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ON THE FRACTIONATION OF AGGLUTININS AND ANTITOXIN.

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E. P. Pick[1] in 1901 associated a number of antisubstances individually with the one or the other of the two serum globulin fractions of the Hofmeister classification. In the pseudoglobulin (3.4 to 4.6 saturated ammonium sulphate solution)[2] group of antibodies he placed the diphtheria and tetanus antitoxins and the typhoid agglutinin of horse serum; the lower or euglobulin fraction (2.9 to 3.4 saturated) comprises diphtheria and tetanus antitoxin and cholera lysin in the goat, rabbit and guinea pig, and finally cholera agglutinin in the horse and goat. It becomes possible, according to Pick, to separate the individual specifically reacting antisubstances by fractioning appropriate mixtures of sera. Such a possibility suggested the application of this method to the further study of certain antibodies, especially of the relation of specific and group agglutinins developed by immunization against a single strain of organism. Preliminary experiments in the course of our investigation indicated the unreliability of Pick's differentiation, and attention was accordingly directed to the actual possibility and practicability of distinguishing between antibodies by fractionation of the globulin. The availability of polyagglutinative sera for the work gave a chance for making numerous and extended observations of the distribution of these antibodies in the fractions.[3]

[1] *Beitr. z. chem. Physiol. u. Path.*, i, p. 351, 1901.

[2] The degrees of saturation, as here expressed, indicate a concentration equivalent to a content in 10 cc. of the precipitated solution of 3.4 and 4.6 cc. of saturated ammonium solution respectively.

[3] A preliminary account of our results was published several months ago in the *Proceedings of the Society for Experimental Biology and Medicine*, iv, p. 15, 1906–1907.

The literature on the fractional precipitation of the antibodies is not extensive. Ide and Lemaire[1] (in 1899) found the precipitation limits of diphtheria antitoxin in horse serum to be from 2.8–4.4 saturation. Fuld and Spiro[2] (1900) associated the antirennin of horse serum with the pseudoglobulin, while a milk-coagulating action was possessed by the eu-fraction. The failures of Brodie and of Atkinson (of this laboratory) to separate diphtheria antitoxin from the accompanying serum globulins are referred to in the following paper. Porges and Spiro[3] without giving any of their experimental protocols divide, according to the distribution of the antibodies, the serum globulin into three distinct fractions; the ammonium sulphate precipitation boundaries of these overlap unless the serum is greatly diluted. Landsteiner[4] found that the antitryptic action of blood serum is possessed by the albumin precipitated by complete saturation with ammonium sulphate after removal of the globulin. Cathcart[5] also observed the antitrypsin to be associated with the albumin but not with the globulin fraction. Glaessner[6] states that the euglobulin fraction inhibits the action of trypsin, but the typical protocol which he publishes and his statement of the Hofmeister classification show a misconception and confusion of the identity of his fractions.[7] Glaessner apparently found that the globulin remaining in solution on dialysis was antitryptic.

Very recently Simon, Lamar and Bispham[8] found that the opsonic substance in blood serum was precipitated with the serum

[1] Ide and Lemaire: *Arch. internat. d. pharmacodyn.*, vi, p. 477, 1899.
[2] Fuld and Spiro: *Zeitschr. f. physiol. Chem.*, xxxi, p. 133, 1900.
[3] Porges and Spiro: *Beitr. z. chem. Physiol. u. Path.*, iii, p. 277, 1903
[4] Landsteiner: *Centralbl. f. Bakt.*, xxvii, Abt. I, p. 357, 1900.
[5] Cathcart: *Journ. of Physiol.*, xxxi, p. 497, 1904.
[6] Glaessner: *Beitr. z. chem. Physiol. u. Path.*, iv, p. 79, 1904.
[7] The error on his part has not heretofore been noted. Glaessner states (p. 82): "Das Globulin des Blutserums lässt sich nach den in Hofmeisters Laboratorium in mindestens 3 Fractionen zerlegen: in das bei 25 Proz. Sättigung mit Ammonsulfat ausfällbare Fibrino-globulin, in das Euglobulin, das bei einer Sättigung von 33 Proz. ausfällt und bei der Dialyse in Lösung bleibt, und endlich in das bei 38 Proz. Sättigung ausfällbare bei der Dialyse unlösliche Pseudoglobulin." The confused nomenclature of the degrees of salt saturation is discussed in the following paper (p. 254).
[8] Simon, Lamar and Bispham: *Journ. of Exp. Med.*, viii, p. 651, 1906.

proteids which separated out on dialysis. Opie and Barker[1] observed that proteolysis in an alkaline medium by the enzyme of the leucocytes is inhibited by the serum albumin.

