5,5'-Diamino-4,4'-dihydroxydiphenylmethane.—The reduction of 5,5'-dinitro-4,4'-dihydroxydiphenylmethane (2.9 g.) was effected on treating it with the solution of stannous chloride (15 g.) and concd. hydrochloric acid (13 cc.) in glacial acetic acid (50 cc.) at 80° for two hours. The product gave colorless needles from ether, m. p. 220–222° (dec.) with preliminary darkening.

Anal. Calcd. for $C_{13}H_{14}O_2N_2$: C, 67.83; H, 6.09; N, 12.17. Found: C, 67.98; H, 6.32; N, 12.27.

The hydrochloride gave colorless prismatic needles, m. p, >305°. *Anal.* Calcd. for $C_{13}H_{14}O_2N_2 \cdot 2HCl$: HCl, 24.09. Found: HCl, 23.34.

5,5'-Tetraacetyldiamino-4,4'-diacetoxydiphenylmethane.—A mixture of 5,5'-diamino-4,4'-dihydroxydiphenylmethane (0.65 g.), anhydrous sodium acetate (0.7 g.) and acetic anhydride (13 g.) was refluxed for fifteen hours. The product formed prismatic needles from dilute alcohol, m. p. 142–143°; yield, 0.8 g.

Anal. Calcd. for $C_{25}H_{26}O_8N_2$: N, 5.81. Found: N, 5.90.

4,5,4',5'-Dimethylbenzoxazolmethane (II).—On submitting the acetyl compound (0.7 g.) to distillation, the distillate soon turned to colorless prisms (0.4 g.). It was water washed and crystallized from ether into colorless prisms, m. p. 103–105°. It is easily soluble in the usual organic solvents but insoluble in dilute acid and alkali.

Anal. Calcd. for $C_{17}H_{14}O_2N_2$: C, 73.38; H, 5.04; N, 10.07. Found: C, 73.16; H, 5.12; N, 10.05.

The author thanks Professor Hata for his interest in the work.

Summary

It was ascertained that 4,4'-dimethoxydiphenylmethane on treatment with fuming nitric acid gives 5,5'-dinitro-4,4'-dimethoxydiphenylmethane.

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[CONTRIBUTION FROM THE PHYSICO-CHEMICAL LABORATORY OF THE NEW YORK STATE AGRICULTURAL EXPERIMENT STATION]

The Optical Rotation and Dissociation of Casein[1]

BY D. C. CARPENTER

In studies dating from 1927, the writer[2] was able to show, by use of the ultracentrifuge, that casein prepared by the classical methods, respectively, of Hammarsten and of Van Slyke and Baker, was a mixture of protein molecules of different molecular weights. It was also shown that these different protein species could be separated from one another by fractionation from suitable solvents and the molecular weights of the pure monomolecular species established. The species were found to have molecular weights, respectively, of 96,000,[3] 188,000 and 375,000. Subsequent work[4] on the content of various amino acids in the 96,000 species, showed that a value of 98,000 for the molecular weight was compatible with the analyses. These molecular weight values obtained by two totally different lines of attack are in excellent agreement with one another. Serological tests[5] on the three protein species separated from casein showed that they were different from one another.

With reference to the old "casein" being a complex mixture, work in Sörensen's laboratory[6] has substantiated our own findings and lately Cherbuliez[7] has separated crude casein into four fractions by ammonium chloride and acetone. Groh[8] has fractionated crude casein by three different procedures. The homogeneity of the various individual fractions obtained by the various workers above has been ascertained only in the materials described by the writer, and it is therefore impossible to say whether clear-cut separations have been obtained by these other workers or not.

Recently Jones and Gersdorff[9] have shown that dissolving crude casein in very dilute alkali and reprecipitating with dilute acid reduces the cystine content of the protein. After five successive repetitions of the operation of dissolving in alkali followed by reprecipitation with acid, these workers record a decrease in cystine content from an initial value of 0.336 to 0.033%. In the course of our work on casein with the ultracentrifuge, we have found that it was impossible

(1) Approved by the Director of the New York Agricultural Experiment Station for publication as Journal Paper No. 45.

(2) T. Svedberg, L. M. Carpenter and D. C. Carpenter, THIS JOURNAL, **52**, 241 and 701 (1930).

(3) D. C. Carpenter, unpublished data.

