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The Preparation and Some Biological Properties of the Asparagine Analog L-2-Amino-2-carboxyethanesulfonamide

BY HANS HEYMANN, T. GINSBERG, Z. R. GULICK, E. A. KONOPKA AND R. L. MAYER

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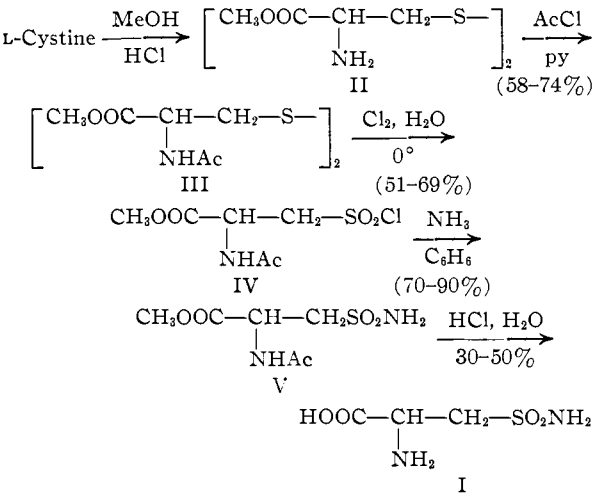
L-2-Amino-2-carboxyethanesulfonamide has been prepared *via* L-2-acetylamino-2-carboethoxyethanesulfonyl chloride which is obtainable from the corresponding disulfide by the action of aqueous chlorine. The sulfonamide inhibits the action of serum asparaginase and depresses or abolishes growth of an asparagine-requiring mutant of *Neurospora crassa*. Further, it reduces growth of *Eremothecium ashbyii*. The compound was without effect on *Mycobacterium tuberculosis in vitro* and *in vivo*, nor did it inhibit growth *in vitro* of several other pathogenic microörganisms.

The growth of *Mycobacterium tuberculosis* and of certain fungi on synthetic media is markedly increased by asparagine, which is therefore a regular constituent of several media.[1] It is generally considered that asparagine serves as a particularly suitable source of nitrogen for the mycobacteria rather than as an essential metabolite.[2] On the supposition that this utilization of asparagine by tubercle bacilli might be susceptible to inhibition by an asparagine antimetabolite, the preparation of 2 - amino - 2 - carboxyethanesulfonamide (I) was undertaken, which was of additional interest in view of the scarcity of data on asparagine antagonists and asparagine metabolism. No report of the synthesis of compound I was found in the literature, although Perdigon, *et al.*,[3] have mentioned it, in passing, as a possible antituberculous agent in a paper devoted to the study of cysteic acid.

To ascertain whether compound I possessed any capability whatsoever of competing with asparagine in biological systems, we investigated its effect on the activity of serum asparaginase, on the growth of an asparagine-requiring mutant of *Neurospora crassa* and on the growth of *Eremothecium ashbyii*. Growth of the last-named yeast is stimulated by a number of amino acids, notably asparagine.[4] The compound was examined for activity against *M. tuberculosis in vitro* and *in vivo*, as well as against several other microörganisms.

The sequence of reactions employed for the preparation of L-2-amino-2-carboxyethanesulfonamide is outlined below. The key step is the preparation of the sulfonyl chloride IV in cold aqueous medium; the chloride, though rapidly decomposed by water at room temperature, is sparingly soluble in the cold and may be isolated and quickly dried on clay *in vacuo;* the dry product is quite stable. The selective hydrolysis of the ester amide sulfonamide V was investigated extensively. Our intention to employ amino acid acylase[5,6] for removal of the acetyl group of V, following saponification, was vitiated by the formation of an unexpected acidic product of as yet unidentified structure under even the mildest, basic conditions. Possibly an acetamidoisothiazolidinone-S-dioxide was formed. Compound V itself did not react with amino acid acylase in the presence or absence of chymotrypsin. Acidic hydrolysis under a variety of conditions furnished an optimum yield of about 50% of the desired product I, which was separated by ion exchange chromatography from accompanying cysteic acid.

A sample of D,L-2-amino-2-carboxyethanesulfonamide was prepared by the same route.