In a paper just published on the relation of diphtheria antitoxin to the serum globulin, Ledingham[2] finds that the pseudoglobulin of horse serum contains the greater part of the antitoxin. The repeatedly precipitated euglobulin, however, in one horse still contained fully 10 per cent of the antitoxin; in a second horse, the euglobulin similarly treated contained practically none of the antitoxin. Single precipitations (without further purification) showed that large amounts of antitoxin may be carried down with the lower fraction; with the one horse, judging in part from the tests on the pseudoglobulin fraction, over half the units must have precipitated with the euglobulin.[3] Ledingham also confirms our own observation here reported that the diphtheria antitoxin of the goat is not invariably linked to the euglobulin fraction as maintained by Pick.

Recently observations have been made by Ruediger[4] on the relation to the blood proteids of streptolysin, the hemolytic substance produced by the development of streptococci in heated serum. The lysin was precipitated with the globulin by saturation with magnesium sulphate; it was found with both the euglobulin and pseudoglobulin of the fractioned undiluted serum and also in both the insoluble proteid and the filtrate of the dialyzed half-

[1] Opie and Barker: *Journ. of Exp. Med.*, ix, p. 207, 1907.

[2] Ledingham: *Journ. of Hygiene*, vii, p. 65, 1907.

[3] Brieger (*Festschrift für R. Koch*, Jena, 1903) and also one of us (Gibson) have already reported similar experiences. In some as yet unpublished experiments carried on for another purpose by one of us (Gibson) and E. J. Banzhaf of this laboratory, it has been found that if undiluted horse serum be precipitated with half its volume of saturated ammonium sulphate solution and allowed to stand for 18 hours, the euglobulin precipitate may contain over two-thirds of the total serum globulin. Precipitation under the same conditions except that the precipitated mixture is 5 or 10 times the volume of the original serum gives a euglobulin figure only of from a fifth to a third the total globulin. The euglobulin at a dilution of 1:10 is noticeably smaller than at 1:5. This fact explains the diminished antitoxic content of reprecipitated or washed euglobulin fraction and makes difficult any hard and fast division into eu- and pseudoglobulins.

[4] Ruediger: *Journ. of Infect. Diseases*, iv, p. 377, 1907.

saturation ammonium sulphate precipitate. Moll,[1] however, has shown that such heating suffices to alter the chemical composition and to change the precipitation characters of the blood proteids.

Owing to the difficulty of making a hard and fast division of the serum globulins into the eu- and pseudo-fractions, our experiments were not planned to be interpreted especially from the quantitative occurrence of the agglutinins in the one or the other fraction of specific agglutinating sera. We have aimed, however, to determine if the relative proportion of the agglutinins of polyagglutinative sera in the fractions remained constant as regards the proportional distribution of all the agglutinins of the serum in the eu- and pseudo-globulins. In a word, by a difference of precipitation limits to ammonium sulphate, it should be expected that the bulk of one or more of the agglutinins would appear in the one fraction, as contrasted with the larger proportion of each of the remaining agglutinins occurring in the other fraction. Attention is particularly directed in studying the results from our standpoint to the pseudoglobulin fraction (filtrate from the euglobulin fraction). Any loss from the euglobulin fraction through solubility in the wash solution has been considered only in the case of the antitoxins. Such loss may be interpreted as due to the resolution of the mechanically precipitated proteids of the more soluble fraction; it may be considered just as well in part as a not absolute insolubility of the eu-fraction in 3.4 saturation ammonium sulphate. The content of agglutinins in the washed euglobulin fraction is of interest, however, as it is more highly "purified" than the pseudoglobulin, so that any relative differences in the distribution of the agglutinins should be from this standpoint the more pronounced for the low fraction.[2] The limitations in determining the agglutinative potency of the serum and of the fractions, however, make difficult at times the interpretation of the readings obtained, and do not permit conclusions being drawn from a single experiment.

[1] Moll: *Beitr. z. chem. Physiol. u. Path.*, iv, p. 563, 1904.

[2] The euglobulin of 2 cc. of serum precipitated at a final dilution of 1 : 5 was washed usually by being three times thoroughly suspended in 10 cc. of 3.4 saturation ammonium sulphate solution and recentrifuged.

It was found repeatedly in our experiments with rabbit and goat sera that the agglutinins for the dysentery group of organisms (Flexner Manila and Shiga), typhoid, colon and cholera, were not confined to either the pseudoglobulin of the washed (with 3.4 saturated ammonium sulphate solution) euglobulin fractions; they were either split by the fractioning, the larger portion occurring in the pseudoglobulin, or almost the entire amount of the agglutinating substances recovered were in this higher fraction in the original quantitative proportion to one another. With antidysentery horse serum, the dysentery (Shiga and Flexner) and *B. coli* agglutinins were fairly quantitatively split between the pseudo- and euglobulin fractions, the latter containing the lesser amount. With an anticholera and antityphoid horse serum, the pseudoglobulin (two experiments) and also the filtrates from two additional 3.6 and 3.8 saturation precipitations contained the bulk of both the agglutinins. In subsequent experiments with sera from other bleedings as well as with the sera used above, the typhoid agglutinin was divided between the two fractions with a somewhat larger proportion occurring in the pseudoglobulin.

