CCLXXIII. CHEMISTRY OF ANTIBODIES AND SERUM-PROTEINS.

I. NITROGEN DISTRIBUTION AND AMINO-ACIDS.

II. PROTEIN CARBOHYDRATE GROUPS.

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Antibodies are associated with the globulin fractions of the plasma-proteins of immune animals and are apparently inseparable therefrom. When, as a result of hyperimmunisation, large amounts of antibody appear in an animal's blood the globulin fractions frequently increase considerably. The question arises as to whether antibodies are specially synthesised proteins quite distinct from normal serum-globulins, or whether the antibodies are derived from the ordinary proteins by some slight modification enabling them to combine specifically with antigens. Our very considerable ignorance concerning the chemistry of antibodies is emphasised in most reviews of immunology [Hartley, 1931; Topley, 1933; Marrack, 1934].

In order to determine whether or not antibodies are distinguishable from normal serum-proteins a number of chemical methods may be enlisted. Success depends upon the possibility of recognising and characterising any particular protein, and it is necessary to be assured of the individuality and uniformity in behaviour of the various serum-protein fractions. It must be emphasised that negative results cannot be conclusive; that is to say, even if all the criteria applied fail to reveal any difference between an antibody and a normal serum-globulin fraction, nevertheless the two may not be identical but may differ in some respect not yet investigated. This difficulty must remain as long as the detailed constitution of the proteins remains undetermined. Notwithstanding this obvious fact the accumulation of even negative results is of considerable significance and narrows down the possibilities of difference.

Some preliminary work on the characterisation of horse serum-protein fractions and comparisons with antitoxin preparations form the substance of this communication.

Serum-proteins during immunisation.

It is not proposed to deal in the present paper with the effect of immunisation (or, more accurately, hyperimmunisation) on the concentration of proteins present in serum [Ledingham, 1909; Hartley, 1914, 1]. It is sufficient to say that an increase in the globulin fractions is frequently observed during immuni-

Table I. *Effect of immunisation with soluble toxins on horse serum-proteins.*

Protein concentrations in g. per 100 ml.

	C. diphtheriae immunisation		Haemolytic streptococcus immunisation	
	Before	After	Before	After
Globulin	4·1	9·1	4·1	7·9
Total protein	7·2	11·2	7·5	9·8

sation although a direct relationship between antibody production and globulin increase has not been traced. As examples of the results obtained, observations may be cited on two horses which yielded potent antitoxins (Table I).

Protein, antitoxin and floccule preparations.

Both sodium sulphate and ammonium sulphate were used to salt out the various globulin fractions, and the antitoxin pseudoglobulin solutions were prepared by the sodium sulphate process [MacConkey, 1924]. The antitoxins were prepared from the plasma of horses immunised with either diphtheria toxoid (formalinised toxin), or with haemolytic streptococcus toxin. Albumin fractions were crystallised or precipitated from the globulin mother-liquors. Some detailed fractionations are described later. Contrary to statements generally made in the literature, diphtheria antitoxin prepared by the sodium sulphate process still flocculates with toxin if the presence of excess acid has been avoided during the fractionation. Floccules were prepared by incubating a neutral mixture of diphtheria toxin and antitoxin. In a typical experiment 7·79 ml. of a pseudoglobulin solution containing 3400 diphtheria antitoxic units per ml. were incubated at 52° with 900 ml. of a toxin containing about 30 Lf units per 1 ml. Flocculation occurred after 40 minutes, and incubation was continued for a further 20 minutes. The mixture was then centrifuged in four 250 ml. tubes, the supernatant fluid was discarded, and the precipitate was rinsed into a 50 ml. centrifuge-tube and washed thoroughly with several changes of saline and distilled water. The floccules did not pack down into a compact solid but remained in the form of a gelatinous mass occupying a greater volume than would be expected from its low solid content (about 0·3 g.). One sample of floccules was prepared from a stock batch of antitoxin, another sample from a batch carefully freed from the last traces of euglobulin, and a third sample from an antitoxin rich in euglobulin. In the first two cases 100,000 units of antitoxin corresponded to 1·1 g. of floccules and in the last case 100,000 units corresponded to 1·4 g.

I. NITROGEN DISTRIBUTION AND AMINO-ACIDS.

Analytical methods.

The experimental procedures followed were such as to be applicable to very small quantities of material, since large quantities of floccules are not readily available.

