The Apparent Arginine Content of Human Plasma Proteins by the Sakaguchi Reaction

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(Received 19 March 1948)

The Sakaguchi (1925) reaction consists in the formation of an unstable red compound on treatment of a protein with sodium hydroxide, α -naphthol and sodium hypochlorite. It is given by certain guanidine derivatives, notably arginine. Sakaguchi found that the intensities of the colours developed by serum albumin and globulin were approximately equal under the conditions he employed; but no conclusions could be drawn as to the actual arginine content of these unhydrolyzed proteins, since some proteins known to contain very little arginine gave a relatively stronger reaction, weight for weight, than others rich in the amino-acid. A modification of the reaction was applied quantitatively to protein hydrolysates by Weber (1930), who substituted sodium hypobromite for hypochlorite and stabilized the colour by adding urea. Weber also observed that the colorimetric determination of arginine in unhydrolyzed proteins was not possible, since the colour values found were much less than would be expected from the actual arginine content (cf. also Calvery, 1945). A modification of Weber's hypobromite method was applied to protein hydrolysates by Jorpes & Thorén (1932), who found that the apparent arginine content decreases linearly with increase in the amount of arginine used for analysis: extrapolation to zero concentration would thus be expected to give a close approximation to the true arginine value. Using this technique, which is stated to give results in satisfactory agreement with those obtained by isolation of the amino-acid as the mono- and di-flavianates, Brand and his co-workers (Brand & Kassell, 1942; Brand, Kassell & Saidel, 1944; see also Brand & Edsall, 1947) found the following values for the arginine content of human plasma proteins (in g./100 g. of protein): albumin 6.2, γ -globulin 4.8, β -globulin 6.8, α -globulin 7.7, fibrin 7.9. Other workers found somewhat lower values by the application of isolation procedures (Block, 1938), e.g. 5.3% for albumin and 4.7% for globulin (Murrill, Block & Newburgh, 1940).

The present investigation was undertaken as a result of the recent publication by Albanese and his co-workers (Albanese & Frankston, 1945; Albanese, Irby & Saur, 1946; Albanese, Saur & Irby, 1947) of a modification of the Sakaguchi reaction which they used for the estimation of unhydrolyzed plasma

proteins. They claimed that when alkaline solutions of plasma proteins were submitted to this modified Sakaguchi reaction and compared with an arginine standard, values were obtained which, when multiplied by a factor, gave figures closely approximating to those obtained by the micro-Kjeldahl method. The present findings with plasma proteins differ from those of Albanese and his colleagues in several respects.

METHODS

The Albanese method consists in taking a 1 ml. sample, containing about 3 mg. of protein in 2.5N-NaOH, adding 5 ml. of water and 1 ml. of α -naphthol (0.1% in 95% ethanol) with vigorous mixing, and allowing to stand for 5 min.; then adding 1 ml. of 0.06N-NaOCl and, exactly 1 min. later, 2 ml. of 20% (w/v) urea; and finally reading in a photoelectric colorimeter after 5 min. A standard arginine solution is similarly treated. For the differential estimation of the plasma proteins the Na_2SO_4 -ether technique of Kingsley (1940) is used. Albanese took a sample of the Na_2SO_4 solution for the 'albumin'; and for the 'globulin' determination he dissolved the globulin disk in NaOH and tested a sample.

Carrying out the test, as described by Albanese, a proportionality of colour over the normal range of plasma protein values was not obtained until the hypochlorite concentration was increased from 0.06 to 0.15N.[†] Even with this concentration, however, there is a slight falling off in proportionality above the upper limits of the normal range of plasma protein values. It is worth remarking that Albanese employed a commercial preparation known as 'Clorox', which might possibly have contained activators of some sort. We used British Drug Houses Ltd. NaOCl solution. (A dilution of 'Milton' gave similar results.) In determining how closely the colour developed was correlated with the N content, we modified the procedure slightly, so as to allow of more precision by avoiding the cumulative error involved in pipetting. To the 1 ml. sample of protein in NaOH (in a 10 ml. volumetric flask), 4.5 ml. of water were added, followed by the other reagents and water to a final volume of 10 ml. The extinction was measured with a Hilger Spekker absorptiometer, with Ilford colour filter 604 (spectrum green). As colour development is influenced by temperature, all tests were carried out in a water bath at 22°.