The results of exhaustion experiments on the two globulin fractions were the same as those that would be obtained in the use of the native serum, and failed to give any reason for believing that we were dealing with a separation of group and specific agglutinins through fractioning.[1]

[1] Immunization with certain bacteria results in the development of "group" or "common" and of "specific" agglutinins in the serum of the animal immunized. Group agglutinins are agglutinating substances which are effective both on the homologous organism and on some allied strains of bacteria; specific agglutinin is effective on the organism used for immunization. An immune serum developed by immunization against *B. dysenteriæ* (Shiga) might contain, for the sake of illustration, simply (1) a group agglutinin effective for Shiga, Flexner Manila, Pfeiffer and *B. coli*, and (2) an agglutinin specific for the Shiga strain. When immunization has been developed simultaneously against two or three of the above organisms instead of the Shiga strain alone, the number and agglutinating scope of the agglutinins resulting becomes more complex, various group agglutinins and the specific agglutinins for each organism being present. The existence of the two types of agglutinins is demonstrated by the agglutinating characters of the diluted serum after the agglutinins for any desired strain of organism have been exhausted by adding sus-

Precipitation of antidiphtheria goat serum (three experiments) showed that less than half the antitoxin remained in the pseudoglobulin; practically none was found in the euglobulin while the 3.4 saturated ammonium sulphate solution washings contained the larger part. Our results with the antitoxic horse serum at a dilution of 1:5 are essentially identical with Pick's.

The results of the work accomplished have demonstrated the untrustworthiness of any such differentiation of the antibodies as those contained in the euglobulin and those of the pseudoglobulin. No evidence has been adduced from our experiments to show that the agglutinins developed in the rabbit, goat and horse can be classed as belonging to either globulin, or that these antibodies can be separated from one another by ammonium sulphate fractioning of polyagglutinative sera.[1]

A description of the experimental procedure is given with the protocols which follow:

FRACTIONATION OF POLYAGGLUTINATIVE RABBIT SERUM.

Combined immunization against Flexner Manila dysentery, Shiga dysentery, Pfeiffer,[2] colon and cholera. Rabbit bled II/27/06. Serum fractioned II/29/06.

Five cc. of the rabbit serum diluted with 11.5 cc. of distilled water, were precipitated by 8.5 cc. of saturated ammonium sulphate solution. After standing 2 hours, 12.5 cc. of the uniform mixture were removed and centrifuged in stoppered tubes. The supernatant fluid contains the "pseudoglobulin" fraction. The

pensions of washed organisms; after contact for some hours the agglutinated and excess of free organisms are removed by filtration. The agglutinins for several related strains can thus be successively withdrawn. In the example given above, exhaustion with either *B. coli*, Flexner Manila or Pfeiffer would remove only the group agglutinin (1) so that the serum would still agglutinate the Shiga, though at a diminished dilution; exhaustion with the Shiga would take out both the group and the specific agglutinins, and this serum would no longer have agglutinating properties for any of the above organisms. Cf. Castellani: *Zeitschr. f. Hyg. u. Inf.*, xl, p. 1, 1902; also Park and Collins: *Journ. Med. Research*, xii, p. 491, 1904.

[1] In the paper by Banzhaf and Gibson following this article, it will be shown that the globulins of the serum do differ markedly in their content of antitoxin per gram proteid.

[2] The original Pfeiffer strain of *B. typhosus*.

precipitate ("euglobulin") was washed three times by being thoroughly suspended in 3.4 saturated solution and centrifuged; it was once more suspended and the volume made up to 12.5 cc. The agglutinating properties were then ascertained of (1) the original serum; (2) the total globulin, a uniform sample of the precipitated serum; (3) the pseudoglobulin fraction, and (4) the washed 3.4 saturation precipitate or euglobulin fraction. Agglutinations were determined microscopically and control slides were examined. Dilutions are in terms corresponding to the original serum. The characters of the agglutinations at the various dilutions are indicated as follows:

+ + + +	agglutination with no free organisms.
+ + +	agglutination with relatively very few free organisms.
+ +	agglutination but with numerous free organisms.
+	incomplete agglutination, small loose groups and many free bacteria.
±	tendency to agglutinate.
−	no agglutination.
o	observation lost.

The procedure was essentially unchanged in the other experiments. The serum used in this and the following experiments was roughly tested for orientation before the final agglutinations were made. A **+ + +** agglutination indicates usually the highest dilution for an observed positive reaction. It should be remembered that the character of the agglutination at any dilution is often difficult to decisively determine; *the actual observations are by no means so exact as would be inferred from the published experiments of Pick and of others.*

The agglutinating properties of this rabbit serum (Table I) were too low to be entirely satisfactory for fractioning, the weakest agglutinating action (1:50) being manifested on the Shiga strain. The agglutinin for this organism drops out in the euglobulin fraction. This lost agglutinin is not found in the pseudo fraction. It is conceivable from this experiment that the agglutinin of the Shiga dysentery is more soluble than that of the other five strains; however, the Shiga shows no such differences in the two following precipitation experiments on later bleedings of the same rabbit. It is more likely that the content of serum in agglutinin was originally so low that in the fractioned and washed

euglobulin the dilution of 1:20 failed to show the proportion of agglutinin present. With the Flexner Manila dysentery, the Pfeiffer typhoid strain, the colon and the cholera—all contained in greater concentration than the Shiga agglutinin—the major portions of the agglutinins occur in the pseudoglobulin, a smaller amount being held by the low fraction.

