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The Spectrophotometric Determination of Protein at 210 mμ

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The absorption spectra of proteins in the region above 240 mμ are well known and extensively used in analytical procedures. The peak in the region of 280 mμ may be used for the analysis of tyrosine and tryptophan (Beaven & Holiday, 1952), and measurement of this peak provides, for relatively homogeneous proteins, a simple and accurate method of determining relative concentrations (e.g. Tombs, 1957). With more complex mixtures such as serum the various proteins will contain widely varying amounts of tyrosine, tryptophan and phenylalanine, and measurements at 280 mμ can no longer be used as a reliable index of total protein content, even where a comparison between one serum and another is all that is required.

Below 230 mμ the absorption of both proteins and peptides rises steeply. However, Goldfarb, Saidel & Mosovich (1951) have shown that there is a definite maximum near 190 mμ which is due mainly to the specific absorption of peptide bonds. If this is so one might expect that all proteins would have a similar specific absorption in this region, since they all have similar peptide-bond contents. Goldfarb *et al.* (1951) report figures which suggest that this is indeed the case for human and bovine albumin and γ-globulin, egg albumin and gelatin. It seemed possible therefore that measurement in the neighbourhood of this peak might offer a rapid and simple method for the estimation of proteins.

A method involving measurement at 215 and 225 mμ has recently been proposed by Waddell (1956) and Bendixen (1957). The choice of a suitable wavelength in this region depends partly on instrumental limitations and partly on the possible contribution of the aromatic amino acids to the specific absorption of individual proteins. Results described here and briefly reported elsewhere (Tombs, Souter & Maclagan, 1959) suggest that 210 mμ is the optimum wavelength for our conditions of measurement.

EXPERIMENTAL AND RESULTS

Nitrogen estimations. Total protein from 0·1 ml. of serum was precipitated with aluminium tungstate (Lorant, 1957), and nitrogen in the precipitate was determined by the micro-Kjeldahl method. A factor of 6·25 was used to convert nitrogen content into protein content.

Absorption measurements. These were made with a standard Hilger Uvispek spectrophotometer, but similar results have also been obtained with a Unicam SP. 500 spectrophotometer. Paired 1 cm. silica cells were used with solvent as blank. A slit width of 1·4 mm. was used at 210 mμ. Cells were cleaned in conc. HNO_3 and cell-matching was checked at frequent intervals.

Solvents. Measurements made in water, 0·9% NaCl soln. and phosphate buffers were identical, but 0·9% NaCl was generally adopted to avoid the possibility of precipitating abnormal globulins. Gramicidin, however, had to be dissolved in absolute ethanol, and its absorption was measured against ethanol as blank; the absorption of absolute ethanol at 210 mμ was sufficiently low (0·45) to permit its use as a solvent.

Collection and storage of serum. Serum samples were taken from those used for normal routine laboratory estimations. They were either used on the day of collection or stored in the frozen state at −15°; repeated freezing and thawing had no effect on the absorption at 210 mμ.

Previous work suggested that 205 mμ might be the wavelength of choice, but with our instrument it was found that 210 mμ was the lowest practicable. Table 1 shows some data which, although not exhaustive, indicate that at this wavelength a considerable variety of serum proteins have specific extinctions close to 200 although there are wide differences between their specific extinctions at 280 mμ.

Gramicidin (40% of tryptophan) shows much greater absorption at both wavelengths, and it is evident for reasons given below that this feature would be still more evident at wavelengths longer than 210 mμ. Subsequent measurements were therefore made at this point.

Instrumental limitations. Measurements made on the same instrument over a period of several months showed a steadily increasing degree of deviation from linearity, particularly at extinctions over 1·0 (Fig. 1), and a marked reduction in absorption at wavelengths below 210 mμ (Fig. 2). These effects are characteristic of stray radiation, accentuated by deterioration of optical components and ageing of the lamp (Scott, 1955). Although correction for stray radiation can be made it is laborious for a routine method. It is preferable to use the instrument in conditions where stray radiation is unimportant, which can be done by cleaning the optical faces and replacing the lamp as soon as any deviation from Beer's Law is noted. We have found it essential to employ the two standards described below with each batch of measurements to ensure that a linear response is obtained, and it is desirable to avoid the use of extinction values over 1·0.

