is 4.0 per cent with a standard deviation of 2.8.

Procedure for dynamics of lysis with endotoxin

The concentration of endotoxin, expressed in terms of final concentration, was 100 μ g. per ml., ten times the concentration used in vivo to enhance antibody production.

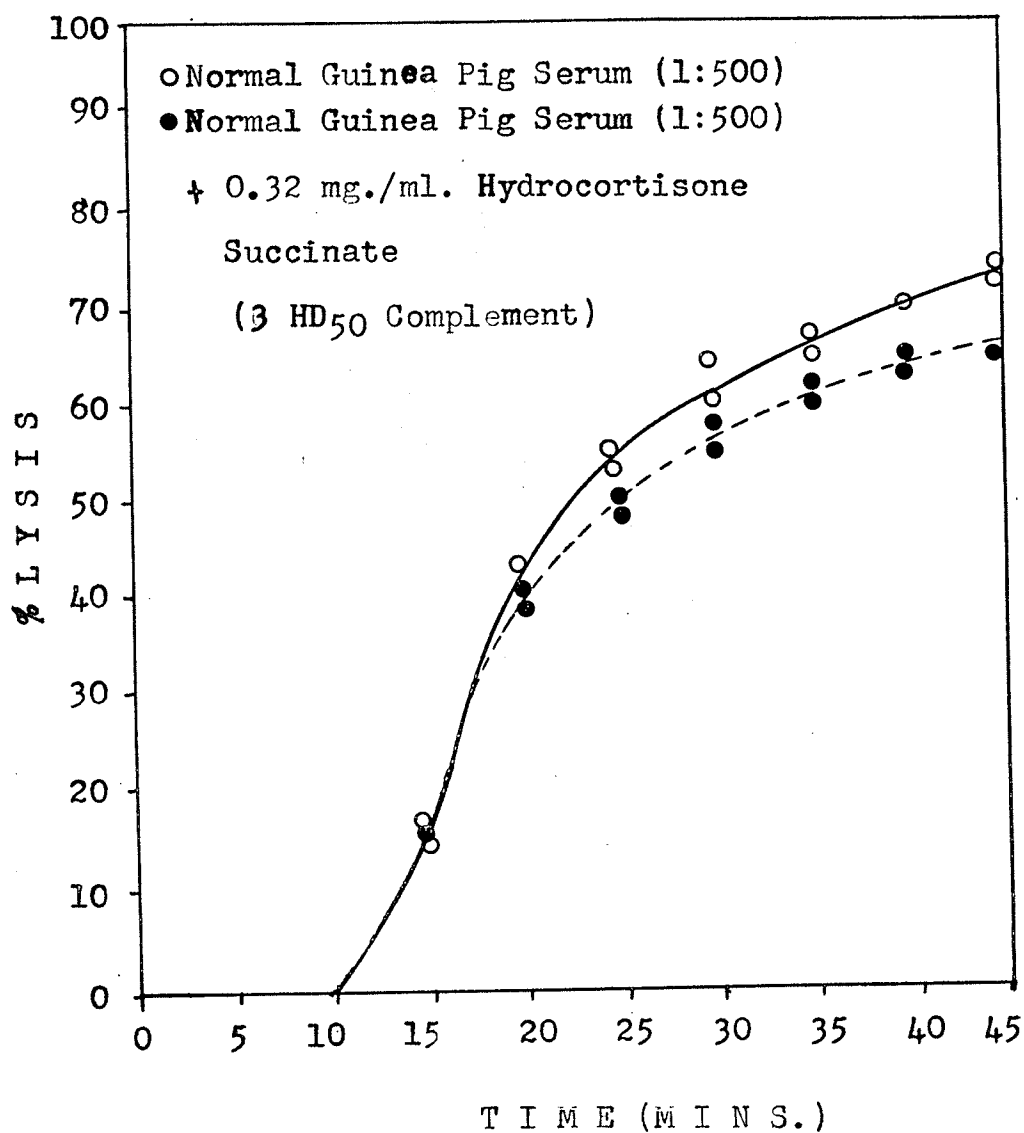


Fig. 5. Dynamics of complementary lysis in the presence of normal guinea pig serum, with and without hydrocortisone succinate.

TABLE VII

REDUCTION OF LYSIS BY HYDROCORTISONE SUCCINATE
IN THE PRESENCE OF NORMAL SERUM

Experiment number	Dilution of normal serum (3 HD ₅₀ complement)	Reduction of lysis after 45 minutes incubation (%)
1.	1:20	0
2.	1:10	4
3.	1:10	0
4.	1:500	6
5.	1:500	6
6.	1:9	5
7.	1:9	5

Results

Figure 6 shows the lysis curves with and without endotoxin. There was no difference in lysis in the presence of endotoxin and hence no apparent effect on the equilibrium. This experiment was repeated once with the same result.

Procedure to test effect of normal serum with endotoxin

The above experiment was repeated with 1:10 normal guinea pig serum in place of antisensitizer to ensure that there was no interaction of endotoxin with complement. Such an interaction might obscure an effect of endotoxin on the