TABLE I. FRACTIONING OF POLYAGGLUTINATIVE RABBIT SERUM.

Bleeding of II/27/06; fractioning II/29/06.

Organism.	Fraction.	20	50	100	200	500	1000
Flexner Manila..	Original serum	++++	++++	++++	**++++**	++	±
	Total gbl.	++++	++++	++++	**++++**	++	−
	Pseudogbl.	++++	++++	**++++**	+	±	−
	Eugbl.*	++++	++++	**++++**	±	−	−
Shiga ...	Serum	++++	**++++**	++	±	−	−
	Total gbl.	++++	**++++**	+	−	−	−
	Pseudogbl.	**++++**	±	−	−	−	−
	Eugbl.*	−	−	−	−	−	−
Pfeiffer ..	Serum	++++	++++	**++++**	++	±	−
	Total gbl.	++++	++++	**++++**	++	−	−
	Pseudogbl.	++++	**++++**	+	±	−	−
	Eugbl.*	++++	**++++**	+	−	−	−
Colon....	Serum	++++	++++	++++	**++++**	±	−
	Total gbl.	++++	++++	**++++**	++	±	−
	Pseudogbl.	++++	++++	**++++**	+	±	−
	Eugbl.*	++++	**++++**	+	−	−	−
Cholera...	Serum	++++	++++	++++	**++++**	±	−
	Total gbl.	++++	++++	++++	**++++**	±	−
	Pseudogbl.	++++	++++	**++++**	±	−	−
	Eugbl.*	++++	**++++**	±	−	−	−

*Tested III/1/06 with a fresh culture.

Exhaustion of the two globulin fractions (Table II) with suspensions of the Flexner Manila strain has not withdrawn the agglutinins for the typhoid from either fraction. Exhaustion with the Pfeiffer likewise is not complete for the dysentery in either pseudo- or euglobulin.

This later bleeding of the same rabbit shows by far the greater part of all the agglutinins in the pseudoglobulin (Table III), and small though relatively proportional amounts of each in the low fraction. The absolute dropping out of the Shiga does not occur as in the preceding fractioning. The results are also more uniform.

TABLE II. EXHAUSTION EXPERIMENT. POLYAGGLUTINATIVE RABBIT.

Exhaustion of the Original Serum and the Fractions (cf. Table I).

Exhaustion with Flexner Manila.

Organism.	Fraction.	20	50
Flexner Manila.....	Serum	+	−
	Pseudogbl.	−	−
	Eugbl.	−	−
Pfeiffer.....	Serum	+ + + +	+ + + +
	Pseudogbl.	+ + + +	+ + + +
	Eugbl.	+ + + +	+ + + +

Exhaustion with Pfeiffer.

Flexner Manila.....	Pseudogbl.	+ + + +	−
	Eubgl.	+ + + +	−
feiffer	Pseudogbl.	−	−
	Eugbl.	−	−

TABLE III. FRACTIONING OF POLYAGGLUTINATIVE RABBIT SERUM.

Serum from bleeding IV/16/06; serum fractioned IV/18/06.

Organism.	Fraction.	50	100	200	500	1000	2000
Flexner Manila...	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**	+
	Pseudogbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	+
	Eugbl.	+ +	+	−	−	−	−
Shiga.....	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + +**	+ +
	Pseudogbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	±
	Eugbl.	+ +	+	−	−	−	−
Cholera....	Total gbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +
	Pseudogbl.	+ + + +	+ + + +	+ + + +	+ + + +	**+ + + +**	+
	Eugbl.	+	−	−	−	−	−
Pfeiffer	Total gbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+ +	+
	Psuedogbl.	+ + + +	+ + + +	+ + + +	**+ + + +**	+	±
	Eugbl.	±	−	−	−	−	−

The third precipitation (Table IV) shows apparently a recovery of all the agglutinins in the high globulin fraction. The pseudo-globulin dilutions for the Flexner Manila, in fact, were read slightly higher than were those of the total globulin.

Pick's single observation by the test-tube method on typhoid rabbit serum, agglutinating at 1 : 20,000, gave the limit of agglutination of the pseudoglobulin at 1 : 3000, of the euglobulin 1 : 20,000; after reprecipitating the fractions three and four times, respectively, the limits of dilution for agglutination were at 1 : 20 and 1 : 8000.

TABLE IV. FRACTIONING OF POLYAGGLUTINATIVE RABBIT SERUM.

Serum from bleeding on V/23/06.