Experimental Part[7]

(a) Preparative Experiments

L-N,N'-Diacetylcystine Dimethyl Ester (III).—The compound was prepared essentially according to Pirie[8] from L-cystine dimethyl ester dihydrochloride (II).[9] In a 3-necked, round-bottom flask equipped with stirrer, gas inlet tube and dropping funnel protected by a calcium chloride tube was placed 13.6 g. of II and 100 ml. of anhydrous pyridine (distilled from barium oxide). A slow current of dry nitrogen was passed over the liquid and the mixture was cooled to 0°. An excess (8 ml.) of acetyl chloride was added slowly so that the temperature remained between 10–15°. After the addition, the mixture was allowed to remain at room temperature for 3 hr.; the solid was collected on a sintered-glass funnel and washed with a little pyridine. The filtrate was diluted with an equal volume of water and the pyridine was removed at 40° *in vacuo* or on a rotary evaporator. The remaining solution was adjusted to *p*H 6.0 with strong alkali, treated with charcoal and evaporated as above until crystals sepa-

(1) For example, modified medium of B. Proskauer and M. Beck, *Z. Hyg. Infektionskrankh.*, **18**, 128 (1894).

(2) *Cf.*, *e.g.*, W. F. Drea and A. Andrejew, "The Metabolism of the Tubercle Bacillus," C. C. Thomas, Springfield, Illinois, 1953.

(3) E. Perdigon, F. Bouquet, M. T. Mazaudier and F. Godard, *Ann. Inst. Pasteur*, **72**, 573 (1946).

(4) See, *e.g.*, T. W. Goodwin and S. Pendlington, *Biochem. J.*, **57**, 631 (1954).

(5) S. M. Birnbaum in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, 115 ff.

(6) We thank Dr. S. M. Birnbaum for his kind donation of a sample of acylase I.

(7) (a) All melting points are corrected. (b) Microanalyses and determination of optical rotation were performed in the laboratory of Mr. L. Dorfman. (c) We thank Dr. G. deStevens and Miss P. Wenk for the preparation of an additional quantity of intermediate V.

(8) N. W. Pirie, *Biochem. J.*, **25**, 618 (1931).

(9) E. Fischer and U. Suzuki, Hoppe Seyler's *Z. physiol. Chem.*, **45**, 406 (1905).

rated. The solid was collected and further crops were secured from the mother liquors. A total of 10.44 g. (74.5%) of III was obtained in four crops, melting between 125 and 129°. The material may be recrystallized from water or ethyl acetate. Similar conditions were employed to acetylate 107 g. and 140 g. of I with yields of 68.5 and 58%, respectively.

The same method was employed to acetylate D,L-cysteine methyl ester hydrochloride. The product was purified *via* the copper mercaptide and forms needles from hexane, m.p. 80–81°.

L-2-Carbomethoxy-2-acetylaminoethanesulfonyl Chloride (IV).—A solution of 10 g. of III in 400–500 ml. of water was placed in a 1500-ml. beaker and surrounded by a cooling bath (ice–salt). The solution was vigorously agitated by means of a glass or tantalum stirrer until the temperature reached 0°. A stream of chlorine was passed in at such a rate that the temperature did not exceed 9°. Soon a voluminous precipitate appeared. The reaction is completed when the mixture shows a yellow tinge of excess chlorine and when the temperature drops spontaneously while chlorine is still being added. A vigorous stream of nitrogen was passed through to entrain excess chlorine, and the solid was collected rapidly on sintered glass, washed with ice-water, transferred to a clay plate and dried in a vacuum desiccator equipped with sulfuric acid and alkali. Yields varied from 7 to 9.6 g. (51–69%). The duration of chlorination varied from 10 to 47 minutes depending on the temperature maintained; it appears that longer exposure at low temperatures favors higher yields (*e.g.*, 47 minutes with $0.5° < T < 2.2°$).

The highest melting point observed for the chloride was 129–131°, often samples melt lower and with decomposition. The dry material appears to be stable when stored in absence of moisture.

The compound dissolves on agitation with water at room temperature and undergoes hydrolysis. A sample of 101.7 mg. consumed, after dissolution, 0.801 meq. of alkali; calculated 0.835 meq.

D,L-IV was prepared analogously by chlorination of D,L-N-acetylcysteine methyl ester.

Anal. 73.1 mg. consumed 6.00 ml. of 0.100 *N* NaOH. Calcd. for $C_6H_{10}NO_5SCl$ (243.5): 0.601 meq. The solution was acidified with 2 ml. of 6 *N* nitric acid and treated with 10 ml. of 0.1 *N* silver nitrate. Calcd. for $C_6H_{10}NO_5SCl$: Cl, 14.14. Found: Cl, 14.0.

