TRANSFORMATION OF PSEUDOGLOBULIN INTO EUGLOBULIN

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INTRODUCTION

In several publications Banzhaf[2] states that when diphtheria serum is heated as it is in the preparation of antitoxin, part of the pseudoglobulin is transformed into euglobulin. Details regarding the methods of analysis or the analytic data on which Banzhaf based his conclusion were not found in the publications examined.

This transformation has both a practical and a theoretical interest. It facilitates the concentration of the antitoxin present in the serum by removing protein without removing any of the antitoxin, so that the final product contains all the antitoxin associated with much less protein. This is desirable because certain of the serum proteins have very little therapeutic value. In applying the heat treatment for the first time to a serum such as anthrax serum, it is obviously desirable to be certain that a similar transformation takes place. Otherwise, there would be no need of the heat treatment, and, besides, the heating may cause loss of potency.

On the theoretical side, the fact that pseudoglobulin can be transformed into euglobulin without affecting the total number of antitoxic units is almost conclusive proof that the antitoxin is a substance separate from pseudoglobulin. That this transformation may take place in some serums, but not in all, is indicated by the following experiments, in which the heating of the serum was carried out under carefully controlled conditions and the analytic data obtained by improved methods. In all, four horse serums were used, as follows:

Anthrax 48.—Serum obtained from horse 48; used in a previous work.[3] Blood drawn January 6, 1916. Potency high.

Anthrax 96.—Serum obtained from horse 96; used previously. Blood drawn January 6, 1916. Potency lower than serum 48.

Diphtheria 1.—A mixture of 500 c. c. of serum, potency 1,400 units per cubic centimeter when bled November 4, 1915, and 700 c. c. of serum, potency 250 units per cubic centimeter when bled October 29, 1915. From two horses.

Tetanus 1.—A mixture of 750 c. c. serum, potency 200 units per cubic centimeter when bled February 14, 1916, and 250 c. c. serum, potency 175 units when bled February 14, 1916. From two horses.

[1] The thanks of the writer are due to Dr. A. Eichhorn, Chief of the Pathological Division, for many valuable suggestions and his interest in the work.

[2] Banzhaf, E. J. The further separation of antitoxin from its associated proteins in horse serum. *In* Proc. Soc. Exp. Biol. and Med., v. 6, no. 1, p. 8–9. 1908.

—— The further separation of antitoxin from its associated proteins in horse serum. No. III. *In* Collect. Stud. Bur. Lab. Dept. Health, city of New York, v. 7, 1912–13, p. 114–116. [1913.]

—— and Famulener, L. W. The proteins and antitoxin in the serum of goats immunized against diphtheria. *In* Collect. Stud. Bur. Lab. Dept. Health, city of New York, v. 8, p. 208–212. [1915.]

[3] Eichhorn, Adolph, Berg, W. N., and Kelser, R. A. Immunity studies on anthrax serum. In Jour. Agr. Research v. 8, no. 2, p. 37–56, 1 fig. 1917.

When received at the Pathological Division these serums were in their native state, without preservative, and had not been filtered. Shortly after their receipt they were Berkefeld filtered and preserved with 0.5 per cent chloroform in a refrigerator.

With the analytical technic described in the previous publication by Eichhorn, Berg, and Kelser,[1] experiments were made on serums anthrax 48 and 96, diphtheria 1, and tetanus 1. The experiments on anthrax 48 and 96 were made in connection with the preparation of globulin for therapeutic use. After filtering the precipitated euglobulin, with or without heating, analyses were made of the filtrates. Obviously the more pseudoglobulin that is converted into euglobulin and precipitated, the less total protein should remain in the filtrate. A large number of analyses obtained on such filtrates, together with those obtained in the experiment in which diphtheria 1 and tetanus 1 were treated with ammonium sulphate, with and without heat, are omitted here because they were inconclusive. In analyses in which 10-c. c. portions of serum or filtrates corresponding to this amount are used, the unavoidable or unknown errors were great enough to obscure the effect of the heat treatment.

HEATING THE SERUM

In the following experiments the serums were heated in a water bath maintained at 61° C. The bath was heated by gas and was provided with a thermoregulator and an electrically driven stirrer. Numerous blank experiments (8) were made in which flasks containing water or one-third saturated ammonium-sulphate solution were heated. One standard thermometer was used for taking the temperature of the water in the bath, while two others were in the flasks being heated. The bath temperature varied from 61° C. by a few tenths of a degree, the bath having been so adjusted that during the experimental heating of the serum the temperature did not rise beyond 61.2° C. nor fall below 60.5° C. This drop was caused by the introduction of the flasks at room temperature, after which the bath temperature rose to almost 61° C. The bath contained about 15 liters of water.

