IV. ON THE ASSOCIATION OF ANTITOXINS WITH THE PROTEINS OF IMMUNISED HORSE SERUM.

BY ANNIE HOMER.

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THE data compiled from results obtained in the routine concentration of antitoxic horse sera show that the average loss of antitoxin, due to its association with "salt-insoluble" globulin, is greater for antitetanic than for antidiphtheritic plasma or serum thus:

Method of concentration employed	Percentage loss of antitoxin experienced with	
	Antitetanic plasma	Antidiphtheritic plasma
Banzhaf-Gibson	22	17
Banzhaf (1913)	18.5	12.5
Homer (1916, 1918)	7.5	2.5

In each of the above processes the proteins of the plasma or serum, prior to their fractional precipitation by ammonium sulphate, are subjected to a heat-denaturation which not only increases their precipitability by sulphates but also leads to a conversion of "salt-soluble" protein into a "salt-insoluble" condition.

A comparatively low value for the extent of the denaturation renders the proteins of the euglobulin-pseudoglobulin zone "salt-insoluble"; the progressive increase in the denaturation causes a corresponding conversion of the fractions of the pseudoglobulin precipitated at successively increasing concentrations of ammonium sulphate and, finally, with the further increases in the extent of the denaturation the albumin fractions of the serum proteins are also rendered "salt-insoluble."

From these observations it is evident that, in the concentration of sera, provided there has been no destruction of antitoxin during the heating of the serum, the losses of antitoxin are regulated by the extent to which the heat-denaturation has converted the antitoxin-bearing proteins into a "salt-insoluble" condition.

My previous work has demonstrated that, in the techniques adopted in the above-mentioned routine methods, the heat-denaturation is sufficient to convert the proteins of the euglobulin-pseudoglobulin zone into a "salt-insoluble" condition. These conclusions, taken in conjunction with the inferences to be drawn from the data recorded above, indicate that the association of anti-

toxin with the unheated proteins of the euglobulin-pseudoglobulin zone is greater in antitetanic than in antidiphtheritic plasma.

In order to furnish further evidence on this point a detailed study has been made of the proportional precipitation of antitoxin and protein by the addition of progressively increasing amounts of sulphate to *antitetanic* and to *antidiphtheritic* plasmas respectively.

It was found that the labour entailed in the investigation was considerably shortened by the substitution of sodium sulphate for ammonium sulphate in the fractionation of the plasma. For, with the use of sodium sulphate in contradistinction to the more toxic ammonium sulphate, there was no necessity for the dialysis of the protein precipitates or of the filtered sulphated plasmas prior to the estimation of their antitoxin content.

The method of procedure was as follows: to separate volumes of unheated antitetanic and antidiphtheritic plasma was added solid anhydrous sodium sulphate in amounts ranging from 9 to 12 per cent., i.e. in amounts sufficient to precipitate the proteins of the euglobulin and of the euglobulin-pseudoglobulin zones. The stoppered bottles containing the sulphated plasmas were heated in a water-bath at 35° until complete solution of the sulphate had been effected. The liquids were then filtered, the respective precipitates being washed on the filters with an aqueous solution of sodium sulphate of the same concentration as that of the sulphated plasma thus filtered.

The antitoxic contents of the filtrates and of the precipitates, the latter being emulsified in a measured volume of water, were estimated in the usual way and, after making the necessary corrections for dilution, etc., data were obtained as regards the percentage precipitation of the respective antitoxins at each stage with the sulphate.

Recent work has shown that the precipitation of the serum proteins and of the antitoxin at a given concentration of ammonium sulphate or of sodium sulphate is somewhat greater from plasma containing cresylic acid than from non-cresylised plasma. I have therefore included in Table I the data obtained from the precipitation of cresylised and of non-cresylised samples of antidiphtheritic and antitetanic plasmas respectively.

Table I. *Showing the percentage precipitation of the antitoxins of antitetanic and of antidiphtheritic horse plasma by anhydrous sodium sulphate.*

Percentage addition of sodium sulphate to the plasma	Percentage precipitation of antitoxin with the protein precipitated by the sulphate from			
	Non-cresylised plasma		Cresylised plasma	
	Antitetanic	Antidiphtheritic	Antitetanic	Antidiphtheritic
9	16	0	16	0
10	16	5	16	4
11	16	5	27	4
12	30	16	35	21

The study of the results given in the table show that both in cresylised and in non-cresylised plasma the precipitation of antitoxin with the proteins

of the euglobulin-pseudoglobulin zone is greater in antitetanic than in antidiphtheritic plasma.