For total proteins, 0.5 ml. of plasma was mixed with 5 ml. of water and the proteins precipitated by the addition of 5 ml. of 20% (w/v) trichloroacetic acid. After 15 min. the proteins were spun down and the tube inverted and drained.

[†] Dr G. M. Bull, working independently in this School, also found it necessary to increase the NaOCl concentration.

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The protein was then dissolved in 10 ml. of 2.5N-NaOH at room temperature. (No significant amount of NH_3 is lost under these conditions.)

For the differential protein estimation, 1 ml. of plasma was added to 15 ml. of 23% (w/v) Na_2SO_4 , and the mixture shaken vigorously with 6 ml. of ether for 2 min. On centrifuging, the 'globulin' collects as a disk at the junction of the water and ether layers. The aqueous ('albumin') layer was decanted; by careful decantation the 'globulin' disk could be made to adhere to the side of the tube. After draining, the 'globulin' was thoroughly washed with 10% trichloroacetic acid, centrifuged, and finally dissolved in 2.5N-NaOH (usually 10 ml.).

A portion of the 'albumin' filtrate was mixed with trichloroacetic acid and the precipitate spun down. After decanting the supernatant liquid and draining, the 'albumin' was washed with trichloroacetic acid and again spun and drained. Finally it was dissolved in 10 ml. of 2.5N-NaOH.

In some cases the total protein, 'albumin' and 'globulin' were precipitated not with trichloroacetic acid but with molybdic acid which gave a sharper precipitation.

The washing of the 'albumin' and 'globulin' was necessary to remove Na_2SO_4 , which was found to enhance the colour produced under the conditions of the test. Thus, the addition of 1 ml. of 23% Na_2SO_4 increased the extinction

Table 1. Colours produced by the Sakaguchi reaction with (total) plasma proteins

Specimen	Diagnosis	Drum reading (E)	Plasma protein (N x 6.25) (g./100 ml.)	Protein in test (mg.)	Specific extinction coefficient
Ed	Uraemia	0.586	6.55	3.275	0.1790
Ba	Hyperthyroidism	0.582	6.71	3.355	0.1732
Ru	Gastric ulcer	0.539	6.07	3.035	0.1776
Hu	Sprue	0.409	4.55	2.275	0.1798
Sp	Pneumonia and chronic nephritis	0.600	6.80	3.400	0.1765
Ho	Addison's disease	0.620	7.12	3.560	0.1742
Ju	Haematemesis	0.614	6.87	3.435	0.1787
Ca	Myxoedema	0.584	6.71	3.355	0.1741
Sp	Lung abscess and chronic nephritis	0.588	6.75	3.375	0.1743
Hu	Sprue	0.470	5.25	2.625	0.1790
St	Acute intestinal obstruction	0.619	7.04	3.520	0.1759
Wi	Rheumatoid arthritis	0.623	7.19	3.595	0.1733
Sp	Lung abscess and chronic nephritis	0.635	7.21	3.605	0.1762
Ed	Uraemia	0.574	6.41	3.205	0.1791
Go	Hypertension	0.492	5.64	2.820	0.1744
Gr	General paralysis of the insane	0.527	6.09	3.045	0.1731
Sp	Lung abscess and chronic nephritis	0.593	6.78	3.390	0.1750
Ke	Normal	0.590	6.85	3.425	0.1724
Ab	Normal	0.614	7.20	3.600	0.1705
Va	Normal	0.630	7.24	3.620	0.1740
Ma	Normal	0.580	6.64	3.320	0.1747

Av. 0.1755

Standard deviation 0.0015; coefficient of variation 0.9; maximum deviation from average 2.9%.

