Immunology, 1961, 4, 289.

The Effects of Guinea-Pig Gamma Globulins on Capillary Permeability and Serum Complement

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(*Received 9th November*, 1960)

Summary. Gamma globulins, isolated from guinea-pig serum by five different methods, increased capillary permeability when injected intradermally into guinea pigs. None of the methods of isolation caused aggregation of the globulins as judged by ultracentrifugal analysis. The γ-globulin activity was not altered by heat (56° for 30 minutes) but was inhibited by albumin, soya-bean trypsin inhibitor and di-isopropylphosphofluoridate. All preparations inhibited serum complement but only when they had been freeze-dried.

INTRODUCTION

We have previously shown that the main γ-globulin fraction isolated by chromatography of guinea-pig serum on diethylaminoethyl cellulose would increase capillary permeability when injected intradermally into guinea pigs (Davies and Lowe, 1960). This action of γ globulin had been briefly mentioned by Wilhelm, Miles and MacKay (1955) in their studies on the isolation of PF/Dil. and by Ishizaka, Ishizaka and Campbell (1959), and Ishizaka and Campbell (1958), who reported that when rabbit serum was separated by zone electrophoresis on starch blocks, 'it was possible to separate a non-specific irritating substance which occurred in the fast moving component of the γ globulins of some sera'. More recently Ishizaka and Ishizaka (1959) and Christian (1960 a and b) have described the ability of aggregated human γ globulins to increase capillary permeability and to fix serum complement. A possible relationship between permeability effects and inhibition of serum complement was suggested by Osler, Randall, Hill and Ovary (1959) who found that formation of anaphylatoxin in rat serum was associated with destruction of component 3 of complement.

The objects of the present work were to study the influence of the method of isolation of γ globulins on their ability to increase capillary permeability and inhibit complement, and also to investigate the relationship between these two properties.

METHODS

Isolation of γ globulins. Guinea-pig blood was withdrawn from the heart on the day it was required for fractionation. Serum was separated and the γ globulins isolated by the following five procedures:

1. Sodium sulphate precipitation (Kekwick, 1940).

2. Ethanol precipitation using the Goldstein and Anderson (1957) modification of the Cohn procedure.
3. Ammonium sulphate precipitation (Friedman, 1958).
4. Zone electrophoresis on Munktell ethanolysed cellulose (A. Gallenkamp & Co. Ltd.) using the apparatus described by Porath (1956) with a column size of 95×4.5 cm. A potential of 1200 volts produced a current of 80 mA using veronal buffer of ionic strength 0.03. Runs usually lasted 24 hours.
5. Chromatography on diethylaminoethyl-cellulose (DEAE — from either Eastman Kodak Ltd., or Whatman). The serum was equilibrated against 0.005 M phosphate buffer, pH 7.0, and the small amount of euglobulins formed was separated by centrifugation and discarded. The equilibrated serum was placed on a column of DEAE-cellulose previously equilibrated with the same buffer. Usually 10 ml. serum were successfully fractionated on 10 g. DEAE-cellulose with a column size 2×15 cm. Under these conditions the main γ-globulin fraction was not adsorbed and was washed down the column by 0.005 M phosphate buffer.

The majority of the γ-globulin preparations were dialysed against distilled water and then freeze-dried. Some preparations were, however, concentrated by dialysis against Carbowax 20 M (Kohn, 1959); they were then dialysed against distilled water and adjusted to the required volume. Protein nitrogen was determined by the micro-Kjeldahl method.

SEDIMENTATION VELOCITY MEASUREMENTS. Sedimentation velocity runs were carried out by Dr. W. E. F. Naismith of Fibres Division, Imperial Chemical Industries Limited, Harrogate, using a Spinco Model E analytical ultracentrifuge. The γ globulins (0.5 per cent w/v) were dissolved in phosphate buffer, $\mu = 0.1$, pH 7, and centrifuged at 59,780 rev./min. (250,000 **g**).

ESTIMATION OF INCREASED PERMEABILITY. The ability of the γ globulins to increase capillary permeability was determined, as described by Davies and Lowe (1960), by injection of 0.1 ml. amounts of solution intradermally into guinea pigs which had been injected intravenously with Pontamine Sky Blue (0.12 ml./100 g. of a 5 per cent solution in 0.45 per cent saline) 5 minutes previously. Fifteen minutes after the intradermal injection the animals were killed by a blow on the head and portions of skin containing the injection sites were removed and pinned, inside uppermost, on a cork mat. The maximum and minimum diameters of the blue areas were measured on the following day.