Nitrogen partition (Hausmann numbers). The method used was based on those of Thimann [1926] and of Narayana and Sreenivasaya [1928]. For the hydrolysis, solutions containing about 0·1 g. of protein were heated on an electric sand-bath for from 18 to 48 hours with 25 ml. of 25 % hydrochloric acid. The hydrolysis was conducted in a 100 ml. pyrex Kjeldahl flask fitted with a simple glass tube air condenser with a bulb resting in the neck of the flask without cork or rubber bung. The hydrolysate was evaporated almost to dryness *in vacuo* on a hot water-bath; the residue was dissolved in water and filtered, and the volume was made up to about 20 ml. with distilled water. The figure for humin-nitrogen appeared to be fortuitously variable and without significance, hence the humin filtered off was not further investigated. Total nitrogen in the filtrate was determined by the Kjeldahl method and the usual protein : nitrogen factor of 6·25 was used for the calculations for the sake of uniformity although a somewhat higher figure is probably more accurate [Smith *et al.*, 1932].

1 or 2 ml. of the filtrate were taken for cystine determination (next paragraph), and ammonia was determined in duplicate aliquot portions, after making alkaline with sodium hydroxide, by the Folin aeration method. Vigorous aeration was found necessary for accurate results. The residual fluid was acidified with 0·6 ml. of concentrated sulphuric acid and boiled; then, to the boiling liquid in a 50 ml. centrifuge-tube were added 6 ml. of 20 % phosphotungstic acid containing 5 % of sulphuric acid. The mixture was allowed to stand in the ice-chest overnight and was then centrifuged, the solid being washed with ice-cold dilute phosphotungstic acid. The monoamino- and nonamino-nitrogen figure was obtained from the Kjeldahl-nitrogen content of the combined supernatant fluid and washings. The centrifuged solid was dissolved in dilute sodium hydroxide solution, and the nitrogen of the solution was determined by the Kjeldahl method to give the diamino-nitrogen content.

Cystine. The cystine content was determined, using a 1 or 2 ml. sample of the filtrate from the hydrolysis described above, by a modification of Folin and Marenzi's [1929] micro-method based upon Tompsett's [1931] method. A sample containing about 2 mg. of nitrogen was taken for each determination and the colour compared with that produced by 0·5 mg. of cystine, the volume being made up to 25 ml. The reagents used were 0·5 ml. of 30 % sodium bisulphite, and after 1 minute, 5 ml. of 10 % sodium bicarbonate and 2 ml. of the uric acid reagent (freed from phenol reagent). No lithium salts were found necessary. The step photometer was used successfully in one or two cases to assist in matching the blue colour in the presence of yellow pigments.

Tyrosine and tryptophan. For the determination of tyrosine and tryptophan Folin and Marenzi's [1929] procedure was followed except that the use of 50 ml. colorimetric solutions in place of 100 ml. accelerated the determination. In the tryptophan determination the addition of cyanide to the colorimetric solutions was abandoned as being of no advantage. An electric sand-bath proved a convenient heat-source for the alkaline hydrolysis.

Results.

Using the methods outlined above 0·2 g. of a protein preparation was sufficient for the determination of amide-nitrogen, monoamino- and nonamino-nitrogen, diamino-nitrogen, cystine, tyrosine and tryptophan. The results are summarised in Table II.

Table II.

	Crystalline albumin		Globulin fractions		Diphtheria floccules	
	Range %	Mean %	Range %	Mean %	Range %	Mean %
Amide-nitrogen	4·7 – 5·4	5·1	8·1 – 9·4	8·9	8·4 – 9·5	9·1
Monoamino-N	58·4 –61·9	60·0	61·9 –72·9	66·9	63·8 –70·5	67·8
Diamino-N	29·7 –33·0	31·6	20·8 –29·4	24·3	22·0 –23·7	22·9
Cystine	5·35– 5·45	5·4	1·87– 3·54	2·76	1·8 – 2·38	2·05
Tyrosine	4·7 – 4·7	4·7	6·39– 6·80	6·53	5·55– 6·2	5·85
Tryptophan	0·44– 0·52	0·48	1·45– 2·32	1·78	1·67– 1·90	1·80

