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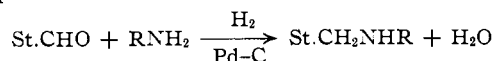
## Biologically Active N'-Alkylstreptomycylamines<sup>1</sup>

By W. A. WINSTEN, C. I. JAROWSKI, F. X. MURPHY AND W. A. LAZIER

Using the paper chromatographic technique, there has been demonstrated<sup>2</sup> the presence of streptomycin derivatives having antibiotic activity in solutions of streptomycin and primary amines subjected to reductive alkylation.

From the method of synthesis used, it was proposed that these derivatives are N'-substituted streptomycylamines. This paper describes the preparation, isolation, purification and proof of structure of streptomycylamine and several N'-n-alkyl derivatives.

The general reaction is represented by the equation

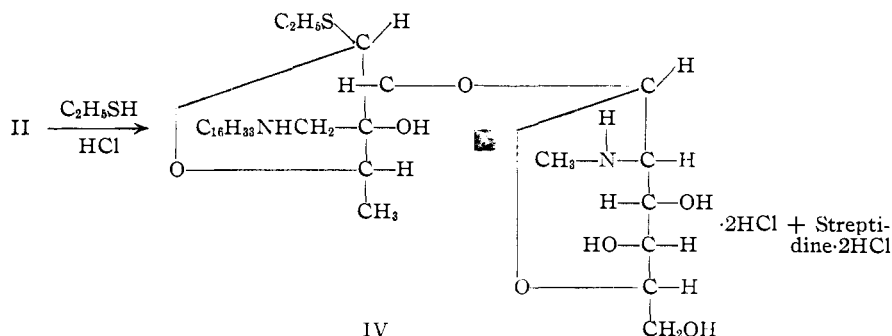


where St. equals streptomycin minus the aldehyde group.

The compounds in Table II were prepared by hydrogenating a solution of the base and streptomycin calcium chloride double salt<sup>3</sup> in absolute methanol. Five per cent. palladium-on-carbon was used as the catalyst under 1000 p. s. i. of hydrogen at 70–85°. The derivatives were purified by fractional crystallization of their helianthates. The fractions which were found to be devoid of streptomycin and dihydrostreptomycin, as determined by paper chromatography, were combined and recrystallized from methanol. The analytically pure tetrahelianthates were converted to tetrahydrochlorides by a method already described.<sup>4</sup>

The general formula I was assigned to these substituted streptomycylamines after a study of the degradation of N'-n-hexadecylstreptomycylamine tetrahydrochloride (II). When II was treated with acid by procedures described for the degradation of streptomycin into streptidine and streptobiosamine,<sup>5,6</sup> only starting material was re-

covered. It was necessary to heat a solution of II in normal sulfuric acid on a steam cone for one hour before hydrolytic cleavage occurred. Thus the insertion of a nitrogen atom in the streptose moiety of the streptomycin molecule strengthens the hemiacetal union between streptidine and streptobiosamine. Although this is not strictly analogous, it recalls to mind the resistance shown by D-glucosaminides to similarly mild hydrolytic treatment.<sup>7</sup> The only product isolated by this acid hydrolysis of II at elevated temperatures was streptidine sulfate (III). The other fragment underwent extensive degradation. Nevertheless, the isolation of III shows that the alkylamino group in II must be attached to the streptobiosamine portion. Additional proof was provided by mercaptolysis<sup>8</sup> and chromatographic purification of the reaction mixture using acid-washed alumina. An amorphous product (IV) was obtained, the analysis of which conformed to the scheme



The antibacterial activity of the N'-alkylstreptomycylamines against *Escherichia coli* and *Bacillus subtilis* is listed in Table II. Preliminary studies indicate that the acute mouse toxicity for all these compounds is higher than that of streptomycin. Replacement of the newly-formed hydroxyl group of dihydrostreptomycin by an amino group lowers the antibacterial activity and raises the acute toxicity in mice. Potency reappears when the N'-alkyl substituent is the n-decyl group. Insofar as *E. coli* potency is concerned, the most effective member of the series studied is N'-n-dodecylstreptomycylamine. This derivative is even more effective than streptomycin against this organism.