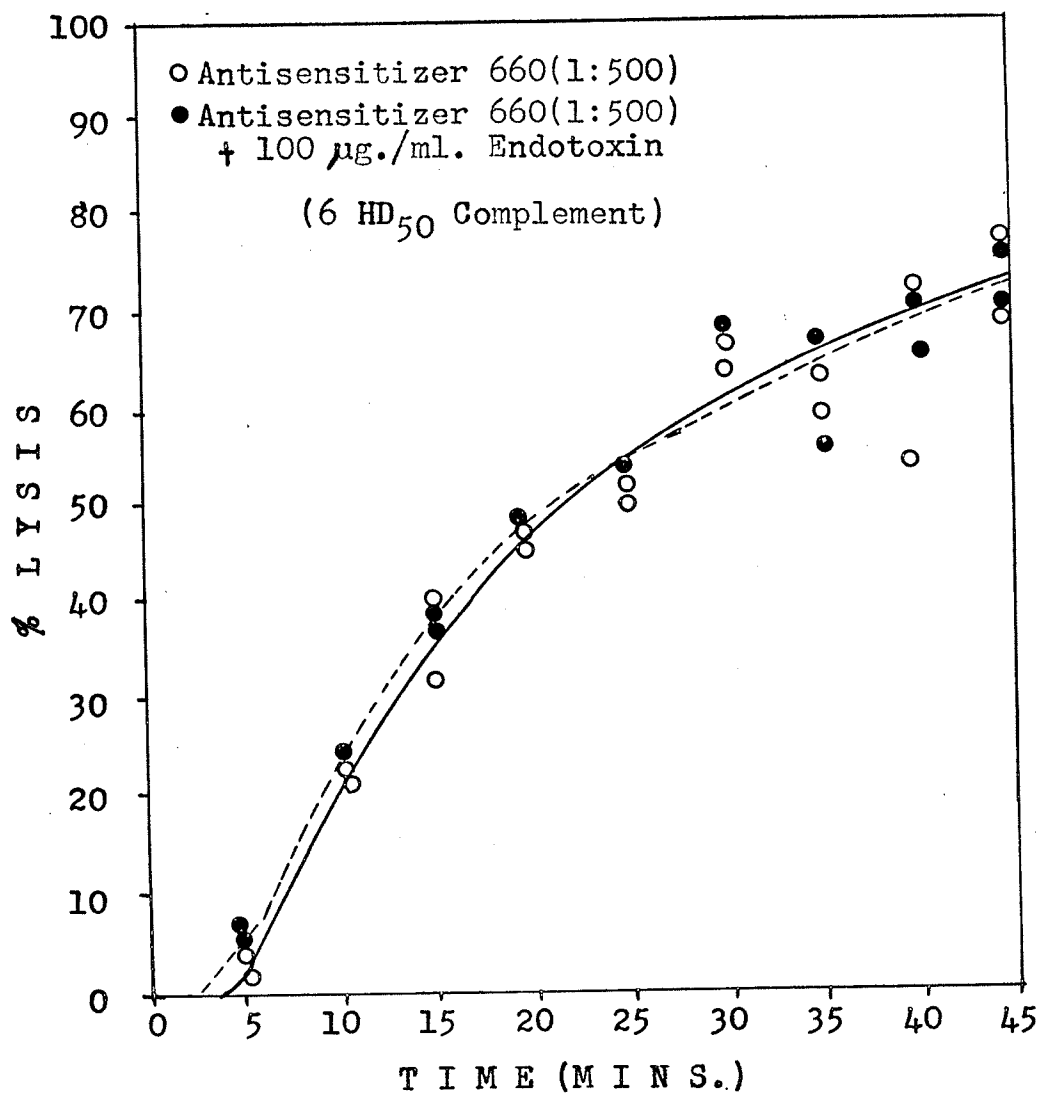


Fig. 6. Dynamics of complementary lysis in the presence of antisensitizer, with and without endotoxin.

equilibrium.

Results

Figure 7 shows that there was no change in lysis in the presence of endotoxin and normal serum and hence no interaction with complement.

Procedure to test reversal of hydrocortisone effect by endotoxin

Since endotoxin reverses the inhibitory effect of hydrocortisone on the immune response in vivo, as shown by Ward and Johnson (1959), an experiment was performed to determine whether reversal of hydrocortisone's inhibitory action on immune lysis could be accomplished. Dynamic studies were performed in the presence of both hydrocortisone and endotoxin at their usual concentrations.

Results

Figure 8 shows that the inhibitory effect of hydrocortisone on lysis was not reversed by endotoxin. This experiment was repeated once with the same result.

Procedure for dynamics of lysis with pertussis vaccine

The experiments with endotoxin were repeated with pertussis vaccine as additive. The vaccine was diluted 1:5, the final dilution containing 0.75 billion organisms per ml. (1:20), and dynamic studies were performed. The in vivo

