CXI. THE AMINO-GROUPS OF THE PROTEINS OF HUMAN SERUM. ACTION OF FORMALDEHYDE AND NINHYDRIN.

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THE object of the present study was to determine quantitatively the free amino-groups in the proteins of human serum using the Sørensen titration [Obermaeyer and Wilhelm, 1913], and to investigate the reaction of ninhydrin with proteins. A product of this reaction with definite physical and chemical properties has been isolated.

EXPERIMENTAL.

Preparation of proteins free from amino-acids. 15 ml. of clear human serum were treated with 15 ml. of 10 % trichloroacetic acid. The proteins were separated on the centrifuge, completely redissolved by adding 4-5 ml. of *N* NaOH, to make slightly alkaline (phenolphthalein) and the solution made up to 30 ml. The process was then repeated using 30 ml. of 10 % trichloroacetic acid, and the redissolved proteins were made to 30 ml. Final adjustment to the phenolphthalein end-point was made with *N*/10 HCl or NaOH, with care to keep the protein in solution. Any traces of free amino-acids were found to be completely removed by this treatment.

Formaldehyde titration. 5 ml. of protein solution, adjusted to phenolphthalein as above, were treated with 2 ml. of neutral 35 % formaldehyde and 2 or 3 drops of phenolphthalein. *N*/10 NaOH was then quantitatively introduced until the medium turned reddish again. Subsequent addition of neutral formaldehyde did not change the final alkalinity. The dry weight of protein was simultaneously determined: a 5 ml. aliquot was precipitated with 5 ml. of 10 % trichloroacetic acid, centrifuged and rinsed with distilled water with care to remove no protein; the coagulated proteins were dried at 105° for 24 hours and weighed. It was found (as an average of 20 determinations showing slight individual variations) that a formaldehyde titration amounting to 0.417 ml. *N*/10 NaOH was given for every 100 mg. dry protein. Since each sample was itself a mixture of many individual sera, the result represents an average composition of human serum.

If the formaldehyde-treated protein itself was precipitated and weighed, an increase of weight was always shown. This larger weight however was not a suitable basis for quantitative comparisons.

Sørensen [1907] has shown that the formaldehyde titration is never fully quantitative and has defined conditions such as indicator end-point and formaldehyde concentration on which it depends. Under conditions approximating ours, estimation of amino-acids was about 95 % complete. Recently, Kekwick and Cannan [1936] have emphasised the importance of formaldehyde concentration on the dissociation curve of egg albumin. In our titration, the formaldehyde concentration was greater than 3 *M*, which gave us consistent figures at the phenolphthalein end-point. Much lower figures were obtained for very small concentrations (about 1/20). By applying our routine titration procedure to

pure amino-acids in order to get a comparative empirical figure, we found that 1 ml. of $N/10$ NaOH corresponded to 1.52 mg. of amino-N. From this, it appears that the average human serum contains 0.635 mg. of amino-N per 100 mg. of desiccated proteins.

To discover whether the possible denaturation of proteins by trichloroacetic acid could affect this result, the titration was repeated with proteins purified from amino-acids by ultrafiltration. A mixture of numerous sera was ultra-filtered under a pressure of 20 atmospheres N and the separated proteins redissolved in pure water and treated with formaldehyde. Then they were precipitated by trichloroacetic acid and compared with a control precipitate of an untreated portion. From these experiments an average figure of 0.65 mg. of amino-N was found for 100 mg. of desiccated proteins.

Formaldehyde-treated proteins differ from normal proteins in some properties. When once precipitated by trichloroacetic acid, they cannot be brought back into solution again. They are less sensitive to alcoholic precipitation than normal proteins. They do not react with ninhydrin.

Albumins and globulins. A mixture of sera was treated with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ at room temperature. Various fractions of proteins were separated by centrifuging at successive intervals during the following 24 hours. Every fraction was carefully purified from adsorbed $(\text{NH}_4)_2\text{SO}_4$ by repeatedly dissolving and precipitating with trichloroacetic acid, until the filtrate gave no reaction with Ba salts or Nessler reagent.