Organism.	Fraction.	50	100	200	500	1000
Flexner Manila .	Total gbl.	++++	++++	++++	++++	+++
	Pseudogbl.	++++	++++	++++	++++	++++
	Eugbl.	−	−	−	−	−
Pfeiffer. . .	Total gbl.	++++	++++	++++	++++	+++
	Pseudogbl.	++++	++++	++++	++++	+++
	Eugbl.	−	−	−	−	−
Cholera. . .	Total gbl.	++++	++++	++++	+++	++
	Pseudogbl.	++++	++++	++++	+++	++
	Eugbl.	±	±	±	−	−

POLYAGGLUTINATIVE GOAT SERUM.

Immunization against Flexner Manila, Shiga, Pfeiffer, cholera and *B. coli*.

The proportion of the agglutinins for each organism is much greater in the pseudoglobulin than in the euglobulin fractions (Table V). This conclusion is confirmed for the Shiga, Pfeiffer and cholera by the redetermination of the agglutinations in the exhaustion experiment (Table VI). The Flexner Manila dysentery strain has not exhausted the agglutinins for the other organisms completely from the eu- or from the pseudoglobulin fractions; nor have the agglutinins apparently been withdrawn to a relatively greater degree from the one than from the other fraction.

Fractioning of the polyagglutinative serum (of lessened agglutinating power) from the second bleeding of the goat immunized against the mixed cultures showed that the agglutinins were almost quantitatively contained in the pseudoglobulin fraction (Table VII).

TABLE V. FRACTIONING OF POLYAGGLUTINATIVE GOAT SERUM.

Serum from bleeding II/27/06; fractioned III/9/06.

Organism.	Fraction.	50	100	200	500	1000
Flexner Manila	Serum	++++	++++	++++	+++	++
	Total gbl.	++++	++++	++++	+++	+
	Pseudogbl.	++++	++++	+++	++	−
	Eugbl.	++++	+++	−		
Shiga	Serum	++++	++++	++++	++++	++
	Total gbl.	++++	++++	++++	++++	+
	Pseudogbl.	++++	++++	++++	++++	−
	Eugbl.	++++	+++	−		
Pfeiffer...	Serum	++++	++++	++	++	+
	Total gbl.	++++	++++	+++	++++	±
	Pseudogbl.	++++	++++	++	+	±
	Eugbl.	++++	+	−		
Cholera...	Serum	++++	++++	++++	++++	++++
	Total gbl.	++++	++++	++++	++++	+++
	Pseudogbl.	++++	++++	++++	++	±
	Eugbl.	++	+	+		
Colon	Serum	++++	++++	++++	++++	+++
	Total gbl.	++++	++++	++++	++++	+
	Pseudobgl.	++++	++++	++++	++++	±
	Eugbl.	++++	++	−		

TABLE VI. EXHAUSTION EXPERIMENT. POLYAGGLUTINATIVE GOAT SERUM.

Exhaustion with Flexner Manila of Fractions (Table V) on III/7/06.

Fraction.	Organism.	20	50	100	200	500	1000
Total gbl.	Flexner Manila	++++	−	−	−	−	−
	Shiga	++++	++++	++++	++++	++++	+++
	Pleiffer	++++	++++	++++	++++	++++	+++
	Cholera	++++	++++	++++	++++	++++	++++
Pseudogbl.	Flexner Manila	−	−	−	−	−	−
	Shiga	++++	++++	++++	++++	++	
	Pfeiffer	++++	++++	++++	++++	+	
	Cholera	++++	++++	++++	++++	+++	
Eugbl. ...	Flexner Manila	−	−	−	−	−	
	Shiga	++++	+++	+	−	−	
	Pfeiffer	++++	+++	−	−	−	
	Cholera	+	+	−	−	−	

Pick's corresponding experiment may be summed up as follows: An antityphoid goat serum agglutinating at 1:2600 was precipitated in a series of progressively increasing concentrations of ammonium sulphate. The initial precipitation was at 2.6 saturation with a dilution of 1:5, agglutination being evident at 1:500 of the unfiltered precipitated mixture. At 3.4 saturation over half and at 3.6 saturation all the agglutinin was precipitated. Again, 20 cc. of the same serum were precipitated with 10 cc. of saturated ammonium sulphate solution; the euglobulin then agglutinated at 1:2400 (in terms of original serum) the pseudoglobulin at 1:20. After two reprecipitations the eu-fraction still reacted at 1:2400.

TABLE VII. FRACTIONING OF POLYAGGLUTINATIVE GOAT SERUM.

Bleeding of III/18/06; fractioned III/20/06.

Organism.	Fraction.	100	200	500	1000
Flexner Manila.....	Total gbl.	++++	++++	+++	±
	Pseudogbl.	++++	**++++**	++	−
Shiga.....	Total gbl.	++++	++++	0	−
	Pseudogbl.	++++	++++	**++++**	±
Pfeiffer.....	Total gbl.	++++	**+++**	±	−
	Pseudogbl.	++++	**++++**	−	−
Cholera.....	Total gbl.	++++	**++++**	+	−
	Pseudogbl.	++++	**++++**	+	−
Colon.....	Total gbl.	++++	**+++**	++	−
	Pseudogbl.	++++	**+++**	−	−

POLYAGGLUTINATIVE HORSE SERUM.