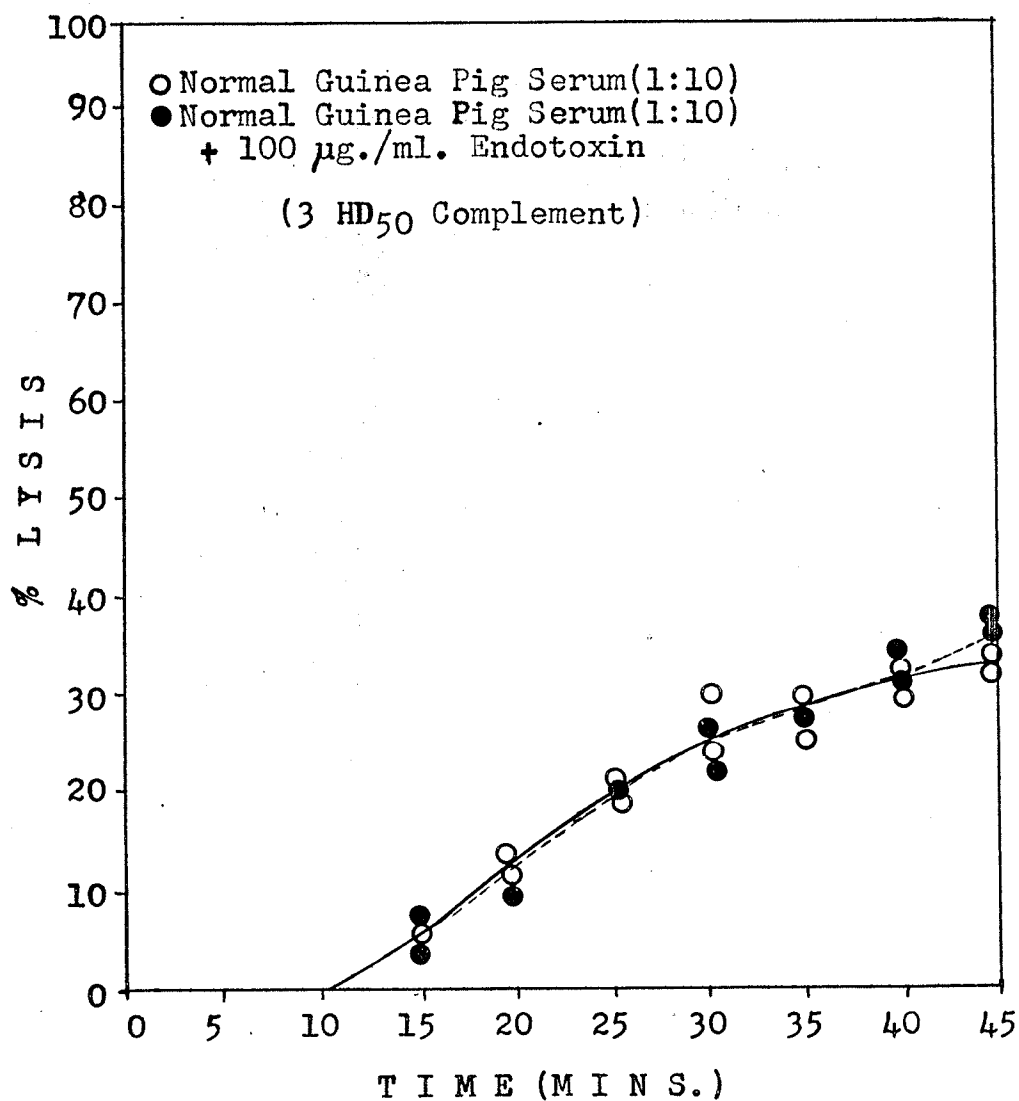


Fig. 7. Dynamics of complementary lysis in the presence of normal guinea pig serum, with and without endotoxin.

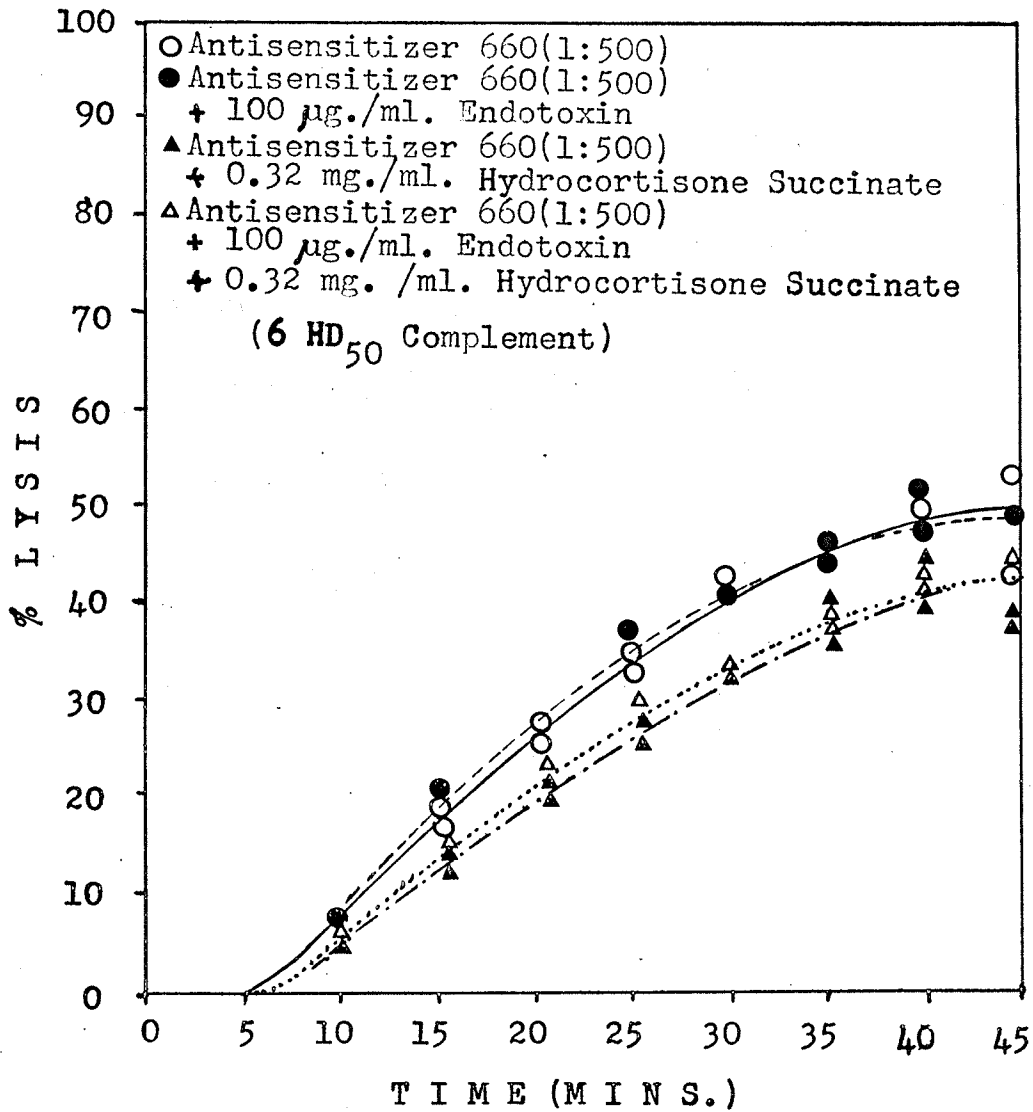


Fig. 8. Dynamics of complementary lysis in the presence of antisensitizer, with and without hydrocortisone succinate and endotoxin.

concentration used to enhance encephalomyelitis production was a 1:5 dilution.

Results

Figure 9 shows that lysis was not changed in the presence of the vaccine and hence there was no apparent effect of the vaccine on the equilibrium. The experiment was repeated once with the same result.

Procedure to test effect of normal serum with pertussis vaccine

The above experiment was repeated in the presence of 1:500 normal guinea pig serum in place of antisensitizer to ensure that the vaccine was not interacting with complement.

Results

Figure 10 shows that there was no change in lysis in the presence of pertussis vaccine and normal serum and hence no interaction of the vaccine with complement.

Procedure to test reversal of hydrocortisone effect by pertussis vaccine

A dynamic study was performed with both pertussis vaccine and hydrocortisone to see if the vaccine would reverse the inhibitory effect of the steroid on immune lysis. The vaccine was diluted 1:2, the final dilution containing 1.9 billion organisms per ml. (1:8), and the usual hydrocortisone concentration was used.