Table 2. Colours produced by the Sakaguchi reaction with plasma 'albumin' and 'globulin'

Specimen	Diagnosis	'Albumin'/ 'globulin' ratio	Specific extinction coefficients			
			'Albumin'	'Globulin'	Total	
					Calc.	Found
Ha	Ptois	1.67	0.1580	0.1868	0.1688	—
Gr	General paralysis of the insane	1.73	0.1658	0.1830	0.1720	0.1731
Br	Ulcerative colitis	0.57	0.1612	0.1838	0.1756	—
Ta	Hodgkin's disease (?)	1.02	0.1584	0.1830	0.1706	—
Key	Normal	1.43	0.1654	0.1868	0.1744	0.1724
Gi	Rheumatoid arthritis	1.00	0.1668	0.1846	0.1758	—
Ab	Normal	1.42	0.1624	0.1838	0.1713	0.1705
Va	Normal	1.47	0.1771	0.1744	0.1760	0.1740
Ma	Normal	0.95	0.1726	0.1736	0.1732	0.1747
Kem	Hypertension	1.36	0.1710	0.1860	0.1772	—
He	Uraemia and alkalosis	1.15	0.1550	0.1808	0.1670	—
Ur	Normal	1.98	0.1677	0.1840	0.1730	—
	Av.		0.1651	0.1826	0.1729	0.1727
	S.D.		0.0067	0.0030	0.0033	0.0017
	Coefficient of variation		4.1	1.6	1.9	1.0
	Max. deviation from average (%)		7.3	4.9	3.4	1.0

coefficient by about 8%, whether 0.15 or 0.06N-NaOCl was used, a blank test containing Na_2SO_4 being carried out at the same time.

In each determination, 1 ml. of the NaOH solutions of total protein, 'albumin' and 'globulin' was taken for the colour test, and 3 ml. for the micro-Kjeldahl.

The result was recorded as a specific extinction coefficient, i.e. the extinction coefficient corresponding to 1 mg. of protein ($N \times 6.25$) in the 10 ml. of test solution, in a 1 cm. cell:

$$\left(\frac{\text{reading with 1 cm. cell}}{\text{mg. protein in test}} \right).$$

The specific extinction coefficient is thus a measure of the ratio of the apparent arginine content to the total nitrogen.

RESULTS

Table 1 shows the results obtained for total proteins in 21 specimens from 16 cases, including 4 normals.

Table 2 shows the results obtained for 'albumin' and 'globulin'. It will be seen that the specific extinction coefficients in all but two cases were different for 'albumin' and 'globulin', and also that the coefficient for total proteins appears to be the resultant of those for 'albumin' and 'globulin'. (The two cases having the same coefficient for 'albumin' as for 'globulin' were both Indians: one had always been on a vegetarian diet, and the other had been on such a diet until a year previously, though whether this is of any significance is not known.)

DISCUSSION

These results do not confirm those of Albanese and his co-workers, who found that the same factor for the conversion of arginine to total protein, namely 19.2, was applicable to 'albumin' and 'globulin' as well. This factor is based on figures for the arginine content of body proteins given by Block & Bolling (1945); but in fact the figures given by those authors do not appear to justify the adoption of this factor for albumin and globulin.

The results given above, on the other hand, appear to be more in keeping with the figures of Brand and his colleagues, according to which the arginine content of the 'globulin' fractions, calculated from that of the electrophoretic α -, β - and γ -globulin components of plasma and their relative proportions, is higher than that of 'albumin'. In one case, the specific extinction coefficient for fibrin measured by the present author gave a value of 0.2220. This also is in agreement with Brand's figures, which show a considerably higher arginine content for fibrin than for the other protein fractions.

It must be emphasized that the specific extinction coefficient, as defined above, depends not only on the

'arginine' but also on the nitrogen content of the protein, which, according to Cook (1946), is somewhat lower for 'globulin' than for 'albumin'. If it be assumed that the arginine content of serum albumin is the same as that of globulin, then the specific extinction coefficients for these fractions would be expected to be in inverse ratio to their relative nitrogen contents, i.e.

$$\frac{\% \text{ N of albumin}}{\% \text{ N of globulin}} = \frac{15.4}{14.3} = 1.08.$$

The ratio ('globulin'/'albumin') of the average specific extinction coefficients found by us is $0.1826/0.1651 = 1.105$. The ratio of the arginine content of (electrophoretic) globulin and albumin according to Brand's figures is 1.06.

These results appear to suggest that the colour intensity developed under the conditions employed is proportional to arginine content even in unhydrolyzed proteins; but it is deemed advisable to refer to 'apparent arginine' content as the matter cannot be regarded as settled. In view of Brand's figures, and those reported here, the claims for an identical arginine: nitrogen ratio for albumin and globulin cannot be accepted.