The hydrochlorides listed in Table II are white, amorphous solids. All but the parent member of the series, streptomycylamine, may be extracted into n-butanol from aqueous solutions at pH 11.5. This property provides a convenient method for

(1) N' designates the nitrogen atom introduced into the streptomycin molecule by catalytic hydrogenation in the presence of primary amines.

(2) (a) Winsten and Eigen, *THIS JOURNAL*, **70**, 3333 (1948).

(b) Winsten, paper presented at the 114th meeting of the American Chemical Society in Washington, D. C., August 1948.

(3) Peck, Brink, Kuehl, Flynn, Walti and Folkers, *ibid.*, **67**, 1866 (1945).

(4) Kuehl, Peck, Hoffhine, Graber and Folkers, *ibid.*, **68**, 1460 (1946).

(5) Peck, Graber, Walti, Peel, Hoffhine and Folkers, *ibid.*, **68**, 29 (1946).

(6) Carter, Clark, Dickman, Loo, Meek, Skell, Strong, Alberi, Bartz, Binkley, Crooks, Hooper and Rebstock, *Science*, **103**, 53 (1946).

(7) Fried and Wintersteiner, *THIS JOURNAL*, **68**, 2096 (1946).

(8) Kuehl, Flynn, Brink and Folkers, *ibid.*, **68**, 2096 (1946).

TABLE I  
 TETRAHELANTHATES OF STREPTOMYCYLAMINE AND N'-ALKYL DERIVATIVES

| Amine derivative<br>St.-CH <sub>2</sub> -N $\begin{matrix} \text{H} \\ \diagdown \\ \text{R} \end{matrix}$ | M. p.,<br>°C. | Formula  | Carbon, % |       | Hydrogen, % |       | Nitrogen, % |       |
|--|---------------|--|-----------|-------|-------------|-------|-------------|-------|
|  |               |  | Calcd.    | Found | Calcd.      | Found | Calcd.      | Found |
| H  | 222-224 dec.  | C <sub>77</sub> H <sub>102</sub> O <sub>23</sub> N <sub>20</sub> S <sub>4</sub>                    | 51.1      | 50.9  | 5.6         | 6.0   | 15.5        | 15.4  |
| <i>n</i> -Octyl  | 203-205 dec.  | C <sub>85</sub> H <sub>118</sub> O <sub>23</sub> N <sub>20</sub> S <sub>4</sub> ·H <sub>2</sub> O  | 52.7      | 52.6  | 6.2         | 6.2   | 14.4        | 13.8  |
| <i>n</i> -Decyl  | 223-224 dec.  | C <sub>87</sub> H <sub>122</sub> O <sub>23</sub> N <sub>20</sub> S <sub>4</sub> ·3H <sub>2</sub> O | 52.2      | 52.3  | 6.4         | 6.5   | 14.0        | 13.3  |
| <i>n</i> -Dodecyl  | 208-209 dec.  | C <sub>89</sub> H <sub>126</sub> O <sub>23</sub> N <sub>20</sub> S <sub>4</sub>                    | 54.2      | 54.5  | 6.4         | 6.8   | 14.2        | 14.7  |
| <i>n</i> -Tetradecyl   | 223 dec.      | C <sub>91</sub> H <sub>130</sub> O <sub>23</sub> N <sub>20</sub> S <sub>4</sub>                    | 54.6      | 55.2  | 6.5         | 6.6   | 14.0        | 14.4  |
| <i>n</i> -Hexadecyl  | 222-223 dec.  | C <sub>93</sub> H <sub>134</sub> O <sub>23</sub> N <sub>20</sub> S <sub>4</sub>                    | 55.1      | 55.0  | 6.6         | 6.9   | 13.8        | 13.9  |