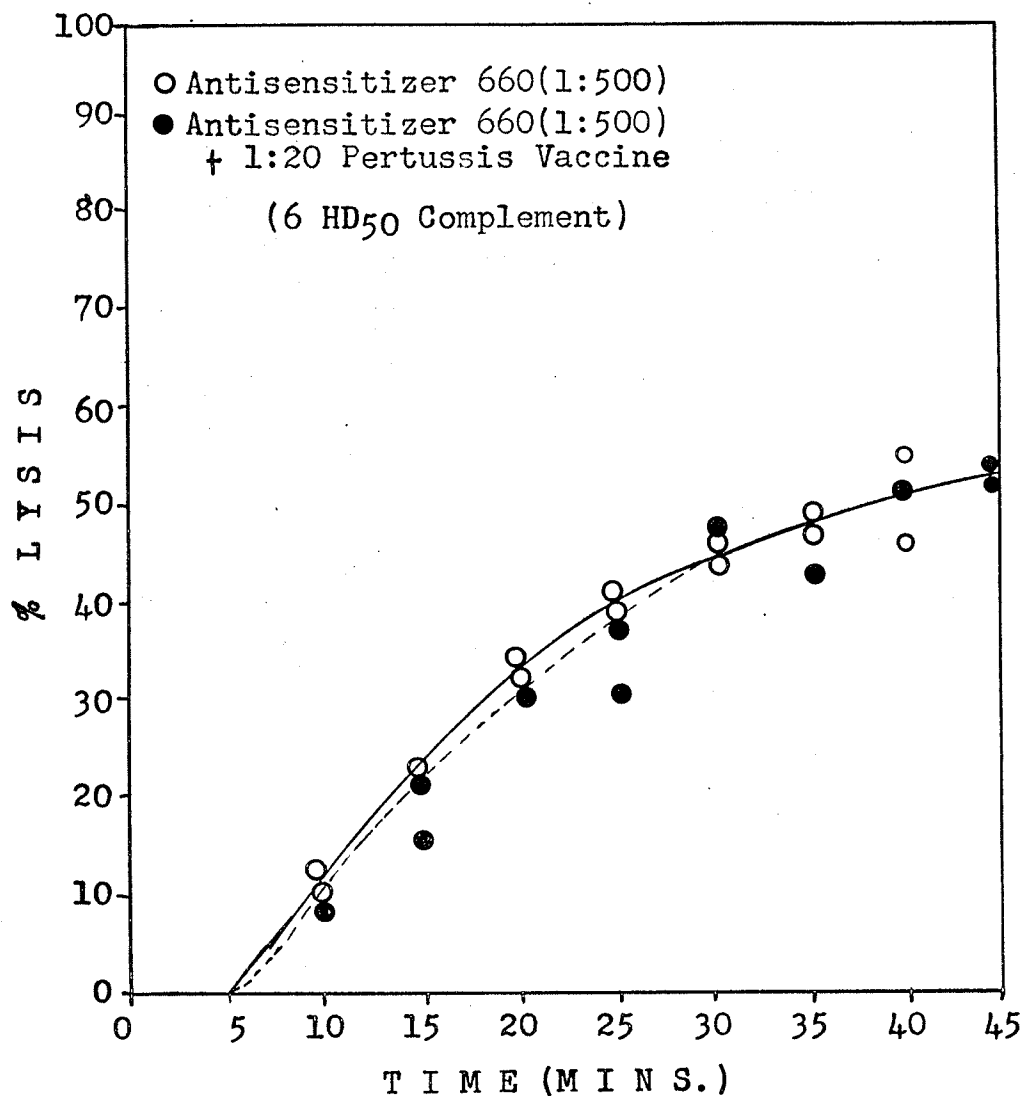


Fig. 9. Dynamics of complementary lysis in the presence of antisensitizer, with and without pertussis vaccine.