L-2-Acetylamino-2-carbomethoxyethanesulfonamide (V).—Benzene (1500 ml.) was boiled to remove moisture. The chloride IV (12 g.) was suspended in the cooled benzene with the aid of a Waring Blendor, and dry ammonia was passed through for about ten minutes while the suspension was gently agitated. The solid was collected, sucked dry and extracted by agitation with 200 ml. of acetone at room temperature. No appreciable amounts were extractable after two treatments. The extracts were evaporated at 40° in a rotary evaporator, affording from 7.7–10.5 g. (70–95%) of crude product, m.p. 135–138°.

The product contained impurities which were removed by recrystallization from hot water (about 2 ml./g.). Thus, batches of IV amounting to 64.6 g. had given 45.5 g. (76.5%) of crude V, which on recrystallization afforded 33.7 g. of V (57%) melting 146–147° and 144–145°.

The reasons for the considerable variability in yield and quality of products and the nature of the by-products have not been determined, nor is it known under which conditions a sample will melt quietly or with effervescence.

The analytical sample melts 146.2–147.4°.

Anal. Calcd. for $C_6H_{12}N_2O_5S$: C, 32.13; H, 5.40; N, 12.49. Found: C, 32.31; H, 5.67; N, 12.17. $[\alpha]^{26}D$ $-30° \pm 0$(EtOH); $-27° \pm 0$ (H_2O).

The D,L-compound was obtained as fine needles from acetone–ether; m.p. 142.0–143.8°.

Anal. Found: C, 32.13; H, 5.79; N, 12.70.

L-2-Amino-2-carboxyethanesulfonamide (I).—A solution of 10.95 g. of V in 150 ml. of 2 *N* hydrochloric acid was heated in a 70°-bath for 8 hr. and evaporated to a sirup on a rotary evaporator at 40°. The evaporation was repeated twice after addition of two 100-ml. portions of water. The sirupy residue was dissolved in water to give 100 ml. of solution and a sample was found to indicate the presence of about 95 meq. of acidity (phenolphthalein).

Amberlite resin IR 4 was allowed to swell overnight in 1 *N* hydrochloric acid, washed with water and used to prepare a resin column 24 × 120 mm. (55 ml.). The column was washed with 1 *N* sodium hydroxide until converted to the free base form and washed with water until the effluent was nearly neutral.

The hydrolysis mixture was added to the column at the rate of 0.004 meq./ml. resin/minute, in the present case approximately 0.2 ml./minute. After the solution had been added, water was passed through the column at the same rate and the effluent was examined by spot tests on filter paper for the presence of ninhydrin-positive solutes. After collection of eight column volumes (400 ml.), the ninhydrin reaction became feeble, and examination of an additional 200 ml. of eluate revealed no appreciable amounts of solute.

The cysteic acid is largely retained by the column, but there is a detectable amount of leakage. The bulk of the sulfonic acid is rapidly eluted by 1 *N* hydrochloric acid. The yellow-colored eluates were treated with charcoal and evaporated to a small volume. Addition of acetone caused gradual precipitation of a solid (258 mg.) which was recrystallized twice from aqueous alcohol.

Anal. Calcd. for $[C_3H_7NO_5S]_2 \cdot H_2O$ (356.33): C, 20.22; H, 4.52; N, 7.86. Found: C, 20.20; H, 4.63; N, 7.80. Calcd. for $C_3H_7NO_5S$: C, 21.27; H, 4.16; N, 8.26. Found: (sample dried *in vacuo* at 80°): C, 21.32; H, 4.13; N, 8.18. $[\alpha]^{25}D$ $+9.2 \pm 1°$ (solvated sample, *c* 1, H_2O).

The aqueous eluates containing ninhydrin-positive material were evaporated at 40° *in vacuo*, affording 4.20 g. (51%) of crude I. The crystals, and particularly the mother liquors, contain as principal impurities cysteic acid and an unidentified amino acid. Compound V crystallizes well from hot water and chromatographic examination after one such recrystallization showed that the unknown amino acid had been removed and that cysteic acid was present to the extent of less than 0.5%. The material decomposes with discoloration and vigorous gas evolution at 190–200°.

Solubility: A saturated aqueous solution at 25° contains 24.9 mg./ml. *Anal.* Calcd. for $C_3H_8N_2O_4S$ (168.17): C, 21.42; H, 4.79; N, 16.66. Found: C, 21.06; H, 4.79; N, 16.30. $[\alpha]^{25}D$ $-16.3 \pm 0°$ (*c* 1.97, H_2O). $pK'_1 = 2.35$; $pK'_2 = 7.88$. Found: (D,L-compound): C, 21.35; H, 4.59; N, 16.07, 15.87.

