I

ATTEMPTS TO ISOLATE THE Rh ANTIGEN FROM RED BLOOD CELLS

II

THE FRACTIONATION OF AN ANTI-Rh ANTISERUM

III

A COMPARISON OF THE VASOCONSTRICTOR ACTIONS OF ADRENALINE AND NOR-ADRENALINE

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1951

ACKNOWLEDGEMENT

I am deeply indebted to Professor Dan H. Campbell and to Professor A. van Harreveld for their guidance and encouragement. I should also like to acknowledge the assistance of Dr. J. R. Cann and Dr. R. A. Brown, who carried out the electrophoretic work of Part II, and of Dr. S. J. Singer, for his helpful discussion of some phases of this work.

ABSTRACT

Part I

Attempts to obtain a substance with Rh antigenic activity from human red blood cells are described. At first, some extractions of the cells using ethanol, methanol, acetone, ether, dioxan, chloroform, and toluene, under different conditions, were performed. Later work involved treatment of the cells with aqueous reagents to degrade the cell wall, followed by attempts to obtain a purified, active extract by precipitation or solution. None of these methods yielded a pure, specifically active Rh antigenic material.

Part II

The fractionation of an anti-Rh serum by electrophoresis-convection, and the immunologic characterization of these fractions, are described. Evidence is presented to show that certain anti-Rh antibodies may exist in globulin fractions other than the γ-globulin.

Part III

Experiments are described in which the vasoconstricting actions of adrenaline and of nor-adrenaline on rats were compared. It was concluded that there was but little difference in their action in this respect. Other experiments indicated that the administration of these drugs to lightly anaesthetized rabbits did not affect the metabolic rate of the subjects.

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PART I

ATTEMPTS TO ISOLATE THE Rh ANTIGEN FROM RED BLOOD CELLS

INTRODUCTION

In 1940, Landsteiner and Wiener (1) showed that when a rabbit was injected with rhesus monkey erythrocytes, its serum agglutinated the red blood cells of certain humans. This indicated that there was some relationship between the rhesus monkey erythrocyte and human erythrocytes, and led to the identification of a new specific blood factor, which was called by these workers the Rh factor. About the same time, Levine and Stetson (2) and Wiener and Peters (3) described an agglutination reaction between a few human serums and the red blood cells of certain individuals; this reaction bore no apparent relationship to those of the ordinary specific blood types. It was recognized shortly thereafter that the Rh factor was the antigen which was ultimately responsible (through the production of specific antibodies) for both of the above agglutination reactions. Subsequent work showed that approximately 85% of white Caucasians had erythrocytes which were agglutinated by these few human serums - i.e. they were "Rh positive." The cells of the remaining 15% were not agglutinated, and they were therefore "Rh negative." Several different antigenic Rh components are now recognized, and these give rise to many subgroups and combinations of subgroups.

This antigen has been found to occur mainly in the erythrocyte. Small amounts have been found in human saliva

(Wiener and Forer, 4), in some organs of the body (Boorman and Dodd, 5) and in the amniotic fluid of some Rh positive infants (Witebsky and Mohn, 6).

One of the chief distinctions between the Rh antigen and the classical blood type antigen lies in the nature of their specific antibodies. Normally Rh negative individuals do not have circulating anti-Rh antibodies; these are only built up as a result of an immunization with an Rh antigen. In contrast, persons with type A blood cells normally have anti-B antibodies in their serum; persons with type B cells have anti-A antibodies, and those of type O contain antibodies for both A and B antigens. The existence of these specific antibodies does not require an antigenic stimulus, but is instead a result of an inherited condition.

When the present research program to isolate the Rh antigenic factor was begun, but few attempts had been made to isolate this material. From some preliminary studies, Wiener believed that the material was soluble in alcohol, and hence was lipid in nature. Campbell and Fisk (unpublished) also concluded from some preliminary experiments that the material was of lipid nature, but were unable to obtain reproducible results. Calvin et al (7) were able to isolate a large molecular weight material which was fairly soluble in water. The following is a brief resume of their investigation:

To one volume of pooled red blood cells there were added eight volumes of distilled water at 5°C. The pH of the hemolysed cells was adjusted to 5.5 - 6.0, and the precipitated stroma were washed at this pH to remove the hemoglobin. A cloudy solution was formed from the stroma when the pH was adjusted to 7 - 8. Centrifuging at 50,000 G yielded a precipitate which was called "elenin". This dissolved at pH 7.5 - 8.0 to give a milky solution, with marked streaming birefringence.

The elenin lost its immunological specificity after being heated to 56°C. for five minutes. Electrophoresis of a 0.1% elenin solution, at 2°C., in phosphate buffer at pH 7.8 and ionic strength 0.2, showed two components:

1. About 80% of the material had a mobility of 6×10^{-5} $cm.^2/v./sec.$

2. About 15% of the material had a mobility of 8×10^{-5} $cm.^2/v./sec.$

From elenin, about 40%-50% of lipid material could be extracted by an alcohol-ether mixture. In some cases, an ether soluble material which would give an indication of Rh activity was obtained. This material was thermostable for 60 minutes at 56°C.

Elenin solutions from Rh positive cells showed good specific Rh activity as judged by agglutination inhibition tests. However, no specific anti-Rh antibodies could be built up by elenin injection into guinea pigs or rabbits.

Injection of this material into human subjects failed to increase any existing antibody titer. Intramuscular injection of large quantities (1 to 1.5 grams) failed to effect a change in the titer of the circulating antibodies.

Perhaps the greatest amount of interest has been centered on Carter's investigations (8,9). The material which she isolated had lipid properties, and was obtained in the following way:

Pooled cells were lysed by the addition of half a volume of water, and then 5 volumes of 95% ethanol were added. The mixture was allowed to stand in the cold overnight, the alcoholic solution was then filtered off and the reddish residue was dried in air. This product was washed twice with aqueous alcohol solutions, and re-dried. Five volumes of ether were added and extraction was allowed to continue in the cold for periods of up to 5 days. Separation and evaporation of the ether extract yielded a yellowish, waxy residue which was soluble in alcohol. This was claimed to be active in complement fixation tests, in agglutination inhibition reactions, in the production of antibodies in guinea pigs when used with a carrier, and in neutralization of antibodies in vivo.

Price et al (10) have reported a purification of Carter's alcohol soluble material. Phospholipids were removed from an ether solution of the crude material by a precipitation with acetone. The soluble portion was

chromatographed from pentane saturated with 95% methanol, on a silica gel column. Fractionation and re-crystallization from ether-pentane or chloroform-pentane yielded glistening needles, with a melting point of 156.9° - 157.2°C.

The material was soluble in alkali, but precipitated upon the addition of CO_2. It had a neutral equivalent of 230±5, and a pK of 10±0.5. It gave no color with ferric chloride, it sublimed unchanged, and it was recovered unchanged after boiling for 1 hour with alcoholic alkali, after refluxing with pyridine-acetic anhydride and after treatment with semicarbazide acetate for 2 days. Analysis showed 58.39% of carbon and 7.79% of hydrogen. This fraction showed a high complement-fixing activity with anti-Rh serum. However, since no study was made of similar products from Rh negative cells, no conclusions may be made as to the specificity of the product.

On the other hand, extracts prepared according to Carter's directions in other laboratories (Wiener, 11: Howe and Rustigian, 12) have not shown any specific activity, as judged by agglutination inhibition tests, complement fixation tests, and attempts at antibody formation.

Spielman (13) has also claimed to have isolated a substance with specific activity. Rh positive erythrocytes were extracted for 8 days at 37°C. with 5 volumes of ethanol. The alcoholic filtrate was evaporated to dryness at 25°C.

in a vacuum, and was then suspended in water and extracted with ether. The ethereal extract was washed with dilute acid and water, and finally dried with Na_2SO_4. It was concentrated by evaporation and a precipitate was obtained by the addition of acetone. A cyclohexane solution of this precipitate was passed through an aluminum oxide column, and a zone which contained inactive materials was noted. This part of the column was discarded; the rest was refluxed with ethanol, and a final product consisting of a light brown, sticky, sometimes waxy material was obtained. Activity in agglutionation inhibition tests, in complement fixation tests and in antibody neutralization tests in guinea pigs was claimed. However, identical extracts made from negative cells gave similar results, so it is very questionable whether a specific substance has been extracted by this procedure.

A water-soluble material which exhibited some Rh activity, as judged by agglutination inhibition reactions, has been found in the amniotic fluid of certain Rh positive infants by Mohn and Witebsky (6). They attempted, unsuccessfully, to purify the substance by precipitation with alcohol, acetone and other agents which were not specified, and therefore concluded that the specific factor was very sensitive to the action of such agents. The material was, however, stable to lyophilization.

Chemistry of the A and B Factors: The Rh factor is at least morphologically related to the A and B factors, and it is therefore of interest to consider briefly some chemical aspects of these latter, better-known factors. Like the Rh factor, the A and B factors are found on the erythrocyte. However, in marked contrast to the Rh factor, substances which show A and B activity are found in some body fluids and organs in large quantities and in a readily extractable form. It is from such sources (notably hog stomach and the gastric juice of certain appropriate individuals) that most of the material which has been studied has been obtained. It should be noted that the substances which are not obtained from the erythrocyte are not necessarily the same as the material which is on the erythrocyte, but there must be some very close antigenic relationship.

Hallauer (14) obtained material with A and B specific activity by alcoholic extraction of erythrocytes, and later Kosjakow and Tribulev (15) reported a similar extraction of a specific material, with a subsequent purification through the removal of protein material by precipitation with trichloracetic acid.

Witebsky and Klendshoj (16), by means of alcoholic precipitation in the presence of sodium acetate, and subsequent deproteinization, were able to obtain group specific substances from certain gastric juices.

Morgan and King (17) obtained a water-soluble A substance from hog gastric mucin by precipitation with sodium sulfate. They were also able to dissolve the A substance in mucin with 90% phenol. A precipitation with ethanol and further purification yielded an immunologically active product.

From all these different sources, the materials prepared by various workers have fairly constant properties. The composition of A substance is about as follows:

C	44.6% - 47.2%	Acetyl group	9.4% - 12.1%
H	6.5% - 6.9%	Hexosamine	21.5% - 38.6%
N	5.5% - 7.1%	Reducing substance (calculated as glucose)	45% - 84%
Ash	0% - 2.4%		

D-galactose and N-acetyl D-glucosamine have been identified, and in some extracts L-fucose has been found. The presence of D-mannose is uncertain. Numerous amino acids have been found.

The acetyl group is apparently necessary for A substance activity. If this group is removed, activity is lost, but can be restored by acetylation. Some of the products have been found to be unstable to alkali. One preparation derived from hog stomach was serologically stable for two days at 37°C. between pH 1.07 and 10.7. At higher temperatures, the pH range over which the substance was stable narrowed, but the extract always showed a greater sensitivity to denaturation by base.

The general conclusion is that the A and B factors are rather similar to each other in their chemical nature, and that the haptenic groups are probably polysaccharides. A molecular weight of about 70,000 has been estimated for the specific A substance from hog gastric mucin. Some amino acid structures are also present, and they may play an important part in the serological specificity of these group specific substances (Landsteiner and Harte, 18). The A substance has been shown to be immunologically related to the alcohol-soluble Forssman hapten.

Practically nothing is known of the chemical nature of some of the other blood factors, such as the M, N, S factors, the Kell factor, or the P, Lewis, or Lutheran factors.

The work to be reported describes some attempts to extract the Rh antigen and to investigate some of its chemical and serological properties. In the first series of experiments, Rh positive, type O erythrocytes were treated with some different organic solvents, and the material which was extracted was tested for activity. In no cases could a specific Rh activity be demonstrated.

A second series of experiments was performed in which some fractionation of the stroma of the red cell with aqueous reagents was attempted. While some breakdown of the cell wall was apparently achieved, no satisfactory fractionation of the protein which comprises this wall

could be obtained. By using the partly degraded material, the stability of the Rh site to some denaturation agents was studied, and some attempts were made to obtain specific anti-Rh antibodies in animals.

EXPERIMENTAL

Materials and Methods

Red Blood Cells. Red blood cells were required for two procedures in this study; they were a source of the Rh antigen, and they were used in the serological tests.

Those cells used for the antigen source were Rh positive, type O erythrocytes which had been obtained from Hyland Laboratories.* In most cases, from three to six samples of blood were pooled for the extraction. These cells were usually three to four days old when the experiment was started; in a few cases they were only a day old.

Extractions were generally done on Rh positive cells only. If a preliminary test indicated any degree of activity in the product, another preparation was prepared from Rh negative cells in the same way. Parallel tests were then run on the two samples.

Red blood cells which were used for the serological testing were usually obtained from the Pasadena Clinical Laboratory.** From one to four samples of Rh positive,

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** 65 N. Madison Ave., Pasadena.

type O cells which were less than two days old were pooled and were washed three to four times with 0.9% saline. They were then made up to a 2% - 5% (by volume) suspension in saline.

Anti-Rh serum for serological tests. Most of the antiserums used for the tests were supplied by Dr. S. Haberman, of the William Buchanan Blood Center, Baylor Hospital, Dallas, Texas. These anti-serums caused agglutination of appropriate cells in a saline medium, and were of the type Rh_o or Rh'_o.

An anti-serum of type Rh_o agglutinates the red cells of 85% of white Caucasians, and it is on the basis of this agglutination test that a person is classified as clinically "Rh positive" or clinically "Rh negative". Some serums can also agglutinate the cells of an additional 1% of white Caucasians. The cells are not agglutinated by the anti-Rh_o serum, and are classified as Rh'. Most of the anti-serums which agglutinate Rh' cells also show Rh_o activity; they are therefore classified as anti-Rh'_o serums.

These anti-serums are all from human sources. The production of Rh antiserums in animals is found to be very unsatisfactory, since high anti-Rh titers cannot be produced, and the specificity of the action of the antibodies is uncertain.

Serological Tests. The activities of the preparations were tested by means of the agglutination inhibition test.

An agglutinating anti-Rh serum was diluted with 0.9% saline so that a series of final concentrations ranging from 1/5 to 1/160 of the original antiserum strength was obtained. To a series of test tubes there were added 0.02 milliliters of each of these dilutions, as well as 0.02 milliliters of the antigenic material which was to be tested. A similar series of antiserum dilutions was set up and 0.02 milliliters of a control material were added. The mixture was incubated at 37°C. for 30 minutes, and then the erythrocyte suspension was added. Incubation was continued for 10 minutes at 37°C., and for 20 minutes at 27°C. The tubes were then lightly centrifuged, and examined to see if agglutination of the test red cells had occurred. Usually the test tubes were re-centrifuged and re-examined, and in doubtful cases a microscopic check was made to determine the agglutination. The results of a typical test are shown in Table I. It is seen that added specific antigen has combined with some of the antibody and thus effectively weakened the antiserum - i.e. there has been inhibition.

The stock antigen solution for the lipid antigen was usually prepared by adding 1 volume of a saturated alcoholic solution of the antigen to 2 volumes of 0.9% saline at 37°C. In cases where the procedure was different, the method which was followed is outlined in the explanatory notes. In preliminary tests, controls consisted of a

solution of 1 part of ethanol to 2 parts of saline. As previously indicated, controls were also run in which lipid extracts of Rh negative cells were used.

When aqueous suspensions of an antigenic material were obtained, they were used without further treatment. Control experiments used 0.9% saline, isotonic buffers, or a preparation from Rh negative cells.

Table I

Agglutination Tests with Added Antigen

Antigen Added	Antiserum Dilutions 1:5	1:10	1:20	1:40	1:80	1:160
From Rh positive cells	+++	++	+	-	-	-
From Rh negative cells	+++	+++	++	++	+	-
Saline	+++	+++	++	++	+	±

+++	Strong agglutination - few large clumps of cells upon shaking
++	Medium agglutination - several small clumps upon shaking
+	Slight agglutination - many small clumps upon shaking
±	Trace of agglutination - small clumps, barely visible
-	No agglutination - no visible clumps.

Organic Solvent Extracts

Ethanol. After considerable preliminary work on extractive procedures, a series of ethanol extractions was performed. Different ratios of amounts of solvent to the amounts of cells were used, as well as different conditions with respect to initial lysis, buffers, pH, added enzymes, or re-extraction with another solvent. These conditions are summarized in Table II. The explanatory notes which follow the table give more complete details about the procedure used, and on the different conditions of the experiment. The number on the first column of Table II corresponds to the number which designates the experiment in the added notes. All extractions were performed at 5°C. - 8°C.

The measured yields of the ethanol extract varied from 1.8 to 8.2 grams per liter of packed cells. If this value is divided by a factor equal to the ratio of the volume of alcohol used to the volume of packed cells, a corrected value of from 1.1 grams per liter per unit volume ratio to 1.8 grams per liter per unit volume ratio is obtained.

The yields of the ether extracts which were obtained after the ethanol treatment varied from 1.2 grams per liter of cells to 3.1 grams per liter of cells. The values, adjusted to the volume of ether used, varied from 0.2 grams per liter per unit volume ratio to 0.8 grams per liter per unit volume ratio.

Table II

Summary of Conditions of Extraction with Ethanol

Preparation Number	Volume Ratios Aqueous Medium : Alcohol : Cells	Aqueous Medium	Enzyme Treatment	Second Solvent	Ratio of Volume Second Solvent / Original Cell Volume
1	1 : 1		No	ether	3.3:1
2	2 : 1		No	-	-
3	5 : 1		No	ether	5:1
4	5 : 1		No	base plus ether	5:1 KOH 5:1 ether
5	5 : 1		No	ether	5:1
6	5 : 1		No	methanol ethanol ether acetone chloroform toluene dioxan	See notes
7	1 : 2 : 1	water	No	ether	
8	1 : 3 : 1	water	No	ether	5:1
9	1 : 3 : 1	water	No	ether	3:1
10	1 : 5 : 1	water	No	ether	5:1

Table II (continued)

Preparation Number	Volume Ratios Aqueous Medium	Alcohol	Cells	Aqueous Medium	Enzyme Treatment	Second Solvent	Ratio of Volume Second Solvent / Original Cell Volume
11	5	7	1	water	Yes	ether	10:1
12	see notes	5	1	acid or base	No	ether	5:1
13	1	5	1	acid or base	No	ether	5:1
14	1	5	1	buffer, saline, acid, base	No	ether	5:1
15	1	5	1	base	No	ether	5:1
16	2	5	1	buffer, acid	No	ether	5:1
17	1	5	1	Phosphate Buffer pH 7.4	Yes	ether	6:1
18		5	1		Yes	ether	6:1
18	1	5	1	Phosphate Buffer pH 7.4	Yes	ether	5:1
19	1	5	1	"	Yes	ether	6:1
20	1	5	1	"	Yes	alcohol	5:1
20	1	5	1	"	Yes	alcohol-ether	5:1
21		5	1 (stroma)			ether	5:1

Preparation 1. To 1500 milliliters of cells there were added with stirring 1500 milliliters of ethanol. The mixture stood 9 days and was then filtered. Five liters of ether were added to the residue and extraction proceeded for 18 days. The alcoholic extract gave slight activity in one test, and none in another. In two separate tests, the ether extract gave no activity.