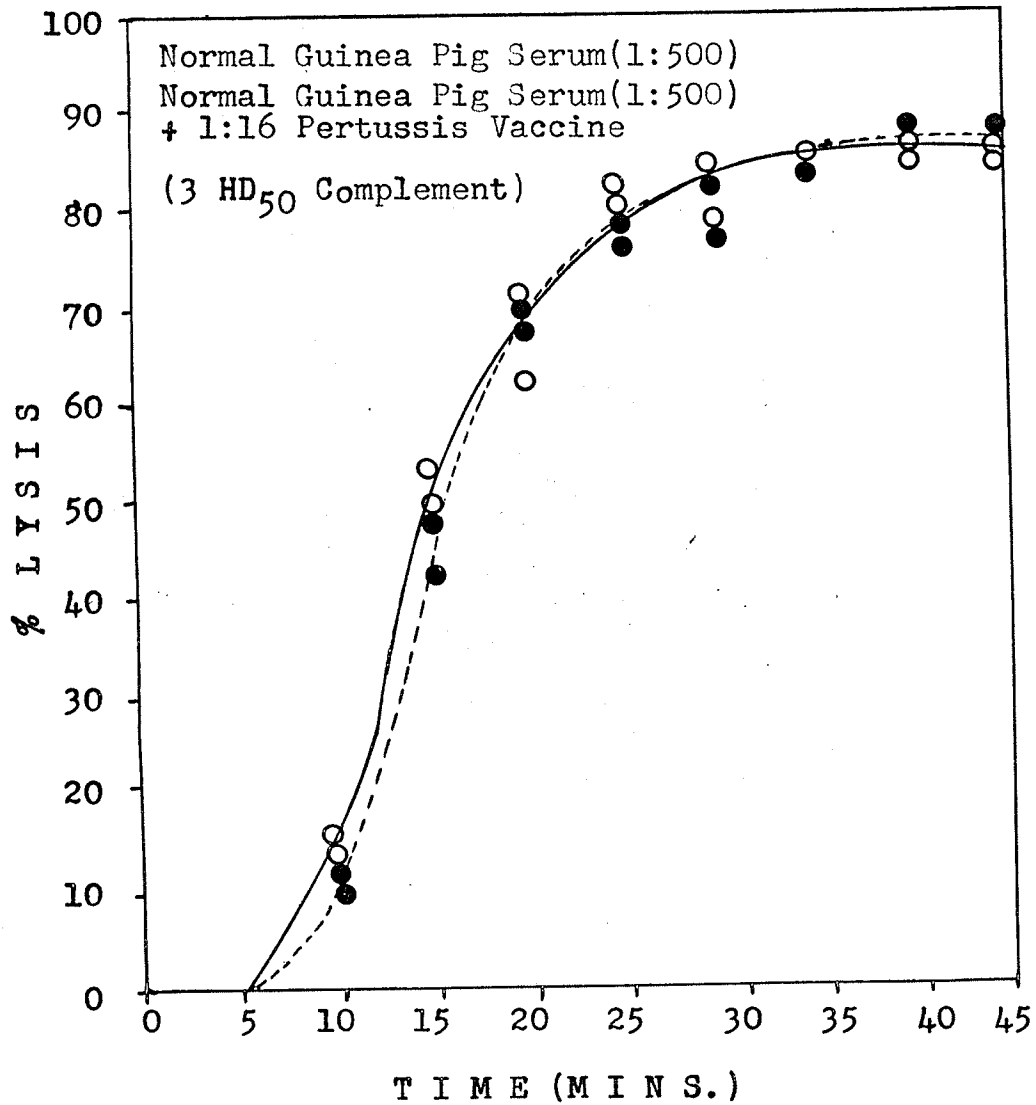


Fig. 10. Dynamics of complementary lysis in the presence of normal guinea pig serum, with and without pertussis vaccine.

Results

Figure 11 shows the same lysis in the presence of both hydrocortisone and pertussis vaccine and hence there was no reversal of the inhibitory effect.

Procedure for dynamics of lysis with Freund's adjuvant

Freund's adjuvant was found to be apparently anti-complementary at concentrations up to approximately 1:1000. Lysis was prevented at higher concentrations and decreased at lower concentrations when complement titrations were performed. At a final concentration of 1:2560, there was no effect on lysis in complement titrations and a dynamic study was performed at this level of adjuvant. Equal parts of Bayel F and Arlacel A were added to BES to give a final concentration of 1:2560.

Because antisensitizer #660 was no longer available, another serum, #638, was used and this serum required a higher dose of complement to give a suitable range of lysis. Thirteen BD_{50} complement was used along with a 1:2000 dilution of antisensitizer.

Results

Figure 12 shows the lysis curve in the presence and absence of adjuvant. There was no change in lysis in the presence of adjuvant and thus no apparent effect of adjuvant on the equilibrium.

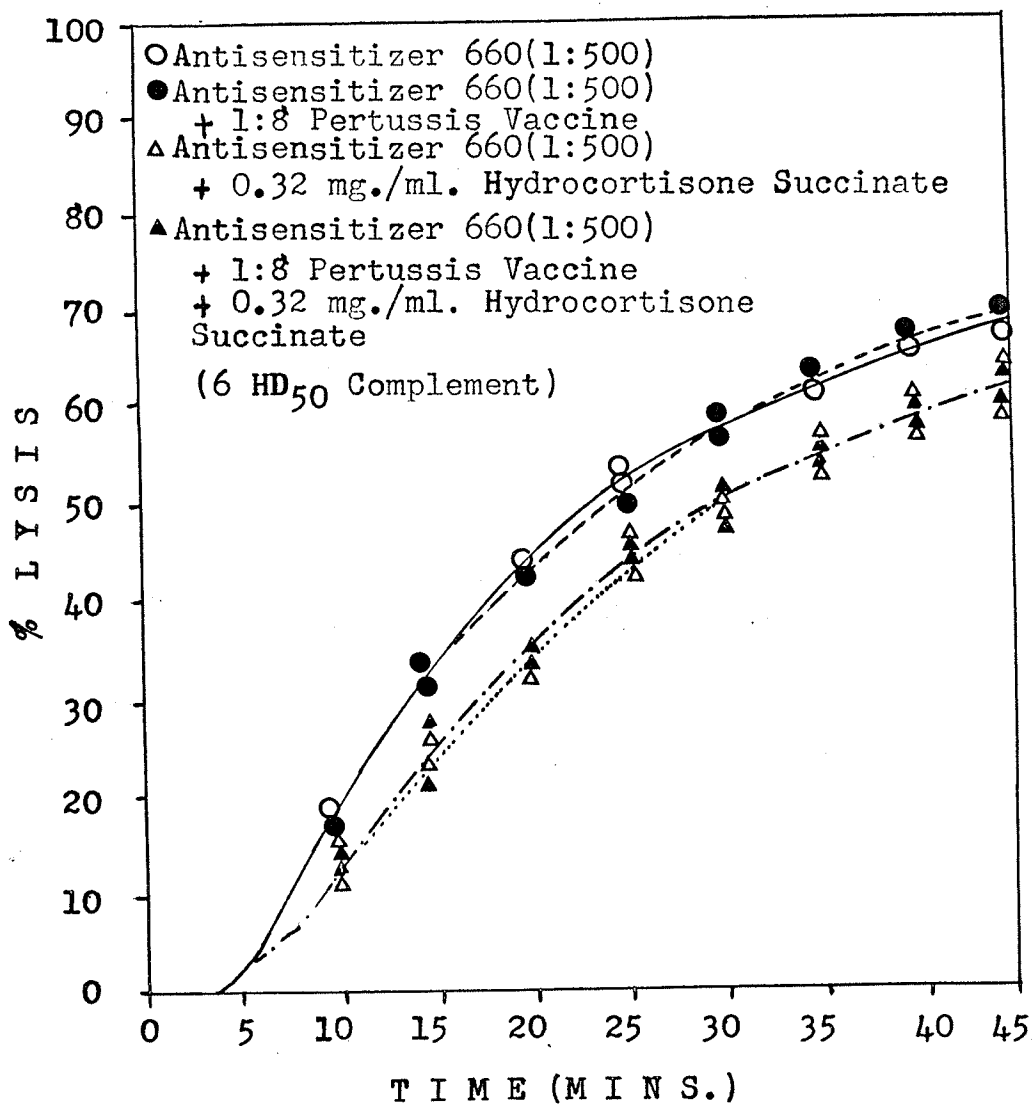


Fig. 11. Dynamics of complementary lysis in the presence of antisensitizer, with and without hydrocortisone succinate and pertussis vaccine.