(b) Biochemical Experiments

Materials and Methods.—A solution of partially purified *Guinea Pig Serum Asparaginase* was prepared according to Meister[10] and parcelled out in 1.4-ml. portions, which were frozen. The assay method was essentially that described in reference 10, followed by aeration of the ammonia[11] and Nesslerization.

The asparagine requiring mutant S 1007 of *Neurospora crassa* is the one isolated by Tanenbaum, Garnjobst and Tatum.[12] Cultures of this mutant and of the parent wild strain were kindly given us through the courtesy of Dr. Alton Meister. The strains were propagated on agar slants prepared according to Beadle and Horowitz,[13] or Beadle and Tatum,[14] fortified with 500 γ/ml. of L-asparagine for the mutant stock cultures. Growth experiments were performed in 250-ml. erlenmeyer flasks employing the basal media given in references just cited. After an average of three days' growth, the mycelia were harvested, pressed off on absorbent paper, dried at 90–100° and weighed.

A 48-hour culture of *Eremothecium ashbyii* (identification number, E 186) was obtained through the courtesy of Dr. Walter Nickerson. The organism was maintained on 1.5% agar slants of McLaren's[15] medium, fortified with 0.5% glycerol. The basal medium (double strength) for growth experiments followed the recommendation of Goodwin and Pendlington.[4] For use the solution was diluted 1:1 with water, other additives as desired and peptone solution to give a final peptone content of 0.4%.

To prepare inocula, 1 ml. of a 24-hour culture of *E. ashbyii* (in McLaren's medium fortified with 0.5% glycerol) was introduced into 15 ml. of basal medium and incubated

(10) A. Meister in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, 383 ff.

(11) O. Folin and C. J. Farmer, *J. Biol. Chem.*, **11**, 493 (1912).

(12) S. W. Tanenbaum, L. Garnjobst and E. L. Tatum, *Am. J. Botany*, **41**, 484 (1954).

(13) J. Beadle and N. H. Horowitz, *J. Biol. Chem.*, **150**, 325 (1943).

(14) J. Beadle and E. L. Tatum, *Am. J. Botany*, **32**, 678 (1945).

(15) J. A. McLaren, *J. Bacteriol.*, **63**, 233 (1952).

TABLE I

WEIGHTS (MG. AVERAGE OF TWO FLASKS OF DRY MYCELIA OF *N. crassa S* 1007 GROWN AT VARYING RATIOS OF L-ASPARAGINE AND L-2-AMINO-2-CARBOXYETHANESULFONAMIDE

L-Asparagine, γ/ml.		0	1	2	5	10	20	50	100	200	500	1000
L-2-Amino-2-carboxyethane-	0	..	5	12.5	15.5	23.5	27	45	57	62	79	123
sulfonamide, γ/ml.	20	..	1	1	13.5	21	32.5	47		64	87	121
	27.2	..	0.5	5.5	14.5	21	33.5	47	59.5	77.5	88	117
	50	..	..	4	14	27	33	48	61	71	82	
	100	..	..	..	13	22	37.5	49	68	69	77	126
	200	..	..	..	7.5	24.5	34.5	63	59	68	86	120
	500	..	..	..	..	14	32	46	61	81	86	115
	1000	..	..	..	..	..	3.5	47	70	85	87	101

for 48 hr. on a rotary shaker at 26–28°. The growth was broken up by means of glass beads, centrifuged, washed twice with distilled water. After dilution 1:5 with distilled water, 0.1-ml. portions served as inocula for the growth flasks, each of which contained 15 ml. of medium. The flasks were incubated on a rotary shaker as above for 5–6 days. The growth was collected on tared filter papers, dried at 60° and weighed. Riboflavin production was insufficient for meaningful analyses.

The compound was tested for activity against *M. tuberculosis* H37Rv on Proskauer–Beck[1] and on Kirchner medium and in mice infected with *M. tuberculosis* H37Rv. Tests for *in vitro antibacterial* and *antifungal* activity were performed by Dr. F. C. Kull of this Department.

Results

I. *Neurospora crassa* **Strain S1007.**—Table I shows the mycelial weight obtained when the mutant was grown on basal medium supplemented with L-asparagine and/or L-aminocarboxyethanesulfonamide in varying ratios.