(*a*) *Antidysentery Horse Serum.* Horse 284; immunized against the Shiga, Flexner Manila and the Mount Desert strains. Bleeding of X/3/06; fractioned X/4/06.

Here (Table VIII) the agglutinins are split between the fractions, the larger part of each occurring in the pseudoglobulin.

(*b*) *Anticholera and Antityphoid Horse Serum.* Horse 254; combined immunization against the original Pfeiffer and cholera. Serum from bleeding on III/27/06; fractioned IV/1/06 and refractioned V/10/06. The results are given in Table IX.

With the cholera-typhoid serum, the agglutination values of which for each organism were in the neighborhood of a dilution

of 1:1000, the bulk of the agglutinins was found in the pseudoglobulin (Table IX); the greater portion was also in the high fraction when the serum was precipitated at 3.6 and again at 3.8 saturation. There is no evidence presented here that the precipitation limits of the cholera agglutinin are in any way different from that of the typhoid. In the second fractionation (V/10/06) it is seen that both the Pfeiffer and the cholera are increased in the eu-fraction as contrasted with the result of the first precipitation at 3.4 saturation.

TABLE VIII. FRACTIONING OF ANTIDYSENTERY HORSE SERUM.

Organism.	Fraction.	50	100	200	500	1000	2000
Flexner Manila....	Serum	++++	++++	++++	++++	+++	++
	Total gbl.	++++	++++	++++	+++	+	+
	Pseudogbl.	++++	++++	+++	+++	+	−
	Eugbl.	++++	++++	+++	±	−	−
Shiga	Serum	++++	++++	++++	++++	+++	−
	Total gbl.	++++	++++	++++	++++	±	−
	Pseudogbl.	++++	++++	++++	−	−	−
	Eugbl.	++++	++++	+++	−	−	−
Colon.....	Serum	++++	++++	++++	++++	++++	0
	Total gbl.	++++	++++	++++	+	−	−
	Pseudobgl.	++++	++++	++++	±	−	−
	Eugbl.	++++	+++	++	±	−	−

The fractionation of the anticholera and antityphoid horse serum is of especial interest because an experiment of this nature is the most striking of E. P. Pick's observations. Pick had found that the typhoid agglutinin was precipitated with the pseudoglobulin fraction in horse serum; the cholera agglutinin, on the contrary, came down with the euglobulin. Pick, therefore, mixed equal volumes of a typhoid and a cholera serum, and progressively precipitated 2 cc. amounts of the mixed sera at 2.8, 3.0, 3.2, etc., saturation (with a final volume of 10 cc. in each case). A well marked separation of the cholera agglutinin into the euglobulin and the typhoid agglutinin into the high fraction resulted. As given in Pick's tables, the observed agglutination values are twice too much, since each serum was diluted a half by the mixing. A direct separation at 3.4 saturation of the agglutinin in a goat cholera serum (agglutinating at a dilution of

TABLE IX. FRACTIONING OF ANTICHOLERA-ANTITYPHOID HORSE SERUM.

1. Precipitation at 3.4 saturation.

Organism.	Fraction.	50	100	200	500	1000
Pfeiffer.....	Total gbl.	++++	++++	++++	++++	+++
	Pseudogbl.	++++	++++	++++	+++	±
	Eugbl.	+	+	−	−	−
Cholera.....	Total gbl.	++++	++++	++++	++++	++++
	Pseudogbl.	++++	++++	+++	+++	+
	Eugbl.	+	+	−	−	−

2. Precipitation at 3.6 saturation.

Organism.	Fraction.	50	100	200	00	1000
Pfeiffer.....	Total gbl.	++++	++++	++++	++++	++
	Pseudogbl.	++++	++++	++++	+++	−
Cholera.....	Total gbl.	++++	++++	++++	++++	++++
	Pseudogbl.	++++	++++	++++	++++	++++

3. Precipitation at 3.8 saturation.

Organism.	Fraction.	50	100	200	500	1000
Pfeiffer.....	Total gbl.	++++	++++	++++	++++	±
	Pseudogbl.	++++	++++	+++	+++	±
Cholera	Total gbl. .	++++	++++	++++	++++	++++
	Pseudogbl.	++++	++++	++++	+++	±

4. The same serum was again fractioned on V/10/06.

Organism.	Fraction.	50	100	200	500	1000	2000
Pfeiffer...	Total gbl.	++++	++++	++++	++++		
	Pseudogbl.	++++	++++	++++	+++		
	Eugbl.	++++	++++	++++	−		
Pfeiffer* .	Total gbl.	++++	++++	++++	++++	+++	++
	Pseudogbl.	++++	++++	++++	+++	±	−
	Eugbl.	++++	++++	−	−	−	−
Cholera ..	Total gbl.	++++	++++	++++	++++	+++	
	Pseudogbl.	++++	++++	++++	+++	++	
	Eugbl.	++++	++++	+++	±	−	

* A second fractionation.