Reference standard. Any known protein solution will serve as a suitable standard. We have found bovine albumin (6·0 g./100 ml.) to be suitable. Two dilutions of 1/1500 and 1/3000 (v/v) were

Table 1. *Specific absorption of some typical serum proteins*

Protein preparation	$E^{1\%}_{1\,cm.}$ 280 mμ	210 mμ
Total serum proteins	About 10	205 (±1·4)
Human albumin	6	203
Human γ-globulin	15	213
Human siderophilin	14	200
Mixed α- and β-globulin	13	214
Bovine albumin	6·8	204
Gramicidin	62	342

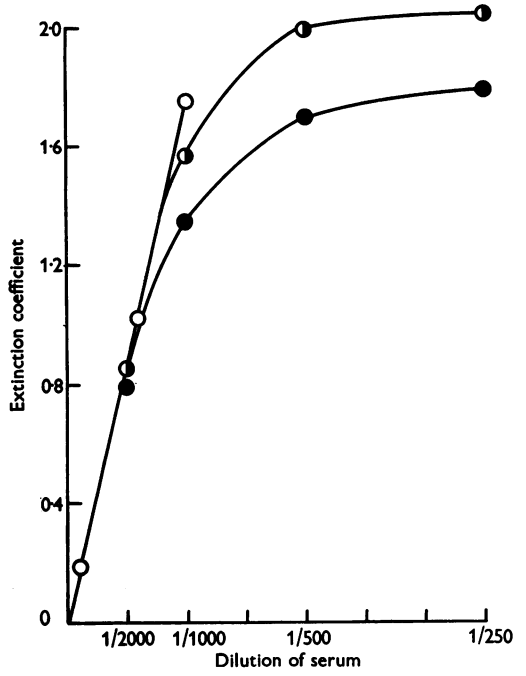

Fig. 1. Deviations from Beer's Law due to ageing of the hydrogen lamp. ○, New lamp; ●, same lamp after about 600 hr. use; ◑, reactivated lamp.

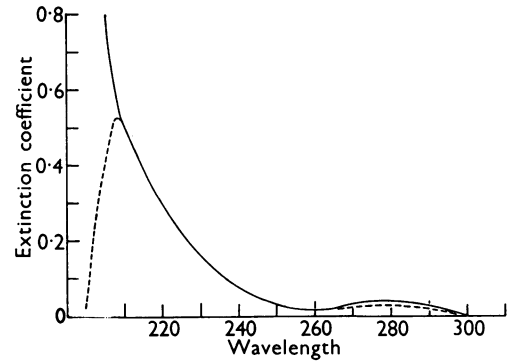

Fig. 2. Absorption spectrum of 1/1000 dilutions of two sera showing the effect of using an aged hydrogen lamp. —, New lamp; - - - -, aged lamp (approx. 600 hr.). The curve measured with the new lamp has been adjusted so as to coincide with the other over the range 210–240 mμ.

used for reasons given below. The stock solution remained stable for at least a week at 4°.

Procedure for total serum-protein determination. Serum (0·1 ml.) was diluted to 200 ml. with 0·9% NaCl soln. and the extinction was measured at 210 mμ within 2 hr. of dilution.

Results were considered acceptable if twice the low standard reading was within 3% of that given by the high standard. Total serum protein was then calculated by simple proportionality from the serum and standard readings.

Serum proteins. Isolated fractions were obtained by column chromatography on cellulose derivatives (Sober, Gutter, Wyckoff & Peterson, 1956) by unpublished methods.

The specific extinction of the total proteins of 40 normal and pathological sera at 210 mμ, $E^{1\%}_{1\,cm.}$ was 205 ± 1·4 (S.E.), range 194–226. This was based on Kjeldahl nitrogen determinations and agrees well with Goldfarb *et al.* (1951), who found for human γ-globulin 208 and for albumin 201, determined on a weight basis. The conclusion that the majority of serum proteins have closely similar specific extinctions at 210 mμ was reinforced by the results given in Fig. 3, which show a linear relationship between extinction at 210 mμ and protein nitrogen in 26 miscellaneous sera. Linearity in the extinction range 0–1·7 was checked with more dilute protein solutions. Although the results shown in Fig. 3 were measured at 1/1000 dilution of serum and the instrument was satisfactory at this time, it was subsequently found that deterioration of the lamp made values measured at a 1/1000 dilution more liable to error than at a 1/2000 dilution. For this reason a 1/2000 dilution of serum was adopted as standard.

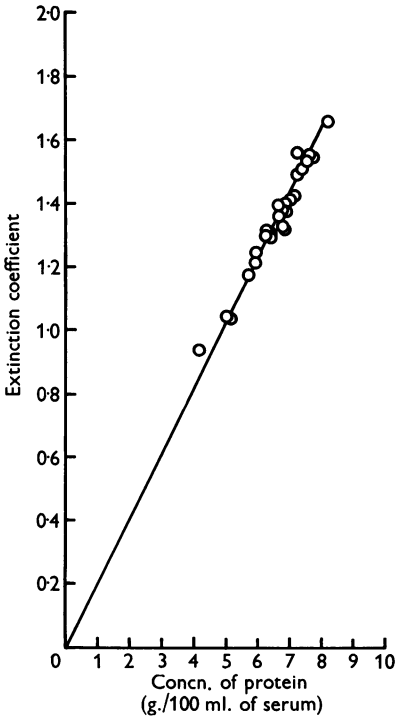

Fig. 3. Total serum protein determined by Kjeldahl analysis, plotted against the extinction coefficient of a 1/1000 dilution of serum, in 26 samples.