To the left of the line bisecting the table is the area, where compound I significantly suppressed growth below the level reached in response to a given dose of asparagine. To the right of the line, growth is not influenced measurably by the amount of antimetabolite present. The effective ratio sulfonamide: asparagine ranges between 10:1 and 50:1 (w.)/(w.)

The parent, wild strain of *N. crassa* was grown on the basal medium in the presence or absence of 1000 γ/ml. of compound I, while the asparagine level was varied from 0 to 2500 γ/ml. The growth of the organisms as measured by the dry mycelial weight was not significantly influenced by either variable.

Since the sulfonamide I is the S-amide of cysteic acid, it was thought desirable to examine the effect of this sulfonic acid on the growth of the mold. The mutant S 1007 was grown at a constant asparagine level of 50 γ/ml. and cysteic acid was incorporated to give a range from 0 to 1000 γ/ml. For the wild strain, both asparagine (0 and 50 γ/ml.) and cysteic acid (0 to 1000 γ/ml.) were varied. Neither the wild nor the mutant strain was inhibited in growth by cysteic acid under the conditions stated.

II. Serum Asparaginase.—The results of the inhibition studies are presented in Table II.

III. *Eremothecium ashbyii.*—Table III and IV give data obtained on growing *E. ashbyii* on basal medium, in the presence of asparagine, and in the presence of both asparagine and the sulfonamide.

Riboflavin production in all cultures was rather poor and in the inhibited runs lay below a level that could be measured photometrically with sufficient accuracy. Even though the inhibited cultures obviously contained less of the vitamin than those growing normally, the data did not allow us to make a reliable estimate of the crucial quan-

TABLE II

INHIBITION OF GUINEA PIG SERUM ASPARAGINASE BY L-2-AMINO-2-CARBOXYETHANESULFONAMIDE

Substrate, 0.04 *M* L-asparagine; buffer, 0.01 *M* sodium borate *p*H 8.5; enzyme preparation, freshly thawed stock solution diluted 1:3.5; incubation mixture, 2 vol. of buffer or 1 vol. of buffer and 1 vol. of buffered inhibitor solution, 1 vol. of substrate and 1 vol. of enzyme preparation. The reaction was stopped by 1 vol. of 15% w./v. trichloroacetic acid. Controls showed that no ammonia was produced from enzyme and/or inhibitor solution in the absence of substrate.

Molar ratio sulfonamide to asparagine	30 min. μmoles of NH_3 per ml. of enzyme stock soln. Inhibitor Present	Absent	Inhibition, %	60 min. μmoles of NH_3 per ml. of enzyme stock soln. Inhibitor Present	Absent	Inhibition, %
1	43.7	55.0	20.6	94.0	107.0	12.2
1[a]	45.8	57.0	19.6	83.0	94.0	11.7
2[a]	39.0	50.3	22.8	78.0	97.0	19.7
6[a,b]	24.5	39.3	38.0	48.0	80.0	40.0
6[a,b]	18.7	32.0	42.0	37.5	63.0	40.5

[a] Average of duplicate measurements. [b] Highest ratio obtainable using buffer saturated with inhibitor.

TABLE III

GROWTH OF *E. ashbyii* IN RESPONSE TO SULFONAMIDE I IN PRESENCE AND ABSENCE OF ASPARAGINE

Sulfonamide I, γ/ml.	Dry mycelial wt. (mg.), Av. of duplicate flasks Asparagine None	940 γ/ml.
0	11.5	19.1
660	1.9	10.8
133	8.6	17.4
13.3	11.9	17.6
1.3	12.0	17.6

TABLE IV

GROWTH OF *E. ashbyii* IN RESPONSE TO ASPARAGINE IN PRESENCE AND ABSENCE OF SULFONAMIDE I

Asparagine, γ/ml.	Dry mycelial wt. (mg.), Av. of duplicate flasks 2-Amino-2-carboxyethanesulfonamide None	1320 γ/ml.
0	11.3	..
400	20.2	..
940	23.0	0.7
1200	24.6	1.4
1600	29.1	2.9
2000	30.7	1.7

tity, *i.e.*, vitamin produced per unit weight of mycelium.

IV. Antibacterial Tests.—The antimetabolite, 2-amino-2-carboxyethanesulfonamide (I), did not affect the growth, *in vitro*, of a variety of bacteria and fungi, including *Staph. aureus*, *E. coli*, *C. albicans*, *N. asteroides*, in concentrations as high as 1% in some cases. *M. tuberculosis* strain H37Rv grown on Proskauer–Beck[1] or Kirchner medium was not inhibited by compound I.

