CCLXVI. THE COMBINATION CURVES, HYDROGEN ION REGULATING POWERS AND EQUIVALENTS OF LACTALBUMIN, AND ITS NON-IDENTITY WITH SERUM- ALBUMIN.

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MILK-PROTEINS were formerly thought to be identical with the corresponding blood-serum-proteins. For example, immunological reactions showed that colostrum-globulin was identical with serum-globulin, and Hamburger [1901], by precipitation methods, demonstrated the close relationship between blood-serum- and milk-proteins. Schlossman and Moro [1903] concluded that serum-albumin and lactalbumin were identical, while Bauer and Engel [1911], by complement fixation, deduced the identity of serum- and milk-proteins. Hence physiologists assumed a simple transference of proteins from the blood-stream to the mammary secretion.

Chemical methods confirmed the biological results in the case of the globulins, but not in the case of the albumins. Thus Hartley [1914] found that lactoglobulin was identical with serum-globulin while Crowther and Raistrick [1916], by the Van Slyke procedure, deduced that the amino-acid contents of serum-globulin and lactoglobulin are essentially the same.

Sebelien [1885] found a large difference in specific rotations of the albumins; Hartley [1914] and Crowther and Raistrick [1916] found marked differences in the percentages of basic amino-acids and Woodman [1921] differences in the NaOH racemisation curves, specific rotations of alkaline solutions being plotted against time.

EXPERIMENTAL.

Lactalbumin was prepared from mixed skim-milk less than 12 hours old. 10 % acetic acid was added very gradually with gentle stirring [Moir, 1931], until a further addition to the filtrate from a test portion gave no precipitate (p_H 4.2). The caseinogen precipitate was filtered off and washed with cold water and the washings added to the filtrate. An equal volume of saturated ammonium sulphate was then slowly added, with stirring, to the clear filtrate; after leaving overnight, the liquid was siphoned off from the precipitated lactoglobulin, which was washed with the strongest concentration of ammonium sulphate with which the filtrate did not give a precipitate. The clear filtrate was saturated with solid ammonium sulphate and the precipitate filtered off after leaving overnight in the refrigerator. The precipitate was dissolved in water to a volume equal to that of the milk taken and an equal volume of saturated ammonium sulphate again added. The filtrate was then saturated with $(NH_4)_2SO_4$. This process was repeated twice, the last saturation being with $MgSO_4$. The lactalbumin was then obtained in a micro-crystalline condition by adding 10 % acetic acid, and leaving overnight in the refrigerator [Allen, 1932].

The lactalbumin was dialysed for 24 days against distilled water, until the dialysate gave no test for ammonia or sulphate after leaving for 48 hours. The dialysed liquid was filtered and had (in 2 % concentration) specific conductivity 0.85×10^{-4} ohms⁻¹, an ash content of 0.002 g. per g. dry protein and a refractive index increase (1 % in water, 20°) of $a=0.0018$; it gave negative Molisch and phosphorus tests and had p_H 4.8.

The measurements with the hydrogen electrode and the calculation of the results were as described formerly [Prideaux and Woods, 1932, 1, 2]. The isoelectric point was taken to be at p_H 4.55 [Csonka *et al.*, 1926].

Four concordant titrations with acid and alkali are summarised in Table I.

Table I.

1 % albumin.					
Acid ml. 0.1 <i>N</i>	p_H	Acid ml. 0.1 <i>N</i>	p_H	Acid ml. 0.1 <i>N</i>	p_H
0.5	4.45	3.68 (4.0)	3.45	5.85 (8.0)	2.8
1.05	4.15	4.28 (5.2)	3.15	6.5 (12.0)	2.35
1.45 (1.5)	4.05	4.65 (5.5)	3.15	6.6 (16.0)	2.15
2.27 (2.5)	3.75	5.05 (6.4)	3.0	6.3 (26.6)	1.9
2.85 (3.2)	3.6				
Alkali ml. 0.1 <i>N</i>	p_H	Alkali ml. 0.1 <i>N</i>	p_H	Alkali ml. 0.1 <i>N</i>	p_H
0.9	5.0	5.0	7.65	10.0 (10.53)	10.55
1.5	5.3	5.25	7.9	11.27 (12.0)	10.7
1.8	5.45	5.55	8.25	12.85 (14.0)	10.95
2.1	5.65	6.0	8.55	13.13 (14.75)	11.05
2.5	5.85	6.3	8.9	14.3 (16.1)	11.15
3.15	6.0	7.0	9.3	16.65 (20.0)	11.4
3.26	6.4	7.1	9.4	17.2 (21.05)	11.45
3.5	6.5	7.6	9.6	17.9 (22.2)	11.5
4.0	6.8	8.3 (8.42)	10.05	19.1 (25.0)	11.65
4.2	6.9	8.7 (8.95)	10.15	21.5 (31.6)	11.8
4.35	7.2	9.65 (10.0)	10.35		

The results of all titrations are expressed in a uniform manner as ml. 0.1 *N* acid or alkali added to 1 g. of albumin.

The acid and alkali additions which gave solutions of low or high p_H are corrected for titrant blank by the potentiometrically determined values of Atkin and Douglas [1924].

DISCUSSION OF RESULTS.

It is at once obvious from the titration curves that lactalbumin is not identical in its amino-acid make-up with serum-albumin, as shown in other ways by Crowther and Raistrick [1916] and Woodman [1921].