The serum was heated in 200-c. c. Erlenmeyer flasks in quantities of 50 c. c. Two such flasks that were lightest and therefore thinnest were chosen from two dozen. These were always used for heating the serum mixtures. The object was to bring the content up to 60° C., hold it there for exactly 30 minutes, and then rapidly cool it. These two flasks were provided with perforated rubber stoppers, each carrying a thermometer graduated in whole degrees and tested by the Bureau of Standards. The thermometer used in the water bath was tested by the Physikalisch-Technische Reichsanstalt, and was graduated in tenths of a degree. Experiment A was on anthrax 48 and 96, both

[1] Eichhorn, Adolph, Berg, W. N., and Kelser, R. A. Op. cit.

being run at the same time. Experiment B was on diphtheria 1 and tetanus 1, both also run at the same time. On account of the breaking of a centrifuge tube containing a heated diphtheria mixture, the heating was repeated next day in duplicate, so that data were obtained on the heated and unheated single portions of anthrax 48, 96, and tetanus 1, and in duplicate portions of diphtheria 1.

The procedure with a single serum was as follows: Into each of two Erlenmeyer flasks of 200-c. c. capacity, 50-c. c. portions of the serum were pipetted. One of these flasks was always a thin flask used specially for heating. To each flask 25 c. c. of water and 32 c. c. of saturated ammonium-sulphate solution were added, making a total volume of 107 c. c. This procedure was then repeated with the second serum, as it was found convenient to run two together. The two flasks not to be heated were stoppered and set aside. The two to be heated were stoppered with stoppers carrying the thermometers. In the meantime the water bath had been in readiness at 61° C., and the two flasks were introduced. All three thermometers were carefully watched and the temperatures recorded at intervals not exceeding five minutes. The temperatures inside the flasks rose from room temperature, about 27° C., to 56° in the first five minutes of the heating, then to 59° in the next five minutes. After 10 minutes' heating the temperatures inside the flasks were exactly at 60° or below it by only a small fraction of a degree. The heating was then continued for exactly 30 minutes, during which time the temperature inside the flasks did not exceed 60°. It dropped a fraction of a degree for a few minutes on three occasions, but the bath temperature during this time was between 60.7° and 61°, and consequently for all practical purposes the serums may be regarded as having been heated for exactly 30 minutes at exactly 60°, with a preheating lasting 10 minutes. At the end of the heating period the flasks were transferred to a pan containing cold water. This brought the temperature down to that of the room in about five minutes. It is believed that this is as severe heating as is necessary in the preparation of globulin. In preparing large amounts of antitoxin, Banzhaf[1] brings the temperature of the mixtures up to just 60° which required two hours' heating in a water bath kept at 66°.

METHOD OF ANALYSIS

The next step is the separation and estimation of the precipitated euglobulin. In the following scheme of analysis the precipitations of euglobulin and pseudoglobulin are made under exactly the same conditions as in the antitoxin (pseudoglobulin) preparation, and consequently the analytic data may be applied to the corresponding globulin or antitoxin preparations without error. All four mixtures were transferred to 100-c. c. centrifuge tubes, which held about 110 c. c. when

[1] Banzhaf, E. J., 1913. Op. cit., p. 115.

filled to the top. After carefully draining the flasks the small amounts remaining in them were disregarded. The tubes were centrifuged for 25 minutes at about 2,500 revolutions per minute. The sedimentation was perfect and the euglobulin was firmly packed at the bottom of the tubes. The supernatant liquids were poured into 100-c. c. volumetric flasks. They may be poured through filter paper to make certain that no particles are poured off; but this is not necessary, as the euglobulin is sticky and firmly adheres to the bottom of the tube, which may be inverted without loss of precipitate. These 100-c. c. flasks should be weighed when dry and graduated in whole cubic centimeters on the neck, as the volume of liquid poured off may be more or less than 100 c. c. by 2 or 3 c. c. The volumes should be noted to be certain that they are the same for both the heated and unheated mixtures of the same serum.

The saturated ammonium-sulphate solution used was neutral to alizarin sulphonate, and it had been filtered through cotton and hard filter paper. The specific gravities of the saturated, one-half saturated, and one-third saturated aqueous solutions of ammonium sulphate were determined with a Westphal balance and found to be as follows at 26° C.: 1.250, 1.142, and 1.089. These figures were of value in calculating from the weight of the flask and contents the amount of supernatant liquid obtained. The supernatant liquid was used for the estimation of (1) pseudoglobulin, (2) albumin, and (3) these two together, in the form of total coagulable protein.