Mice infected with *M. tuberculosis* strain H37Rv were treated for 21 days with daily doses of 10 mg./mouse given subcutaneously; the treated animals did not differ from the control group.

For the sake of completeness, we add that the material was not cytotoxic to chick fibroblasts at 200 γ/ml., nor did it affect vaccinia virus in eggs.

Discussion

The results demonstrate that the asparagine analog, 2-amino-2-carboxyethanesulfonamide, possesses definite ability to compete with asparagine. The action of serum asparaginase was inhibited by 40% when assayed in the presence of six molecules of the antimetabolite for each molecule of natural substrate. The data obtained with the amount of material available for enzyme kinetic studies are insufficient for analysis according to Lineweaver and Burk[16]; in the absence of direct evidence to the contrary, one may reasonably assume this inhibition to be of the competitive type.

The compound is toxic for an asparagine-requiring strain of *Neurospora crassa* when eight to forty molecules of sulfonamide are present for every molecule of asparagine. The growth inhibition caused by a given concentration of I is reversed by addition of asparagine; this holds true for levels of I ranging from 20 γ/ml. to 1000 γ/ml. Since the parent and mutant strains do not differ in asparaginase content,[12] the inhibition of this enzyme is not a likely mechanism for the growth depression of mutant cultures by compound I. Possibly, the sulfonamide competes with asparagine transport across the cell wall.

Compound I was not toxic for the parent, wild strain of *N. crassa*, growth of which does not depend on an exogenous supply of asparagine. Though asparagine could be demonstrated in the filtrates from such cultures, we have no information about the intracellular concentrations of asparagine and of compound I. Thus, we cannot rule out the simplest explanation for the lack of effect of I on the wild strain, *viz.*, that effective ratios of I to asparagine were not reached.

In the case of *E. ashbyii*, Table IV shows that the sulfonamide I suppresses growth at a concentration between 133 to 660 γ/ml. when asparagine is absent. In the presence of 940 γ/ml. of asparagine, the toxicity of 660 γ/ml. of the sulfonamide (molar ratio approximately 2:1) appears less pronounced, since half-maximal growth occurred, indicating the possibility that the effect may be due to antimetabolic competition. However, when the organisms were exposed to a higher concentration of the sulfonamide (1320 γ/ml.), the toxicity could not be reversed by increasing the asparagine concentration up to the same molar ratio of 2:1. Moreover, since *E. ashbyii* responds favorably to several amino acids other than asparagine, the effect noted here may well be one of general toxicity rather than of antimetabolic competition.

SUMMIT, NEW JERSEY

(16) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, STATE UNIVERSITY OF IOWA]

Electrophoresis of Adsorbed Protein

BY DIPTI K. CHATTORAJ[1] AND HENRY B. BULL

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The electrophoretic mobilities of bovine serum albumin and of egg albumin adsorbed on inert particles exhibit over a substantial *p*H range two maxima as a function of protein concentration which are related to structural changes in the adsorbed protein monolayers. Comparison of the electrophoretic mobilities of dissolved and of adsorbed bovine serum albumin is consistent with the view that the electrical double layer associated with the adsorbed protein molecules can be treated as a plane plate condenser, although the nature of the underlying surface influences the electrophoretic mobility of adsorbed protein in important ways.

Attention has been called recently to the fact that the effective electrophoretic radii of protein molecules adsorbed on microscopically visible glass particles are very much larger than their solution radii.[2] There are a number of additional and interesting problems associated with the electrophoresis of adsorbed proteins and some of these problems we have now considered. We have studied the electrophoretic mobilities of Pyrex glass particles as well as of particles of liquid (Nujol) and of solid paraffin particles as functions of protein concentration and of *p*H at constant ionic strength and are able to present certain conclusions from these investigations.

Experimental

The crystalline bovine serum albumin (B.S.A.) was obtained from Armour and Co. The egg albumin (E.A.) was prepared from fresh hens' eggs by the method of Kekwick and Cannan.[3] Both proteins were exhaustively dialyzed against water and the concentrations determined by dry weight.

The microelectrophoretic measurements were made in a flat cell oriented laterally, each experimental point being the

(1) Chemistry Department, Jadavpore University, Calcutta, India.

(2) H. B. Bull, THIS JOURNAL, **80**, 190 (1958).

(3) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936).