 TABLE II  
 TETRAHYDROCHLORIDES OF STREPTOMYCYLAMINE AND N'-ALKYL DERIVATIVES

| Amine derivative<br>St.-CH <sub>2</sub> -NHR<br>R = | M. p.,<br>°C. | Formula   | Carbon, % |       | Hydrogen, % |       | Nitrogen, % |       | Bioassay<br>gamma/mg.<br><i>E. coli</i> <i>B. subtilis</i> |     |
|---|---------------|---|-----------|-------|-------------|-------|-------------|-------|--|-----|
|   |               |   | Calcd.    | Found | Calcd.      | Found | Calcd.      | Found |  |     |
| H   | 164-166 dec.  | C <sub>21</sub> H <sub>46</sub> O <sub>11</sub> N <sub>8</sub> Cl <sub>4</sub>                    | 34.6      | 35.0  | 6.3         | 6.7   | 15.4        | 14.7  | 465  | 385 |
| <i>n</i> -Octyl                                     | 169-170 dec.  | C <sub>29</sub> H <sub>62</sub> O <sub>11</sub> N <sub>8</sub> Cl <sub>4</sub> ·H <sub>2</sub> O  | 40.5      | 40.6  | 7.5         | 7.5   | 13.0        | 13.3  | 238  | <12 |
| <i>n</i> -Decyl                                     | 180-181 dec.  | C <sub>31</sub> H <sub>66</sub> O <sub>11</sub> N <sub>8</sub> Cl <sub>4</sub>                    | 42.8      | 42.9  | 7.6         | 7.4   | 12.9        | 12.9  | 635  | <12 |
| <i>n</i> -Dodecyl                                   | 146 dec.      | C <sub>33</sub> H <sub>70</sub> O <sub>11</sub> N <sub>8</sub> Cl <sub>4</sub>                    | 44.1      | 44.5  | 7.8         | 8.0   | 12.4        | 12.5  | 1000   | <12 |
| <i>n</i> -Tetradecyl                                | 191-192 dec.  | C <sub>35</sub> H <sub>74</sub> O <sub>11</sub> N <sub>8</sub> Cl <sub>4</sub>                    | 45.4      | 45.6  | 8.1         | 8.1   | 12.1        | 12.2  | 765  | <12 |
| <i>n</i> -Hexadecyl                                 | 175-177 dec.  | C <sub>37</sub> H <sub>78</sub> O <sub>11</sub> N <sub>8</sub> Cl <sub>4</sub> ·4H <sub>2</sub> O | 43.3      | 43.2  | 8.1         | 8.1   | 10.8        | 10.8  | 240  | <15 |
| Streptomycin·3HCl                                   |               |   |           |       |             |       |             |       | 841  | 841 |

the separation of these N'-alkyl derivatives from streptomycin and dihydrostreptomycin.

In addition to the compounds listed in the tables, the following amines were satisfactorily coupled with streptomycin under similar conditions: *n*-butylamine, cyclohexylamine, 2-amino-5-diethylaminopentane, ethanolamine, L-lysine, 2-aminoheptane, sulfanilamide, benzylamine, *p*-aminosalicylic acid,  $\beta$ -phenethylamine, 2-aminopyridine, 3-aminoquinoline, D-glucosamine,  $\alpha$ -naphthylamine and  $\beta$ -cyclohexylethylamine. Although these derivatives were not purified, their existence in predominant amount was proved by paper chromatography of the crude reaction mixtures.