Preparation 2. Fifty milliliters of red blood cells were shaken with 100 milliliters of cold ethanol, and the mixture was set aside at 5°C. for 2 days. The alcoholic solution was taken off and lyophilized, and the dry product was suspended in 1 ml. of 0.9% saline. This stock was used for the inhibition tests. Very erratic results were obtained in all tests.

Preparation 3. Following the work of Carter (8,9), 1 liter of cells was added to 5 liters of ethanol, the whole was stirred for 2 hours and then allowed to stand overnight. The yellowish filtrate was removed and the solvent was evaporated under vacuum, with temperatures up to 31°C. The residue from the alcohol extraction was washed with an alcohol-ether mixture, and then re-extracted with 5 liters of ether. After 13 days in the cold, with occasional stirring, the ether supernatant was removed and evaporated, with temperatures up to 37°C. For testing, the extracts were dissolved in alcohol, and the solution was added to an equal volume of saline, as in Carter's procedure. In this

experiment, the alcohol extract gave slight signs of activity, while the ether extract gave no such indication.

Preparation 4. An intermediate alkali treatment was then introduced into the extraction. Twenty milliliters of red cells were added to 100 milliliters of ethanol, and the mixture was allowed to stand for 6 days in the cold. The solvent was filtered off, the residue was partly dried in air, and then shaken with 100 milliliters of 0.03 N KOH. After 4 days in the cold, 100 milliliters of ether were added, and the mixture was allowed to stand for 7 days. The supernatant gave a slight activity in one test. The alcohol extract gave slight activity in one test, and none in two other tests.

Preparation 5. To 100 milliliters of cells there were added with shaking 500 milliliters of ethanol. The whole was stirred at 5°C. overnight. The solvent was then filtered off, and the precipitate washed first with 600 milliliters of 50% (by volume) of ethanol in water, and then with 600 milliliters of 25% (by volume) of ethanol in water. The washed precipitate was partially air dried, and then 600 milliliters of ether were added. Extraction proceeded for 4 days, after which the ether solution was removed and evaporated in a current of air. The precipitate was re-extracted with 300 milliliters of ether for 5 days, and then the ethereal solution was removed and evaporated. Neither extract gave an indication of specific activity.

Preparation 6. A series of extracts of whole cells with ethanol was made. One hundred milliliters of cells were extracted with 500 milliliters of ethanol, in the cold, for 3 days. The residue was divided into seven portions of approximately 15 grams each, and 45 milliliters of one of the following solvents were added to each portion: (a) methanol, (b) ethanol, (c) ether, (d) acetone, (e) chloroform, (f) toluene, (g) dioxan. Extraction proceeded for at least 22 days at 5°C. when the solvents were filtered off, evaporated at room temperature, and tested for activity. None of them showed the presence of an Rh factor.

Preparation 7. One hundred milliliters of cells were lysed by the addition of 100 milliliters of water, and 200 milliliters of ethanol were then added. The mixture was shaken and stored in the cold. After filtration, the residue was re-extracted with ether, to give a filtrate which was evaporated to dryness and suspended in saline for testing. No consistent results could be obtained. The filtrate from the alcohol treatment was treated with an equal volume of acetone, to yield a white precipitate. This was washed, suspended in saline, and tested. No activity was found.

Preparation 8. Eight hundred fifty milliliters of cells were lysed by the addition of 500 milliliters of water, and then three liters of ethanol were slowly added, with shaking. After storage in the cold for 12 days, the mixture

was filtered, and the alcoholic filtrate evaporated to dryness, with temperatures up to 50°C. The residue from the original alcohol extraction was re-extracted with 4 liters of ether for 36 days in the cold. The ethereal extract was evaporated to dryness at temperatures up to 63°C. The alcohol extract gave no activity, whereas the ether extract gave only a weak indication.

Preparation 9. One and one half liters of cells were lysed by the addition of an equal volume of water, and the whole poured into 5 liters of ethanol. This was done at room temperature. After 24 hours in the cold the precipitate was removed, partly dried, and stored at 5°C. for 6 days. Four and one half liters of ether were added, and the mixture was stirred for 3 days, when the ether extract was filtered off. Both extracts were evaporated to dryness. The maximum temperature reached by the alcohol extract was 27°C., that of the ether extract 8°C. The ether extract showed a slight activity.

Carter had stated that the ether extract, if dissolved in alcohol and then poured into saline, gave a cloudy suspension, from which a clear, active supernatant could be obtained upon centrifugation. The ether extract of this product was so treated, but no indication of activity could be obtained from the clear supernatant.

Preparation 10. One liter of cells was lysed with 1 liter of water, and this was added, in two batches, over a period

of 3 days, to 5 liters of ethanol. The mixture was allowed to stand in the cold for 5 days, and then the precipitate was removed. The filtrate was evaporated to dryness, with temperatures at a maximum of 32°C. To the precipitate was added 4½ liters of ether, and the extraction was allowed to proceed in the cold for 20 days. The ether solution was removed, evaporated, and tested. Neither extract gave any activity.

Preparation 11. It is known that if Rh positive cells are treated suitably with trypsin, they are agglutinated much more readily by anti-Rh serums. The reason for this is not known, but it might be through an uncovering of additional sites on the cell surface, by the digestion and removal of some sort of protein layer. In view of this effect, it was decided to make some extractions using trypsin in the course of the preparation. Twenty milliliters of red cells were lysed with 100 milliliters of water, and 5 milliliters of a trypsin solution which contained 2 milligrams of trypsin* per milliliter were added. The mixture was allowed to stand at 37°C. for 15 minutes. One hundred fifty milliliters of ethanol were added and the mixture was put aside at 5°C. for 6 days. After filtration, the residue was air-dried as room temperature, and then 200 milliliters of ether were added. After 9 days of extraction in the cold the ether solution was removed and evaporated. The alcohol

*Nutritional Biochemicals Corporation, 1-300.

extract gave an indication of activity in one test and no indication in another. The ether extract showed slight activity. Experiments 17, 18, 19, and 20 describe further experiments with trypsin treated cells.

Preparation 12. To 10 milliliters of cells were added 50 milliliters of ethanol, and then sufficient 0.1 N HCl or 0.1 N NaOH was added to give pH values of 3.2, 5.8, 6.9, 9.2. (The pH of the unaltered solution was measured at 8.0.) After 5 days in the cold, the mixtures were filtered, and the residues were air-dried. Fifty milliliters of ether were added to each residue, and after 14 days in the cold, the supernatants were removed. The ether extracts from the two most acid starting mixtures showed no activity, the material from the extraction at pH 6.9 showed a trace of activity, and the last extract showed no activity in one test and slight activity in another.

Preparation 13. To 10 milliliters of cells and 50 milliliters of ethanol, there were added 10 milliliters of either 0.1 N KOH, 0.01 N KOH, water, 0.01 N HCl, or 0.1 N HCl. The samples were shaken, stored 2 days at 5°C. and then filtered. The residue was air-dried at room temperature, and 50 milliliters of ether were added. This mixture stood 2 days at 5°C. and the ether extracts were removed and evaporated. In several tests of these preparations, the neutral extract never showed any activity, while the others showed some slight activity.

Preparation 14. Another experiment was run in order to check the results of preparations 4 and 11. To 10 milliliters of cells and 50 milliliters of ethanol there were added, in different samples, 10 milliliters of water, 10 milliliters of phosphate buffer of pH 7.43, 10 milliliters of 0.9% saline, 10 milliliters of 0.1 N KOH, and 10 milliliters of 0.1 N HCl. These were allowed to stand for 1 day, and then the mixture was filtered. The precipitate was washed in 20 milliliters of ether and to the precipitate was added 50 milliliters of dry, peroxide-free ether. The mixture stood 4 days at 5°C., the ether solution was removed, and the precipitate was washed with ether. Washings were combined with the filtrates, and the whole evaporated. In this case, the buffered extract and the alkali extract gave no activity, while the acid, saline, and water extracts gave a slight indication of activity.

Preparation 15. In another run, there was added to the alcohol-cells mixture, 50 milliliters of 0.01 N KOH in one case, and 10 milliliters of the same reagent in another case. After extraction for a period of 3 days at 5°C. the mixture was filtered and the residue was air-dried at room temperature. Fifty milliliters of ether were then added and the extraction continued for another 14 days at 5°C. The ether extracts gave no activity.

Preparation 16. As a further test of the effect of pH upon

the extraction, a series of runs was performed in which 10 milliliters of red blood cells were added to 50 milliliters of ethanol, and then 20 milliliters of veronal buffer were added, as well as sufficient 0.1 N HCl to give the following pH values: 3.1, 5.16, 6.11, 7.02, 7.38. These extractions were allowed to stand for 6 days at 5°C. and were then filtered. To the residues were added 50 milliliters of ether. This extraction lasted 13 days at 5°C. Solvents were evaporated in a current of air, with the aid of an infra-red lamp. None of the alcoholic or ether extracts gave any inhibition.

Preparation 17. One hundred milliliters of cells were added to 100 milliliters of phosphate buffer, of pH 7.4. Ten milliliters of a 0.2% trypsin solution were added. This was incubated for 15 minutes at 37°C., then a total of 500 milliliters of ethanol were added, with shaking, and the whole was then stored at 5°C. overnight. After filtration, the precipitate was first washed with 600 milliliters of 50% (by volume) of ethanol in water, and then with 600 milliliters of 25% (by volume) of ethanol in water. The precipitate was partially dried, and 600 milliliters of ether were added. Extraction proceeded for 4 days at 5°C. when the ether solution was removed and evaporated in a current of air. The precipitate was re-extracted with 300 milliliters of ether for 5 days, and then this solution was evaporated. Neither extract gave any specific activity.

Preparation 18. The above experiment was repeated with a fresh batch of cells, but the second ether extraction was not performed. As a variation, a parallel run was made in which the trypsin was added directly to the cells, without the addition of buffer. Alcohol was added later, as above. Another parallel run, which actually duplicated the conditions of one of the former runs (preparation 3), was made in which the trypsin and the buffer were both omitted, and alcohol was added directly to the cells. This is described under preparation 5. No activity was found in any of the extracts.

Preparation 19. In the course of another series of experiments, the conditions of preparation 17 were set up, but with 1/10 the amounts of materials. The first ether extract was examined for activity, but none was found.

Preparation 20. Two extractions were performed under the same initial conditions described for preparation 17 - i.e. to 1 volume of cells, plus 1 volume of phosphate buffer at pH 7.4, there was added 1/10 of a volume of trypsin solution. Incubation and extraction with 5 volumes of ethanol followed. In both cases, the alcoholic extract was inactive. The precipitate was extracted with 5 volumes of ethanol in one case, and in the other case with 5 volumes of an ethanol-ether mixture (ethanol:ether in volume ratio of 9:1). Neither extract was active.

Preparation 21. Five milliliters of cells were lysed in ice-cold water which had been saturated with carbon dioxide, and the resulting stroma were centrifuged off as a heavy precipitate. They were washed three times with cold, CO_2-saturated water, and then extracted at 5°C. for 1 day with 25 milliliters of ethanol. The residue from the extraction was re-extracted with 25 milliliters of ether for 11 days. No activity was found in either of the extracts.

Methanol. A small number of extractions were made in which methanol was the initial solvent. These experiments are summarized in Table III, and the corresponding notes follow the table.

Table III

Summary of Conditions of Extraction with Methanol

Preparation Number	Volume Ratios H_2O:MeOH:Cells	Second Solvent	Ratio of Volume Second Solvent / Volume Cells
1	5 : 1	ether	5:1
2	1 : 5 : 1	ether	5:1
3	5 : 1	methanol ethanol ether acetone dioxan chloroform toluene	See notes below

Preparation 1. Ten milliliters of cells were mixed with 50 milliliters of methanol, shaken, and extracted in the cold for 8 days. This yielded 0.07 grams of dried extract, or 1.4 grams per liter of cells per unit volume ratio. The first residue was re-extracted with 50 milliliters of ether, to give another 0.03 grams of material, or 0.6 grams per liter of cells per unit volume ratio. Both extracts were inactive.

Preparation 2. The experiment was later repeated using the same conditions as mentioned above, but in addition a parallel run was made in which the cells were first lysed by the addition of an equal volume of water. In both cases, the ether extracts were inactive.

Preparation 3. One hundred milliliters of cells were shaken up with 500 milliliters of methanol and stored in the cold for 3 days. After the methanol solution was removed, the residue was partially air-dried, and divided into seven portions of about 15 grams each. Each portion was extracted with 45 milliliters of one of the seven solvents indicated, for at least 22 days. The residues obtained from the evaporation of the solvents were inactive.

Acetone. The following notes summarize the two extractions in which acetone was the initial solvent.

Preparation 1. Nine hundred milliliters of packed cells were extracted with 2 liters of acetone in the cold for 4

days. The acetone extract was lyophilized, but gave no indication of activity. The residue from this first extraction was re-extracted with five liters of ethanol in the cold for 26 days. This too yielded no activity after lyophilization. The yield of acetone extract was 2.1 grams, or 1.0 grams per liter of cells per unit volume ratio; that of the ethanol extract was 3.4 grams, or 0.7 grams per liter of cells per unit volume ratio.

Preparation 2. One hundred milliliters of cells were shaken up with 500 milliliters of acetone in the cold. Extraction proceeded for 3 days, when the acetone fraction was removed. The residue was air-dried and 45 milliliters of each of the seven different solvents as indicated in the previous experiments were added to 7-gram portions of dried residue. Extraction continued for at least 22 days. No activity was obtained from any of the residues which resulted from the separation of the solution and evaporation of the solvent.

Dioxan. Extractions with dioxan are indicated in Table IV and in the amplified notes.

Preparation 1. Ten milliliters of cells were extracted with 50 milliliters of dioxan, in the cold for 10 days. The residue was re-extracted with 50 milliliters of ether for 2 months in the cold. The dioxan extract gave no activity, but the ether extract gave an apparent activity,

Table IV

Summary of Conditions of Extraction with Dioxan

Preparation Number	Volume Ratios H_2O:Dioxan:Cells	Second Solvent	Ratio of Volume Second Solvent / Volume Cells
1	5 : 1	ether	5:1
2	½ : 5 : 1	ether	5:1
3	5 : 1	methanol ethanol ether acetone dioxan chloroform toluene	See notes

which on the basis of the results of the following experiment was probably non-specific. The yield of the dioxan extract was 1.6 grams, or 32 grams per liter of cells per unit volume ratio; that of the ether extract was 0.3 grams, or 6 grams per liter of cells per unit volume ratio.

Preparation 2. A second dioxan extract was made on 10 milliliters of cells to which 5 milliliters of water had been added. Fifty milliliters of solvent were used, and extraction proceeded for 6 days in the cold. The solvent was filtered off, and 50 milliliters of ether were added to the residue. The mixture was stored in the cold for 5 days, after which the ether solution was separated and the solvent evaporated. Neither extract gave any activity.

Preparation 3. Following the procedure previously outlined,

100 milliliters of cells were extracted with 500 milliliters of dioxan for 3 days in the cold. The residue was divided into seven fractions of approximately 15 grams each, for re-extraction with 45 milliliters of the seven solvents as in the former procedures. No activity was found in either the first extract or any of the subsequent re-extractions.

Ether. MacFarlane (19) showed that lipoproteins could be extracted from serum proteins by a treatment at very low temperatures. This suggestion was followed, and these extractions were carried out.

Preparation 1. Three milliliters of a stroma suspension were frozen in a dry ice-methyl cellosolve bath. To this, 10 milliliters of ether which had been pre-cooled to under -20°C. were added. This was allowed to thaw slowly, and the ether layer was removed for evaporation and testing.

Preparation 2. In a second experiment, an attempt was made to remove much of the water in the raw material before the extraction with ether. Therefore the following procedure was undertaken. To 3 milliliters of suspension there were added 5 milliliters of chilled acetone. The mixture was quickly centrifuged, and the supernatant poured off. Another washing with acetone was quickly performed, and then 10 milliliters of pre-cooled ether were added, the mixture thawed, the ether layer removed, and the solvent evaporated. No activity was obtained in either extract.

Preparation 3. According to Block (20) lipoproteins may sometimes be extracted by successive treatments with ether, alcohol, and then ether again.

To 10 milliliters of a stroma slurry, 50 milliliters of ether were added, with shaking. The whole stood 1 day in the refrigerator, when the ether was poured off, and another 50 milliliters of ether was added to the residue. Extraction was allowed for 1 day in the cold, when the ether was taken off and combined with the first ether extract. The residue was taken up with 50 milliliters of ethanol and extracted in the cold for 2 days. The ethanol extract was removed. The residue was washed with 5 milliliters of ethanol, followed by two ether washings of 5 milliliters each. Thirty milliliters of ether were then added, and after 1 day in the cold the supernatant was poured off and another 30 milliliters of ether were added. After a day for extraction, the ether layer was taken off and combined with the preceding ether solution. All extracts were evaporated to dryness and tested. None showed any activity.

Ether-Alcohol. The above low-temperature type of experiment was repeated, but different ether-alcohol mixtures were used for the solvent. In all cases, 5 milliliters of suspension were extracted with 20 milliliters of solvent. The experiment was divided into two parts. In Part I, the stroma

samples were frozen and extracted with the different solvents. In Part II, the samples were washed twice, rapidly, with acetone, before the solvents were added. Temperatures in all cases were under -20°C. The different solvents were (a) 100% ether, (b) ether-alcohol (9:1 by volume), (c) ether-alcohol (5:5 by volume), (d) ether-alcohol (1:9 by volume), (e) 100% alcohol. None of these extracts showed the slightest trace of activity.

Phenol. An attempt was made to extract with phenol. Twenty milliliters of stroma were mixed with 100 milliliters of phenol, and the mixture stored in the cold for 3 days. The total volume of the solution was 100 milliliters. Fifty milliliters were extracted with 3 portions of 25 milliliters each of phosphate buffer, pH 7.36. The combined extracts were dialysed against water, and then evaporated to concentrate them. No activity was found.