1:1000) from the typhoid agglutinin in horse serum (1:20,000) was made. The euglobulin was precipitated by adding a half volume of saturated ammonium sulphate solution directly to the mixed sera. The high agglutination values are again given as *direct* observations, though probably calculated for the original undiluted sera. The figures for the reprecipitated euglobulin are for the typhoid, 1:3000, and for the cholera 1:1600, (an increase over the value of the original goat serum); the pseudoglobulin (3.3 saturation filtrate) agglutinated the typhoid at 1:16,000, the cholera at 1:20.

TABLE X. FRACTIONING OF ANTICHOLERA-ANTITYPHOID HORSE SERUM.

Horse 254; bleedings of II/27/06 and V/29/06; fractioned X/8/06.

Fractions tested with the Mt. Sinai culture of typhoid.

Centrifuged.	Fraction.	100	200	500	1000	2000
	II/27/06					
2 hrs. after precipitation...	Total gbl.	++++	++++	++++	+++	+
	Pseudogbl.	++++	+++	+++	±	
	Eugbl.	++++	+++	+	±	
After 12 hrs.	Total gbl.	++++	++++	++++	++++	+++
	Pseudogbl.	++++	+++	+++	±	
	Eugbl.	++++	+++	+	±	
	V/29/06.					
	Serum	++++	++++	++++	++++	+
After 2 hrs....	Total gbl.	++++	++++	++++	+++	+
	Pseudogbl.	++++	++++	++++	++	±
	Eugbl.	++++	++++	++++	++	±
After 12 hrs....	Total gbl.	++++	++++	++++	++++	+
	Pseudogbl.	++++	+++	+++	+	
	Eugbl.	++++	++	±		

In the following observations it is shown that a relatively large proportion of the typhoid agglutinin may occur in the euglobulin fraction.[1]

Six cc. of each serum were diluted with 13.8 cc. of water and precipitated by the gradual addition of 10.2 cc. of saturated ammonium sulphate solution. Uniform samples (15 cc.) of each

[1] On resuming this problem in the fall of the year, it was found that our cholera culture was spontaneously agglutinating; it could not therefore be employed in testing the agglutination values of the fractions.

were centrifuged after two hours' standing. The euglobulin precipitates were washed by thoroughly suspending the proteid in 15 cc. of 3.4 saturation ammonium sulphate solution and again centrifuging; the precipitates were washed three times in this fashion. Exactly similar precipitations were made on the two sera but the precipitated mixtures were centrifuged after twelve instead of two hours standing. The results are given in Table X.

A high typhoid agglutinin content in the euglobulin was also obtained on fractioning the last bleeding of the cholera-typhoid horse. The results are shown in Table XI.

TABLE XI. FRACTIONING OF ANTICHOLERA-ANTITYPHOID HORSE SERUM.

Horse 254; bled X/3/06; fractioned X/4/06; tested only with typhoid.*

Organism.	Fraction.	100	200	500	1000	2000
Mt. Sinai Typhoid.....	Total gbl.	++++	++++	++++	**++++**	++
	Pseudogbl.	++++	++++	++++	**+++**	−
	Eugbl.	++++	++++	**+++**	−	−
St. Typhoid...	Total gbl.	++++	++++	**++++**	+	+
	Pseudogbl.	++++	++++	**++++**	++	−
	Eugbl.	++++	++++	**+++**	−	−

*The Pfeiffer strain agglutinated spontaneously. The Mount Sinai strain has always shown the same agglutinations as the Pfeiffer in numerous other observations.

FRACTIONATION OF DIPHTHERIA ANTITOXIC GOAT AND HORSE SERUM.

Fresh goat serum and serum from horse 307, VII/5/06, were fractioned as follows: 3 cc. of serum diluted with 6.9 cc. of water were precipitated with 5.1 cc. of saturated ammonium sulphate solution, and the precipitate from a 10 cc. sample of each obtained by centrifuging. The precipitates were suspended in 3 cc. of 3.4 saturated ammonium sulphate solution and again centrifuged; the washing was twice repeated and the wash solutions united and made up to 10 cc. The precipitates were suspended as usual in 3.4 saturated ammonium sulphate and made up to 10 cc. The results calculated per cc. of the original undiluted serum follow:

Fraction.	Goat.	Horse.
Serum	90 units	250 units
Total globulin	90 "	250 "
Pseudoglobulin	35 "	+200 "
Euglobulin	5 "	0* "
Wash solution	50 "	+25 "

* Tested for 5 units against 100 m.l.d.; the guinea pig died in 12 hours, autopsy showing a typical diphtheria toxin picture.

The same results were obtained in two similar experiments. The two sera certainly show a different behavior towards ammonium sulphate precipitation. A relatively large proportion of the goat antitoxin is precipitated with the euglobulin. The facility with which the antitoxin can be washed out almost completely (in a total united volume of wash solution less than the original volume of the precipitated mixture) shows that the antitoxin is not invariably linked to the euglobulin.