As an average, proteins collected after a few minutes (globulins) contained about 0.5 mg. amino-N per 100 mg. dry weight, whereas proteins still in solution after 24 hours (albumins) contained 0.85 mg. An actual analysis, including intermediate fractions, is illustrated in Table I. The amino-N content of the total protein (3.686 mg. per 571 mg. = 0.646 mg. per 100 mg.) closely approximates to that already recorded.

Table I.

Time of contact with ammonium sulphate	Weight of proteins collected mg.	Amino-N mg.	mg. amino-N per 100 mg. dried protein
6 min.	103.6	0.504	0.486
2 hours	123.8	0.590	0.476
6 hours	38.6	0.152	0.394
24 hours {ppt. {solution	131.0	0.760	0.580
	173.8	1.680	0.965
Total	570.8	3.686	—

The action of ninhydrin. To 5 ml. of neutral protein solution freed from amino-acids as described, 0.6 ml. of $N/10$ NaOH and 40 mg. of ninhydrin (dissolved in 2 ml. of water) were added, and the mixture kept stoppered at 37° . In a few hours, some insoluble material (amounting to about 6 mg. per 100 mg. protein and containing small variable traces of nitrogen) appeared and was removed by centrifuging. Thereafter, the mixture remained homogeneous at 37° for many days. Two days after treatment the proteins were precipitated by addition of 1.5 ml. $N/10$ HCl and centrifuged. The supernatant liquor contained no protein, provided that a demonstrable excess of ninhydrin was present. The precipitate was of a dark violet colour and could be redissolved by addition of about 2 ml. $N/10$ NaOH and again reprecipitated by $N/10$ HCl. Treatment with 10% trichloroacetic acid destroyed the compound.

Ninhydrinised proteins apparently do not react with formaldehyde. When viewed against a strong electric light, a slightly alkaline solution of ninhydrinised

proteins has a reddish colour in presence of phenolphthalein. On addition of $N/10$ HCl, a grey turbidity appears suddenly at the neutral point. Addition of formaldehyde did not seem to produce an increased acidity as usual since no turbidity became visible in a solution of ninhydrinised proteins brought exactly to the neutral point, even though a single drop of $N/10$ HCl was sufficient to produce it immediately after.

Some ninhydrinised protein has been prepared in a greater state of purity, by using ultrafiltration to avoid the use of coagulating agents. Proteins, prepared by ultrafiltration, were treated with ninhydrin as above. It was notable that the usual small precipitate was hardly apparent. Then the whole of the liquid was again ultrafiltered. In the ultrafiltrate an excess of free ninhydrin was demonstrated. The remaining protein was a violet powder which readily dissolved in pure water and gave with formaldehyde no reaction whatever. It was more readily precipitated by HCl from aqueous solutions than were normal proteins.

DISCUSSION.

In this paper, the basis of comparison has been the weight of protein as precipitated by trichloroacetic acid and dried. Such precipitates are heavier than those obtained by addition of two volumes of alcohol or of acetone. The last two are mutually similar and represent, within a somewhat narrow range of variations, about 92 % of the trichloroacetic acid precipitates. Although they presumably represent more accurately the actual weight of proteins, they cannot be redissolved in water and so were unsuited to our purpose. If reference to such alcohol or acetone precipitates is desired, the results quoted throughout should be multiplied by 1.085.

The significance of the amino-N estimation is of course restricted by the ordinary limitations of a Sørensen titration. Only amino-groups and the imino-groups of proline and hydroxyproline are ordinarily determined. It may be remarked that the total N of a solution of proteins is not altered by reaction with either formaldehyde or ninhydrin.

The difference in amino-N content of successive fractions of the serum proteins suggests that amino-N determinations might assist in the purification of proteins.

The modified proteins here described might be interesting material for studying the effect of blockage of amino-N on the antigenic behaviour of a protein. An immunological study of these proteins is now being carried out.

SUMMARY.

A quantitative study of the amino-N in the various proteins of human serum by formaldehyde titration is reported. Albumin contains more amino-N than globulins.

The existence of definite reaction products of ninhydrin with proteins from human serum is established. Purification and properties of these products are described.

The physical properties of proteins are considerably altered by combination with formaldehyde or with ninhydrin. The ninhydrin-protein compound no longer reacts with formaldehyde, and *vice versa*.

REFERENCES.

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