Immunization Experiments. Some attempts were made to specifically immunize animals against the Rh factor, using organic solvent extracts as antigens. A carrier protein was injected along with the solution of the extract. No specific antibodies could be demonstrated.

Experiment 1. One half gram of an extract which had given a weak indication of activity (see preparation 8 of the ethanol extractions) was dissolved in 75 milliliters of

ethanol at 56°C., and passed through a Seitz filter. Seventy-five milliliters of saline were added through the filter. Eighty-two milliliters of a re-constituted sheep serum (8% dried serum in water) which had been Seitz filtered and centrifuged, were added to this, and the mixture stored in the cold.

Six guinea pigs and 6 rabbits were injected with this antigen, over the course of two weeks. Rabbits received 4 injections of 2 to 3 milliliters each, intravenously, and 2 injections of 2 to 3 milliliters intraperitoneally. The guinea pigs received 4 injections of 2 milliliters and 2 injections of 1 milliliter, all intraperitoneally. At the end of this period, the guinea pigs had built up no titer, while the rabbits had developed agglutinins which were not specific for the Rh factor. After a 12 day rest, the injection of the guinea pigs was resumed, and over a period of a month they were given 10 injections of 1 milliliter each, intraperitoneally. They developed agglutinins to a titer of 256 against human red cells, but these were not specific for the Rh factor, as shown by tests with both Rh positive and Rh negative cells.

Experiment 2. A second series of injections was performed, with a different carrier protein and different conditions of concentration and mixing. A stock extract solution was made by dissolving sufficient extract in alcohol to give a concentration of 0.5% by weight, and filtering this solution

through a Seitz filter. A serum solution was made by heating 35 milliliters of hog serum to 56°C. for 30 minutes, diluting with saline, and passing through a Seitz filter. Boiled saline was added to bring the total volume to 360 milliliters, and 4½ milliliters of a 10% phenol solution were added as a preservative.

The rabbits for the test were divided into four groups of 3 animals each, and 6 intraperitoneal injections were given each animal during 2 weeks.

Group I received 1 milligram of extract each. One volume of extract solution was mixed with 25 volumes of serum solution, and after standing 1 hour, the proper volume to give the dose indicated was injected. The two solutions were mixed thus before each injection.

Group II received 1 milligram of extract. One volume of extract solution was mixed with 25 volumes of serum solution. The solutions were mixed at the beginning of the experiment, and this stock was used throughout for Group II.

Group III received 10 milligrams of extract. Ten volumes of extract solution were mixed with 25 volumes of serum solution, 1 hour before each injection, and then the proper amount was injected.

Group IV received 10 milligrams of extract. The proper amounts of extract solution and serum solution were mixed at the beginning of the experiment, and this stock was used throughout for Group IV injections.

Two weeks after the last injection, the animals were bled. No specific agglutinins could be demonstrated in any of the sera.

Conclusion. On the basis of agglutination inhibition tests, it appears that organic solvents will not extract the undenatured antigen from the erythrocyte. The failure of some attempts to develop specific agglutinins lends support to this statement. The significance of these results will be discussed later.

Extracts from Treatment with Aqueous Reagents

Since the methods of extraction by organic solvents had not yielded an active product, some other methods of cell degradation and solution were tried.

The Rh factor must be a structure on the surface of the cell wall, and any methods of cell solution must allow this complete site to pass into solution in some sort of undenatured form. This condition is presumably attained when A and B factors are extracted, but the evidence thus far is that the Rh site is not so readily removed in intact form. If it is a more or less integral part of the cell wall structure, and not something of the nature of a "side chain" on the cell wall, then a considerable breakdown of the red cell ghost will be necessary in order to put this structure

into solution.

The main constituent of the cell wall is a protein which has been called stromatin. This has been recognized as a "protein sui generis" by Jorpes (21) on the basis of its amino acid composition. Evidence shows that it is very difficult (short of drastic procedures) to put all of the cell wall into solution. Mild treatment can rupture the wall, and apparently fragments of different sizes may be torn off. Some lipid constituents may be removed in the process of ordinary hemolysis; such material is mainly neutral fat and cholesterol esters (Ponder, 22). A globulin and an albumin are also reported to have been removed from the surface of the cell wall with mild treatment (Ponder, 23).

Furchgott (24) tried to dissolve stroma in dilute alkali, but he could not obtain any satisfactory solution. He also tried, without success, different concentrations of NaCl and KCl, in buffered phosphate. Following a procedure of Boehm (25) he treated lysed cells with 2.5 M $LiClO_4$ buffered to pH 7.8. The cells ultimately broke into small fragments which he estimated to be 0.1 μ in diameter and 1 μ long.

Ballantine and Parpart (26) treated erythrocytes with trypsin at pH 6.8 for 24 hours, and found that there was no change in the permeability of the cell wall, as compared with normal cells. Presumably there had been but little degradation of the cell surface. On the other hand,

treatment with pancreatic lipase resulted in increased permeability, as measured by the susceptibility to hemolysis.

The work of Calvin and co-workers has already been mentioned (page 2, this thesis). Their elenin is presumably a lipo-protein fraction which is separable from stroma under the conditions mentioned.

Stern et al (27) obtained stroma by freezing red blood cells. This product was suspended in a buffer, and then centrifuged. The supernatant solution showed two electrophoretic components (other than hemoglobin) and gave a good Tyndall effect. The slow component, which had approximately the same mobility as the slower of Calvin's components, was considered to have consisted of particles of undegraded stroma. The faster component was not further identified.

Sigurdson (28) claimed to have isolated small particles from stroma, after lysing the cells with hypotonic saline. Heating increased the degree of breakdown.

These results seem to indicate that in most cases there has been nothing more than a fragmentation of the cell wall. Several large pieces are broken off, or the wall may be broken into comparatively large portions, depending on the severity of the treatment. The smaller pieces are difficult to centrifuge, and may be readily dispersed, probably to form a colloidal suspension.

In undertaking the present work, it was thought that

a mild alkali treatment might break down some of the lipoprotein complexes, and that some separation by salt fractionation might then be achieved. Subsequently, other fractionation and extraction procedures, including some which had proved useful in the separation of bacterial antigens, were applied to the product from the initial treatment. No satisfactory further fractionation was obtained.

Preparation. Ten milliliters of packed, Rh positive, type O erythrocytes were mixed with 20 milliliters of 0.01 N KOH and the mixture was stored in the cold. After 12 hours, no precipitate could be obtained after centrifuging at 3,000 r.p.m. for 10 minutes. The pH was 7.2. Thirty milliliters of saturated ammonium sulfate (SAS) were added, and a flocculent precipitate was obtained. The pH was now about 6.4. The mixture stood 3 hours in the cold, and then the precipitate was centrifuged off and dialysed against water until no more sulfate was detectable in the dialysate. The precipitate was suspended in saline, and was found to give good inhibition of agglutination. Similar material prepared from Rh negative cells gave no inhibition. This effect was checked a number of times, on different stocks of cells.

Properties. In a fresh precipitate, the particles were small enough and could be so readily dispersed in a saline or buffer solution that an active, cloudy supernatant was left after centrifuging the suspension for 10 minutes at

3,400 r.p.m. In old preparations or in one which had been suspended in water, a similar centrifuging gave a clear supernatant in which no activity could be detected.

In Table V are shown the results of an experiment in which an inhibitor sample was suspended in water (inhibitor A) or in phosphate buffer of pH 7.4 (inhibitor B). The supernatant fractions from a centrifugation as mentioned above were used for the test.

Table V

Activity Comparision of Different Inhibitor Suspensions

	Antiserum Concentration 1/10	1/20	1/40	1/80
Supernatant A	±	++	++	++
Supernatant B	-	-	-	-
Negative preparation	+	++	++	+

There was a sufficient excess of antibody so that a prozone was beginning to show in the case of the most concentrated antiserum. Inhibitor A, when it was later suspended in saline, gave inhibition, thus showing that it had not been denatured. With longer centrifuging, even in saline suspension, the activity of the supernatant material decreased.

A comparison of the activities of stroma, and of the preparation derived from stroma by the treatment described, is shown in Table VI. Both the stroma and the preparation

derived from it were made from the same stock and were of the same age. Apparently, the stroma has denatured more rapidly, or is less active, than the prepared material. The inhibitor material thus prepared, rather than stroma, was therefore used in subsequent tests. It will be referred to as the product of the KOH-SAS treatment.

Table VI

Comparison of Activities of Stroma and Preparation

	Antiserum Concentration 1/40	1/80	1/160	Milligrams of protein per milliliter
Stroma				
Positive cells	++	++	-	2.8
Negative cells	+++	+	-	2.7
Preparation				
Positive cells	-	-	-	4.7
Negative cells	++	++	-	3.8

Effect of pH Change. A number of 3 milliliter samples of a freshly prepared inhibitor which had been checked for activity were adjusted to the pH values indicated in the following table, with 0.1 N KOH or 0.1 N HCl. They were then heated for 1 hour at 37°C., and the pH was re-checked. All samples were brought to a pH of between 7.0 and 7.5 by addition of acid or base. In two of the samples, where but little of the reagent was necessary to obtain neutrality, a small amount of salt solution was added to make a correction

for the volume change and ionic strength change which was attendant upon the neutralization of the other samples. Table VII shows the pH values in the different phases of the test; Table VIII shows the results of the inhibition tests with the treated preparations.

Table VII

Conditions of Digestion

Inhibitor Sample	Initial pH	pH after digestion	Adjust to pH
5	4.72	4.68	7.01
6	6.07	6.36	7.25
7	7.14	7.25	7.18
8	7.92	7.81	7.00
8a	7.95	7.85	7.20*
9	9.20	8.88	7.18
9a	9.18	8.78	7.26**
10	9.96	9.51	7.16
11	11.00	10.42	7.42

* Add 1 milliliter 0.5% NaCl
**Add ½ milliliter 0.5% NaCl

Table VIII

Agglutination with Treated Inhibitors

Inhibitor Sample	Antiserum Concentration 1/10	1/20	1/40	1/80
5	+++	+++	++	++
6	++	-	-	-
7	±	-	-	-
8	±	-	-	-
8a	-	-	-	-
9	±	-	-	-
9a	+	-	-	-
10	+++	++	+	+
11	+++	++	+	+
saline control	+++	+++	+++	+

+++ strong agglutination of test erythrocytes

++ medium agglutination of test erythrocytes

+ slight agglutination of test erythrocytes

± trace of agglutination of test erythrocytes

- no agglutination of test erythrocytes

The conclusion was that this extract had a maximum stability in the region of pH 8. There was an indication that at pH values 1 unit on either side of this, some denaturation occurred.

An experiment to determine the rate of denaturation was set up. A sample of a preparation was suspended in buffer,

and heated to 37°C. Samples were removed at various times and used in agglutination inhibition tests. Similar extracts from Rh negative cells were used for controls. The decrease in the inhibitory activity of the preparation was followed, and thus a rough idea of the extent of denaturation of the antigenic site was obtained. Precise measurements were not possible, due to the inherent large error in the serial dilution technique. However, it appeared that at pH 7.1, in phosphate buffer, nearly all the activity was lost in 2 hours. At pH 5.9, the activity was lost in 30 minutes.

Effect of Ethanol. The stability of the extract to ethanol was determined in the following way. Inhibitor stock was added to phosphate buffer, pH 7.34, and then different amounts of ethanol were added. Saline was added as necessary to bring samples to a definite volume. As a control, similar mixtures of buffer, ethanol, and saline were made up, with water added to take the place of the inhibitor. The actual constitution of the various solutions was as shown in Table IX. These solutions were heated for 30 minutes at 37°C., and were then tested for activity. The results are shown in Table X. In another run, a similar series was made up, with all reagents at 8°C. The inhibitor stocks stood with the alcohol in the cold overnight. Samples were then incubated with antiserum for 1½ hours in the cold. Cells were added, and the mixture kept in the cold for another 40 minutes. The tubes were then centrifuged and read. The results of this test are shown in Table XI.

Table IX

Composition of Inhibitor Suspensions

Sample	Volume of Inhibitor Added (ml.)	Phosphate Buffer pH 7.4 (ml.)	EtOH (ml.)	Saline (ml.)	H_2O (ml.)	% EtOH
i	1.5	1.0	-	0.50	-	0
ii	1.5	1.0	0.05	0.45	-	1 1/3
iii	1.5	1.0	0.10	0.40	-	2 2/3
iv	1.5	1.0	0.25	0.25	-	6 3/4
v	1.5	1.0	0.50	0.00	-	13 3/4
vi	-	1.0	-	0.50	1.5	0
vii	-	1.0	0.10	0.40	1.5	2 2/3
viii	-	1.0	0.25	0.25	1.5	6 3/4
ix	-	1.0	0.50	-	1.5	13 3/4

Table X

Agglutination with Treated Inhibitors

Ethanol %		Antiserum Concentration 1/20	1/40	1/80
0	Inhibitor i	-	-	-
	Blank vi	+++	+++	-
1 1/3	Inhibitor ii	-	-	-
2 2/3	Inhibitor iii	-	-	-
	Blank vii	+++	+++	++
6 3/4	Inhibitor iv	+++	+	±
	Blank viii	+++	++	+
13 3/4	Inhibitor v	+++	+++	++
	Blank ix	+++	+++	±

Table XI

Agglutination with Treated Inhibitors

Ethanol %		Antiserum Concentration 1/20	1/40	1/80
0	Inhibitor xiii	-	-	-
4	Inhibitor x	-	-	-
	Blank xiv	+++	++	+
8	Inhibitor xi	±	-	-
	Blank xv	++	++	-
17	Inhibitor xii	+	+	-
	Blank xvi	+++	++	-

From these two experiments, the following conclusions may be drawn:

(a) At 37°C., for 30 minutes, ~ 3% ethanol does not destroy the antigen, ~ 6% ethanol destroys all but a small amount, ~ 14% ethanol destroys all noticeable activity.

(b) At 8°C., for 12 hours, ~ 4% ethanol does not destroy the antigen, ~ 8% ethanol may give slight denaturation, ~ 17% ethanol gives considerable denaturation. (In the case of the 17% ethanol, at 8°C., the effective titer of the antiserum is the same for both the blank and the inhibitor. The conclusion which is drawn for this case is based on the observation of the difference in the strength of the agglutination.)

Oxidation. Equal volumes of 1/1000 hydrogen peroxide and the inhibitor were allowed to stand at room temperature

(28°C.) for 5 minutes. Antiserum was added, and the mixture was heated for 30 minutes at 37°C., as usual, before the addition of cells. Activity was retained after this mild oxidation. The presence of the peroxide did not interfere with agglutination, as shown by a suitable control.

Lyophilization. Lyophilization is a convenient method of preserving protein preparations, provided that the process does not denature them.

1. A sample of the KOH-SAS preparation was lyophilized. Activity was lost.

2. A sample of a stroma suspension was checked for activity, and then lyophilized. The dried material was subjected to the KOH-SAS treatment, and again, the product was found to be inactive.

Extraction with Organic Solvents. Though previous work had indicated that there was little chance of obtaining any active Rh substance by solvent extraction, nevertheless the following experiments were tried.

The method mentioned by Block (20) was employed, with the following sources of antigen: (a) 5 milliliters of Rh positive, type O cells; (b) 2 milliliters of a preparation from positive cells; (c) 2 milliliters of another preparation from positive cells; (d) 2 milliliters of a preparation from negative cells.

These materials were extracted twice with 2 volumes of ether, followed by two extractions with 2 volumes of ethanol, and two more extractions with 4 volumes of ether. Each of

the extracts was evaporated and the product was tested. None of the materials gave any active extracts.

In another experiment, a sample of a preparation was lyophilized, and two samples of 100 milligrams each were extracted with ether in one case, and with a mixture of ether and ethanol (9:1 by volume) in another case. Extractions were made at -25°C., in accordance with the recommendations of MacFarlane (19). Neither of the evaporated extracts yielded any activity.

Fractionation

Ammonium Sulfate. After an initial KOH treatment, different preparations were treated with different concentrations of ammonium sulfate. A concentration of this salt equal to about 1/3 saturation was the minimum amount which would yield a precipitate. Such a precipitate was active. Further addition of ammonium sulfate gave an additional precipitate, of about the same degree of activity. No marked fractionation, therefore, was obtained in this manner.

Concentrated Salt Solution. An attempt was made to break up the stroma to a greater degree by an intermediate treatment with 10% NaCl in the preparation of the antigenic material. Ten milliliters of a stroma suspension were treated with 20 milliliters of a 0.01 N KOH solution for 4 days in the cold. The resulting precipitate was centrifuged down, and made up to 15 milliliters with water. NaCl

was then added to give a final concentration of salt of 10%. After the mixture had stood overnight in the cold, it was centrifuged. The supernatant was brought to half saturation with ammonium sulfate and the resulting small precipitate was centrifuged off, washed, and re-suspended. A slight activity was shown. The first precipitate from the concentrated salt solution had more activity. Apparently but little break-up of the stroma had been effected.

A further attempt was made to fractionate the product of the KOH-SAS treatment. Fifty milliliters of a suspension of this product were adjusted to a concentration of 10% NaCl, and the pH adjusted to 7.89 with 0.1 N KOH. The mixture was held at 37°C. for 30 minutes, when 20 milliliters were taken off and centrifuged at 17,000 r.p.m. for 30 minutes. The supernatant was dialysed against saline. The precipitate was suspended in 20 milliliters of phosphate buffer, at pH 7.36. The supernatant had but slight, if any, activity, whereas the precipitate showed a strong inhibitory action.

A similar test was run in which a sample of the same suspension was used for the starting material. Sodium chloride was added to a final concentrate of 10%, and the solution divided into 2 portions. One portion was adjusted to pH 6.18, and another to pH 8.0. Both were held at 5°C. for 3 days, and then were centrifuged at 17,600 r.p.m. for 25 minutes. The supernatants were dialysed against saline, and then tested for activity. None was found, so apparently

the treatment did not further degrade the cell.

Alcohol Fractionation. Half of the precipitated fraction from 50 milliliters of cells was suspended in sufficient saline to bring the total volume to 30 milliliters. Twenty milliliters of phosphate buffer, pH 7.4, were added, followed by 30 milliliters of ethanol. A precipitate which could be readily centrifuged down was obtained. About 25 milliliters of this packed precipitate were added to 100 milliliters of the above buffer. Meanwhile, the supernatant from the alcohol precipitation, after standing 2 hours in the cold, yielded another heavy precipitate which was removed and suspended in buffer. The supernatant from this precipitation was brought to half saturation with ammonium sulfate, and a third precipitate was obtained. This was taken off, washed, and re-suspended in buffer. All of the precipitates, and the first two supernatants, were tested for activity. None was found. Subsequent work indicated that the alcohol had probably denatured the active sites.