Comparison with ovalbumin and serum-albumin.

The resemblance between the lactalbumin curve and the curves for ovalbumin and serum-albumin is not so close as that which exists between the two latter. The curve for lactalbumin lies entirely below those of the other albumins. There is little uncertainty in the region of p_H 7-9. The chief difference however is the attainment of a maximum combining capacity with acid at p_H 2.6 and 6.5 ml. 0.1 *N* acid combined per g.

This is almost exactly equivalent to the free amino-group of the lysine (9.89 %), which suggests that this is the only free basic group present in lactalbumin. The equivalent weight on the acid side is thus 1540. The corresponding equivalent of gelatin is 1180, and that of serum-albumin 1150 (770), whilst ovalbumin has no maximum acid-combining capacity.

Even above p_H 2.6 lactalbumin requires much less acid to bring it to a given p_H than either ovalbumin or serum-albumin. Thus to bring 1 g. to p_H 3 lactalbumin requires 3.0 ml. and serum-albumin and ovalbumin 6.5 to 7.0 ml.; hence a mixture of lactalbumin and one of the other two could be estimated by titrating with acid to two definite p_H values according to Harris's method [1925].

Acid titrations.

Above p_H 2.6 the acid branch extends to p_H 3.1 as a straight line, thence with increasing slope through the isoelectric point to p_H 6.

Table II presents a comparison of the buffering power against acid of lactalbumin over various ranges with those of serum-albumin and ovalbumin.

Table II.

$$B = \frac{d(\nu)}{d(p_H)} = \frac{\text{ml. of titrant added}}{\text{corresponding } p_H \text{ change}}.$$

Lactalbumin		Serum-albumin		Ovalbumin	
p_H range	10^4 B.	p_H range	10^4 B.	p_H range	10^4 B.
2.75-3.6	3.65	2.3-3.0	—	—	—
2.6-4.4	3.2	2.6-4.4	4.6	2.3-3.0	6.1
3.6-4.55	2.9	3.0-4.0	5.1	2.6-4.4	5.1
3.0-4.0	3.5	3.0-5.0	5.8	3.0-4.0	4.7
3.0-5.0	3.0	4.0-4.7	4.1	3.0-5.0	3.5
4.0-4.55	3.0	4.7-6.0	2.8	4.0-4.7	2.6
4.55-6.0	1.95	—	1.25	4.7-6.0	1.7
6.0-8.0	0.8	6.0-8.0	0.75	6.0-8.0	0.67
7.8-9.0	1.17	—	—	—	—
9.0-10.2	1.9	9.0-10.5	2.85	9.0-10.5	2.5
					(curvature changing)
10.5-11.3	6.25	10.0-11.0	4.0	10.3-11.3	4.0
11.25-11.8	11.6	10.4-11.6	5.85	—	—

It is evident that, except near the isoelectric point, on the alkaline side from the inflection at p_H 7 up to p_H 10.5, the buffering power of lactalbumin is less than that of serum-albumin or ovalbumin, while above p_H 10.5 it is much greater.

Ratio of acidic and basic equivalents, and the numbers of free acidic and basic groups.

Equivalent as base, p_H 2.75-3.6 = 1580 (1540 from the inflection).

Equivalent as acid, p_H 10.5-11.3 = 920.

The ratio is 1.72 (1.67 from the titration equivalent). This does not agree with the percentages of fission products, even assuming that lysine provides the only free basic group; but naturally all the basic and acidic groups are not combining at these p_H values. Also combination at peptide linkages has to be taken into account.

Free amino-nitrogen.

The free amino-N, as liberated in the Van Slyke micro-apparatus in 30 minutes, agrees with the lysine content (9.89 %) assuming that the ϵ -amino-group of this amino-acid is the only one reacting.

Free amino-N 0.96, 0.94, 0.95 % mean 0.95, giving lysine 9.93 %.

Heat-denaturation.

Samples of 1 % solution were heat-denatured by suspension in the steam of a boiling water-bath for periods of 5-10 minutes. The p_H was changed only from 4.8 to 4.95 showing the absence of hydrolytic fissions, and the liquid became

turbid. Proof of practically complete denaturation was obtained by bringing a cooled sample to the isoelectric point, leaving for 30 minutes and filtering. Most of the protein coagulated; the filtrate possessed a refractive index scarcely greater than that of distilled water.

The other samples were rapidly cooled, brought to the isoelectric point, made up to the original volume and titrated immediately.

There appears to be some slight chance of the denatured protein reverting to the undenatured form during titration. This point was tested by bringing the p_H of a sample back to that of the isoelectric point, after titration with acid or alkali. The denatured protein was reprecipitated, showing that reversion had not taken place to any large extent.

The titration of lactalbumin which had been denatured, but not coagulated, by heat gave a curve which coincided, within the limit of experimental error, with the curve for natural lactalbumin (Fig. 1).

Fig. 1. The combination of 1 % lactalbumin with acid and alkali.
x Natural; o heat-denatured.

Thus, as proved for ovalbumin by Prideaux and Woods [1932, 1, 2] and confirmed by Loughlin [1933] and for serum-albumin by Prideaux and Woods [1932, 2], heat-denaturation without coagulation produces no change in the number of acidic and basic groups. This agrees with the theory postulating the hydrolysis of some especially labile non-peptide linkages, rather than with the theory of condensation between amino- and carboxyl groups.

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