### Experimental<sup>19</sup>

**General Procedure for Reductive Alkylation.**—Two hundred milliliters of a methanol solution containing 14.9 g. (0.01 mole) of the calcium chloride double salt of streptomycin and 9.4 g. (0.06 mole) of *n*-decylamine was reduced under 1000 p. s. i. of hydrogen at 80-85° in the presence of 5% Pd-C (5 g.). After six hours, heating was discontinued and the hydrogenation was continued for 18 hours. The catalyst was filtered off, washed with methanol and the combined filtrate and washings were poured into 10 volumes of absolute ether. The precipitate was filtered off, washed with ether and dissolved in distilled water. The solution was adjusted from pH 8 to 5.5 with hydrochloric acid, frozen and lyophilized. The crude residue weighed 13.2 g. It assayed as follows: maltol,<sup>10</sup> 0  $\gamma$ /mg.; streptidine,<sup>11</sup> 660  $\gamma$ /mg. The negative maltol test for crude N'-*n*-decylstreptomycylamine (VI) signifies that no streptomycin was present. Such a value would be given by dihydrostreptomycin or any N'-alkylstreptomycylamine. The streptidine assay, a colorimetric determination of guanidino groups, was carried out by a procedure developed in this laboratory.

**Preparation of Helianthates.**—The following description is typical of the procedure used in preparing helianthates. Crude VI (24.6 g., 0.028 mole) was dissolved in 100 ml. of

water and added to 48 g. (0.146 mole) of sodium helianthate (methyl orange) in 12 liters of water. The precipitate was separated by filtration, washed with acetone and dried (53.2 g.). This material was extracted with 3 liters of boiling methanol, filtered free of insoluble crude calcium helianthate and cooled. The cooled filtrate deposited 8.3 g. of crystalline product melting at 216-218°. The mother liquor was concentrated to 1 liter and cooled. This yielded 4.3 g. of product of similar purity. Further concentration yielded 6.9 g. of less pure material. The first two fractions were combined and recrystallized from methanol to yield the tetrahelianthate of VI, m. 223-224° (dec.). This product was shown by paper chromatography to be free of streptomycin and dihydrostreptomycin. After drying at 100° under reduced pressure (0.2 mm.) it was submitted for analysis.

**Sulfuric Acid Hydrolysis.**—One gram (0.001 mole) of II was dissolved in 25 ml. of *N* sulfuric acid and heated on a steam cone for one hour. The solution was cooled and 200 mg. of crystalline precipitate was removed by filtration. A single recrystallization from water yielded pure streptidine sulfate monohydrate. *Anal.* Calcd. for C<sub>8</sub>H<sub>18</sub>O<sub>4</sub>·H<sub>2</sub>SO<sub>4</sub>·H<sub>2</sub>O: C, 25.4; H, 5.9; N, 22.2. Found: C, 25.7; H, 5.7; N, 22.2.

**Mercaptolysis.**—One gram of II was suspended in 50 ml. of ethyl mercaptan saturated with hydrogen chloride. The mixture was placed in a stoppered flask and shaken mechanically overnight. The solvent was allowed to evaporate at room temperature. The residue was dissolved in methanol and placed on a column of acid-washed alumina. Development was continued with methanol-ether (3:2). The 8  $\times$  200 ml. fractions collected were freed of solvent. The sirupy residues in each instance gave no test for guanidine groups with streptidine reagent. They were combined, dissolved in water, the solution frozen and lyophilized. The residue was a light brown glass (300 mg.). After drying *in vacuo* at 100° for six hours, it was analyzed. *Anal.* Calcd. for C<sub>31</sub>H<sub>64</sub>O<sub>7</sub>N<sub>2</sub>Cl<sub>2</sub>S: C, 54.8; H, 9.4; N, 4.1; S, 4.7. Found: C, 55.3; H, 9.5; N, 4.5; S, 5.1.

**Paper Chromatography.**—Paper strip chromatography<sup>12</sup> was routinely used for the analysis of the crude products obtained. *Micrococcus pyogenes* var. *aureus* 9996 or a strain of *B. subtilis* was used to seed the agar plates. In addition, a modification of the technique of Horne and Pollard<sup>12</sup> was employed for developing chromatograms.

(9) All melting points are uncorrected.