Formamide. The procedure of Morgan and Partridge (28) was followed, except that the starting material was a suspension, rather than a dried material, and heating was avoided. Ten milliliters of a slurry of the KOH-SAS product was treated with 87 milliliters of formamide. This mixture stood 3 hours in the cold, and then 200 milliliters of ethanol were added. The mixture was allowed to stand overnight in the cold. The slight precipitate which

formed was taken off, suspended in phosphate buffer, pH 7.3, and dialysed against the same buffer. To the supernatant, another 100 milliliters of ethanol were added, and the mixture allowed to stand for 12 hours. The new precipitate which formed was dialysed against a saline-buffer mixture. Neither of the suspended precipitates gave any inhibition.

Trichloracetic acid. An extractive procedure as given in Kabat and Mayer (29) was followed in this experiment. Twenty-five milliliters of a suspension of the KOH-SAS product (about 1.7% by weight) were mixed with 25 milliliters of 0.2 N trichloracetic acid. Centrifuging at 14,000 r.p.m. for 10 minutes gave a precipitate which, in another experiment, was shown to be inactive. The supernatant was then dialysed against water to get rid of the acid. Cold ethanol was then added to give a final concentration of 68% ethanol (by weight). Evaporation in a current of air eventually yielded a precipitate. This was centrifuged, and to the ¼ milliliter of packed precipitate which resulted were added 1 milliliter of saline and 1 milliliter of phosphate buffer, pH 7.4. This suspension gave no activity.

In another similar experiment, the supernatant which resulted after the addition of the trichloracetic acid and subsequent centrifugation was dialysed against saline, and evaporated to yield an inactive precipitate, and a non-specifically active supernatant.

Trypsin Treatment. The products of the KOH-SAS treatment were treated with trypsin in an attempt to obtain a further degradation.

Experiment 1. Five milliliters of a packed inhibitor solution were suspended in 15 milliliters of phosphate buffer, pH 7.3, and 0.5 grams of trypsin* were added. The mixture was allowed to stand for 2 days at 5°C., and then the pH was adjusted to 8.3 with 0.1 N KOH. After this mixture had stood another 12 hours at 5°C., 100 milligrams of trypsin were added, and the whole was kept at 5°C. for another 3 days. A centrifuging at 3,000 r.p.m. for 10 minutes brought down a small precipitate which was discarded. The supernatant was brought to half saturation with ammonium sulfate, allowed to stand for 12 hours at 5°C. and then centrifuged at 36,000 G for 15 minutes. The precipitate thus obtained was suspended in 10 milliliters of phosphate buffer at pH 7.37, and re-centrifuged at 40,000 G for 30 minutes. One milliliter of the supernatant obtained from this procedure was dialysed against 3 milliliters of the phosphate buffer. A sample taken from inside the dialysing bag which contained the supernatant still showed activity. The outer (3 milliliter) portion, which would contain the dialysable material, showed no activity. Centrifuging another portion of the supernatant at 900 r.p.s. for 2000 seconds yielded a supernatant which could be carefully

*Nutritional Biochemicals Corporation, 1-300.

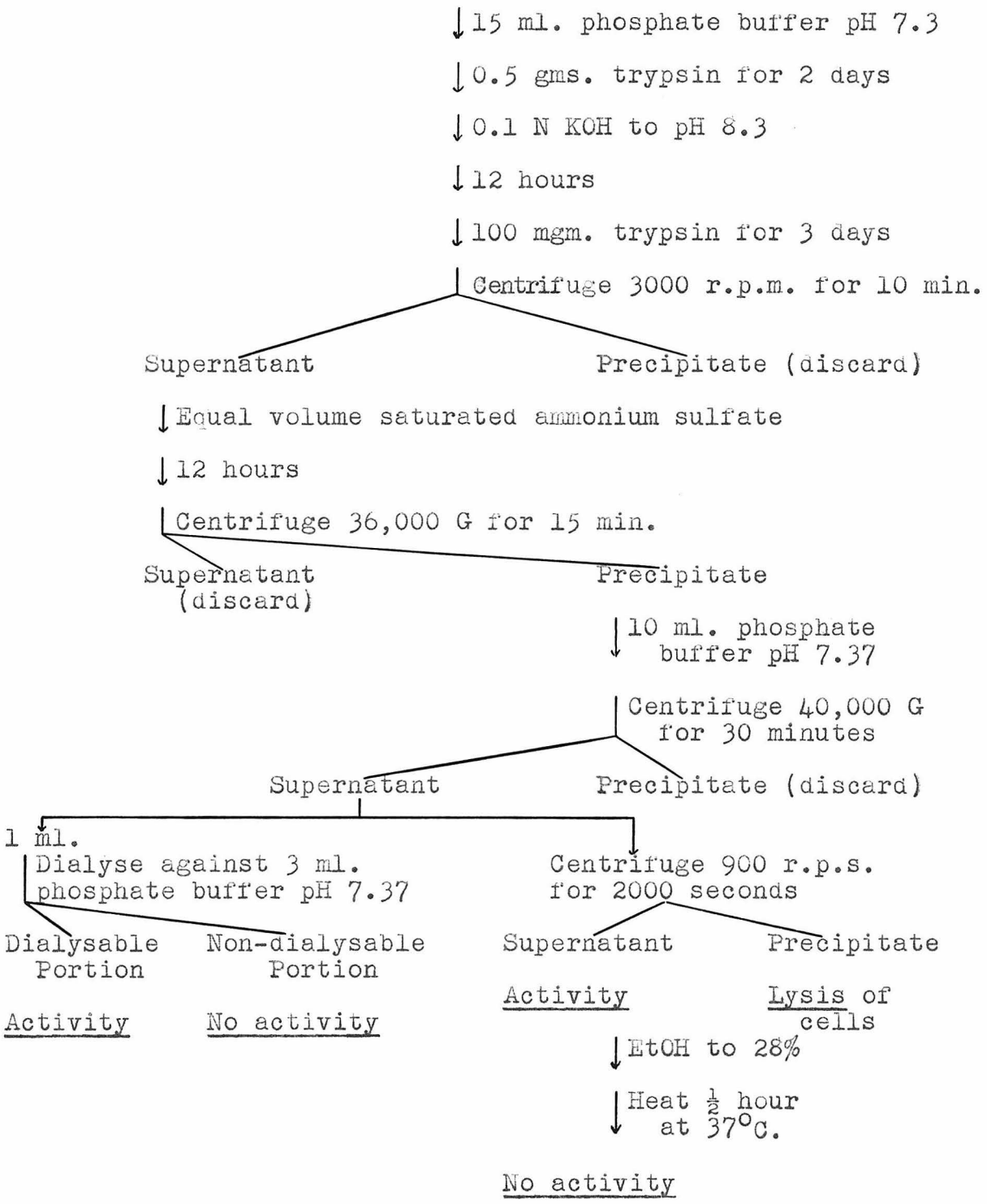
separated from a precipitate, which gave good inhibitory action. Addition of ethanol to the supernatant to a final concentration of 28%, and heating for 30 minutes at 37°C. destroyed the activity. The precipitate, when suspended in buffer and used in an inhibition test, caused lysis of the added erythrocytes. This may have been due to the action of some of the original trypsin which may have been carried along in the procedure. A flow sheet of this procedure is shown in figure I.

Experiment 2. The specificity of the action in the above experiment was questioned, so another experiment was set up in which both Rh positive and Rh negative cells were used. Smaller quantities of trypsin were used.

The KOH-SAS treatment was done as usual on 20 milliliters of cells, and the product was treated with 0.05 grams of trypsin for 3 days. The supernatant from a light centrifugation, after a mild alkali treatment as above, and another similar trypsin treatment, was again lightly centrifuged. The new supernatant was treated with an equal volume of saturated ammonium sulfate and re-centrifuged at 38,000 G. This gave a precipitate which was suspended in phosphate buffer, and re-centrifuged at 40,000 G for 30 minutes. The supernatant was inactive. The re-suspended precipitate was active in a non-specific manner, as indicated by a test with the product from Rh negative cells. However, a greater inhibitory action was obtained with the

Figure I

Flow Sheet of Trypsin Treatment



product from Rh positive cells, so some specific activity is presumed to have existed.

Experiment 3. In another preparation, using only Rh positive cells, 30 milliliters of the packed KOH-SAS product were suspended in 60 milliliters of phosphate buffer at pH 7.36, and 10 milliliters of this material were treated with 100 milligrams of trypsin after being brought to pH 8.5 with 0.1 N KOH. After heating for 15 minutes at 37°C., followed by 6 hours in the cold, the product was lightly centrifuged, and the supernatant re-centrifuged at 13,500 r.p.m.* for 20 minutes. The precipitate from this centrifugation was suspended in 10 milliliters of buffer, and another 100 milligrams of trypsin were added. After 1 hour in the cold, this was centrifuged at 13,500 r.p.m.* for 20 minutes. An attempt was made to rid the supernatant of the trypsin by two precipitations at 0.4 saturation of ammonium sulfate, with subsequent centrifugation and resolution of the precipitate. The trypsin should remain in the supernatant fraction. The final step was dialysis to get rid of the ammonium sulfate. This product was active in an inhibition test.

The supernatant from the high speed centrifuging after the first trypsin treatment was brought to 0.4 saturation with ammonium sulfate and re-centrifuged. The precipitate was washed with 0.4 saturated ammonium sulfate and dialysed against saline. After another re-precipitation, solution,

* 23,000 G

and dialysis, the product was tested, and found to be active. A flow sheet of this operation is shown in Figure II. Material derived from a re-precipitation of the trypsin stock did not show any inhibition.

Experiment 4. As another check on the specificity of the action, another experiment using Rh positive and Rh negative cells was performed. Two trypsin treatments were done. Approximately the same quantities of material were used as in Experiment 3. After each treatment, light centrifuging of the material under investigation yielded a supernatant which was used for the next step. After the last trypsin treatment, a precipitate was obtained by bringing the solution to 1/3 saturation with ammonium sulfate. This was dialysed against phosphate buffer, and was found to be active. The results were as shown below. Product II was the material before the trypsin treatment. Product III was that obtained after one trypsin treatment. Product IV followed the last trypsin treatment and ammonium sulfate precipitation. In all cases, parallel runs were done with similar products derived from negative cells. The results of the titrations with inhibitors prepared from such cells were uniformly the same as the one which is shown. It is seen that the enzyme treatment slowly destroyed the activity of the inhibitor.

Figure II

Flow Sheet of Trypsin Treatment

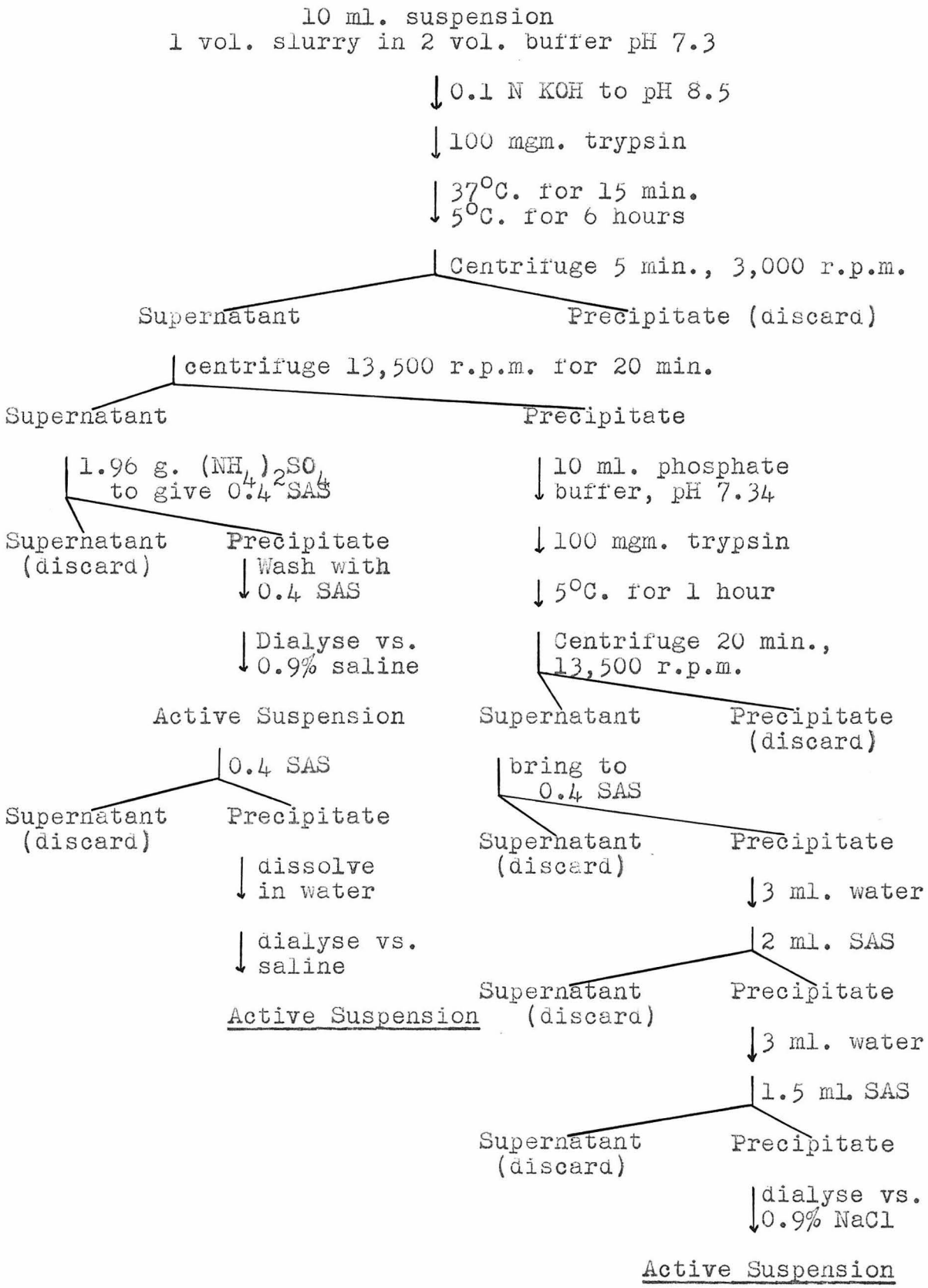


Table XII

Effect of Trypsin Treatment upon Inhibitor Activity

Inhibitor	Antiserum Concentration 1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Product II	++	+	±	-	-	-	-
Product III		+++	+	-	-	-	-
Product IV		+++	++	+	-	-	-
Negative Product		+++	+++	++	+	-	-
Saline		++	+++	++	+	+	-

Immunization. An attempt to develop a rabbit anti-Rh serum was made, with a sample of the KOH-SAS preparation injected along with a protein carrier, or adjuvant, as the antigen.

The general procedure as given in Kabat and Meyer (30) was followed. One hundred milligrams of dried mycobacterium butyricum were suspended in 15 milliliters of mineral oil. Twenty milliliters of aquaphor were warmed to melting and mixed with 20 milliliters of a suspension of a preparation of the degraded stroma. This preparation contained 8.7 milligrams of protein material per milliliter of suspension. The bacterial suspension was added, and 10 milliliters of mineral oil were added to make the mixture more fluid. Merthiolate to give a final concentration of 1:5000 was

added as a preservative.

Twelve rabbits were injected intraperitoneally with ½ milliliter of this suspension. Seven injections were given over a period of 2 weeks, and then the animals were rested for 2 weeks. Another 4 injections were given, and 10 days after the last injection the animals were bled.

A sample of the pooled serums was serially diluted and agglutination tests were run on Rh positive and Rh negative cells. The end points were approximately the same with either cell sample, at a dilution of 1/81 of the serum. Two tenths of a milliliter of the serum were then diluted with 2 milliliters of saline and absorbed with 0.2 milliliters of packed Rh negative, type O cells, for 90 minutes at room temperature. An agglutination test on this absorbed serum, with Rh positive and Rh negative cells, did not show any indication of specific anti-Rh agglutinins. A 1 milliliter sample of this absorbed serum was then again absorbed with 0.2 milliliters of packed Rh negative, type O cells. An agglutination test with both types of cells showed a very slight indication of the presence of some anti-Rh agglutinins, but the effect was too small to be significant. A similar test was made with trypsin treated cells, and no real difference was seen. These results are summarized in the table below. It was concluded that no specific antibodies were developed by this procedure.

Table XIII

Effect of Absorption of Antiserum upon Specific Rh Agglutination

Agglutination	Antiserum Concentration (Unabsorbed) 1/9	1/27	1/81	1/243	1/729
Positive cells	+++	+++	±	-	-
Negative cells	+++	+++	+	-	-
	Antiserum concentration (Absorbed once) 1/10	1/20	1/40	1/80	1/160
Positive cells	++	+	+	-	-
Negative cells	++	+	+	±	-
	Antiserum concentration (absorbed twice) 1/10	1/20	1/40	1/80	1/160
Positive cells	+	-	-	-	-
Negative cells	±	-	-	-	-
Positive cells (treated)	+++	+++	++	+	-
Negative cells (treated)	+++	+++	+	+	-

Conclusion. Stroma could be degraded to smaller particles which gave a strong and specific Rh activity. They were not readily refractionated to yield a true solution of a hapten. Treatment with trypsin seemed to denature the Rh site slowly. While there was some inhibitory activity in the supernatant fractions which were obtained after a trypsin treatment, it is felt that the specificity of this

action is still questionable, and should be investigated further before any conclusions may be drawn. Denaturation by heat occurred readily, especially if the pH of the suspension of the inhibitor material were below 7 or above 9. Ethanol rapidly denatured the specific site. Lyophilization destroyed its activity, but mild oxidation did not affect it.

Rabbit anti-Rh antibodies were not developed when this material was injected intraperitoneally along with a carrier, mycobacterium butyricum.

DISCUSSION

From the action of different reagents upon cells and upon stroma, with respect to Rh activity, some conclusions may be drawn.

Th Rh hapten is not like the Forssman hapten, or like A and B substance, in solubility or stability characteristics. It is not soluble in methanol, ethanol, acetone, dioxan, ether, or ether-alcohol mixtures. A preliminary treatment of erythrocytes with either ethanol, methanol, acetone or dioxan does not render the antigen soluble, in undenatured form at least, in such lipid solvents as methanol, ethanol, ether, chloroform, acetone, dioxan, or toluene. The active portion is, therefore, probably not entirely lipid or polysaccharide in nature.