(4) D. C. Carpenter, THIS JOURNAL, **53**, 1812 (1931).

(5) D. C. Carpenter and G. J. Hucker, *J. Infect. Dis.*, **47**, 435 (1930),

(6) Linderström-Lang, *Z. physiol. Chem.*, **176**, 76 (1928); *Compt. rend. trav. Lab. Carlsberg*, **17**, No. 9 (1929).

(7) E. Cherbuliez and M. L. Schneider, *Helv. Chim. Acta*, **15**, 597 (1932); E. Cherbuliez and F. Meyer, *ibid.*, **16**, 600 (1933).

(8) J. Groh, E. Kardos, K. Denes and V. Serenyi, *Z. physiol. Chem.*, **226**, 32–44 (1934).

(9) D. B. Jones and C. E. F. Gersdorff, *J. Biol. Chem.*, **104**, 99 (1934).

to use dilute alkali as a solvent for the protein without irreversibly disrupting the protein molecule. Well buffered solvents such as described in our former publications are required in dealing with proteins.

Data have been collected in Svedberg's laboratory[10] showing the stability *p*H range for several proteins. The breaking up or scission of proteins as a result of acidity or alkalinity results in the formation of fragments one-sixth (amandin and excelsin) to one-half (egg albumin, serum albumin, edestin and legumin) the weight of the original molecule. In the case of the two albumins this is followed by a further breaking up of the material into such small pieces that the centrifuge is not sufficiently powerful to effect sedimentation.

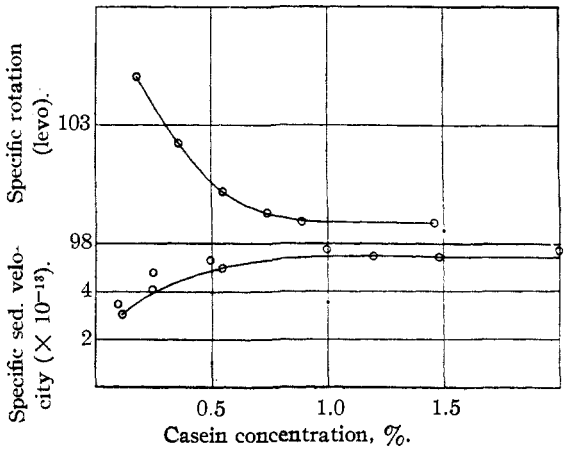

Fig. 1.

It is pointed out that the cases of scission noted above are the result of fairly high acidity or alkalinity as expressed in *p*H units and confirm our results on the hydrolytic scission of casein.[11] Nevertheless they are not to be confused with the experiments hereinafter reported in which the *p*H has been held constant throughout at 6.8 (within the range of stability with respect to *p*H) and only the protein concentration varied.

Experimental Part

The work to be described deals with the optical rotation of the simplest (96,000) species of casein and was undertaken not only to obtain data on a physical constant but to correlate optical properties with results obtained in the centrifuge,

A stock solution of the casein (species 96,000) was prepared in $M/60$ mixed phosphate buffer solution at *p*H 6.8 and a series of dilutions made of the stock solution by the addition of $M/60$ buffer named above and examined in the polariscope at 20°. In order that the errors in observation might be of the same order throughout the series, the concentrated solutions were observed in short tubes and the less concentrated solutions in longer tubes as far as was possible.

The data are recorded in Table I and are shown in Fig. 1 together with curves obtained by centrifuging similar solutions, which latter are taken from one of our previous publications. In Fig. 1 are recorded sedimentation values of two samples of casein, one considerably purer than the other. The curve shown represents the purer sample.

TABLE I

OPTICAL ROTATION OF CASEIN (MOL. WT. 96,000) IN $M/60$ MIXED PHOSPHATE BUFFER AT *p*H 6.8

Casein concn.	$[\alpha]$ levo degrees	l, cm.	$[\alpha]_D^{20}$ levo degrees
1.446	0.715	5.0	98.9
0.867	.858	10.0	99.0
.723	.717	10.0	99.3
.542	1.085	20.0	100.1
.362	0.740	20.0	102.2
.181	.380	20.0	105.1

Discussion

It will be seen from the data that the specific rotation is practically constant at −99° between the range 0.9 and 1.5% protein and rapidly increases in magnitude when the protein concentration is decreased. The sedimentation velocity constant curve behaves in much the same way, being constant ($S_{20°} = 5.56 \times 10^{-13}$) at concentrations between 0.9 and 1.5% and decreasing at concentrations below 0.9%. At 0.1% protein concentration the sedimentation value ($S_{20°} = 3.09 \times 10^{-13}$) indicates that the protein particles have a molecular weight around 32,000, that is, they are one-third as large as the original molecules before dilution. This value is calculated from the equation $M_1/M_2 = [S_1/S_2]^{3/2}$ which we have used before and which involves the assumption of a spherical particle, and therefore represents the lower limit of possible values of the weight of the dissociated molecule deducible from our data.