The nitrogen partition figures are in agreement with the results of Hartley [1914, 2] and also with those of Lustig and Haas [1931] except that the globulin amide-nitrogen figures of the last authors appear to be too low. The albumin amino-acid figures compared with those of Folin and Marenzi [1929] are as follows, the present figures following those of Folin and Marenzi in each case: cystine, 6, 5·4 %; tyrosine, 4·7, 4·7 %; tryptophan, 0·53, 0·48 %. It will be observed that there is good agreement in each case. Comparatively few figures

for serum-globulin are given in the literature; the variations shown in Table II are probably extreme values since very different fractions were employed deliberately in order to obtain the widest possible range of values. It will be seen at once that albumin may easily be distinguished from globulin in respect of each analytical constituent. Although the globulin preparations included highly potent diphtheria antitoxins and haemolytic streptococcus antitoxins, yet these could not be distinguished from each other or from antitoxin-free preparations. Perhaps this is not surprising since only a comparatively small proportion of even a highly potent antitoxic globulin consists of actual antitoxin, and inactive serum-proteins constitute the bulk of the material present. However, the floccules, obtained by incubating a balanced mixture of diphtheria toxin and antitoxin, probably represent an approximation to chemically pure diphtheria antitoxin and three preparations of floccules were subjected to analysis. Even in the case of these floccules the analytical results fall within the range of values obtained for serum-globulins, and no significant difference was observed with the possible exceptions of tyrosine and cystine which appeared to be somewhat less in amount in the floccules. It would not, however, be safe to base any wide conclusions on such comparatively slight differences.

II. PROTEIN CARBOHYDRATE GROUPS.

Protein-carbohydrate determinations with orcinol.

The Tillmans and Phillipi [1929] method in which the protein is heated with orcinol and sulphuric acid has been carefully examined and elaborated by Sørensen and Haugaard [1933]. In the original method the brown colour developed is compared with that produced by a standard glucose solution but the colours produced with serum-proteins do not match the glucose standards well. Sørensen and Haugaard found however that a good match can be obtained if a galactose-mannose mixture is used instead of glucose for colorimetric comparison, the comparison being conducted by using a photometer in place of a simple colorimeter. Rimington [1931] showed that the carbohydrate group in serum-proteins is a complex of two molecules of hexose and one of glucosamine. Glucosamine does not react in the Tillmans and Phillipi reaction. In the present work it will be assumed therefore that the serum-protein carbohydrate complex is galactose-mannose-glucosamine, and no facts encountered were incompatible with this hypothesis [Bierry, 1934].

In the earliest experiments described in this paper the Tillmans and Phillipi directions for the reaction were followed. Later a galactose-mannose solution was substituted for the glucose standard, and finally a photometer was employed and the Sørensen and Haugaard method was followed precisely. An important point which was overlooked for some considerable time, and may easily lead to serious error, is the effect of light on the colorimetric solutions. Even the diffused daylight of a north-lit room is sufficient to interfere seriously with the colorimetric solutions after heating with orcinol and sulphuric acid. The colour remains constant in the dark for a considerable time but the solutions after heating should be cooled in a darkened vessel and stored in the dark until colorimetric comparison is made. In a trial experiment the extinction coefficient for yellow light ($\lambda = 570 m\mu$) of a protein solution heated with orcinol and sulphuric acid rose from 0·06 to 0·17 after standing 2 hours on the bench in a room facing the north sky. There was no perceptible change in a sample kept in a dark-room for the same period. A blank solution in which orcinol was

heated alone with sulphuric acid had an extinction coefficient of 0·03 for yellow light which remained unchanged in the dark but rose to 0·23 after 2 hours in diffused daylight. In these cases the absorption at the red end of the spectrum is affected more than that at the blue end. Having ensured the use of the correct comparison solutions and having shielded the colorimetric solutions from light, the chief remaining difficulty of the method lies in the use of solutions for the "blank" determinations. The two solutions that can be used for the purpose are the protein alone heated with sulphuric acid, and orcinol alone heated with sulphuric acid. The "blank" value of these two is sometimes disproportionately high compared with that of the protein heated with orcinol and sulphuric acid together, and the protein solution tends to fluoresce and to change colour even in the dark. An attempt was made to surmount this difficulty by using for "blank" determinations a protein containing no carbohydrate and heating this with orcinol and sulphuric acid as in an ordinary determination. Gelatin was selected as being the most favourable available protein for the purpose, but, unfortunately, both the laboratory stock specimens and Coignet's Gold Label gelatin appear to contain 0·4 % of a carbohydrate which behaves similarly to serum-protein carbohydrate. Gelatin, therefore, cannot be used for the purpose and the problem of the "blank" remains a difficult one.

Results.