TITRATION OF COMPLEMENT. Guinea-pig serum collected on the day of the experiment was used as a source of complement. The number of 50 per cent haemolytic units ($C'H_{50}$)/ml. was estimated by the method of Pillemer, Blum, Lepow, Wurz and Todd (1956). The sheep cells were sensitized by mixing equal volumes of a suspension containing 5×10^5 cells/cu.mm. and a 1 per cent dilution of 'Wellcome' Haemolytic Serum (haemolytic titre between 1 in 1000 and 1 in 2000). All dilutions were made in veronal buffer containing calcium and magnesium (Pillemer *et al.*, 1956). The same buffer was used to prepare the solutions of γ globulins and inhibitors.

Anti-complementary activities were determined by mixing equal volumes of protein solution and a dilution in buffer of guinea-pig serum of such a strength that the mixture contained about 50$C'H_{50}$/ml. After incubation for 30 minutes at 37° the residual complement was estimated.

RESULTS

CAPILLARY PERMEABILITY

In the initial experiments each γ-globulin preparation was tested separately over a wide range of concentrations, four guinea pigs being used for each preparation. All preparations appeared to be of roughly equal potency. For more accurate comparison, one group of four guinea pigs was injected with two concentrations (1200 and 400 μg. protein/ml.) of each of the five preparations. These results are recorded in Table 1 from which it can

TABLE 1

EFFECT OF INTRADERMAL INJECTION OF GUINEA-PIG γ GLOBULINS INTO GUINEA PIGS

Method of preparation	*Amount injected (μg. protein/ml.)*	*Mean lesion diameter (mm.)*
Ammonium sulphate	1200	15.4
	400	13.3
Sodium sulphate	1200	15.1
	400	12.6
Alcohol	1200	14.4
	400	12.2
DEAE-cellulose	1200	15.0
	400	12.8
Zone electrophoresis	1200	14.1
	400	12.0
	Buffer control	4.6

All samples assayed on same group of guinea-pigs. 0.1 ml. injected.

be seen that all preparations were, in fact, equally potent. This finding suggested that the permeability effects of the γ globulins were not dependent on the method used for their isolation.

TABLE 2

EFFECT OF INTRADERMAL INJECTION OF GUINEA-PIG γ GLOBULINS INTO GUINEA PIGS

Concentration of solution injected (μg. protein/ml.)	*Mean lesion diameter (mm.)*
2500	17.8
1250	15.0
625	13.5
312	12.9
156	11.8
Buffer	5.5

γ globulin was prepared by DEAE chromatography. 0.1 ml. injected.

The potency of the γ globulins can be assessed from Table 2 which gives the results of injecting different quantities of the same preparation into groups of four guinea pigs; significant blueing was caused by 15 μg. of protein.

ANTI-COMPLEMENTARY ACTIVITY

In order to study the rate at which γ-globulin inactivates complement, γ-globulin solution (5 ml. of 500 μg. protein/ml.) isolated by zone electrophoresis, was mixed with 5 ml. of diluted guinea-pig serum containing 1000 C'H50. The mixture was incubated at 37° and aliquots withdrawn at intervals for estimation of residual complement activity.

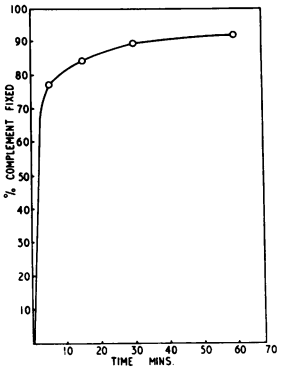

FIG. 1. Relationship between the amount of complement fixed by isolated guinea-pig γ globulin when incubated with guinea-pig serum for different times.

The results, illustrated in Fig. 1, showed that virtually all the complement was inactivated in 30 minutes. Since the object of the experiments was to compare the complement-inactivating power of the various γ globulins it appeared sufficient, and was certainly more convenient, to use these conditions of 30 minutes at 37° rather than the more usual 19 hours at 4°.