Both the sedimentation and optical rotation curves are rapidly changing in the region of 0.1% protein and neither gives any indication of a flattening out or a constant state. It was im-

(10) Svedberg and co-workers, THIS JOURNAL, **50**, 3318 (1928); *Trans. Faraday Soc.*, **26**, 740 (1930).

(11) D. C. Carpenter, *J. Biol. Chem.*, **67**, 647 (1926).

possible to obtain sedimentation data at concentrations below 0.1% with the thickness of the centrifuge cells available when these experiments were done, and if there was any flattening out of the sedimentation curve, corresponding to a state where the molecules were only reduced to one-third their original weight, it was hoped that experiments on optical rotation might indicate such. It proved to be the case, however, that the rotation of the species was not of enough magnitude to permit rotation readings of sufficient accuracy to settle this question unless perhaps the length of the polarizing tube for dilute solutions could be increased to nearly one meter. Such equipment unfortunately was not at our disposal.

A change in optical rotation with concentration of a substance is usually assumed to be the result of dissociation. The results here recorded are compatible with this idea to a certain extent but it is to be pointed out that, so far as our results go, the protein breaks into pieces one-third as large as the original molecule, a totally different type of dissociation than is common among electrolytes. Furthermore, this breaking into three pieces necessitates, according to the amino acid constituents present, that the pieces be unlike as to chemical composition; for instance, as there are only two cystine molecules in the whole protein molecule, it is obviously impossible to distribute two cystine groups between three pieces.

It is to be pointed out that the dissociation here described is entirely different in cause from that of scission of the molecule which latter occurs at high acid or high base concentrations. The dissociation noted occurs practically at neutrality simply by diluting the protein solution with a larger volume of the buffer solution in which the protein is dissolved.

It might be argued that the decrease of the sedimentation velocity on dilution with buffer solution was due to an effect of increased electrical charge on the protein particles. Tiselius[12] however has shown that any such possible electrical effect on sedimentation velocity disappears in the case of phycoerythrin at *p*H 8.1 when the salt concentration of the solvent lies between 1.2×10^{-2} and 3.3×10^{-3} normal. The $M/60$ buffer salt concentration which we have used would presumably give a similar result in eliminating any electrical effect.

It might be supposed that the observed effects are due to the fact that the ratio of available base to protein has been constantly increased as buffer solution is added to increase the dilution. There is no doubt but that an increase in this ratio has taken place. However, if the protein dissociation is due to the usual type of ionization of COOH groups, it is obvious that fresh COOH groups cannot be ionized as the protein dilution proceeds because of the fact that *p*H is held constant by the high buffer salt concentration. In any case such assumed behavior would have no bearing on the breaking up of the protein molecule into three distinct pieces.

The dissociation here described is entirely reversible because of the fact that when the concentration of protein is increased from 0.1% range to 1% again, centrifuging results show that only the 96,000 weight molecule is present, the 32,000 weight pieces having reunited by groups of threes into one particle.

Summary

1. The optical rotation of casein (mol. wt. 96,000) in phosphate buffer solution at *p*H 6.8 has been determined for dilutions between 0.15 and 1.5% protein. In the more concentrated solutions $[a]_D^{20} = -99°$. This value increases to $-105°$ in dilute solutions.

2. Results of ultracentrifugal analysis of these solutions show that the casein molecule is broken down or dissociated by dilution into particles one-third the weight of the original molecule. As long as the whole molecule is intact $[a]_D^{20}$ is constant at $-99°$, but immediately the molecule starts to dissociate into smaller fragments, the rotation increases.

3. The dissociation reaction is completely reversible on increasing the protein concentration, the 32,000 weight particles reuniting to form again the 96,000 weight molecule.

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(12) A. Tiselius, *Kolloid Z.*, **59**, 306 (1932).