The first results obtained with serum-proteins were for crystalline albumin, 0·78 %, and for globulin 3·3 % of carbohydrate, both calculated as galactose-mannose-glucosamine (g.m.g.). This value for crystalline albumin is in good agreement with molecular weight determinations (see Discussion) but the agreement is probably quite fortuitous. With one sample of horse serum the albumin precipitated between the limits of 50 and 60 % saturation of ammonium sulphate had a carbohydrate content of 1·35 % g.m.g. whilst that precipitated at 60–70 % saturation had 4·39 % g.m.g. From another sample of serum, globulins were removed by half saturation with ammonium sulphate. A portion of the albumin in the filtrate crystallised out on adding acetic acid, and the remainder separated out on adding more ammonium sulphate. By a series of fractionations two samples of albumin were isolated of widely different solubilities in salt solutions. Before carbohydrate determinations were carried out the proteins were coagulated by boiling, the coagulum was washed thoroughly in a centrifuge-tube with boiling water, and the denatured protein was dissolved in dilute alkali. After four recrystallisations the less soluble albumin had a carbohydrate content of 0·52 % g.m.g., and after nine crystallisations this had fallen to 0·29 %. This low value is not in accord with any molecular weight figures for albumin and suggests that if the fractionation were continued long enough an albumin free from carbohydrate could be prepared. The more soluble albumin prepared had a carbohydrate content of 4·77 % g.m.g. This fraction had remained in the filtrate four times during the fractionation and on three occasions had been precipitated with ammonium sulphate, before being coagulated and washed.

Globulin fractions also differ widely in carbohydrate content. As an example there is the case of a sample of serum fractionated with sodium sulphate; the globulin precipitated between the limits 14 to 18 % of sodium sulphate contained 2·2 % of carbohydrate (g.m.g.) whilst the 18 to 22 % fraction contained 4·5 %, and the albumin in the 22 % filtrate contained 3·2 % of g.m.g. A sample of serum from a horse immunised with diphtheria toxoid is of some interest in showing the variation of the antitoxin in the different fractions. Both the

variation in carbohydrate content of the fractions and the range in time of flocculation, under standard conditions, from 43 to 100 minutes are very striking. The rapidity of flocculation does not run *pari passu* with the purity of the antitoxin, as measured in units per g. of protein. Barr *et al.* [1931] have reported other serological differences in different fractions of antitoxic plasma.

Table III. *Carbohydrate and antitoxin contents of horse serum-globulin fractions.*

Sodium sulphate fraction g. per 100 ml.	Carbohydrate (g.m.g.) g. per 100 ml.	Diphtheria antitoxin units per 1 g. protein	Time of flocculation mins.
10–14	2·64	5,340	43
14–18	3·45	20,750	70
18–22	4·44	18,640	100
22 filtrate (albumin)	2·61	—	—

The floccules prepared from diphtheria toxin and antitoxin as previously described were examined in order to determine whether they were free from carbohydrate or whether the carbohydrate accompanied the antitoxin in its specific union with diphtheria toxin. The flocculation might well be expected to separate the antitoxin from any material not firmly combined in the protein molecule. The carbohydrate content, in addition, appears to provide a delicate test for the differentiation of protein fractions. It was found that the floccules contained carbohydrate and in about the same range of concentration as serum-globulin fractions. The three samples of floccules were prepared as described previously from differently fractionated antitoxin preparations, and the carbohydrate contents were 2·61, 3·52 and 4·2 % of g.m.g. (mean 3·4 %) as compared with a range of 2·2 to 4·4 % in the different globulin fractions examined. Again, therefore, we find that diphtheria toxin-antitoxin floccules are indistinguishable from ordinary serum-globulins.

Discussion.

The analytical figures obtained (see Table II) for albumins and globulins are consistent and in accord with results reported by other workers. In the case of each constituent the figures are significantly different for the two proteins. It was not however found possible to distinguish diphtheria antitoxin from a streptococcus antitoxin preparation, or to distinguish either from normal serum-globulin. When however a balanced mixture of diphtheria toxin and antitoxin is incubated a specific flocculation occurs and the precipitated floccules contain the greater part of the toxin and antitoxin originally present [Ramon, 1923; Hartley, 1926] although they constitute less than 1 % of the total material present in the balanced mixture and less than one-third of the protein present in the original antitoxin preparation. Different workers using differently prepared materials unite in finding that about 100,000 units of antitoxin yield about 1 g. of floccules, and the contribution of the toxin to the bulk of the material is small. There is strong presumptive evidence therefore that from the chemical point of view floccules represent very highly purified diphtheria antitoxin, if not completely pure. Marrack and Smith [1930] failed by a spectrometric method to distinguish between floccules and serum-globulin. In the chemical investigations now reported the behaviour of floccules appeared to be identical with that of serum-globulin, as shown by the analytical figures for nitrogen distribution and for various amino-acids (summarised in Table II).