The ready denaturation by alcohol, pH change, or heat

all point to a protein-like structure in the active portion of the site. The destruction by trypsin is added confirmation of this. On the basis of the apparently slow destruction by this enzyme, the absence in the specific Rh region of certain trypsin sensitive linkages might be postulated. The denaturation due to lyophilization indicates the possibility of a lipoprotein structure.

The failure of several methods of fractionation of stroma particles to yield an active extract seems to indicate that the Rh site is a reasonably integral part of the surface of the cell wall. It is probably not a separate, isolable region of a particular protein or lipoprotein configuration, which differs in molecular structure from the rest of the cell wall. Rather, it probably represents a relatively minor difference in the configuration of the molecular constituents of the wall. The properties of this region, with respect to solubility in different media, are probably but little different from the properties of any other region of the wall, and hence its isolation will be very hard.

If the Rh region possesses no outstanding structural characteristics, compared to the rest of the cell, the failure of Rh positive cells, when injected into animals, to yield a high concentration of anti-Rh antibodies may be explained. Antibodies are built up against all the different portions of the cell wall which constitute the

antigen, and there is no preferential manufacture of antibody against the Rh site. Those antibodies which are directed against this site are in such low concentration that they escape detection. In the work with alcoholic extracts, the antigenic site was probably denatured, so any specific antibody formation would be impossible.

REFERENCES

1. Landsteiner, K., and Wiener, A. S., Proc. Soc. Exper. Biol. Med., 43, 223, (1940)

2. Levine, P., and Stetson, R. E., J. A. M. A., 113, 126, (1939)

3. Wiener, A. S., and Peters, H. R., Ann. Intern. Med. 13, 2306, (1940)

4. Wiener, A. S., and Forer, S., Proc. Soc. Exper. Biol. Med., 47, 215, (1941)

5. Boorman, K. E., and Dodd, B. E., J. Path. Bact., 55, 329, (1943)

6. Witebsky, E., and Mohn, J. F., J. Exper. Med., 82, 143, (1945)

7. Calvin, M., et al, Proc. Soc. Exper. Biol. Med., 61, 416, (1946)

8. Carter, B., Am. J. Clin. Path., 17, 646, (1947)

9. Carter, B., J. Immunol., 61, 79, (1949)

10. Price, C. C., et al, J. A. C. S., 70, 3527, (1948)

11. Wiener, A. S., J. A. M. A., 141, 1330, (1949)

12. Howe, C., and Rustigian, R., J. Immunol., 64, 505, (1950)

13. Spielman, W., Z. Naturforschg., 4b, 284, (1949)

14. Hallauer, C., Z. Immunitätsforschg., 83, 114, (1934)

15. Kosjakow, P. N., and Tribulev, G. P., Z. Immunitats-forschg., 98, 261, (1940)

16. Witebsky, E., and Klendshoj, N. C., J. Exptl. Med., 72, 663, (1941)

17. Morgan, W. T. J., and King, H. K., Biochem. J., 37, 640, (1943)

18. Landsteiner, K., and Harte, R. A., J. Exptl. Med., 71, 551, (1940)

19. McFarlane, A. S., Nature, 149, 439, (1942)

REFERENCES (continued)

20. Block, R. J., in Schmidt, "Chemistry of the Amino Acids and Proteins," C. C. Thomas, Second Edition, (1945)

21. Jorpes, E., Biochem. J., 26, 1488, (1932)

22. Ponder, E., "Hemolysis and Related Phenomena," Grune and Stratton, p. 130

23. Ponder, E., ibid., p. 133

24. Furchgott, R. F., Cold Springs Harbor Symposium, 8, 224, (1940)

25. Boehm, G., Biochem. Z., 282, 22, (1935)

26. Ballantine, R., and Parpart, A. K., J. Cell. and Comp. Physiol., 16, 49, (1940)

27. Stern, K. G., et al, J. B. C., 161, 731, (1945)

28. Morgan, W. T. J., and Partridge, S. M., Biochem. J., 34, 169, (1940)

29. Kabat, E. A., and Mayer, M. M., "Experimental Immunochemistry," C. C. Thomas (1948) p. 514

30. Kabat, E. A., and Mayer, M. M., ibid., p. 544

PART II

THE FRACTIONATION OF AN ANTI-Rh ANTISERUM

INTRODUCTION

Blood serum may be divided, by salt precipitation methods, into two fractions, the albumin fraction and the globulin fraction. Many experiments have shown that only the latter fraction contains those proteins which are formed as a result of an antigenic stimulus, and are called antibodies. Electrophoretic analyses have shown that the globulin protein may be subdivided into three major groups, the α -, β -, and γ -globulins, which differ among themselves in their molecular weight and also, at a given pH, in their electrical charge. Antibodies have nearly always been found in the γ -globulins, although a recent work by Enders (1) showed that the human antibody to typhoid O antigen, and all of the isoagglutinins, were contained in a fraction of the serum which contained 70% γ-globulin, and 25% β -globulin. He concluded that this antibody was probably in a fraction which differed from the usual γ -globulin, and later Deutsch (2) showed that the antibody was in a subfraction of the γ -globulin which he termed γ_1-globulin. This was the first demonstration of differences, other than serological, in human antibodies.

Different types of Rh antibodies, which have different serological activities, have been recognized. The first type is the one which causes agglutination of Rh positive cells, when the cells are suspended in a saline medium.

A second type, called the blocking antibody, inhibits the agglutination of Rh positive cells by an agglutinating antiserum. A third type, variously called a developing antibody, a third order antibody, or a cryptagglutinoid, is recognized by the following reactions.

This antibody will form a specific complex with an Rh positive erythrocyte, but the formation of this complex does not lead to either agglutination or blocking. However, if there is added a third component which will combine in a specific manner with the complexed cells (but not with the non-complexed cells), agglutination will take place. Such a third component is found in the antibodies which are formed in a rabbit after the animal has been injected with human globulin. These antibodies combine specifically with human globulins, and therefore their union with the developing antibody globulin on the above complex can ultimately result in an agglutination of the cells. It should be noted that both of the first mentioned types of antibody will also give a developing test. However, the antibody which will subsequently be referred to as the developing antibody is that one which causes neither agglutination nor blocking, but gives only this test.

The postulated modes of action of these different antibodies are shown in Figures I - III. In Figure I, the erythrocyte (E) is depicted, with some antigenic sites (A) on its surface. These sites are combined specifically

with corresponding antibody sites on the molecule of the agglutinating antibody (B). If both the erythrocyte and the antibody contain more than one combining site, and these sites are suitably arranged so that steric factors are not a consideration, it can be seen that a large number of erythrocytes may be linked together with the possible ultimate formation of a visible clump.

However, if the antibody molecule has only one combining site on it, no coupling between the complexed cells may be formed, and so no agglutination can take place. If, in addition, the combining sites of the cell are all covered, the agglutinating antiserum will be ineffective when it is added to the system. This is shown in Figure II, in which (C) indicates the blocking antibody. While this picture of the action of a univalent antibody may not be strictly correct, owing to our lack of detailed knowledge of antigen-antibody reactions, it nevertheless may serve as a useful approximation to what is taking place. It assumes that the dissociation constant of the cell-agglutinating antibody complex is at most not much greater than the dissociation constant of the cell-blocking antibody complex, and that the two types of antibodies are present in approximately equal concentrations.

Figure III shows the sequence of reactions in the test for the developing antibody. The erythrocyte (E) combines with the specific antibody (D). In this case, the Rh

antigenic site is marked (A'). Since the developing antibody does not necessarily show a blocking activity, the site of its action on the erythrocyte may differ from the site of action of the other two types of antibodies. Nevertheless, the common Rh specificity seems to point to some sort of relationship between the regions of the erythrocyte which respond to the first two types of antibodies, and those regions which respond to the third type of antibody.

The antibody (D) now, by virtue of its globulin nature, has an antigenic site on it which specifically reacts with the rabbit anti-globulin serum. The antibodies (R) in this antiserum are presumably multivalent; thus the complex of the cell plus developing antibody may be built up to proportions which are visible to the eye.

A separation of agglutinating and blocking antibodies on the basis of their solubilities was made by Witebsky (3) who dialysed an anti-Rh serum against water, to obtain a water-insoluble globulin, and a water-soluble globulin. The former fraction was soluble in saline; this solution gave a typical agglutination test with Rh positive erythrocytes. The water soluble fraction showed the presence of blocking and of developing antibodies.

An attempt to obtain a more precise fractionation of these antibodies through the use of ion-exchange resins was made by Hill and Haberman (4). Blocking antibodies

Figure I

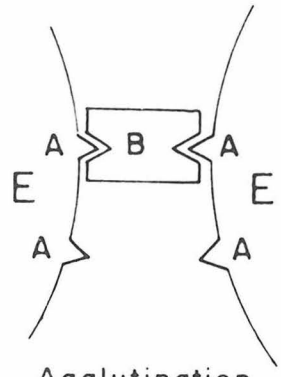
Mechanism of Agglutination Reaction

Figure II

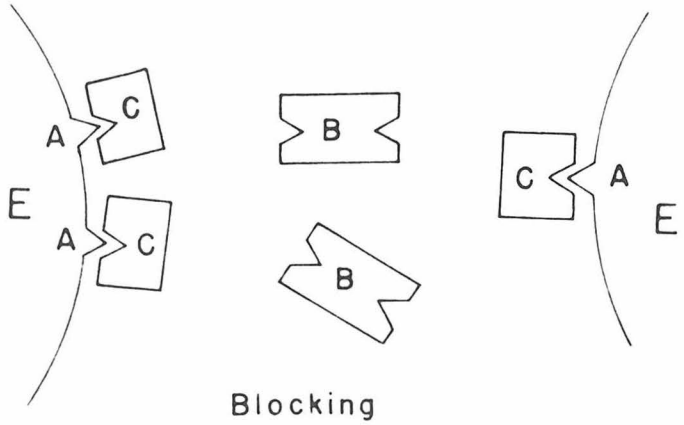
Mechanism of Blocking Reaction

Figure III

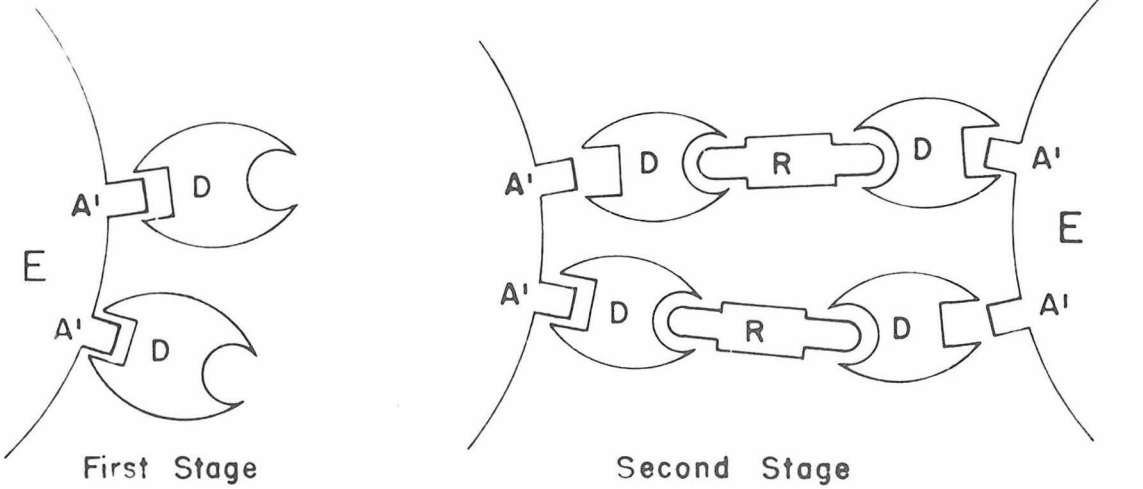
Mechanism of Developing Reaction



Agglutination
Figure I



Blocking
Figure II



First Stage

Second Stage

Developing
Figure III

and some developing antibodies were found in the water soluble globulin fraction. The water insoluble portion, which contained the α -, β -, and γ -globulins, was fractionated by differential solubility in NaCl solutions of different concentrations. Agglutinating antibodies were found in the γ -globulin fraction, while developing antibodies were found mainly in the β -globulin fraction. It should be noted that this final fractionation and characterization is quite rough - e.g. the fraction labelled as a β -globulin probably contains much of the other globulins.

A separation by electrophoresis-convection seemed to offer an excellent means for carrying out a fractionation study. The treatment of the protein is very mild, so denaturation should be at a minimum, in contrast to the ion-exchange process, where the effect of the treatment upon the protein may be considerable. The separation of the serum into its components is much more satisfactory, in that fractions of a higher purity and of known composition may be obtained.

A fractionation of an antiserum by this method had been carried out in these laboratories by Cann et al (5) and it had been concluded that the blocking antibody appeared to be associated with a γ -globulin fraction of low mobility. About 75% of the developing antibody appeared to be in the γ -globulin, but the remaining 25%

appeared to be associated with the α - and/or the β - globulin. A second anti-Rh serum was investigated to study the pattern of the antibody distribution, and the results of this work are presented below.

EXPERIMENTAL

Materials and Methods

Fractionated Antiserum. This was an anti-Rh serum sent here by Dr. R. Day, of New York. The donor was an Rh negative woman, who delivered an Rh positive child which subsequently showed symptoms of erythroblastosis fetalis. The donor had had two blood transfusions as a child, and these may have sensitized her - i.e. caused an antibody production against the (probably) Rh positive cells which would be used in the transfusion. At the time of the delivery, anti-Rh antibodies could be detected in the maternal circulation, and the antiserum sample was taken a few days after the birth of the child. This was antiserum I. Four months later, another sample of blood was taken from the same donor and the serum therefrom was shipped here. This sample was called antiserum II. Both of these samples were fractionated according to the procedure to be described.

Agglutinating Antiserum. This was an antiserum sample obtained from Hyland Laboratories. It caused agglutination

of Rh positive cells at all dilutions up to (i.e. more concentrated than) 1:512.

Rabbit Anti-Globulin Serum. For the tests on fractions from antiserum I, samples of sera from three rabbits which had been immunized with human globulin were pooled and used as a stock. For the tests of fractions from antiserum II, the stock was a sample of frozen antiserum from a similarly immunized rabbit. On each day that tests were run, a portion of the frozen antiserum was thawed out and treated as below.

The antiglobulin serum was diluted with an equal volume of saline, and then absorbed once, at room temperature, for 1 hour, with 2 volumes of packed, well washed erythrocytes. (Adequate washing on the absorbing cells was very necessary, as otherwise traces of normal serum globulins which adhered to the cells partially neutralized the rabbit antiserum.) The erythrocytes were then centrifuged off, and the absorbed antiserum was again diluted with saline until the final concentration was 1/6 that of the original antiserum.

Human Erythrocytes. For tests on antiserum I, a pool of Rh positive, type O cells was used. It was endeavored to obtain cells not more than 2 or 3 days old, at the very most. On antiserum II, the Rh positive, type O cells were drawn from one donor for all the tests. They were never

more than 1 day old at the time that the tests were run. The cells were washed and suspended in isotonic saline, to a final concentration of 2% - 5% by volume.

Agglutination Test. Two one-hundredths of a milliliter of the erythrocyte suspension were incubated in 13 x 100 millimeter test tubes with 0.02 milliliters of dilutions of the fraction to be tested, for 10 minutes at 37°C., and for 20 minutes at room temperature (27°C.). After light centrifuging, the tubes were examined to see if agglutination had taken place. A second centrifuging and re-examination were done as a check. Doubtful cases were examined microscopically. In all cases to be reported, no agglutinating antibodies were found, so further mention of them will be omitted.

Blocking Test. To the cells and test antiserum used in the test above, 0.02 milliliters of a 1/128 dilution (in saline) of the agglutinating antiserum were added. The tubes were re-incubated for 30 minutes at 37°C., and then centrifuged and examined, as above, for agglutination. The reciprocal of the value of the highest dilution which still completely inhibited agglutination was called the titer of the blocking antiserum.

The amount of agglutinating antibody which was added was four times the minimum amount necessary to just give agglutination. In spite of this excess, though, the presence of small quantities of blocking antibody could

be shown. The minimum amount of the latter which was required to give a blocking titer of 1 to the fraction under test was that amount which would completely inhibit agglutination in the first tube of a series of serial dilutions. If there were less of the blocking antibody present, there would be agglutination of the erythrocytes, but this agglutination would be weaker than in a control experiment, in which no blocking antibody had been added. Observations on the strength of agglutination were based on the ease with which a clump of agglutinated cells broke up, with gentle shaking.

Developing Test. Two one-hundredths of a milliliter of the erythrocyte suspension were incubated for 30 minutes at 37°C. with 0.02 milliliters of serial dilutions of the fraction to be tested. The cell suspension was then washed 3 times with 0.5 milliliters of 0.9% saline. After the last washing, as much as possible of the supernatant was removed from the centrifuged cells, and then 0.02 milliliters of saline were added, with 0.02 milliliters of the prepared anti-globulin serum. The tubes were shaken, incubated at 37°C. for 30 minutes, and examined for agglutination after light centrifuging. The reciprocal of the value of the lowest concentration of the test fraction which gave agglutination was taken as the developing titer of the fraction.

Fractionation Procedure. The antiserum was fractionated in the electrophoresis-convection apparatus which has been

described by Kirkwood et al (6). Figures IV and V show the fractionation scheme, as well as the buffers used and the pH values at which the fractionations were done. For all buffers, $\Gamma/2 = 0.1$. The last bottom fraction was salt fractionated into an albumin fraction and a globulin fraction.

Results

The results of the electrophoretic analyses of the various fractions which were obtained are shown in Table I and Table II. Figure VI shows representative electrophoretic patterns from the fractionation of antiserum I.

Table III and Table IV show the results of the tests of the fractions for the different types of antibodies, as well as the volumes of the different fractions and their protein concentrations. Figures VII and VIII show the titer and concentration values in graphical form.

Some of the top fractions were concentrated to see (a) if antibody titers could be raised, (b) if the degree of improvement was proportional to the increase in the concentration, and (c) if some titer could be obtained in those cases where no titer had previously been found. Three concentration methods were used: i. perevaporation with the aid of an infra-red lamp; ii. perevaporation in the cold room; iii. lyophilization.

Tables V and VI show the results of these concentration procedures on those samples which had been treated.

