### Experimental

**Materials.**—The thiophene used in this work was obtained through the courtesy of the Monsanto Chemical Co., Inc., St. Louis, Mo. The 2-chlorothiophene and 2,5-dichlorothiophene were obtained through the courtesy of the Jefferson Chemical Co., Inc., New York, N.Y. Ethyl chlorocarbonate, 3-methylthiophene and anhydrous ammonia were commercial products.

**General Procedure for the Preparation of Ethyl Esters of Thienyl-α,β-acetylenic Acids.**—To freshly prepared sodium amide (0.05 mole) in 350 ml. of liquid ammonia in a three-necked flask there was added 0.05 mole of the thienylacetylene or its derivatives dissolved in a threefold volume of absolute ether. After stirring for an additional one-half hour, the liquid ammonia was allowed to evaporate on a steam-bath, while replacing it with absolute ether. The contents of the flask were cooled to 0–5° and 0.3 of a mole of ethyl chlorocarbonate dissolved in 35 ml. of absolute ether was added slowly. The reaction mixture turned from red-brown to pale yellow. It was allowed to reach room temperature and was stirred for an additional four hours. The sodium chloride was filtered off, the filtrate washed with 5% sodium carbonate solution, finally twice with ice-water, dried over anhydrous sodium sulfate and rectified.

**General Procedure for the Hydrolysis of Ethyl Ester of Thienyl-α,β-acetylenic Acids.**—One gram of ethyl ester of thienyl-α,β-acetylenic acid dissolved in 15 ml. of benzene was added to a solution of 25 ml. of 1.5 *N* sodium hydroxide and the reaction mixture shaken at room temperature for 48 hours. The isolation of the acid was carried out in the usual manner. The yields and elemental analyses of α,β-acetylenic acids are recorded in Table II.

**Attempts to Prepare the Thienyl-α,β-acetylenic Acids *via* the Direct Carbonation of the Sodium Salts of Thienylacetylenes.**—Sodium acetylide was formed in the usual manner using 0.2 g. of iron nitrate plus 0.42 g. of sodium and 200 ml. of liquid ammonia. Ten grams of pure 2-thienylacetylene was added dropwise over a period of one hour. The solution was then stirred ten minutes longer. The three-necked flask was then placed on a steam-bath and the ammonia driven off, using a stirrer and reflux condenser. Then 40 ml. of dry benzene were added from a dropping funnel to be used as solvent in the carbonation reaction. This solution containing the solvent and the sodium acetylide salt was placed in a bomb and carbonated with 200 g. of Dry Ice for 24 hours. The reaction product was acidified with 5 *N* sulfuric acid at 0° and twice extracted with ethyl ether. The combined organic layers were extracted again with 10% sodium carbonate and the aqueous layer cooled to 0° and acidified with 3 *N* sulfuric acid giving the free acid. The product, if recrystallized from carbon tetrachloride, was identified as 2-thenoic acid only.

**Acknowledgments.**—This study was aided in part by a grant from the Office of Naval Research. The analyses were carried out by Drs. G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England.

CONTRIBUTION NO. 295
DEPARTMENT OF ORGANIC CHEMISTRY AND ENZYMOLOGY
FORDHAM UNIVERSITY
NEW YORK 58, N. Y.

## Evidence from Titration Curves on the "Acyl Shift" in Proteins

BY CHARLES TANFORD

RECEIVED OCTOBER 22, 1954

A recent paper by Lumry and Eyring[1] has drawn attention to the "acyl shift" which may occur at peptide bonds adjacent to serine or threonine residues in proteins. This intramolecular rearrangement appears to explain satisfactorily the lability, during acid hydrolysis, of peptide bonds adjacent to serine or threonine residues. Lumry and Eyring make the further suggestion that the acyl shift may be important under conditions much milder than those of acid hydrolysis and that, therefore, "acid titration experiments will almost universally need re-evaluation in light of this finding."

The purpose of this note is to show that such re-evaluation of titration data is not necessary. In fact, titration data show that the acyl shift does not occur, at least in proteins so far carefully examined, in the relatively mild acidities (down to *p*H 2) reached in titration studies.

The connection between the acyl shift and titration curves lies in the fact that an amino group is released during the rearrangement.[1] Each serine or threonine residue capable of undergoing the acyl shift would therefore be expected to contribute a cationic group to the protein molecule. The total number of cationic groups would then be considerably greater than the number calculated from amino acid analysis on the basis that only arginine, lysine, histidine and terminal α-amino residues contribute to the number of cationic groups.