(10) Boxer, Jelinek and Leghorn, *J. Biol. Chem.*, **169**, 153 (1947).

(11) Monastero, method to be published.

(12) Horne and Pollard, *J. Bact.*, **55**, 231 (1948).

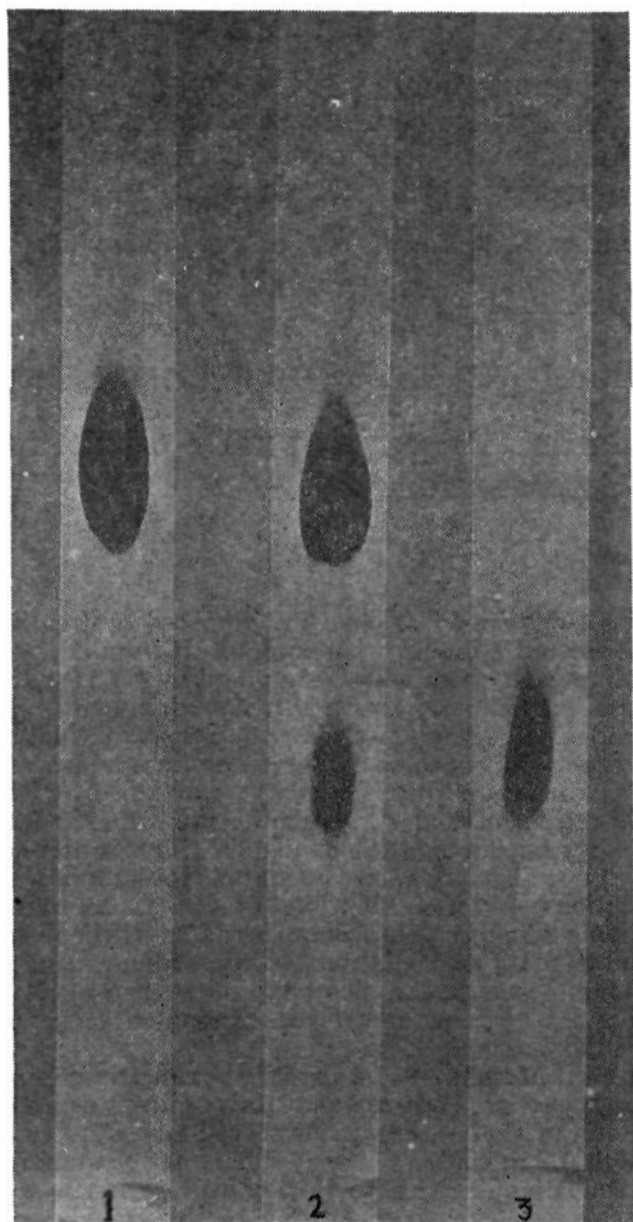


Fig. 1.—Chromatography of streptomycin, dihydrostreptomycin and mixture of the two, period of development two days; mobile phase *n*-butanol-2% piperidine-2% *p*-toluenesulfonic acid; test organism *M. pyogenes*: strip 1, dihydrostreptomycin (10 microliters of a solution containing 600 units per ml. was chromatographed); strip 3, streptomycin (10 microliters of a solution containing 200 units per ml. was chromatographed); strip 2, mixture of streptomycin and dihydrostreptomycin (10 microliters containing 200 and 600 units per ml. of each antibiotic, respectively, was chromatographed).

The dried paper chromatograms were sprayed with a reagent for guanidino groups.<sup>13</sup> Those areas which contained compounds having guanidine groups developed a red color.

The suitability of the paper chromatographic method<sup>1</sup> for analyzing mixtures containing streptomycin and its synthetic derivatives is indicated in Fig. 1 and Fig. 2. Streptomycin, dihydrostreptomycin and a mixture of the two were subjected to paper chromatographic analysis. The resulting strips were laid on an agar plate seeded with *M. pyogenes*. After incubation and removal of the strips, the agar plate presented the appearance shown in Fig. 1. The zone of inhibition due to the slower moving antibiotic is due to dihydrostreptomycin, the chromatographically more rapid antibiotic being streptomycin.