Measurement of sera taken from the same individual before and after feeding showed no detectable effect of ordinary degrees of lipaemia. In one individual there was an apparent increase of 0·3 g. of protein/100 ml. of serum after fat-feeding, but this serum showed an exceptional degree of lipaemia. pH variation between 2 and 10 had little effect on absorption. The extinction of 1/1000 or 1/2000 dilutions of serum remained constant for 2 hr., and then rose by about 2% over the next 4 hr. It is therefore important to make measurements within 2 hr. of dilution.

Albumin determination. Half-saturation with $(NH_4)SO_4$ was employed as follows: 0·1 ml. of serum was added to 4·9 ml. of $(NH_4)_2SO_4$ solution (2·09 M; 276 g./l.). After centrifuging in an angle-head centrifuge at 6000 *g* for 10 min., albumin was estimated on a suitable dilution of the supernatant fluid (usually 0·5 ml. + 9·5 ml. of water). Alternatively, globulins were precipitated by 21% (w/v) Na_2SO_4 (Howe, 1921) and albumin was estimated in the filtrate.

In the determination of albumin:globulin ratios $(NH_4)_2SO_4$ is preferable to Na_2SO_4 for the precipitation of globulins because the mixture can be centrifuged at room temperature. Table 2 shows reasonable agreement between the results obtained when $(NH_4)_2SO_4$ and Na_2SO_4 are used for precipitation, followed by spectrometric estimation of the albumin. Table 3 shows the results of spectrometric estimations with $(NH_4)_2SO_4$ used for precipitation, and routine laboratory measurements with Na_2SO_4 and micro-Kjeldahl nitrogen estimations. The agreement was not exact but was within the reproducibility normally found for this determination, and the two methods showed no systematic difference.

DISCUSSION

The wavelength chosen for this work appears to be better than those previously employed because it diminishes greatly the variations in specific extinction between different proteins. This is especially true of serum proteins since all the examples which we have tested showed closely similar specific extinctions.

There is a large number of possible interfering factors since all carboxylic acid and buffer ions,

alcohols, bicarbonate and aromatic compounds absorb in this region. However, with serum the overwhelming majority of absorbing material is the protein and this, coupled with the very high specific extinction of proteins in this region, reduces the contribution to the absorption from substances other than protein to negligible levels. This was shown by the fact that the absorption of protein-free filtrates of serum prepared by the zinc hydroxide precipitation method was approximately 0·5 % of that of the original serum. (Values up to 0·6 % were, however, obtained with citrated bank plasma, owing to the high level of citrate.) Although a similar method has been successfully employed on cerebrospinal fluid (Waddell, 1956), it cannot be used on urine where the levels of interfering substances are much higher. There are also severe restrictions on permissible solvents, e.g. buffers containing carboxylic acids cannot be used.

Although a large part of the absorption at 210 mμ is due specifically to peptide bonds, the aromatic amino acids, particularly tryptophan, also make an appreciable contribution (approximately one-quarter of the total for human albumin), and it was to be expected that proteins rich in these amino acids would have higher specific extinctions than others. Thus Goldfarb *et al.* (1951) found $E^{1\%}_{1\,cm.}$ for insulin (12 % of tyrosine, 8 % of phenylalanine) to be about 250. A more extreme case is that of gramicidin, containing about 40 % of tryphophan, for which we found a specific extinction of 340 (average for serum proteins 205).

The absorption spectrum of tryptophan rises steeply below 230 mμ to a maximum at 220 mμ, shows a minimum at 205 mμ and then rises steeply again (Saidel, 1955), whereas the curve for proteins shows no minimum in this region. Tyrosine and phenylalanine behave similarly to tryptophan although their specific absorptions are lower. Values around 220 mμ are therefore undesirable, and 205 mμ is the wavelength at which the aromatic amino acids make the least contribution to the total absorption. Gramicidin shows a maximum at 225 mμ, no doubt due to its tryptophan content. For technical reasons (see Experimental and Results), 210 mμ was the lowest wavelength at which we could operate consistently and appears to be the optimum value for the instruments employed. Further, the specific extinction of tryptophan is the same at 210 mμ and about 227 mμ, so that differential measurements near these two wavelengths could be used in principle to eliminate the contribution of tryptophan completely. Waddell (1956) has described a method

Table 2. *Comparison of ammonium sulphate* (2·05 M) *and sodium sulphate* (1·47 M) *for globulin precipitation*

Estimations were made by the spectrometric method (see text). A/G, Albumin:globulin ratio.