The total number of cationic groups of a protein molecule, present at the acid end-point of a titration curve, is equivalent to the number of hydrogen ions bound in going from the isoionic point to the acid end-point.[2] This number can therefore be accurately determined for those proteins for which the isoionic point can be established. For proteins soluble in water at the isoionic point, this point may be established by direct measurement on deionized solutions. In some other proteins, where salt binding is not important, it may be established as the point of intersection of titration curves at different ionic strengths.

The pertinent data are available for five different proteins and the total numbers of cationic groups in these proteins, as determined from titration, are listed in Table I. The figures are compared with analytical data. It is seen that in every case the agreement between analytical and titration data is within experimental error.[3] The titration curves do not show any excess cationic groups due to acyl shift. If all serine and threonine residues were capable of undergoing an acyl shift alkaline to *p*H 2, the number of excess cationic groups would have been 26 for ribonuclease, 16 for lysozyme, 32 for β-lactoglobulin, 50 for ovalbumin, and 46 for serum albumin.

To the five proteins of Table I should be added insulin.[7] In this protein the isoionic point has

(1) R. Lumry and H. Eyring, *J. Phys. Chem.*, **58**, 110 (1954).

(2) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 446.

(3) For the sake of uniformity, all analytical data have been taken from the same compilation (Tristram, ref. 4). To indicate the probable error involved one may examine the data for lysozyme. Here Tristram's compilation gives the analysis of Lewis, *et al.*, (ref. 5). Another analysis by Fromageot and de Garilhe (ref. 6) would lead to a total of 19.8 rather than 17.8 cationic groups. The average of these would correspond exactly to the number found by titration.

(4) G. R. Tristram in H. Neurath and K. Bailey, ed., "The Proteins," Vol. 1, Part A, Academic Press, Inc., New York, N. Y., 1953, p. 181.

(5) J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

(6) C. Fromageot and M. P. de Garilhe, *Biochim. Biophys. Acta*, **4**, 509 (1950).

(7) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2163 (1954).

not been determined independently, but the alkaline end-point of the titration has been established. This enables one to determine the total number of both acid and basic groups by titration. Again there is complete agreement with analysis. No anomalous groups are observed.

TABLE I

NUMBER OF CATIONIC GROUPS

| | Assumed mol. wt. | Analyses[a] Arginine, histidine and lysine | Terminal N groups | Tota | Titration |
|---|---|---|---|---|---|
| Ribonuclease | 13,500 | 17.3 | 1[b] | 18.3 | 19[e] |
| Lysozyme | 14,200 | 16.8 | 1[c] | 17.8 | 19[f] |
| β-Lactoglobulin | 39,000 | 40.6 | 3 | 43.6 | 45[g] |
| Ovalbumin | 45,000 | 41.0 | 0 | 41.0 | 41[h] |
| Human serum albumin | 65,000 | 92.5 | 1[d] | 93.5 | 94[i] |

[a] Except where otherwise indicated analytical data were taken from the compilation by Tristram (ref. 4). See ref. 3. [b] C. B. Anfinsen, R. R. Redfield, W. L. Choate, J. Page and W. R. Carroll, *J. Biol. Chem.*, 207, 201 (1954). [c] Several references are given in ref. *f*. [d] H. van Vunakis and E. Brand, Abstracts, 119th Meeting, Am. Chem. Soc., 1951, p. 28c. [e] C. Tanford and J. Hauenstein, in preparation. [f] C. Tanford and M. L. Wagner, THIS JOURNAL, 76, 3331 (1954). [g] R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, 142, 803 (1942). [h] R. K. Cannan, A. C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, 41, 243 (1941). [i] C. Tanford, THIS JOURNAL, 72, 441 (1950).

As regards the six proteins here discussed, it can therefore be said with certainty that no significant fraction of the theoretically possible sites have undergone an acyl shift during titration to *p*H 2.

**Acknowledgments.**—This investigation was supported by a research grant from the National Science Foundation and by grant H-1619, from the National Heart Institute, of the National Institutes of Health, Public Health Service.