In view of the well-marked effect of sodium sulphate on colour development, and the possibility of other substances also interfering, it is evident that this reaction should not be applied uncritically to the determination of arginine in biological fluids.

SUMMARY

1. The technique of Albanese & Frankston (1945) for the determination of plasma proteins by a modified Sakaguchi reaction gave a proportionality of colour over the normal range of values only when the hypochlorite concentration was increased to 0.15N.

2. A 'specific extinction coefficient' (measure of ratio of apparent arginine to total N) has been measured for total plasma proteins in 21 specimens from 16 cases.

3. In 10 out of 12 cases in which sodium sulphate-separated 'albumin' and 'globulin' were separately examined, the specific extinction coefficient for 'globulin' was, on the average, 13% higher than that for 'albumin'.

4. Both with 0.06N and with 0.15N sodium hypochlorite, the presence of sodium sulphate led to a large increase in the amount of colour developed.

I wish to thank Prof. E. J. King, at whose suggestion this work was done, for his constant help and advice; Dr I. D. P. Wootton for help with the statistical calculations; and the volunteers who contributed blood.

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Amino-acid Metabolism of Tissue Cells *in vitro*

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(Received 19 March 1948)

Ever since the negative results of the investigations by Burrows & Neymann (1917), Carrel & Ebeling (1924) and Baker & Carrel (1926, 1928) the amino-acids have been regarded as being able neither to prolong the life of cells nor to accelerate cell growth in tissue cultures. However, in these investigations normal culture media were used and such media already contain the necessary amino-acids. This point was realized by the present author, and a technique was devised (Fischer, 1941) whereby the compounds of the culture medium were dialyzed against a Ringer's solution containing glucose. It was found that the dialyzed culture medium was completely unable to maintain the life of the cells—they died and disintegrated within 24 hr. With this dialyzed medium as a basis it became possible to make a systematic analysis of the effect of substances of low molecular weight on the maintenance of cells grown *in vitro*.

A study was then made of the effect of an amino-acid mixture composed of nine amino-acids in the same relative proportions as found by Bergmann & Niemann (1936) in fibrin (Table 1). It was found that cystine plays a very important part. Without cystine, the remaining amino-acids are no longer able to save the cells from rapid disintegration within 24 hr. It was also observed that in the cultures of myoblasts and osteoblasts cystine cannot be replaced by methionine, though methionine is an essential acid for the organism as a whole whilst cystine is not. Moreover, both lysine and glutamic acid were found to be of great importance to the cells. Obviously, the amino-acids required by the whole organism are not the same as those necessary

to a pure culture of tissue cells. For example, glutamic acid is essential to myoblasts, but not to the organism as a whole. Our technique of 7 years ago has since been improved, and the present paper presents some of the results obtained.

EXPERIMENTAL

The tissue cells in the present experiments belonged to a pure strain of myoblasts from a 9-day-old chicken embryo. The individual cultures were divided into two halves, one serving as control in the medium described below, the other as the experimental culture, in the same medium, but with the substance under investigation. At intervals during growth, drawings of the tissue cultures were made by means of an Edinger projector which magnified twenty times. The area of each drawing was measured by means of a planimeter and the results are given in Figs. 1–14, expressed as ratios according to the formula $(B-A)/A$, where A = initial area and B = growth area (Fischer, 1925).

The medium consisted of 0.5 ml. dialyzed chicken plasma, 1 ml. Tyrode's solution and 0.1 ml. of the mixture called the basic nutrient (Table 1). Coagulation was produced by adding 1 drop of dialyzed embryo juice. When coagulated, a liquid phase was introduced by adding 0.5 ml. dialyzed serum and 0.1 ml. of the basic nutrient. Carrel flasks type D-3 were used. The pH of the mixture was adjusted by introducing into the flask a gas composed of 8% CO₂, 12% N₂ and 80% O₂. The flasks were sealed with sterile rubber stoppers.

The basic nutrient mentioned (Table 1) was designed by G. Ehrensward, and is a mixture of substances known empirically to be important to the whole organism. It contains three main groups of substances; the first group consists of salts including salts of heavy metals and organic phosphates which can function as phosphorylating agents,