A preliminary study has also been made of the proportional precipitation of the antitoxins of *antidysenteric* and of *antimeningitic* horse plasma with the various protein fractions, and data have been obtained with regard to the association of the respective antitoxins with the "salt-soluble" globulins from the unheated and from the heat-denaturated plasmas.

(a) *The isolation of the "salt-soluble" globulins from the unheated plasmas.*

To a measured volume of plasma was added an equal volume of a saturated solution of ammonium sulphate. The sulphated plasmas were filtered and the precipitates, after having been well washed with a half-saturated solution of ammonium sulphate, drained and well pressed, were emulsified in a small volume of water which was subsequently diluted with a saturated solution of sodium chloride until the volume of the emulsion was approximately twice that of the plasma taken for precipitation. Solid salt was added to the liquid which, after complete saturation with the salt, was allowed to stand at room temperature for 48 hours before filtration. The precipitate, consisting of insoluble protein and salt, was well washed with a saturated solution of salt and to the filtrate and washings was added 0.25 per cent. of glacial acetic acid. The acidified liquid was filtered and the precipitate was pressed and dialysed in the usual way.

(b) *The isolation of the "salt-soluble" globulins from the heat-denaturated plasmas.*

Measured volumes of the plasmas used in (a) were adjusted in the usual way so as to ensure, during the heating process, the conversion of the "salt-soluble" proteins of the euglobulin-pseudoglobulin zone into a "salt-insoluble" condition. The adjusted plasmas, contained in stoppered bottles, were heated in a water-bath at 58° for 5 hours. To each of the heated liquids was added an equal volume of a saturated solution of ammonium sulphate and the ensuing precipitate was then subjected to the same treatment as that described above for the corresponding precipitate from the unheated plasmas.

The comparison of the relative antitoxic contents of the original antidysenteric plasma and of the "salt-soluble" globulin fractions isolated therefrom, was made in terms of a laboratory standard toxin prepared by drying *Shiga bacilli in vacuo*. The determinations of the antitoxic values of the original antimeningitic plasma and of the "salt-soluble" globulins were carried out by the method advocated by Hitchens and Robinson [1916].

The values thus obtained for the titre of the respective liquids cannot be relied upon to the same extent as those furnished by the generally accepted methods for the testing of antitetanic and of antidiphtheritic sera. For this reason the data furnished in Table II are open to criticism. However, that a certain amount of reliance can be placed in these results is shown by the fact

that, as was anticipated from our previous knowledge of the heat-denaturation of antitoxic sera, the extent of the association of antitoxin with the "salt-soluble" globulins is considerably less in the heat-denaturated than in the unheated plasmas.

Table II. *Showing the percentage of the total antitoxins precipitated with the "salt-soluble" globulins of antidyenteric and antimeningitic horse sera.*

Plasma	Percentage of the total antitoxin associated with the "salt-soluble" globulins of the	
	Unheated plasmas	Heat-denaturated plasmas
Antidyenteric	50	30
Antimeningitic	30	10

The results depicted in the table are therefore sufficiently striking to lead us to the conclusion that, contrary to our experience with antitetanic and antidiphtheritic plasma, the bulk of the antitoxins in antidyenteric and antimeningitic plasma is associated with the proteins of the euglobulin and of the euglobulin-pseudoglobulin zones.

Attempts to furnish data in regard to the association of the antitoxins of antidyenteric and of antimeningitic sera with the proteins precipitated at successively increasing concentrations of sodium sulphate have been postponed until more exact methods of testing the titre of these sera are forthcoming.

The results of the present investigation are of interest as they show that the various antitoxins are adsorbed by different protein fractions of horse serum, a phenomenon which can be regarded as an indication of differences in the molecular composition of the respective antitoxins.

REFERENCE.

Hitchens and Robinson (1916). *J. Immunol.*, 1, 345.