The results shown in Fig. 2 were obtained from crude

(13) Snell and Snell, "Colorimetric Methods of Analysis," D. Van Nostrand Co., Inc., New York, N. Y., 1947, p. 400.

Fig. 2.—Chromatography of N'-substituted streptomycylamine preparations, period of development eighteen hours, mobile phase *n*-butanol-2% piperidine-2% *p*-toluenesulfonic acid: strip 1, N'-*n*-octylstreptomycylamine preparation; strip 2, N'-*n*-decylstreptomycylamine preparation.



reaction mixtures prepared as described by one of us,<sup>2</sup> viz., by reductively alkylating streptomycin with either *n*-octylamine or *p*-decylamine, at room temperature, utilizing a platinum oxide catalyst and hydrogen at one atmosphere pressure. The two slower moving antibiotics only partially separated (because of the short period of chromatography) are dihydrostreptomycin and unchanged streptomycin. The triangular-shaped zones of inhibition found farther down the strip chromatograms are due to the new derivatives.

It is of interest to note that upon paper chromatographic analysis of methanol solution of mixtures of streptomycin and different alkylamines, only a streptomycin zone of inhibition was observed. Any aldimine present in solution undoubtedly decomposed almost at once during the course of chromatography using a wet solvent. This is understandable since the formation of aldimines from simple alkyl aldehydes and alkyl amines is a highly reversible reaction, the presence of water favoring the production of free aldehyde and free amine.

**Biological Assays.**—The antibacterial activity against *E. coli* and *B. subtilis* was determined by methods of the Food and Drug Administration.<sup>14</sup> The low potency against *B. subtilis* was attributed to the low diffusibility of these compounds in agar. Antibacterial potency against this organism was demonstrated by the agar-dilution-streak method.<sup>15</sup> By this procedure non-diffusibility of the antibacterial agent was not deleterious. The low *B. subtilis* potency was considered *prima facie* evidence for the absence of any significant quantity of dihydrostreptomycin in the crude reaction product. These diffuse quite well in agar, so that their *B. subtilis* and *E. coli* potencies are equivalent.

(14) *Federal Register*, 12, 2224-2225 (April 4, 1947).

(15) This work was carried out by Mr. K. B. Tate of the Pfizer laboratory.

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### Summary

Streptomycylamine, *N'*-*n*-octyl, *n*-decyl, *n*-dodecyl, *n*-tetradecyl and *n*-hexadecylstreptomycylamine have been prepared, characterized and shown to have antibacterial activity.

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## The Combination of Organic Anions with Serum Albumin. VII. The Protein Sites Involved in the Combination

By J. D. TERESI

The study of complex formation of serum albumin with organic anions of known structure offers promise of obtaining information of protein configuration. Serum albumins are especially suited for this type of study since these proteins have been shown to combine reversibly with many types of organic anions.<sup>1-7</sup>

A large amount of evidence has been reported to support the view that anions combine with positive groups on the albumin molecule. Therefore, anion-protein interaction at pH 7.6 would involve, for the most part, the guanidinium groups of the arginine residues and the  $\epsilon$ -ammonium groups of the lysine residues of the albumin molecule. The study of these interactions after modification of the protein molecule by removal of either of these positive charges may give information concerning the nature and the identification of the binding sites. In this paper the results of anion binding with native and modified bovine serum albumin are reported.

### Experimental

**Native Albumin.**—The crystalline bovine serum albumin used was obtained from Armour Laboratories. The water content was obtained by a dry weight determination on a sample of protein dried at 110° for twenty-four hours.