The precipitated euglobulin in the centrifuge tubes was dissolved in water and transferred to 400-c. c. beakers. These were heated up to the boiling point to coagulate the euglobulin, which separated out in large flocks. The addition of acid was not necessary, although in some instances 1 c. c. of $N/5$ acetic acid was added to favor flocculation. The precipitates were then filtered on weighed papers, washed free from sulphate, washed with small amounts of alcohol and ether, dried to constant weight in the air oven at 100° C., and weighed.[1] In only one instance was there any difficulty in securing flocculation—namely, in a heated diphtheria-euglobulin precipitate. When this occurs, there is, of course, a loss through the passage of unprecipitated protein into the filtrate, and generally the filtrate is very cloudy. That the result would be low in the case referred to (see Tables I and II) was noted before the determination was completed. The heated euglobulin apparently was different from the unheated. The latter dissolved readily in water, forming a clear solution, while the heated euglobulin dissolved much more slowly, forming a milky suspension which became almost water-clear on standing overnight.

Table I contains the results for euglobulin obtained from 50-c. c. portions of serum.

[1] For details see Eichhorn, Adolph, Berg, W. N., and Kelser, R. A. Op. cit.

TABLE I.—*Weights of euglobulin obtained from 50-c. c. portions of serum*

Serum.	Without heat.	With heat.	Increase in weight.
	Gm.	*Gm.*	*Gm.*
Anthrax 48	0.774	1.201	0.427
Anthrax 96	.547	.793	.246
Diphtheria 1	.406	.719	.313
Do	.435	[a].642	.207
Tetanus 1	.243	.304	.061

[a] Result low, probably due to incomplete flocculation.

The figures in Table I show unmistakably that more euglobulin precipitate was obtained from the heated serum than from the unheated. Obviously, it was desirable to ascertain definitely where the excess of euglobulin came from, and to check the correctness of these single results. Concordant duplicates do not prove correctness of results in these analyses; they do prove uniformity of technic and uniformity of error.

In the supernatant liquid (filtrate) poured off the euglobulin precipitates, the following determinations were made: Total coagulable protein (pseudoglobulin plus albumin), pseudoglobulin, and albumin. The method used was as follows:

TOTAL COAGULABLE PROTEIN.—Duplicate portions of the filtrate of 20 c. c. each, containing 9.35 c. c. of serum, were transferred to 400-c. c. beakers; about 300 c. c. of water were added and the mixture brought to a boil. The coagulum, consisting of pseudoglobulin and albumin, flocculated easily. This was filtered on weighed papers as usual, dried, and weighed. The results are tabulated as item C in Table II.

PSEUDOGLOBULIN.—A single portion of 25 c. c. of the filtrate, containing 11.68 c. c. of serum, was used. To this, 10 c. c. of saturated ammonium-sulphate solution were added, resulting in 50 per cent saturation. This was done in a 100-c. c. centrifuge tube and the mixture centrifuged for 20 to 25 minutes at 2,500 revolutions per minute. The pseudoglobulin packed firmly in the bottom of the tube. The supernatant liquid was poured off and its volume noted. This is the solution used for albumin determination. The precipitated pseudoglobulin is dissolved in water, transferred to a 400-c. c. beaker, heated, coagulated, filtered, dried, and weighed as usual. The results are tabulated as item D in Table II.

ALBUMIN.—Of the above fluid from pseudoglobulin precipitation 25 c. c., containing 8.34 c. c. of serum, was diluted and heated, and the coagulated albumin filtered, dried, and weighed as usual. The results are tabulated as item E in Table II.

The figures for euglobulin in Table II, item B, are the same as those in Table I divided by 5. No corrections were made for the volume of the precipitate in any case. The main objects of obtaining these data were, as stated before, to ascertain the source of the excess of euglobulin

in the heated precipitates, and to determine the accuracy of the method of analysis. Theoretically, A should equal the sum of B and C, and C should equal the sum of D and E. Although this equality is absent, the differences appear to be consistent, and indicate a uniformity of error due, possibly, to the absence of corrections for volume of precipitate, solubility of the precipitate in the wash water, occluded ammonium sulphate, etc. These errors, however, do not invalidate a comparison of the data in one column with those of the same serum in the next.

TABLE II.—*Analyses of heated and unheated serums.*

[All weights calculated to grams in 10 c. c. of serum.]