Figure IV

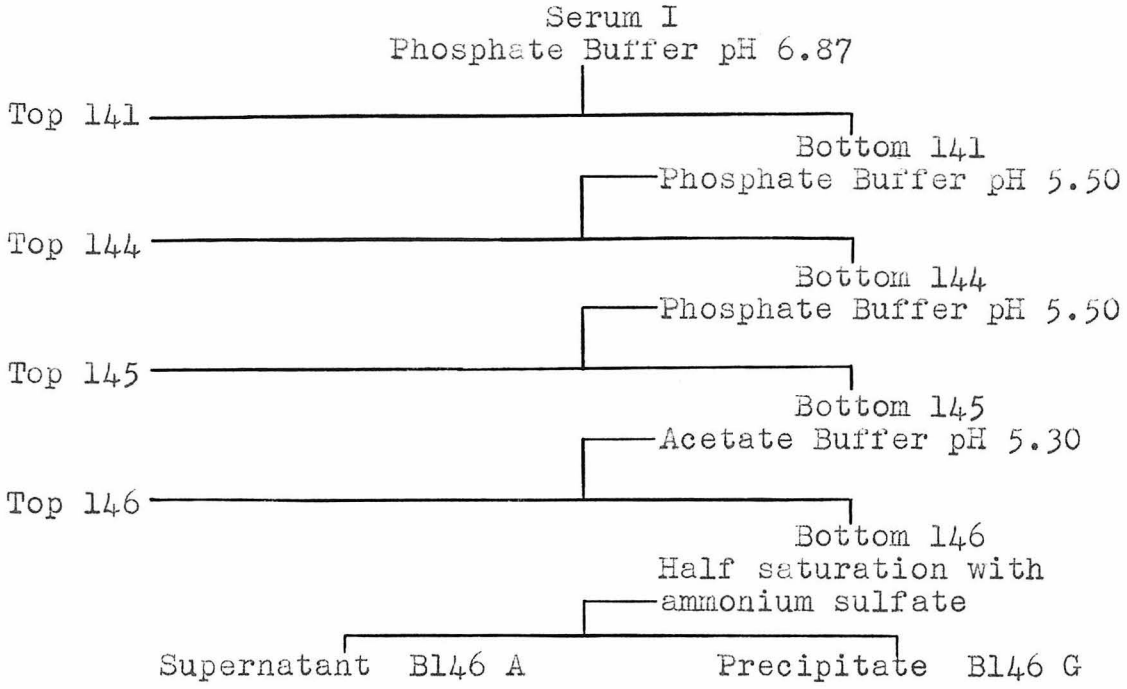


Figure V

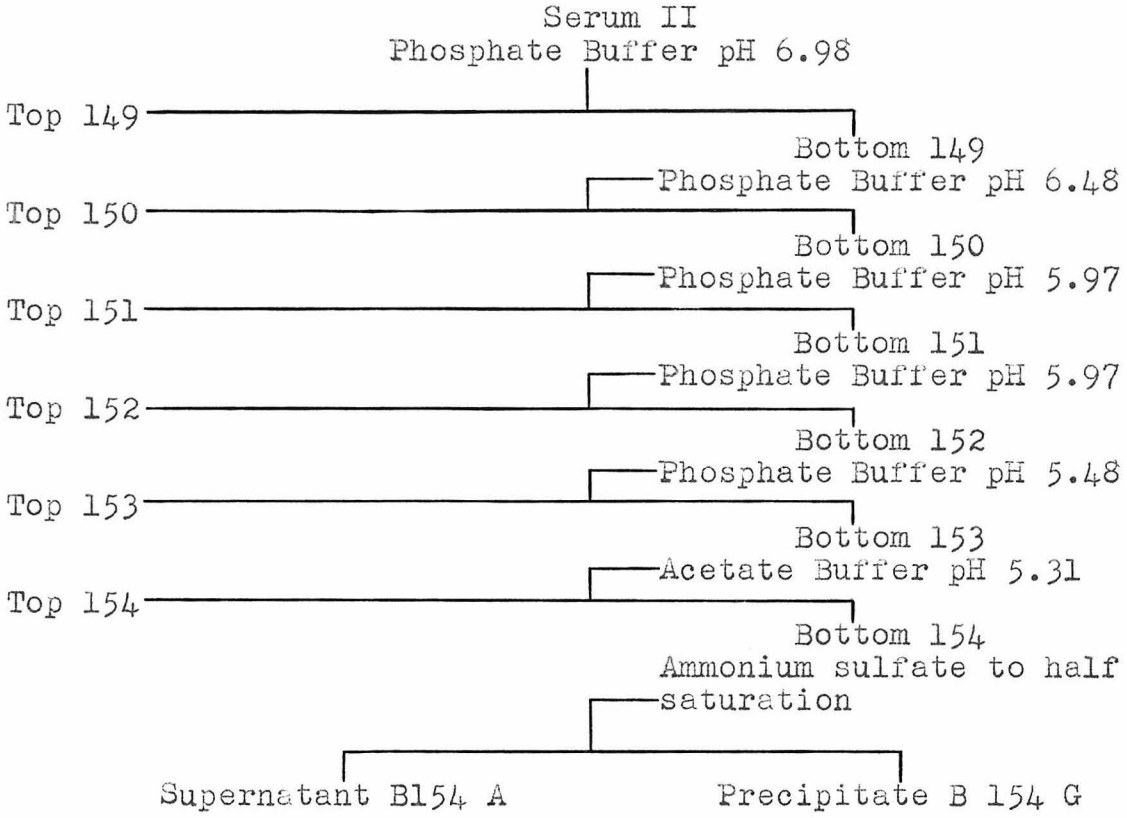


Table I

Analysis of Antiserum I Fractions

Components / Fraction	γ μ	γ %	β μ	β %	a_1 μ	a_1 %	a_2 μ	a_2 %	Albumin μ	Albumin %
Anti-serum I	1.47	11	3.12	16	5.14	8	4.04	20	6.30	45
T141	1.36	98		-		-		-		-
T144	1.54	53	3.27	43	4.81		4			-
T145	1.64	51	3.27	47	4.45		2			-
T146	-	19	3.46	50	4.53		24		6.68	7
B146G	-	-	-	2	5.77	47	4.53	48	-	3
B146A	-	-	3.28	2	5.30	12	4.29	3	6.48	83

Table II

Analysis of Antiserum II Fractions

Components / Fraction	γ μ	γ %	β μ	β %	a_1 μ	a_1 %	a_2 μ	a_2 %	Albumin μ	Albumin %
T149	1.18	97	-	3		-		-		-
T150	1.37	93	3.40	7		-		-		-
T151	1.65	76	3.29	24		-		-		-
T152	1.78	77	3.35	23		-		-		-
T153	1.61	42	3.35	58		-		-		-
T154	1.52	12	3.26	37	4.95		37		6.60	13
B154G	-	-	-	2	4.86	57	4.00	32	6.33	8
B154A	-	-	3.22	3	5.21	4	4.12	2	6.38	88

- - - - - - - - - - - - - - - - - -

μ is in units of 10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$

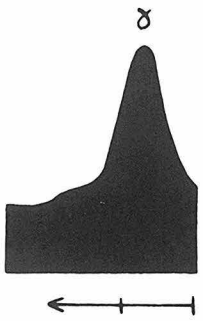
Figure VI

Representative Electrophoretic Patterns from Fractionation of Antiserum

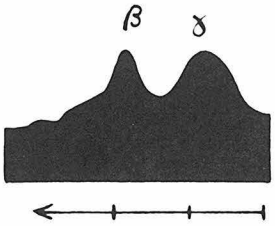
All patterns traced from rising boundaries

Arrow shows direction of migration

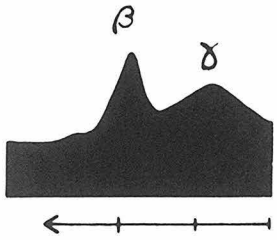
Scale marked in centimeters



Top 141



T 144



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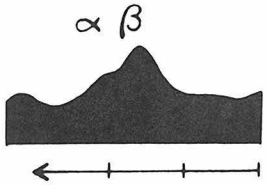
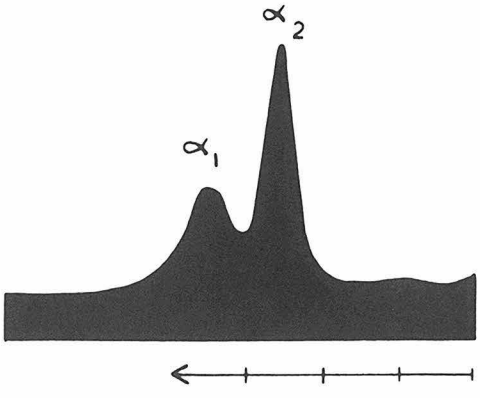
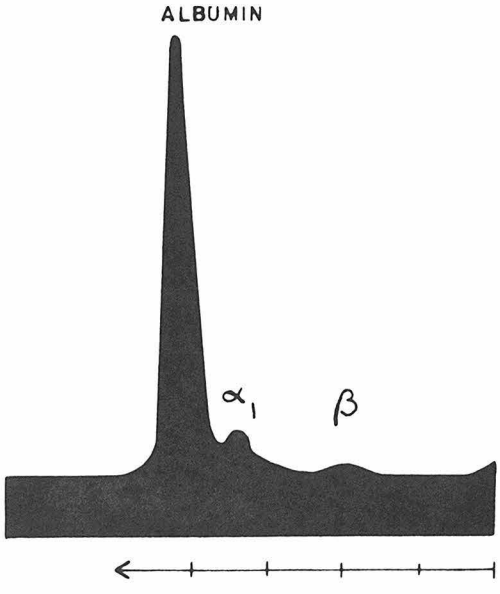


T 146



B 146 G



B 146 A

Table III

Analysis of Antiserum I Fractions

Fraction	Blocking Titer	Developing Titer	Concentration g/100 ml.	Volume
Original serum	8	64	7.0	39
T141	1	8	0.25	61
T144	1	16	0.33	45
T145	0	8	0.17	56
T146	0	8	0.23	50
B141	1	64	3.60	70
B144	-	-	-	71
B145	2	8	2.79	70
B146G	1	8	0.92	50
B146A	0	0	0.34	205

Table IV

Analysis of Antiserum II Fractions

Fraction	Blocking Titer	Developing Titer	Concentration g/100 ml.	Volume ml.
Original serum	8	32	7.0	70
T149	1	8	0.37	62
T150	1	2	0.27	56
T151	0	2	0.22	50
T152	0	1	0.13	45
T153	0	1	0.19	45
T154	0	2	0.40	40
B149	2	32	-	-
B150	2	16	-	-
B151	2	16	-	-
B152	1	8	-	-
B153	0	8	-	-
B154G	2	8	2.40	34
B154A	0	0	0.50	295

Figure VII

Results of Fractionation of Antiserum I

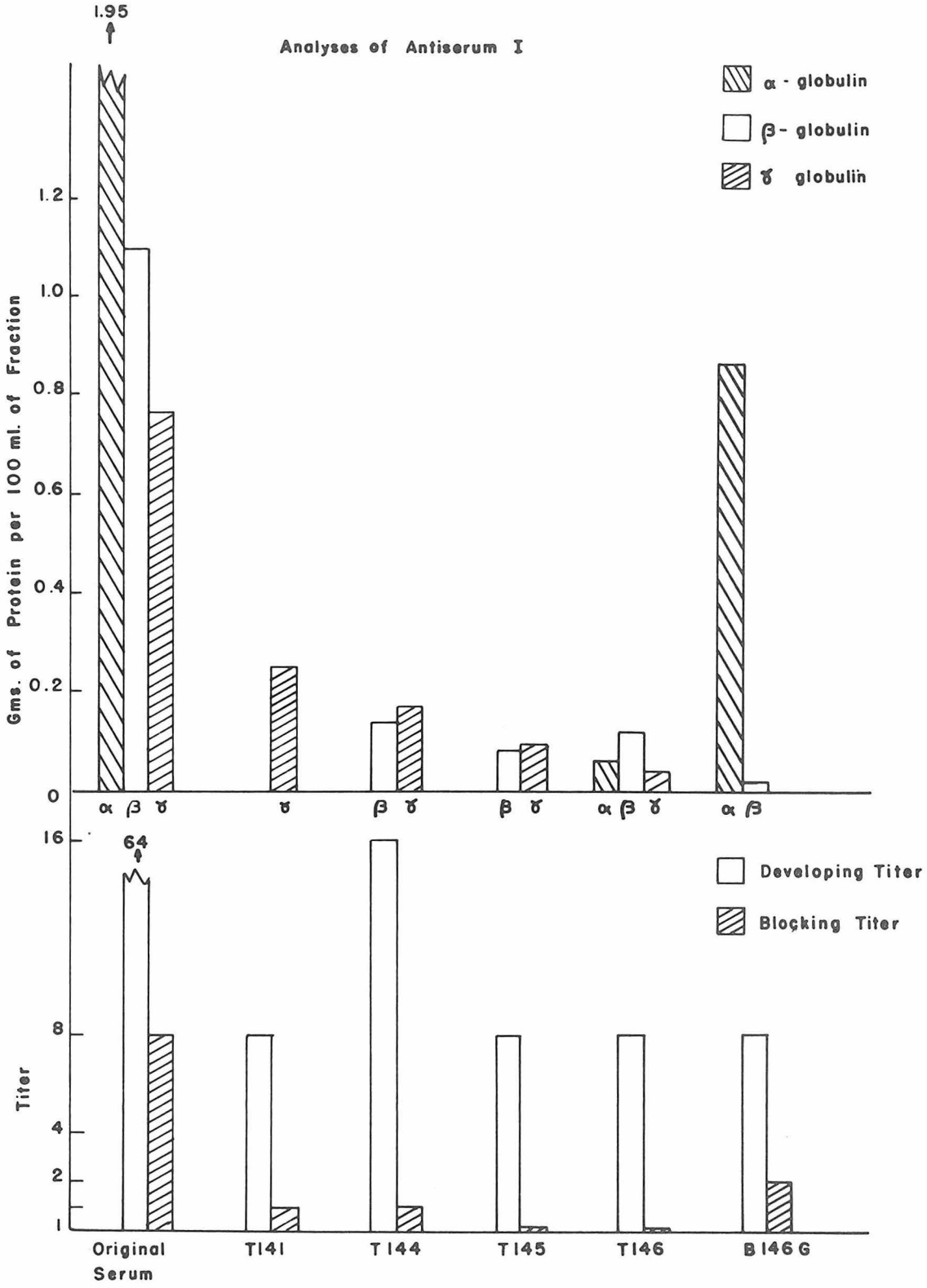

1.95
Analyses of Antiserum I
α - globulin
β - globulin
γ globulin
Gms. of Protein per 100 ml. of Fraction
1.2
1.0
0.8
0.6
0.4
0.2
0
α β γ
γ
β γ
β γ
α β γ
α β
64
16
8
4
2
1
Titer
Developing Titer
Blocking Titer
Original Serum
T141
T144
T145
T146
B146G

Figure VIII

Results of Fractionation of Antiserum II

Analyses of Antiserum II

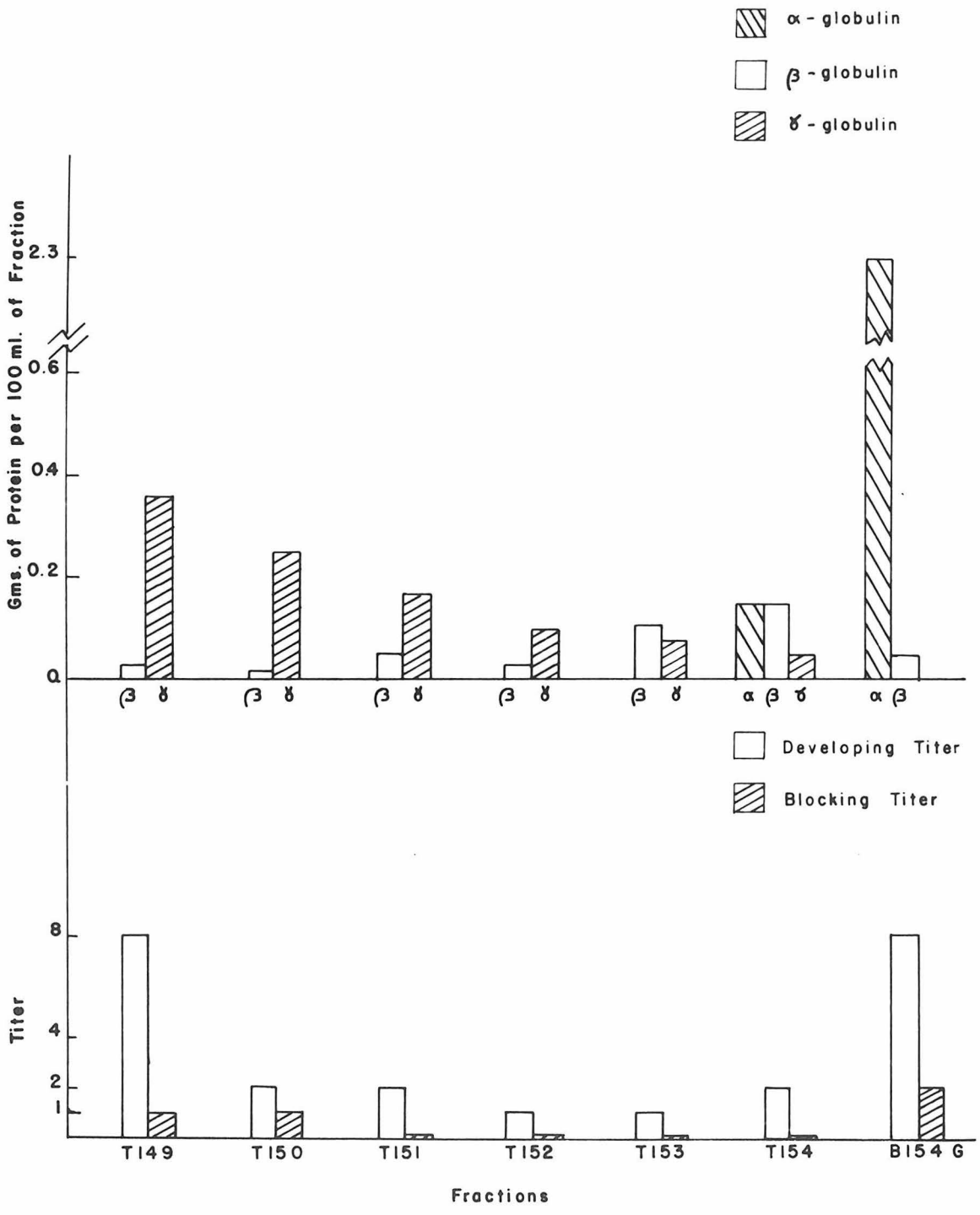


Table V

Serological Activity of Concentrated Fractions of Antiserum I

Fraction	Concentration Method	Concentration Factor	Concentration Final (gms./ 100 ml.)	Blocking Titer	Developing Titer
T141	i, iii	9	2.3	*	**
T145	i, iii	12	2.0	4	**
T146	i, iii	7	1.7	4	**

* Antibody may be denatured, either due to concentration process or to aging (fraction was 2 - 3 weeks old.)
**No test made

Table VI

Serological Activity of Concentrated Fractions of Antiserum II

Fraction	Concentration Method	Concentration Factor	Concentration Final (gms./ 100 ml.)	Blocking Titer	Developing Titer
T149	i	3	1.0	8	8
	iii	4	1.3	4	4
T150	i	3	0.9	2	4
	iii	4	1.2	4	4
T151	i	3	0.7	1	2
	iii	4	0.9	1	0
T152	i	4	0.7	1	1
T153	i	4	0.8	1	1
	ii	3	0.6	0	4
T154	ii	3	0.9	2	2

DISCUSSION

From the results on antiserum I, it is seen that the blocking titer was rapidly lost as the fractionation progressed. The conclusion is that the blocking antibody seemed to be associated with a slowly moving portion of the γ-globulin. The most interesting feature of this study is that there was still a strong developing antibody titer in the B146G fraction, which was 95% α-globulin. This is added confirmation of the results previously obtained by Cann et al, but in the present case the final product was a much more electrophoretically pure α-globulin - i.e. there was less albumin and β-globulin in it.