**Modified Albumin.**—One modified protein was prepared by acetylation of the  $\epsilon$ -ammonium groups of the lysine residues by treating one part of the bovine serum albumin in one-half saturated sodium acetate with 1.2 parts of acetic anhydride at 0 to 5° as described by Olcott and Fraenkel-Conrat.<sup>8</sup> The solution was then dialyzed exhaustively and the protein was then lyophilized. The resulting product was soluble in water. A formal titration<sup>9</sup> indicated that 90 to 95% of the  $\epsilon$ -amino groups were acetylated.

(1) B. D. Davis, *J. Clin. Invest.*, **22**, 753 (1943).

(2) B. D. Davis, *Am. Scientist*, **34**, 611 (1946).

(3) I. M. Klotz, F. M. Walker and R. B. Pivan, *THIS JOURNAL*, **68**, 1486 (1946).

(4) I. M. Klotz and J. M. Urquhart, *J. Biol. Chem.*, **173**, 21 (1948).

(5) Paul D. Boyer, G. A. Ballou and J. M. Luck, *ibid.*, **167**, 407 (1947).

(6) J. D. Teresi and J. M. Luck, *ibid.*, **174**, 653 (1948).

(7) Fred Karush and Martin Sonenberg, *THIS JOURNAL*, **71**, 1369 (1949).

(8) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Rev.*, **41**, 153 (1947).

(9) M. L. Anson and John T. Edsall, "Advances in Protein Chemistry," Vol. II, Academic Press Inc., New York, N. Y., 1945, p. 317.

Another modified protein was prepared by treating the bovine serum albumin with excess 2 *M* formaldehyde for ten minutes at room temperature and at pH 11 with subsequent exhaustive dialysis to remove free and reversible formaldehyde. Under these conditions formaldehyde reacts reversibly with  $\epsilon$ -ammonium groups and irreversibly with guanidinium groups.<sup>8</sup> This was verified by the formal titration method which showed that 95-100% of the  $\epsilon$ -amino groups were still available. This water-soluble protein was also lyophilized and stored for later use.

**Solutions.**—Protein solutions of desired concentration and ionic strength (0.2) were prepared with phosphate buffer at pH 7.6. Solutions of the sodium salts of the compounds reported in this paper were also made up to the desired concentrations with phosphate buffer at pH 7.6 and ionic strength 0.2.

**Spectral Method.**—The spectrophotometric behavior of *o*-nitrophenolate, *p*-nitrophenolate, *m*-nitrophenolate, picrate and methyl orange was observed at pH 7.6 and ionic strength 0.2 in the presence of buffer, native albumin and modified albumin with the aid of the Beckman spectrophotometer. The concentration of the protein solution used in each case was approximately 0.35%.

**Dialysis.**—Commercial sausage casing was used in the preparation of cellophane bags for the dialysis-equilibrium studies. Cellophane bags containing 5 ml. of 0.2 or 0.4% buffered protein solution were immersed in 10 ml. of each anion solution contained in a suitable bottle. Controls containing buffer only inside the bag were prepared in the same manner for each concentration of anion. The bottles were placed in a cold room at 1° for three days. The bags were then removed and the external solutions were analyzed spectrophotometrically for the anion.

### Results and Discussion

**Spectral Studies.**—The spectroscopic behavior of a buffered methyl orange solution is shown by Curve 4 of Fig. 1, which was obtained by plotting the wave length in ångström units against the optical density expressed as  $\log I_0/I$ . The addition of buffered native serum albumin to the methyl orange solution alters the spectrum of methyl orange as can be seen by comparing Curve 4 with Curve 1 of Fig. 1. This displacement has been interpreted by Klotz<sup>10</sup> as an effect due to the formation of a methyl orange-albumin complex. The substitution of acetylated albumin (Curve 2, Fig. 1) or formaldehyde treated albumin (Curve 3, Fig. 1) for the native albumin results in a reversal of the displacement. The experiment represented by Curve 3 was repeated with a carefully treated al-

(10) I. M. Klotz, *THIS JOURNAL*, **68**, 2299 (1946).