Pick's experiment is given briefly for comparison with our results:

Twenty cc. of antitoxic goat serum[1] (neutral reaction) were precipitated with 10 cc. of saturated ammonium sulphate solution. After two hours, the precipitate was pressed out and dissolved in 30 cc. of water; it was reprecipitated at 3.3 saturation and dissolved in 20 cc. of water (euglobulin fraction). The filtrates were united and made up to half saturation, the precipitate dissolved in the original volume (20 cc.) and refractioned between 3.3 and 5.0. The euglobulin then obtained was united with the above euglobulin solution and the mixture precipitated at 3.3 saturation. The serum contained about 10 units per cc. The fractions tested as follows:

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[1] Pick states (p. 361) "Zu dem nun folgenden Trennungsversuche mit Diphtherie immun Ziegenserum stand mir nur ein Ziegenserum zur Verfügung, von dem 0,1 ccm eben im stande war, die 10 fache tödliche Giftmenge eines Toxins zu paralysieren, das in der Dosis von 0,0098 ccm ein Meerschweinchen von etwa 260 g in drei Tagen tötete." This would make the potency of the serum used only 1 unit per cc. The control test made actually gives the strength as 10 antitoxin units per cc.: 0.098 cc. toxin (10 m.l.d.) were neutralized by 0.01 cc. serum; therefore 100 m.l.d. toxin were neutralized by 0.1 cc., or 1 cc. serum neutralized 10 × 100 m.l.d. toxin. Ledingham has passed this over: "The goat serum with which Pick worked had a very low antitoxic value inasmuch as 0.1 cc. was required to neutralize 10 lethal doses of a toxin whose m.l.d. was only about 0.01 cc." Pick, himself, speaks of the antitoxic value of this serum incidentally "Man erkennt trotz der geringen Wertigkeit des Serums...."

Pseudoglobulin:

0.05 cc. + 10 m.l.d. toxin (testing for 2 units). The guinea pig died on the second day.

Euglobulin:

0.01 cc. + 10 m.l.d. toxin (testing for 10 units). Died on the third day.

0.017 cc. + 10 m.l.d. toxin (testing for 6 units). Induration and loss of weight, but survived.

Ledingham states in his conclusions that in the horse serum the relationship of the diphtheria antitoxin to the pseudoglobulin fraction "holds good only when the antitoxin content of the serum is steadily rising." Horse 307 of this department had been subjected to immunization for over five months; it attained a maximum of over 300 units per cc. in three months and had declined to 250 units two months later when the blood of the serum used in our fractionation experiments was drawn.[1] Apparently Ledingham's conclusion (from observations on a single horse) is not of general application.

Tabulating Pick's results by a somewhat different arrangement than the one presented in his paper (p. 384), it is seen from the following—

Animal.	Diphtheria Antitoxin.	Tetanus Antitoxin.	Cholera Lysin (Pfeiffer.)	Typhoid Agglutinin.	Cholera Agglutinin.
Goat	eugbl.	eugbl.	eugbl.	eugbl.	eugbl.
Rabbit				eugbl.	
Guinea pig ..				eugbl.	
Horse	pseudogbl.	pseudogbl.		pseudogbl.	eugbl.

—that there is no evidence of any differences in the precipitation limits of the antibodies in goat, rabbit and guinea pig sera. We should hardly expect *a priori*, then, that a separation of any other antibodies by fractionation of goat and rabbit serum would be possible, and we have not found otherwise. Yet the probability of such a separation for horse serum was suggested by Pick's experiments. With this serum, even, the distribution of the antibodies as determined by Pick, has been similarly homogeneous with the exception of the cholera agglutinin. Pick, accordingly, gives one example, and one only, of an antibody

[1] The horse subsequently was killed as no longer of service for antitoxin production.

differing from other antibodies in the serum of the same species by its precipitation characters toward ammonium sulphate. This observation of Pick's we have been unable to verify when polyagglutinative horse sera have been used. It is probable, however, that the serum globulins of different animals or even of various individuals of the same species may show a different and inconstant behavior quantitatively toward fractional ammonium sulphate precipitation. We have not found that any of the antibodies in goat, rabbit and horse serum were invariably associated with the euglobulin. Our results with goat diphtheria antitoxin have been confirmed by Ledingham. At the same time we have presented repeated observations with several strains of typhoid showing that a large proportion (almost half in some instances) of the typhoid agglutinin of horse serum may be found in the thoroughly washed euglobulin fraction of both old and fresh serum.

The results of our experiments have already been briefly summarized in the preceding portion of the paper.

It is to be hoped that any future work on the fractionation of the antibodies or of the proteids of the blood will not be undertaken without a thorough comprehension of the nature and limitations of the process. Salt fractionation is a valuable method for the purification and preparation of proteid products; the salt concentration precipitation limits, however, are not a reliable means for differently classifying proteids the precipitation characters of which are not widely separated.