TABLE 3

INACTIVATION OF GUINEA-PIG COMPLEMENT BY GUINEA-PIG γ GLOBULINS ISOLATED BY DIFFERENT METHODS

Method of preparation	*Concentration of solution used (μg. protein/ml.)*	*% C'H50 fixed*
Ammonium sulphate	1200	93
	400	82
Sodium sulphate	1200	93
	400	62
Alcohol	1200	93
	400	69
DEAE-cellulose	1200	84
	400	57
Zone electrophoresis	1200	100
	400	95

Originally 45 C'H50/ml. were present.
C'H50 = No. of 50 per cent haemolytic units of complement.

The general plan of the investigation was similar to that used to compare the effects on permeability in that, as a first step, each γ globulin was tested separately over a wide range of concentrations and then, in the same experiment, all preparations were tested at two concentrations (1200 and 400 μg. protein/ml.). These results are summarized in Table 3

from which it is clear that the γ globulins isolated by zone electrophoresis are more potent than the other four preparations in their ability to fix complement.

SEDIMENTATION VELOCITY

The ultracentrifuge results did not reveal any evidence of aggregation. The preparations prepared by DEAE chromatography, zone electrophoresis and precipitation with sodium sulphate were homogeneous, but the samples obtained by precipitation with

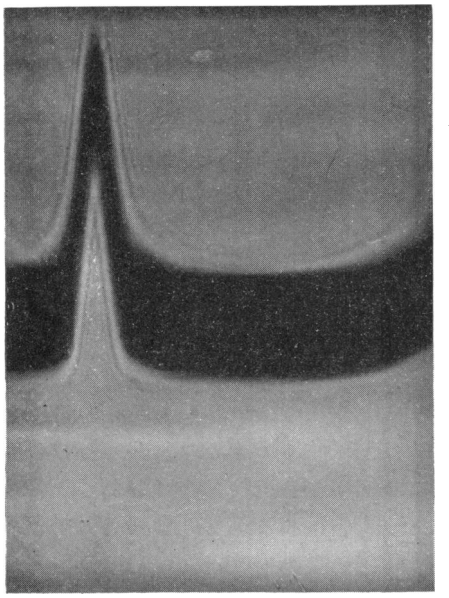

FIG. 2. Sedimentation velocity diagram of guinea-pig γ globulin isolated by chromatography on DEAE-cellulose. The sample was dissolved in phosphate buffer (μ=0.1, pH=7) and centrifuged at 59,780 rev./min. (250,000 g.). Photograph taken after 32 minutes. Direction of sedimentation from left to right.

ethanol and ammonium sulphate contained traces of impurity which sedimented less rapidly than the γ globulins. The corrected sedimentation constants in all cases were between 6.5 and 6.7S. A typical sedimentation velocity diagram is shown in Fig. 2.

ACTION OF HEAT ON CAPILLARY PERMEABILITY AND ANTI-COMPLEMENTARY ACTIVITY

Heating human γ globulins leads to aggregation and consequent increase in permeability. γ-globulin solution (2.4 mg./ml.), was heated at 56° for 30 minutes and then assayed for ability to increase capillary permeability and to fix complement. The results presented in Table 4 clearly indicate that neither activity was affected by this heat treatment.

However, when serum was heated at 60° for 30 minutes and then chromatographed on DEAE-cellulose, the resulting γ globulins did not markedly increase capillary permeability (Table 5).

TABLE 4

EFFECT OF HEAT ON THE CAPILLARY PERMEABILITY AND ANTI-COMPLEMENTARY ACTIVITY OF GUINEA-PIG γ GLOBULINS

DEAE-cellulose fractionated γ globulins (2.4 mg./ml.) divided into two parts. One part heated at 56° for 30 minutes, other kept at room temperature.

Solution injected	*Mean lesion diameter (mm.)*	*% $C'H_{50}$ fixed*
Heated γ globulins	13.8	62
Unheated γ globulins	14.1	62
Buffer	4.5	

Originally 42 $C'H_{50}$/ml. were present.

TABLE 5

EFFECT OF HEAT ON GUINEA-PIG SERUM BEFORE FRACTIONATION

Serum heated at 60° for 30 minutes and then γ globulins isolated by DEAE-cellulose chromatography.

Solution injected	*Mean lesion diameter (mm.)*
γ globulins from heated serum	8.0
γ globulins control (2.4 mg./ml.)	12.6
Buffer	5.1

0.1 ml. injected.
γ globulins from heated serum were at concentrations originally present in serum.