The variations in antibody titer may be satisfactorily explained (within the limits of experimental error) on the assumption that a blocking antibody was present in the slowly moving part of the γ-globulin, while developing antibodies were present in the α-globulin as well as in the γ-globulin. The existence of antibodies in the β-globulin fraction cannot be affirmed or denied, but it is not necessary to assume any antibody in this fraction in order to explain qualitatively the results.

Antiserum II was divided into more fractions, but the trend of the results was the same, with the rapid disappearance of the blocking titer after the removal of the first two top fractions, and the consistent presence of

the developing antibody in all the fractions, including B154G, which was 89% α-globulin.

It will be noticed that the antiserum II had a smaller concentration of developing antibody - for a given concentration, the developing titers in the second sample were uniformly lower than in the first sample. This was probably the result of the usual decrease in antibody titer in a given donor with time, when no further antigenic stimulus is given.

Another difference between the two antibodies is found in the results of the concentration studies. In general, there had been an increase which was roughly proportional to the concentration factor in the blocking titer of a fraction after it had been concentrated, whereas the developing titer had not increased. There was probably some denaturation of the developing antibodies.

The results from fraction T153 show some indication that if the perevaporation is done in the cold, the developing titer is maintained. A similar indication was found in an experiment on another fraction:

One sample of B154G was perevaporated with the aid of an infra-red lamp; another was perevaporated in the cold. Both samples were thus reduced to approximately 1/8 of their original volume; they were then made up to the original volume with saline. (There was some intermediate adjustment of salt concentration to keep the material in solution, but both samples were treated in the same manner.)

The sample which was perevaporated with the lamp had dropped one tube in developing titer; the one which had been concentrated in the cold maintained the original titer. The blocking titers were unaffected in both cases. This one experiment, unfortunately, is not too significant because of the large experimental error.

A useful procedure, and a check on the experiment, is to see whether the total amount of antibody, as judged by the titers, was maintained.

Let the number of antibody molecules in each fraction be $n_1, n_2, n_3 \ldots n_i$. Let the antibody concentration in each fraction be $c_1, c_2, c_3 \ldots c_i$. Let the volume of each fraction be $v_1, v_2, v_3 \ldots v_i$. Let the titer of each fraction be $t_1, t_2, t_3 \ldots t_i$. Assume that in the original serum these functions were n_o, c_o, v_o, t_o. If the titer is assumed to be a function of the concentration of the antibody, then:

$$t_i = kc_i$$

$$n_i = k' c_i v_i$$

therefore $$n_i = k'' t_i v_i$$

$$n_o = n_1 + n_2 + n_3 + \ldots.$$

therefore $$t_o v_o = t_1 v_1 + t_2 v_2 + t_3 v_3 + \ldots.$$

If this is applied to antiserum I, the following results are obtained:

Blocking titer $t_o v_o = 312$ $\quad \Sigma t_i v_i = 156$

Developing titer $t_o v_o = 2496$ $\quad \Sigma t_i v_i = 2456$

The agreement of the blocking titers is satisfactory, if allowance is made for the inherent error in the experiment (a factor of 2) as well as for denaturation and unavoidable loss in each operation. If an estimated 5% was lost during each of the five separations, the reduced $t_o v_o$ is $312 \times (0.95)^5 = 243$ - a better agreement.

The apparently close agreement of the figures for developing titers is not indicative of an excellent check. The value of $t_o v_o$ should be reduced by at least the factor shown above, to allow for loss and possibly a greater denaturation. The reduced value is 1930. Still, the agreement is satisfactory.

The procedure, applied to antiserum II, shows a satisfactory conservation of antibody.

By using titers obtained from the concentrated samples of the different fractions, the results of the blocking tests are:

$$t_o v_o = 560$$

$$\Sigma t_i v_i = 330$$

The reduced $t_o v_o = 390$. This value is calculated on the basis of seven operations.

$\Sigma t_i v_i$ for the blocking titers of the unconcentrated fractions is 186. This is a low value, as some of the fractions, with a blocking titer of zero according to the experimental procedure, nevertheless contained some antibody. This was shown by the observation that the degree of

agglutination, in tubes with the most concentrated samples of such fractions, while never zero, was less than the agglutination in tubes where the sample under test was more dilute. Making a rough correction for this untabulated antibody will raise the value given above somewhat - probably to 200 or 250. However, this low value is within the range of experimental error, when compared to a corrected $t_o v_o$ of 390, so most of the antibody has been preserved.

For the developing titers, the corrected $t_o v_o$ is 1550. $\sum t_i v_i$ 1100. If the $\sum t_i v_i$ is calculated for the concentrated top fractions, and compared to that for the unconcentrated fractions, a great decrease is noted.

$(\sum t_i v_i) = 313$ (concentrated)

$(\sum t_i v_i) = 838$ (unconcentrated)

This may be accounted for on the basis of denaturation.

Differences in amino acid composition of α - and γ - globulins have been reported (7). The different fractions obtained by this method of preparation should be analysed to determine their composition. If a difference is still found, it might mean that different amino acid arrangements might fold similarly to yield similar combining site configurations against a particular antigen. These different arrangements might be a result of specific antibody production by different cells of the reticulo-endothelial system.

Any difference in the structure of such a site might be reflected in the susceptibility of the site to denaturation effects.

REFERENCES

1. Enders, J. F., J. Clin. Invest., 23, 510, (1944)

2. Deutsch, H. F., et al, J. Immunol., 56, 183, (1947)

3. Witebsky, E., and Mohn, J. F., J. Lab. Clin. Med., 33, 1353, (1948)

4. Hill, J. M., Haberman, S., and Guy, R., Am. J. Clin. Path., 19, 134, (1949)

5. Cann, J. R., Brown, R. A., Gajdusek, C., Kirkwood, J., and Sturgeon, P., (unpublished)

6. Cann, J. R., Kirkwood, J., Brown, R. A., and Plescia, O. J., J. A. C. S., 71, 1603, (1949)

7. Edsall, J. T., "Advances in Protein Chemistry", Academic Press, Inc. (1947), Vol. III, p. 464

PART III

A COMPARISON OF THE VASOCONSTRICTOR ACTIONS OF ADRENALINE AND NOR-ADRENALINE

INTRODUCTION

The classical action of adrenaline (epinephrine) in the animal is to cause a considerable rise in the blood pressure. This effect has been considered to be the result of a two-fold action of the drug, viz:

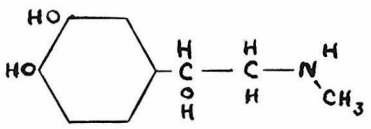
1. Peripheral constriction of the arterioles,
2. Direct stimulation of the heart muscle.

According to Goodman and Gilman (1) the first effect controls, in large measure, the level of the diastolic pressure. Adrenaline administration constricts the blood vessels of the splanchnic bed, as well as those of the pulmonary circulation. Euler (2) reports that the blood vessels of the perfused human placenta are constricted, under the influence of the drug. On the other hand, those vessels supplying the skeletal muscles are dilated (1)(3).

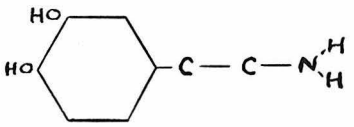
However, when adrenaline is administered in small amounts, there is a fall in blood pressure, presumably due to vasodilation (1)(4). This effect is seen when subcutaneous injections are given and the local concentration of adrenaline at the site of injection constricts the nearby blood vessels. The rate of blood flow through these vessels is consequently reduced, and hence the rate of introduction of adrenaline into the general circulation is also reduced. The concentration levels obtained are then much smaller than those which are attained as a result

of intravenous injection into a larger vessel.

Nor-adrenaline (nor-epinephrine, arterenol) on the other hand, causes vasoconstriction in nearly all cases (1). A vasodilating effect upon the coronary vessels of cats and dogs, after administration of this drug, has been reported by Burn and Hutcheon. The same paper also indicates that small doses of nor-adrenaline will dilate the intestinal blood vessels of these animals (3).



Adrenaline



Nor-adrenaline

Goldenberg et al have made a series of studies on the relative effects of adrenaline and nor-adrenaline upon humans (5)(6). They calculated that there was a drop in the peripheral resistance as a result of administration of therapeutic doses of adrenaline, and so concluded that it acted as an over-all vasodilator. From similar studies on nor-adrenaline, they concluded that it was present as an over-all vasoconstrictor.

In the present work, the action of adrenaline was reinvestigated and compared to the action of nor-adrenaline. In the work cited above, the dosages were of the order of 0.1 microgram of drug per kilogram of body weight per minute. These values were just sufficient to produce and

maintain a desired blood pressure level in the patient. The dosages which were used in the present investigation ranged from 3 to 60 micrograms per kilogram injected over a period of about 30 seconds. A more direct method of measurement was used, and the reflex activities which also influence the blood pressure were eliminated.

Goldenberg obtained peripheral resistance values from calculations based on measurements of arterial pressure and cardiac output, according to the formula:

$$\text{Total peripheral resistance} = \frac{\text{Arterial pressure}}{\text{Cardiac output}}$$

However, according to the calculations of van Harreveld, Feigen and Lerman (7) the peripheral resistance is not the main governing factor of arterial pressure. The latter is mainly dependent on a parameter which is not considered in the above formula. These workers derive a formula:

$$P = \frac{V(R+r)}{C_1 R + (C_1 + C_2) r}$$

V = Volume of circulating blood

A = Arterial pressure

r = A parameter which lumps together some of the conditions of operation of the heart, and which effectively represents the resistance of this pump.

C_1, C_2 are parameters which correspond to a capacity factor, as represented by the elastic properties of the arterial and venous systems.

r is relatively small, and if V is assumed to be constant, it is seen that the arterial pressure is dependent upon C_1 and is but little affected by R.

In the following experiments it was assumed that, under a constant head, the rate of flow of a liquid through a tube was an inverse function of the resistance to that flow. This has been corroborated for liquids which have no formed elements in them, over moderate pressure ranges (8). The perfusion rate of a solution through the circulatory system of a rat was studied, and changes in this rate, upon the introduction of a drug, were assumed to be a reflection of the degree of vasoconstriction, and hence of the resistance to flow, of the blood vessels.

EXPERIMENTAL

A rat was injected with a solution of fastusol, in order to prevent clotting in the subsequent operative process. After about 10 minutes to allow for the absorption and circulation of the fastusol, the rat was anaesthetized with ether. Too much anaesthesia was avoided, as this apparently made the subsequent response less acute. After anaesthesia, the rib cage was cut open to expose the heart. The right atrium and the left ventricle were cannulated, and the blood was flushed out of the circulatory system by perfusion with oxygenated Ringer's solution. This solution was introduced into the ventricle, and taken

out via the cannula in the atrium. The animal was removed to a warm room to keep the temperature at 37°C., and the left ventricle connected to a constant-head input, while the cannula from the right atrium was connected to a drop counter. In this way, all of the circulation, except the coronary and the pulmonary systems, was in the perfusion circuit.

After a steady rate of perfusion was obtained, the drug was introduced, and if there was any effect, a second administration was not made until the perfusion rate had again reached a steady value. Different concentrations of the drugs were used, but in every case the lowest concentrations were used for the initial injection.

Originally, the drugs were introduced by injection with a syringe and needle directly into the rubber tubing which connected the reservoir of Ringer's solution with the animal. However, any handling of the tubing introduced variations in the output rate, so the apparatus shown in Figure I was used.

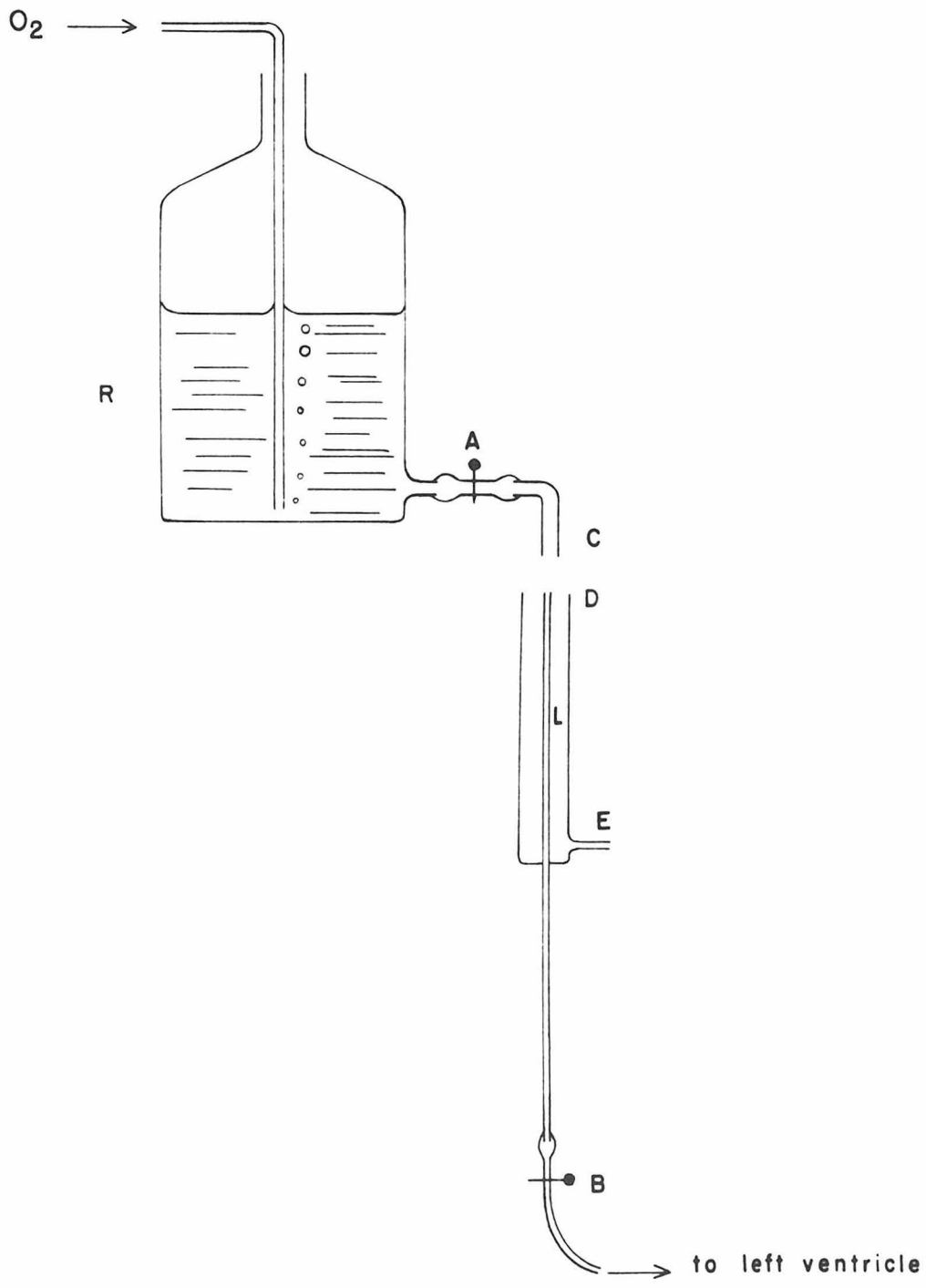
L consisted of a piece of 4 millimeter (outside diameter) glass tubing, about 12 inches long, and which was jacketed by a 6 inch piece of 25 millimeter (outside diameter) glass tubing. The latter had an outlet at E, at one end, and was sealed to the inner tube at this end. Oxygenated Ringer's solution was introduced from R. Clamp B was initially closed, after the system had been filled

with perfusing solution. Clamp A was opened enough to allow a good flow of liquid. B was then carefully opened to allow the Ringer's solution to flow down through the animal, under the head of pressure which was governed by the height at which L was clamped. This height was adjusted to allow, initially, 1 to 3 drops per second of liquid to flow through the animal. Some edema developed as a result of this pressure, but it did not become severe until about an hour after the start of the experiment. It did not interfere with the results for any one drug injection, although it might have been the cause of the general slowing of the drop rate as the experiment proceeded, through the constriction of the vessels by external pressure. With a little practice the pinch clamps could be adjusted so that there was a steady flow of Ringer's solution into the animal, without the introduction of air bubbles at D. Overflow at D, from the necessary slight excess at C, was drained off at E. To introduce the drug solution, a bent needle was inserted at D, and the solution was forced in from a syringe. If the needle tip was held about an inch below D, and the drug solution was colored with fastusol, the injection could be performed without loss of liquid into the overflow.

The dropwise output from the right atrium was allowed to fall across two wire electrodes which were connected in series with a transformer supply and an electromagnet.

Figure I

Apparatus designed to allow the introduction of a drug solution into a perfusing fluid, without causing any change in the pressure under which the latter is fed into a system.


O_2
R
A
C
D
L
E
B
to left ventricle

The armature of the electromagnet marked a kymograph. A clockwork timer gave one-second impulses for calibration purposes.

RESULTS

The number of drops per 10 seconds is plotted, and shown in the following graphs. Before and after any injection, points are plotted for every 10 second interval. When the rate is more steady, points are plotted at half minute or minute intervals.