The only possibly significant differences are in the cystine and tyrosine figures which tend to a lower value in the case of floccules than with globulin fractions. The amide-, monoamino- and diamino-nitrogen figures and the tryptophan contents are, however, very closely similar, and it must be concluded that no difference has been firmly established.

The recognition of the presence in the protein molecule of carbohydrate groups opens up aspects of protein structure which have received very little attention. It is extremely difficult to harmonise the view that normal serum-proteins contain 3 % or even more of carbohydrate with the usual theories of protein chemistry. As the cumulative effect of the work of many investigators over a period of 40 years it is established that the carbohydrate group in serum proteins is probably galactose-mannose-glucosamine (g.m.g.) or some very similar grouping [Rimington, 1929; 1931; Sørensen and Haugaard, 1933]. The quantitative determination of the carbohydrate groups in serum-proteins leads to some interesting conclusions on the homogeneity of the fractions.

By a coincidence the first crystalline serum-albumin examined in the present investigation possessed a carbohydrate content of 0·78 % (calculated as g.m.g.). Assuming that there is one molecule of this group present in each protein molecule the molecular weight of serum-albumin can be calculated as $100/0{\cdot}78 \times 537 = 68{,}840$, a value in good agreement with the commonly accepted values (67,500–69,000) of Svedberg. That this agreement was quite accidental became obvious when, by fractional crystallisation and salting out methods, it was found possible to reduce the carbohydrate content to 0·52 % after four crystallisations and to 0·29 % after nine crystallisations. On the other hand the more soluble fractions had values ranging up to as high a value as 4·77 %. These extreme values of 0·29 and 4·77 % were observed with fractions of the same original sample of albumin. Sørensen and Haugaard [1933] also describe albumin fractions of differing carbohydrate content. Unless it is claimed that the carbohydrate grouping is a fortuitous contamination of the albumin it must be conceded that, by fractionation, albumins of different properties and structure may be isolated. The fact that the carbohydrate accompanies the albumin through salting out operations, crystallisations, dialysis and even coagulation by boiling rules out at least some of the possibilities of impurities in the albumin.

Serum-globulins were also found to be separable into different fractions; by salting out methods, fractions with as low a carbohydrate content as 2·2 % and as high as 4·4 % were isolated, the more soluble fractions again having the higher carbohydrate content. There seems to be however an abrupt break between the carbohydrate contents of the most soluble globulin (over 4 % g.m.g.) and of the least soluble albumin (under 1 % g.m.g.). The tenacity with which the carbohydrate clings to the globulins through various purification processes lends weight against the hypothesis that the protein-carbohydrate combination is a simple adsorption, and the difficulty of hydrolysing the carbohydrate-protein linking provides evidence in the same direction.

The examination of the carbohydrate content of diphtheria toxin-antitoxin floccules is of some importance for two reasons:

(1) If the carbohydrate is not an integral part of the protein molecule a specific serological reaction such as the flocculation reaction may separate the carbohydrate, which would not be expected to accompany antitoxin into its specific union with toxin.

(2) If the carbohydrate does accompany antitoxin in floccules the amount present may provide a delicate test for differentiating antitoxin from ordinary serum-globulins.

Actually it was found that the different samples of floccules contained carbohydrate in a similar range of concentrations (mean value 3·45 % g.m.g.) to that of ordinary serum-globulins. Again, therefore, it must be concluded that the carbohydrate cannot be separated from the protein and that antitoxin cannot be differentiated from serum-globulins.

Evidence has accumulated to support the view that the carbohydrate group forms an integral part of the protein molecule; this, together with the variation in the carbohydrate content of different fractions, presents a strong case for the existence in serum of more than one globulin and more than one albumin, these fractions having different chemical and physical properties. Polarimetric evidence [Hewitt, 1927] supports the view of the existence of different globulins.

Summary.

1. It was not found possible by the chemical methods employed to differentiate antitoxins or diphtheria toxin-antitoxin floccules from ordinary serum-globulins.

2. Serum-globulin and crystalline serum-albumin apparently are not homogeneous entities but can be separated into fractions of widely different carbohydrate content.

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