EFFECT OF FREEZE-DRYING

In some experiments the γ globulins were not dialysed and freeze-dried but were concentrated by the Kohn (1959) technique, dialysed, adjusted to the concentration originally present in serum and then assayed for anti-complementary activity. It was noted that their ability to fix complement was no greater than that of albumin. To test the effect of freeze-drying the γ globulins, the following experiment was set up. Fresh guinea-pig serum (20 ml.) was fractionated on DEAE-cellulose and the eluate containing the γ globulins divided into two equal portions. The first half was dialysed and concentrated to 10 ml. by Kohn procedure, i.e. the γ globulins were now present in the concentration originally found in serum. The second half was dialysed and freeze-dried. Protein was estimated on both fractions and the freeze-dried material reconstituted in buffer to give the original serum concentration. The anti-complementary and capillary permeability activities of these two samples together with a sample of γ globulin prepared in the usual manner by zone electrophoresis and freeze-dried were determined. Table 6 shows that all three

samples have the same capacity to increase capillary permeability but the γ globulins that had not been freeze-dried were much less anti-complementary than the other two; in fact they had about the same potency as albumin.

TABLE 6

EFFECT OF FREEZE-DRYING ON THE ANTI-COMPLEMENTARY AND CAPILLARY PERMEABILITY ACTIVITY OF GUINEA-PIG γ GLOBULIN

γ globulins fractionated by DEAE-cellulose chromatography and divided into two portions:
A. Dialysed and adjusted to concentration originally present in serum.
B. Dialysed, freeze-dried and dissolved in concentration originally present in serum.

Substance tested	% $C'H_{50}$ *fixed*	*Mean lesion diameter (mm.)*
A. γ globulin in solution (1.98 mg./ml)	25	16.7
B. Freeze-dried γ globulin (1.98 mg./ml.)	63	15.0
Control electrophoretic γ globulin (0.5 mg./ml.)	72	13.0
Buffer	None	4.5

The anti-complementary activity seems to be an artefact induced in the γ globulins by freeze-drying whereas the capillary permeability activity may well be a property of the native protein.

EFFECT OF INHIBITORS

Guinea-pig serum does not increase capillary permeability when injected intradermally into guinea pigs, yet the γ globulins isolated from the serum by five different procedures were active in this respect. Two explanations appeared possible, either all five methods of isolation had resulted in some 'activation' of the γ globulin, or there was a natural inhibitor

TABLE 7

INHIBITORY EFFECT OF γ-GLOBULIN-FREE GUINEA-PIG SERUM ON THE CAPILLARY PERMEABILITY INCREASING ACTIVITY OF γ GLOBULINS

Solution injected	*Mean lesion diameter (mm.)*
γ globulin	14.5
Serum − γ globulin	4.7
γ globulin + serum − γ globulin	4.9
Buffer	4.5

Fractions prepared by zone electrophoresis and adjusted to concentrations present in original serum. Mixtures are of equal volumes. 0.1 ml. injected.

present in the serum. This latter explanation seemed the more reasonable since all five preparations were equally potent in increasing capillary permeability and moreover, addition of γ globulin to the remainder of the serum from which it had been originally isolated yielded an inactive mixture (Table 7).

In an attempt to identify the inhibitor in the γ-globulin-free serum a sample of guinea-pig serum was fractionated by zone electrophoresis and the various fractions were tested alone and in combination. Table 8 shows that albumin will considerably reduce the permeability effect of γ globulin when the two proteins are mixed in the ratio present in serum.

TABLE 8

THE CAPILLARY PERMEABILITY ACTIVITY OF GUINEA-PIG SERUM CONSTITUENTS ALONE AND MIXED WITH γ GLOBULIN

Fraction injected	*Mean lesion diameter (mm.)*
γ globulin + buffer	12.8
β globulin + buffer	12.1
α_1 globulin + buffer	5.4
α_2 globulin + buffer	5.7
Albumin + buffer	3.6
γ globulin + β globulin	14.0
γ globulin + α_1 globulin	12.1
γ globulin + α_2 globulin	12.0
γ globulin + albumin	7.1
Buffer	5.0

Fractions prepared by zone electrophoresis and adjusted to concentrations present in original serum. Mixtures are of equal volumes. 0.1 ml. injected.