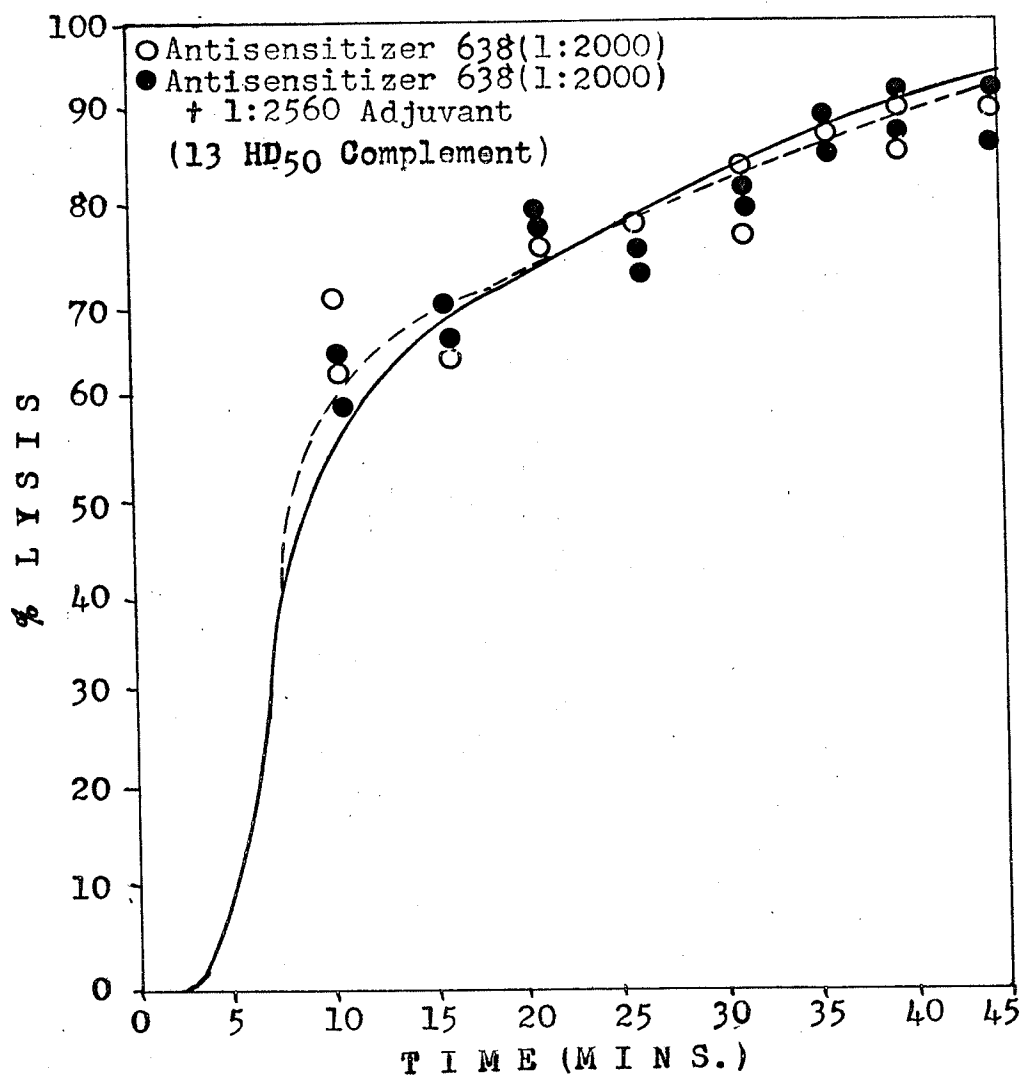


Fig. 12. Dynamics of complementary lysis in the presence of antisensitizer, with and without Freund's incomplete adjuvant.

IV. PRODUCTION OF HOMOLOGOUS ANTISENSITIZER

All previous work was performed with heterologous antisensitizer (guinea pig-produced anti-rabbit). It was thought that homologous antisensitizer, guinea pig-produced anti-guinea pig gamma globulin, might give a different effect in dynamic studies.

Autologous auto-antibody has been produced by Milgrom and Witebsky (1960) using rabbits injected with their own gamma globulin in Freund's complete adjuvant. An attempt was made to produce antibodies against homologous gamma globulin in guinea pigs.

Procedure

Guinea pigs were immunized with one per cent guinea pig gamma globulin in Freund's complete adjuvant. The sera were titrated for inhibition of lysis using sheep red cells sensitized with guinea pig-produced sensitizer.

Results

Four normal guinea pig sera were titrated and the inhibition of lysis curves are shown in Figure 13. Seven sera from immunized guinea pigs were tested and the four which produced more inhibition of lysis than the normal sera are also graphed in Figure 13.