It is seen that qualitatively there is no difference in the effect which the two drugs produce upon the circulation, as far as the resistance to flow is concerned. All reflex mechanisms are cut out, and the effect is apparently a net constriction of the blood vessels. It should be pointed out that in an experiment of this type, vasodilation will not show up. Even when no drug is present, the vessels are relaxed, and probably distended to their full extent under the hydrostatic pressure of the perfusing fluid. Hence the only effect which may be observed will be due to vasoconstriction.

Metabolic Rate. Goldenberg reported that both adrenaline and nor-adrenaline increased the oxygen consumption of

humans. On the other hand, Reale et all (9) reported that injection of l-nor-adrenaline into humans at the rate of 7 - 15 micrograms per minute had no effect upon the oxygen consumption. Lundholm (10) has indicated that adrenaline increases the oxygen consumption of unanaesthetized guinea pigs, but that anaesthetic drugs markedly inhibited this action.

The effects of these two drugs upon lightly anaesthetized rabbits were compared. A rabbit was lightly anaesthetized and a trachea cannula inserted and connected with a metabolism apparatus. The administration of neither of these drugs had any effect upon the oxygen consumption. It is, however, difficult to draw conclusions from a comparison of the effect of the drug upon humans who are not under basal conditions and the effect upon a lightly anaesthetized rabbit.

Figure II

Effect of administration of adrenaline and nor-adrenaline (arterenol) upon perfusion rate

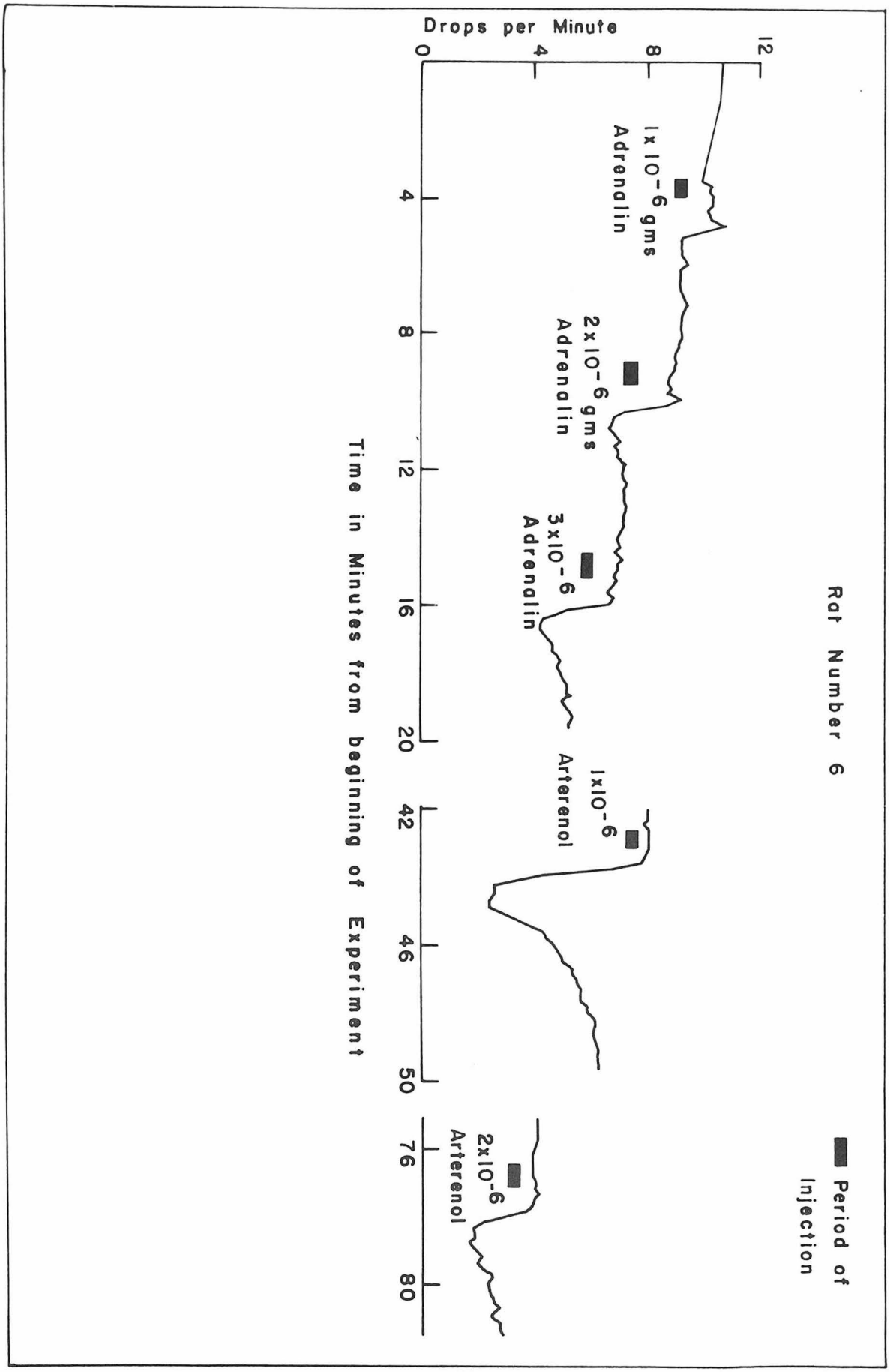

Rat Number 6
Period of Injection
Drops per Minute
0
4
8
12
1x10⁻⁶ gms Adrenalin
2x10⁻⁶ gms Adrenalin
3x10⁻⁶ Adrenalin
1x10⁻⁶ Arterenol
2x10⁻⁶ Arterenol
4
8
12
16
20
42
46
50
76
80
Time in Minutes from beginning of Experiment

Figure III

Effect of administration of adrenaline and nor-adrenaline (arterenol) upon perfusion rate

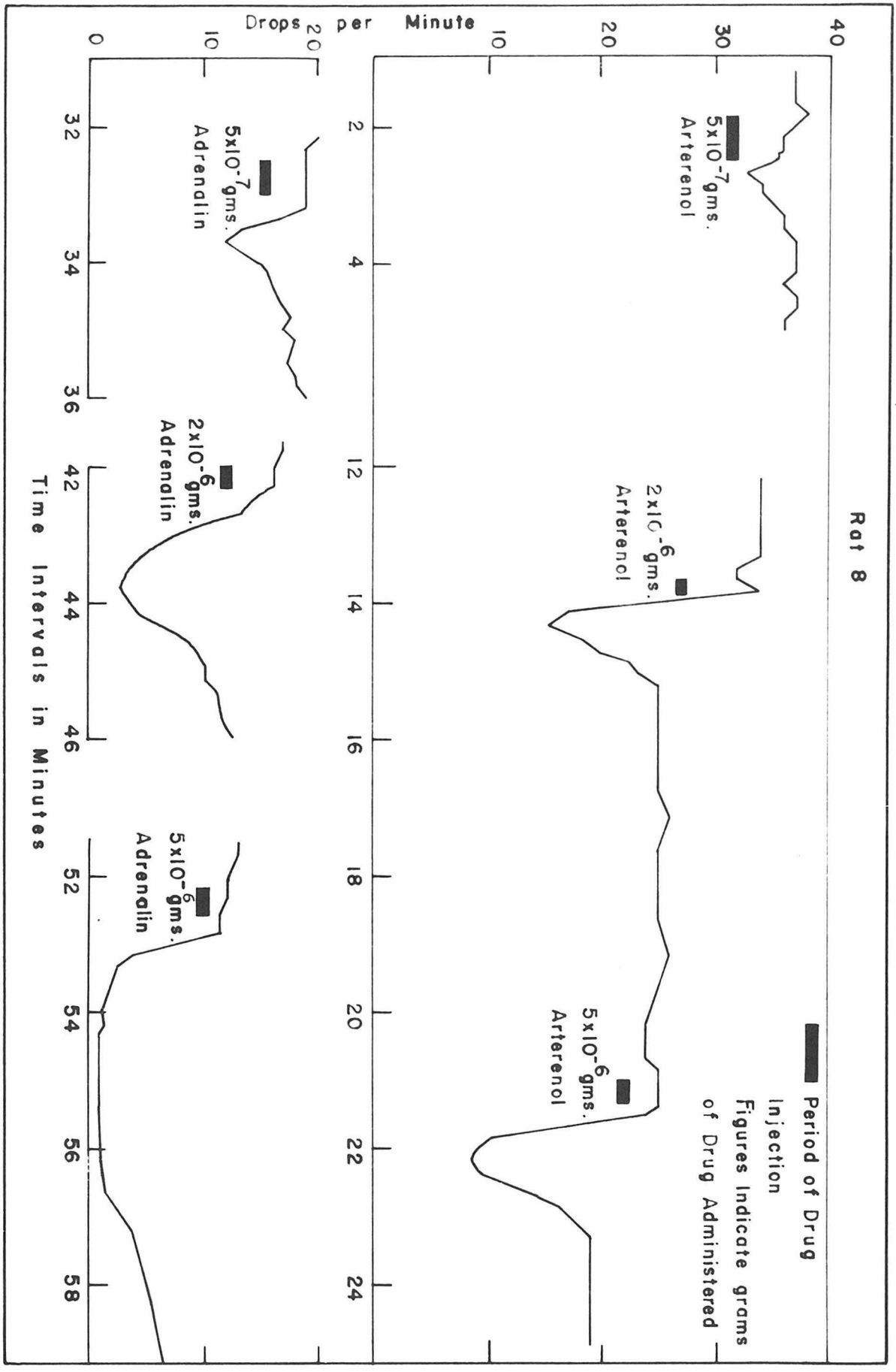
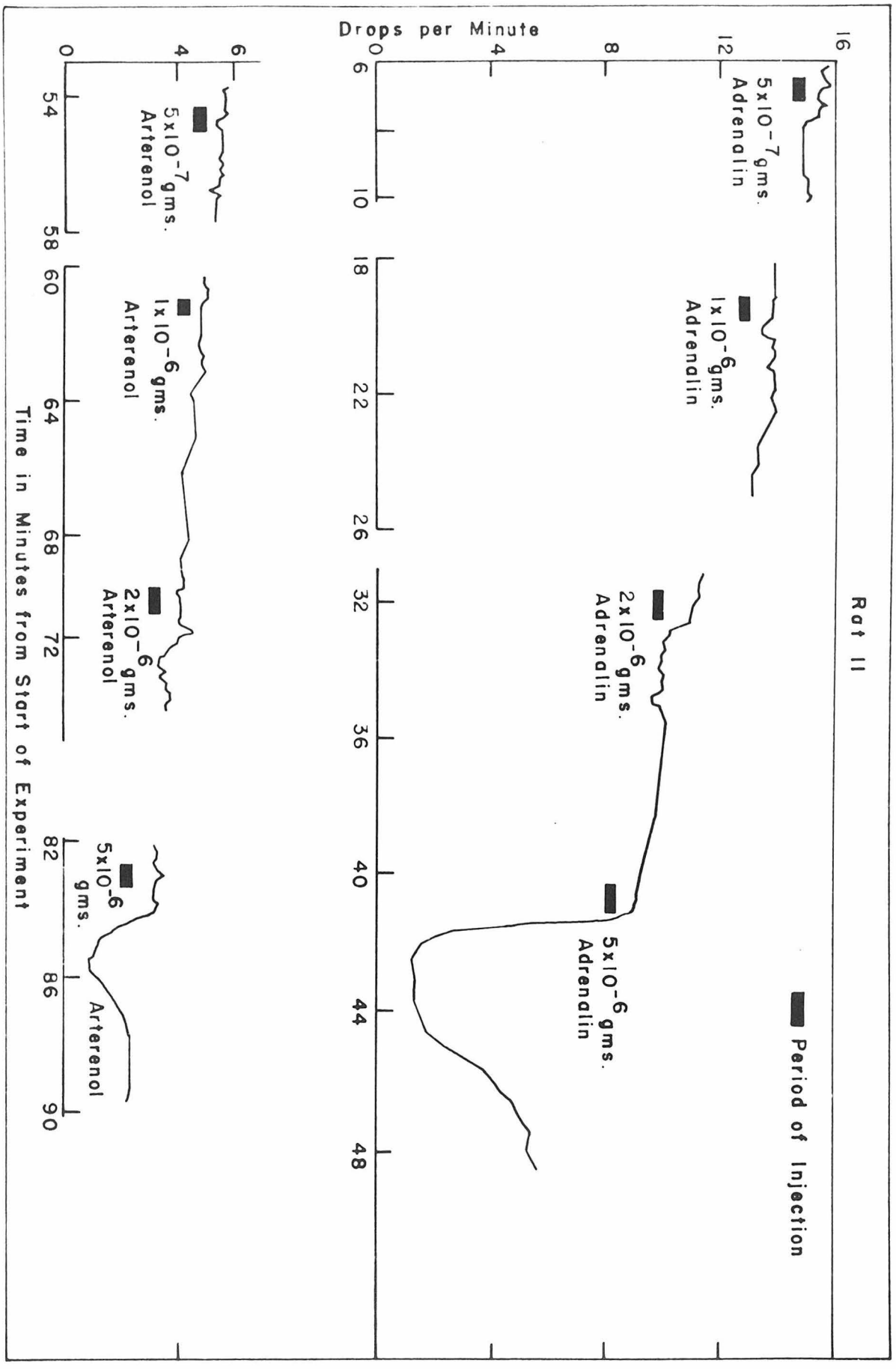

Rat 8
Period of Drug Injection
Figures Indicate grams of Drug Administered
Drops per Minute
40
30
20
10
0
2
4
12
14
16
18
20
22
24
5x10-7gms. Arterenol
2x10-6gms. Arterenol
5x10-6gms. Arterenol
5x10-7gms. Adrenalin
2x10-6gms. Adrenalin
5x10-6gms. Adrenalin
32
34
36
42
44
46
52
54
56
58
Time Intervals in Minutes

Figure IV

Effect of administration of adrenaline and nor-adrenaline (arterenol) upon perfusion rate


Rat 11
Period of Injection
Drops per Minute
5x10⁻⁷gms. Adrenalin
1x10⁻⁶gms. Adrenalin
2x10⁻⁶gms. Adrenalin
5x10⁻⁶gms. Adrenalin
5x10⁻⁷gms. Arterenol
1x10⁻⁶gms. Arterenol
2x10⁻⁶gms. Arterenol
5x10⁻⁶ gms.
Arterenol
Time in Minutes from Start of Experiment

REFERENCES

1. Goodman, L., and Gilman, A., "The Pharmacological Basis of Therapeutics," The MacMillan Co., (1941) p. 396 ff.

2. Euler, U. S. von, J. Physiol., 93, 129, (1938)

3. Burn, J. H., and Hutcheon, D. E., Brit. J. Pharmacology, 4, 373-80, (1949)

4. Starr, I., et al, J. Clin. Invest., 16, 799, (1937)

5. Goldenberg, M., et al, Am. J. Med., 5, 792, (1948)

6. Goldenberg, M., J. A. M. A., 140, 776, (1949)

7. van Harreveld, A., Lerman, L., and Feigen, G., Am. J. Physiol., 157, 168, (1949)

8. Glasser, O., "Medical Physics", The Year Book Publishers, (1944)

9. Reale, A., Kappert, A., Skoglund, C., and Sutton, G., Acta Physiol. Scand., 20, 153, (1950)

10. Lundholm, L., Acta Physiol. Scand., 19, Suppl. 67, 39, (1949)

PROPOSITIONS

1. (a) The Coombs' developing test enables blocking antibodies, which are possibly monovalent, to be detected on the erythrocyte. A modified developing test, using a labelled antigen, may enable reagins to be detected in a precipitin test.

(b) The apparent failure of some animals to develop a high antibody titer in the course of an immunization may be partly due to the presence of a non-precipitating, and possibly monovalent antibody. A modified developing test will show the presence of such antibodies.

2. The formation of an antigen-antibody complex in regions of antigen or antibody excess may be studied by an observation of the effect on the transmission of polarized light through such a system when a transverse electric field, normal to the direction of the light, is applied and removed. (See O'Konski, C. T. and Zimm, B. H., Science, 111, 113, (1950))

3. In a given hemolytic reaction, the effectiveness of the complement varies with the origin of the complement. This variation may be due to some specific reaction between the complement and the antibody. An investigation into the presence or absence of a complex which would result from such a reaction might assist in clarifying this feature of complement action.

4. It has been suggested that blocking anti-Rh antibodies are complexes of agglutinating antibodies with antigen sites

which have been derived from destroyed Rh positive cells. (Itano, H. A., Ph. D. Thesis, 1950) This may be checked by treating a blocking antiserum by a procedure which would dissociate such a complex and denature the antigen site, and then attempting to find a specific agglutinating action in the treated antiserum.

5. Erythrocytes may be marked by a treatment with H_2S, which converts the hemoglobin into sulfhemoglobin, and colors the cells a pale green. The immunologic action of such treated cells is altered but little. (McKerns, K., and Denstedt, O., Can. J. Res. E27, 164, (1949)) Use of these marked erythrocytes will permit a demonstration of an exchange of specific antibodies from one erythrocyte to another. (Mayer, M. M., Croft, C. C., Bowman, W. M., Fed. Proc. 9, 387, (1950))

6. The introduction of a radioactive atom into an antibody or antigen may result in an altered reactivity of the antibody or antigen. The presence of such an effect should be investigated.

7. The reaction between phenaceturic acid and acetic anhydride and sodium acetate yields 2-benzilidene-3-acetyl-5-oxazolone, and this product may be hydrolysed to yield N-acetyl glycine. (Shemyakin, M., Lur'e, S. I., Rodionovskaya, E. I., C. A. 44, 1096^d.) This reaction should be investigated for its applicability to the general case:

$$R_{(1)}\text{-}CH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}COOH \xrightarrow[R_{(2)}\text{-}COO^-]{} R_{(2)}\text{-}CO\text{-}NH\text{-}CH_2\text{-}COOH$$

8. The anomalous reaction of benzyl magnesium chloride with certain reagents to yield o-tolyl derivatives instead of benzyl derivatives may be explained in terms of an intermolecular reaction, rather than an intramolecular rearrangement.

9. The accumulation of potassium ion in nerve and muscle fibres may be explained on the basis of a Donnan membrane equilibrium. (See Boyle, P. J., and Conway, E. J., J. Physiol., 100, 1, (1942))

10. It has been stated that diving mammals such as whales do not suffer from "bends" because they make a complete expiration before diving. (Wiersma, C., personal communication) It is proposed that this situation arises, instead, as a result of the fact that all inspirations are at atmospheric pressure, rather than at a higher pressure.