It is also clear from Table 8 that permeability activity was associated with the β-globulin fraction. Subsequent examination of this fraction by electrophoresis on paper and starch gel revealed the presence of some γ globulin as contaminant. The high activity shown by

TABLE 9

EFFECT OF ALBUMIN ON ANTI-COMPLEMENTARY ACTIVITY OF GUINEA-PIG γ GLOBULINS

Concentration (μg. protein/ml.)	% $C'H_{50}$ *fixed*
γ globulins (2400)	68
Albumins (24,000)	29
γ globulins + albumin	59

Originally 47 $C'H_{50}$/ml. were present.
Fractions prepared by DEAE-cellulose chromatography. Mixtures are of equal volumes; single fractions diluted with equal volumes of buffer.

the β globulins, however, suggests that they too may be classed among the permeability factors.

From the results recorded in Table 9 it can be seen that albumin did not inhibit the anti-complementary activity of γ globulin when the two substances were mixed in the proportions found in serum.

Tables 10 and 11 summarize the results with other inhibitors. Soya-bean trypsin inhibitor (1 mg./ml.), di-isopropylphosphofluoridate (10^{-3} M) and, to a lesser extent, ovomucoid inhibitor, all antagonized the permeability effects of γ globulin. Pre-treatment of the animals with mepyramine maleate (5 mg./kg. intraperitoneally, 15 minutes before

TABLE 10

EFFECT OF INHIBITORS ON CAPILLARY PERMEABILITY INCREASING ACTIVITY OF GUINEA-PIG γ GLOBULIN

Substance injected (μg. *protein/ml.*)	*Mean lesion diameter* (*mm.*)
γ globulin (2400) + buffer	11.8
γ globulin (2400) + DFP	6.0
γ globulin (2400) + SBTI	4.5
γ globulin (2400) + OI	8.9
Buffer	4.3

0.1 ml. injected.
Di-isopropylphosphofluoride (DFP) 10^{-3} M.
Soya-bean trypsin inhibitor (SBTI) 1 mg./ml.
Ovomucoid inhibitor (OI) 1 mg./ml.
Mixtures are of equal volumes.

TABLE 11

EFFECT OF INHIBITORS ON ANTI-COMPLEMENTARY ACTIVITY OF GUINEA-PIG γ GLOBULINS

Mixture	% $C'H_{50}$ *fixed*
0.5 ml. γ globulin + 0.5 ml. buffer	68.3
0.5 ml. γ globulin + 0.5 ml. SBTI	70.6
0.5 ml. SBTI + 0.5 ml. buffer	9.75

Originally 41 $C'H_{50}$/ml. were present.
γ globulin 2.4 mg./ml.
Soya-bean trypsin inhibitor (SBTI) 1 mg./ml.

intradermal injection) had no effect on the action of γ globulin. Soya-bean trypsin inhibitor did not affect the anti-complementary activity of γ globulin.

Di-isopropylphosphofluoride could not be tested since it will itself inhibit complement (Levine, 1955).

DISCUSSION

Ishizaka and Ishizaka (1959) and Christian (1960 a and b) have shown that acquisition by human γ globulins of capillary permeability increasing and anti-complementary properties is associated with aggregation of the globulins. The former workers found that both activities increased when the γ globulins were heated, maximum activation taking place at 63° for 30 minutes. Using a sodium sulphate precipitation method Christian (1960a) fractionated human γ globulins heated at 56° for 30 minutes into two active and

four inactive components. The two active fractions were markedly aggregated and possessed sedimentation constants of 40 and 30S respectively. More recently, Marcus (1960) has shown that human γ globulin caused a marked decrease in the $C^{1}1$ and $C^{1}4$ activity of guinea-pig complement. This anti-complementary activity was enhanced by heating the γ globulins at 56° or by adsorption on bentonite. These three reports on the effect of heat on the anti-complementary activity of γ globulin do not agree with those of Davis, Kabat, Harris and Moore (1944) who found that heating at 56° for 30 minutes greatly diminished the anti-complementary activity of human γ globulin.

In the experiments reported here the γ globulins isolated from fresh guinea-pig serum by five different methods possessed high activity in increasing capillary permeability. Moreover, ultracentrifugal analysis of the five preparations showed no evidence of aggregation so that the permeability property seems to belong to the native protein itself. Two of the γ-globulin preparations (those obtained by precipitations with ethyl alcohol and ammonium sulphate) undoubtedly contained traces of impurity as indicated by the sedimentation velocity diagrams. Augustin and Hayward (1960) have shown that pure γ globulin can be eluted from DEAE-cellulose columns. The fact that two of our γ-globulin samples contained impurities is, however, irrelevant to their effects on capillary permeability since all five preparations were equally potent in this respect, indicating that γ globulin itself is responsible for the activity. The capillary permeability increasing activity did not increase after heating the γ globulins indicating a marked difference from isolated human γ globulins. It has been pointed out by Franklin (1960) that heat does not readily aggregate isolated rabbit γ globulin and presumably guinea-pig γ globulins behave similarly.