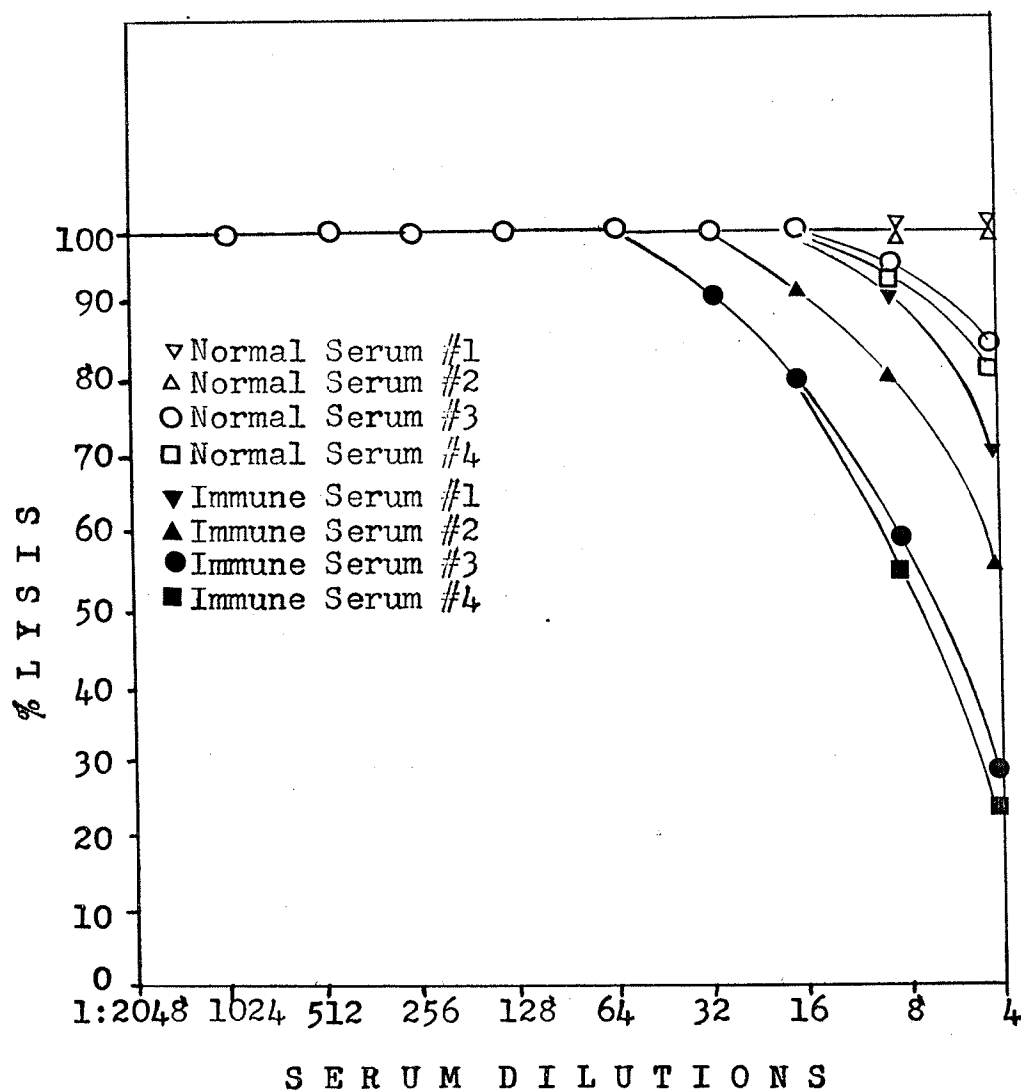


Fig. 13. Titration of guinea pig sera for homologous antisensitizer.

Procedure for dynamics of lysis with
homologous antisensitizer and hydrocortisone

It was thought that if the action of hydrocortisone was on the equilibrium, homologous antisensitizer might give a different effect than heterologous antisensitizer in the dynamic studies. The sera with the highest titres of homologous antisensitizer were pooled (#657), diluted 1:9 and dynamic studies performed at the usual hydrocortisone level and 3 HD_{50} complement.

Results

Figure 14 shows that there is the same inhibition of lysis as in previous experiments.

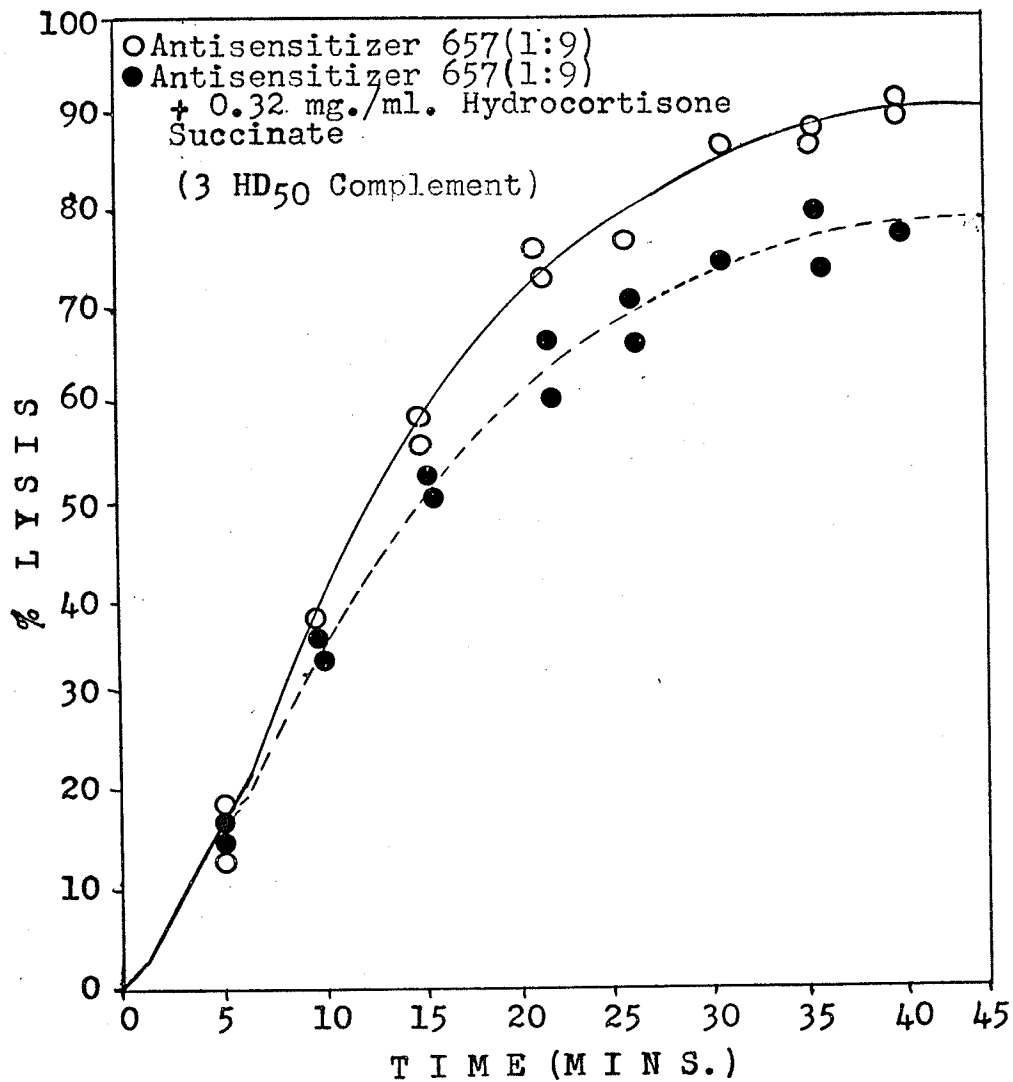


Fig. 14. Dynamics of complementary lysis in the presence of homologous antisensitizer with and without hydrocortisone succinate.