	Pptn. with 2·05 M-$(NH_4)_2SO_4$			Pptn. with 1·47 M-Na_2SO_4		
Serum	Albumin (g./100 ml.)	Globulin (g./100 ml.)	A/G	Albumin (g./100 ml.)	Globulin (g./100 ml.)	A/G
1	4·1	2·5	1·7	4·1	2·5	1·7
2	3·7	2·5	1·5	4·2	2·0	2·1
3	2·6	2·2	1·2	2·8	2·0	1·4
4	4·4	3·3	1·3	4·3	3·5	1·2
5	6·1	1·3	4·7	5·4	1·4	3·9
6	3·4	2·6	1·3	3·1	2·8	1·1
7	1·9	2·8	0·7	1·9	2·8	0·7
8	4·4	2·6	1·7	4·9	2·2	2·1
9	3·5	3·0	1·1	3·7	2·8	1·3
10	4·4	3·9	1·1	4·4	3·8	1·1

Table 3. *Comparison of albumin: globulin (A/G) ratios by Kjeldahl (sodium sulphate) and spectroscopic (ammonium sulphate) methods*

Serum	Albumin (Kjeldahl) (g./100 ml.)	Albumin (spect.) (g./100 ml.)	Globulin (Kjeldahl) (g./100 ml.)	Globulin (spect.) (g./100 ml.)	A/G (Kjeldahl)	A/G (spect.)
1	3·2	2·8	3·1	3·2	1·0	0·9
2	3·3	2·8	5·3	3·9	0·6	0·7
3	3·5	3·5	3·6	3·2	1·0	1·1
4	5·8	6·1	1·6	1·3	3·5	4·7
5	4·4	4·1	3·0	2·5	1·5	1·7
6	2·2	2·3	1·9	2·4	1·1	1·1
7	4·3	4·4	3·5	3·9	1·2	1·1
8	4·7	4·4	3·1	3·3	1·5	1·3
9	4·7	4·6	2·6	2·1	1·8	2·2
10	3·3	3·0	2·6	2·9	1·2	1·0
11	1·9	1·9	3·3	2·8	0·6	0·65

based on the peptide-bond absorption, differential measurements being used in which the extinction at 225 mμ is subtracted from that at 215. The use of these wavelengths would not, however, have the effect of reducing the contribution of the aromatic amino acids, and cannot be applied to cases such as gramicidin, since this peptide actually has a greater absorption at 225 than at 215 mμ.

From our results it seems that measurement in the region of 210 mμ can be used as a reliable, precise and quick method for the estimation of serum proteins. The use of measurements at this wavelength also appears to provide a fairly general method of protein estimation, and serious error is unlikely unless the protein contains exceptionally large amounts of aromatic amino acids, as with gramicidin or insulin.

A further advantage of this method lies in the great sensitivity. When no interfering substances were present it was possible to detect concentrations down to 2 μg. of protein/ml., corresponding to an extinction of 0·04. Advantage was taken of this in experiments with anion-exchange cellulose for the column chromatography of serum proteins. Amounts of serum as low as 0·1 ml. were used satisfactorily and the quantitative interpretation of results was aided by the uniformity of specific absorption in the various fractions.

SUMMARY

1. Absorption measurements at 210 mμ can be usefully employed for protein estimation. At this wavelength the total proteins of 40 normal and pathological sera had a mean specific extinction coefficient $E^{0.1\%}_{1\,cm.}$ of 20·5 ± 0·14. Albumin, α-, β- and γ-globulin all had specific extinction $E^{0.1\%}_{1\,cm.}$ near 20. The method is directly applicable to all proteins studied except those containing unusually large amounts of aromatic amino acids.

2. A method for the estimation of total serum proteins and for albumin:globulin ratios based on the above is described. Bovine serum albumin had almost the same specific extinction coefficient as serum proteins and formed a useful reference standard.

3. The use of the extinction at 210 mμ for the detection of very small quantities of protein (2 μg./ml.) and its application to column chromatography are discussed. In this connexion it is necessary to avoid the use of buffers containing carboxylic acids.

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Some Properties of Uterus Actomyosin and Myofilaments

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Some properties of the actomyosin-like adenosine triphosphatase of the uterus have already been described (see Needham & Cawkwell, 1956). It was shown that this enzyme splits off only one phosphate group and has a pH–activity curve in 0·5M-potassium chloride resembling that of myosin from skeletal muscle. The activity, however, is much lower than that of skeletal-muscle actomyosin; rabbit- and pig-uterus actomyosin (with which most of this work was done) also differ from skeletal muscle actomyosin (which is somewhat inhibited at higher concentrations of potassium chloride) in being less active in media containing 0·1M-potassium chloride than in those containing 0·5M-potassium chloride. Thus at 28°, with Ca^{2+} ion activation, the values for μmoles of inorganic phosphorus liberated/mg. of nitrogen/min. are about 0·2 and 0·6 in media containing 0·1 and