The anti-complementary activity of the guinea-pig γ globulin appears to be an artefact induced in the proteins by the method used for freeze-drying. All protein solutions were freeze-dried from salt-free solutions without the addition of stabilizing agents. The activity may perhaps be due to breakage of some -S-S- linkages liberating an increased number of -SH groups. Many sulphydryl compounds are anti-complementary (Cushman, Becker and Wirtz 1957). A small change of this kind would not readily be detected on the ultracentrifuge using the concentrations of protein employed in this study. It must be emphasized that the anti-complementary activity of these γ globulins is not marked. Direct comparison with other studies on anti-complementary effects of γ globulin is not justifiable because they refer to human γ globulin and the anti-complementary activity has been determined under conditions which differ from ours. Ishizaka and Ishizaka (1959) for example, found that 10 μg./ml. of aggregated human γ globulin and 550 μg./ml. of unheated human γ globulin were sufficient to inactivate 50 $C'H_{50}$ units when incubated for 19 hours at 4°. In contrast, the guinea-pig γ globulin prepared by zone electrophoresis was our most active sample, 400 μg./ml. produced 95 per cent inactivation of complement (Table 3) when incubated according to the conditions described on page 290.

The anti-complementary activity of the guinea-pig γ globulin is not, however, concerned with the permeability effect. There is, in fact, no relationship between capillary permeability increasing and anti-complementary activity, as is further emphasized by the inhibition of permeability but not of anti-complementary activity by albumin and soya-bean trypsin inhibitor.

The capillary permeability increasing activity of guinea-pig β and γ globulins has previously been noted by Wilhelm *et al.* (1955) who proceeded to study the activity of α_2 globulins since they found them to be much more potent (ratio $\gamma:\beta:\alpha_2$ 1:4:24). In our

experiments, separation of α_2 globulin by either zone electrophoresis or DEAE-cellulose did not produce fractions with any marked capillary permeability activity (see Table 8 and Davies and Lowe, 1960). Active fractions were, however, obtained when the α_2 globulins were prepared by the method of Wilhelm, Mill and Miles (1957). It is clear, therefore, that isolation of α_2 globulins by either zone electrophoresis or ion exchange chromatography does not activate the precursor of Wilhelm *et al.* (1955) as does the ether fractionation method used by these workers.

Inhibition of increased capillary permeability by S.B.T.I. and D.F.P. suggests that the permeability effect depends upon an enzymic action possibly involving an esterase. Moreover, since activity is not destroyed by heating for 30 minutes at 56° it appears likely that γ globulins are themselves a substrate for an enzyme at the injection site which could liberate a peptide acting as the ultimate mediator of capillary permeability. There is no evidence that release of histamine was involved, the permeability activity of γ globulin is fully evident in animals treated with mepyramine.

The potency of γ globulins as mediators of increased capillary permeability is not high when compared weight for weight with some of the previously described mediators such as histamine, serum kinin or the most active α_2 globulin of Wilhelm *et al.* (1957) but this comparison may not be relevant for two reasons. First, as we have already suggested, the ultimate mediator may well be a smaller molecule derived enzymically from the γ globulin and secondly there is no need to postulate a substance highly potent on a weight basis if ample supplies of the material are available at the site of inflammation. This situation could easily exist at a site infiltrated with globulin-synthesizing lymphocytes.

The finding that complement-fixing properties of γ globulins become evident only after freeze-drying casts some doubt on studies designed to show relationships between complement-fixing and other biological properties of γ globulins.

A further point emerging from our studies concerns the importance of serum albumin as an inhibitory substance. It appears that the inflammatory potential of serum may be largely governed by the relative amounts of albumin and various globulins.

In the very early phases of inflammation it is probable that increased capillary permeability results in the transudation of whole plasma and it follows that γ globulins are unlikely to function at this time since there would be sufficient albumin present to inhibit them. If γ globulins do play a part in inflammation it must be at a later phase when the relative amount of globulin is increased, perhaps by the influx of globulin-synthesizing lymphocytes.

ACKNOWLEDGMENTS

We should like to thank Miss A. V. Worth, Mr. R. A. Westhead and Mr. A. J. Wood for technical assistance.

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