CHAPTER IV

DISCUSSION

CHAPTER IV

DISCUSSION

This report concerns the dynamic equilibrium of the system consisting of sheep red cells sensitized with rabbit-produced hemolysin, guinea pig complement, and anti-antibody made by the injection of guinea pigs with guinea pig red cells sensitized with rabbit-produced hemolysin. The additives endotoxin, pertussis vaccine, hydrocortisone and Freund's adjuvant were tested for their ability to shift the equilibrium in either direction.

The absorption technique first attempted was thought to be inadequately sensitive because of the heavy suspension of red cells necessary to obtain measurable absorption. There was also difficulty in obtaining similar results from duplicate samples.

The second technique attempted, titration of anti-sensitizer in the presence of additive, was thought to be more sensitive because of the more dilute cell suspension used. However, there was found to be an interaction of additive with complement in some cases which would necessitate a higher complement level in the antisensitizer titrations. This could result in an increase in lysis in

the presence of additive which might be attributed to the higher complement dose and no conclusion could be reached in regard to the action of additive on the equilibrium. This technique was abandoned at this point.

The third and most extensively used technique, a study of the dynamics of complementary lysis of the system in the presence of additive, provided a satisfactorily sensitive means of observing the equilibrium.

The additives endotoxin, pertussis vaccine and Freund's adjuvant had no effect on the lysis of the system and thus no effect on the equilibrium.

From theoretical considerations, hydrocortisone was considered capable of influencing lysis. This was found to be the case although the mechanism remains unknown. Since the lysis is altered by the steroid in the absence of anti-sensitizer, it is possible that the effect is not on the equilibrium but due to an interaction of hydrocortisone with complement.

The work of both Gewurz et al. (1965) and Jennings (1966) appears to confirm such an interaction although there is uncertainty as to the exact nature of the interaction. Gewurz et al. found that pre-incubation of hydrocortisone and complement enhanced the reduction of lysis and concluded that the action of the steroid was a direct action on free complement rather than an action on the red cell membrane.

Jennings found no such enhancement with pre-incubation in his system. He did find, however, that the steroid appeared to block the combination of complement with the sensitized cells. Additional evidence for this apparent action of hydrocortisone on the red cell membrane is given by Jennings' observations that the hormone reduced non-complementary lysis caused by bacterial lysins and ultraviolet radiation.

The other possibility is that the steroid is acting by shifting the equilibrium toward the direction of a smaller supply of free, sensitized cells susceptible to lysis by complement. Lysis inhibition by hydrocortisone appeared to be less in the presence of normal serum than in the presence of antisensitizer, thus apparently providing evidence for some degree of action of the steroid on the equilibrium. However, it is difficult to compare these observations since different concentrations of normal serum were used. Taking the observations in which the same concentration of normal serum as antisensitizer was used, there is probably no significant difference in the reduction of lysis by hydrocortisone, the mean reduction in the presence of antisensitizer being 8.3 per cent and in the presence of normal serum being 6.0 per cent. At higher normal serum concentrations, ranging from 1:9 to 1:20, there was less reduction in lysis, ranging from zero to five per cent. The higher serum level may tend to offer some protection from the action of hydrocortisone.

It is probable, therefore, that hydrocortisone exerts an anticomplementary action and does not affect the equilibrium.

Cewurz et al. caution against applying their results to the in vivo situation since the doses of hormone used were much higher than the physiological concentration which is approximately 40 μ g. per ml., although they suggest that accumulation at appropriate sites may occur in vivo. The present experiments demonstrate that hydrocortisone causes a reduction in lysis at a lower concentration than that shown by Cewurz et al. This concentration, 0.32 mg. per ml., remains approximately ten times the physiological concentration.

REVIEWS

CHAPTER 1

CHAPTER V

SUMMARY

The equilibrium studied involved an immune system consisting of sheep red cells sensitized with rabbit-produced hemolysin, complement, and antisensitizer produced by the injection of guinea pigs with guinea pig red cells sensitized with rabbit-produced hemolysin.

The additives hydrocortisone, endotoxin, pertussis vaccine and Freund's adjuvant were tested for their ability to shift the equilibrium in either direction.

1. The first technique attempted, measurement of antisensitizer before and after absorption on sensitized cells, was considered too insensitive to detect small changes in the equilibrium and was discarded.

2. The second technique used was titration of anti-sensitizer in the presence of the additives pertussis vaccine, endotoxin, hydrocortisone succinate and cortisone acetate. Cortisone acetate and hydrocortisone succinate appeared to decrease lysis in the complement titrations. This interaction of additive with complement would necessitate increasing the level of complement in antisensitizer titrations in the presence of additive. Any subsequent alteration in

lysis might be attributable to this rather than to an effect on the equilibrium and this technique was discarded.

3. The third and most useful technique was the study of the dynamics of lysis in the presence of additives. The presence of hydrocortisone resulted in a reduction of complementary lysis. This reduction:

a) was not due to technical error,

b) was not due to the hydrocortisone vehicle but the steroid itself,

and c) also occurred (though possibly to a somewhat lesser extent) in the presence of normal serum in place of antisensitizer and therefore was probably due to the interaction of hydrocortisone with complement rather than to an action on the equilibrium.

4. Endotoxin:

a) did not cause a change in lysis in the presence of antisensitizer,

b) did not cause a change in lysis in the presence of normal serum,

and c) did not reverse the inhibitory effect of hydrocortisone on lysis.

5. Pertussis vaccine:

a) did not cause a change in lysis in the presence of antisensitizer,

b) did not cause a change in lysis in the presence of normal serum,

and c) did not reverse the inhibitory effect of hydrocortisone on lysis.

6. Freund's incomplete adjuvant did not cause a change in lysis in the presence of antisensitizer.

7. Hydrocortisone gave the same reduction of lysis in the presence of homologous antisensitizer as in the presence of heterologous antisensitizer.

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CHAPTER VI

CHAPTER VI

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CHAPTER VII

APPENDIX