Serum.	Anthrax 48.		Anthrax 96.		Tetanus 1.		Diphtheria 1.			
	Unheated.	Heated.	Unheated.	Heated.	Unheated.	Heated.	Unheated.	Heated.	Unheated.	Heated.
	Gm.	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
A. Total coagulable protein....	0.674	0.674	0.789	0.789	0.558	0.558	0.665	0.665	0.665	0.665
Do.....	.618	.618	.788	.788	.....	.....	.....	.....	.....	.....
B. Euglobulin.....	.155	.240	.109	.159	.049	.061	.081	.144	.087	[a].128
C. Pseudoglobulin and albumin estimated together...	.437	.378	.668	.625	.447	.451	.534	.487	.523	.484
D. Pseudoglobulin.....	.290	.211	.568	.535	.385	.378	.475	.397	.479	.438
E. Albumin.....	.191	.191	.133	.135	.103	.103	.090	.098	.087	.097
Pseudoglobulin converted into euglobulin per cent..	27		6		0.2		16		9	

[a] Result low; see footnote to Table I.

DISCUSSION OF RESULTS

It will be noticed that the figures for albumin are practically identical in the heated and unheated serums. The figures for pseudoglobulin are consistently lower in the heated serums than in the unheated, which is an almost necessary consequence of the transformation of part of the pseudoglobulin into some protein having several of the precipitation characteristics of euglobulin. What is most important for the present investigation is that the loss of pseudoglobulin in the heated serums corresponds almost quantitatively with the gain in euglobulin in the same serums.

Obviously the figures for euglobulin, pseudoglobulin, and albumin are interdependent, and an error in one may cause a corresponding error in another. The figures for total coagulable protein, however, are independent of the others, and an error in them has no direct influence on the figures for the others. The two figures for total coagulable protein in the anthrax serums were obtained January 11, 1916 (upper figures), and May 9, 1916 (lower figures). The comparatively large difference between the two consecutive determinations in anthrax 48 may perhaps be due to the action of serum protease, which continued to digest the serum proteins. Chloroform does not prevent this action when the protease is active. The figures obtained were 0.676, 0.672, and 0.616

and 0.619 gm. The averages of these are the figures recorded in Table II. The corresponding figures for anthrax 96 were 0.756, 0.789, and 0.785 and 0.792. The first figure was rejected, being obviously erroneous. The corresponding figures for the diphtheria and tetanus serums were obtained February 28, 1916. The figures for euglobulin, pseudoglobulin, and albumin were obtained in May, 1916.

The percentage of pseudoglobulin transformed into euglobulin by the heat treatment is calculated by taking the difference between the pseudoglobulin in the unheated and heated serums and dividing by the amount of pseudoglobulin in the unheated serum. These results are tabulated at the bottom of Table II. Thus, for anthrax 48 the figures are $\frac{0.290-0.211}{0.290}$, which equals 27 per cent. However, this is not the only way to calculate this figure. The results for pseudoglobulin may be obtained by subtracting the figures for albumin from those for pseudoglobulin plus albumin. If the percentage of transformation be calculated from the lower values for pseudoglobulin, the figures are 24, 8.4, −1.2, 12.4, and 11.2, respectively, reading across the bottom of Table II. It is expected that further work on the methods of analysis will yield better results.

From Table I it is apparent that the increase in euglobulin is easily determined when a 50-c. c. portion of serum is used. When, however, the filtrate is divided into several portions for the other determinations, the errors in analysis become proportionately large. It is furthermore apparent that in general the amount of pseudoglobulin transformed is not large, considering the long period of heating at a comparatively high temperature—that is, 30 minutes at 60° C. The extremely small amount of the transformation in the tetanus serum leads to the inference that while the transformation may take place when serums are heated, it does not necessarily always take place. This might not be noticed when handling large volumes of mixed serums.

This work was completed several months before the recent investigations of Homer[1] came to the author's attention. Homer states (p. 291) that there is no conversion of pseudoglobulin into euglobulin when serum mixtures were heated to 61° or 63° C. It is probable that the failure to observe the transformation was due to the use of analytical technic that was not delicate enough and to the errors incidental to the handling of large amounts of serum mixtures, as in the routine preparation of antitoxin. Better analytical results can undoubtedly be obtained when using small amounts of serum under conditions adapted to exact analysis and separate from antitoxin preparation.

[1] Homer, Annie. On the concentration of antitoxic sera. *In* Biochem. Jour., v. 10, no. 2, p. 280–307. 1916.

—— An improved method for the concentration of antitoxic sera. *In* Jour. Hyg., v. 15, no. 3, p. 388–400. 1916.

CONCLUSION

The transformation of pseudoglobulin into euglobulin was observed in four serums that had been heated 30 minutes at 60° C. in the presence of 30 per cent saturation ammonium sulphate. In some instances the amounts transformed were considerable, although in one of the serums the amount was so small as to indicate that the transformation does not take place in all serums.

The methods of analysis were improved by the use of the centrifuge as a means of separating globulin precipitates from their filtrates. The precipitations in the analyses were made at the same dilutions as in the precipitations of globulin for therapeutic use.