DEPARTMENT OF CHEMISTRY
STATE UNIVERSITY OF IOWA
IOWA CITY, IOWA

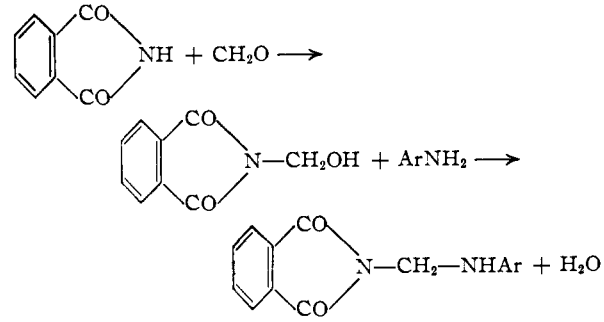
## Identification of Amines. I. N-(Arylaminomethyl)-phthalimides

BY MELDRUM B. WINSTEAD AND HAROLD W. HEINE

RECEIVED DECEMBER 1, 1954

Work in this Laboratory has shown that the reaction of primary aromatic amines with phthalimide and formalin to produce N-(arylaminomethyl)-phthalimides is a general reaction and one that is particularly useful for the identification of aromatic amines. A survey of the literature has revealed that only N-(piperidinomethyl)-phthalimide[1a,1b] and N-(morpholinomethyl)-phthalimide[1a] have been prepared by this method. N-(Morpholinomethyl)-phthalimide also has been synthesized by treating N-hydroxymethylmorpholine with phthalimide.[2] In addition two N-(arylaminomethyl)-phthalimides, N-(anilinomethyl)-phthalimide and N-(phenylhydrazinomethyl)-phthalimide, have been synthesized by the treatment of aniline and phenylhydrazine, respectively, with N-bromomethylphthalimide.[1b]

A possible mechanism of this reaction involves the initial formation of N-hydroxymethylphthalimide which subsequently reacts with the amine, *i.e.*




Thus in one experiment the present investigators treated N-hydroxymethylphthalimide with aniline in an aqueous alcohol solution and produced a 70% yield of N-(anilinomethyl)-phthalimide.

Alternately the formaldehyde may react initially with the aromatic amine to form the N-hydroxymethyl intermediate which would subsequently react with phthalimide to form the desired derivative.[2] Work is in progress to determine which of the two postulated mechanisms is correct.

The N-(arylaminomethyl)-phthalimides are easily made and readily purified. In many cases they precipitate from the reaction medium in a high state of purity and in good yields. All of the derivatives melt above 110° and below 235°. In addition the melting point of each derivative is distinctly different from that of the starting reagents. The melting points of isomeric derivatives vary from thirty to fifty degrees with only three exceptions.

The preparation of N-(arylaminomethyl)-phthalimides of additional primary and secondary aromatic amines as well as N-(alkylaminomethyl)-phthalimides of primary and secondary aliphatic amines now is being investigated.

### Experimental[3]

**Preparation of N-(Arylaminomethyl)-phthalimide Derivatives.**—Three grams (0.0204 mole) of phthalimide is suspended in 35 ml. of boiling 80% ethanol. Two milliliters of 37% formaldehyde is added and the solution is refluxed until all of the phthalimide has dissolved. Next a solution of 0.023 mole of the aromatic amine dissolved in 5–10 ml. of 80% ethanol is added. The alcoholic solution turns yellow or orange almost immediately after the amine has been added. The alcoholic solution is refluxed for one-half hour. If the aromatic amine contains a *meta*-directing group the refluxing period is increased to one hour. In a few cases the derivative is not too soluble in boiling 80% ethanol and begins to precipitate during the refluxing period.

The reaction medium is thoroughly chilled, and the product is filtered and dried. Many of the derivatives are brightly colored compounds which generally precipitate in a high state of purity. The derivative is recrystallized from 80% ethanol. Those derivatives which are only slightly soluble in this solvent are best recrystallized from a mixture of dioxane and petroleum ether (65–110°).

Table I lists the N-(arylaminomethyl)-phthalimide derivatives prepared.

**N-Hydroxymethylphthalimide.**[4]—One hundred and two grams (0.70 mole) of phthalimide, 52 ml. of formalin and

(1) (a) M. B. Moore and R. T. Rapala, THIS JOURNAL, **68**, 1657 (1946); (b) F. Sachs, *Ber.*, **31**, 3233 (1898).

(2) W. I. Weaver, J. K. Simons and W. E. Baldwin, THIS JOURNAL, **66**, 222 (1944).

(3) All melting points are corrected.

(4) S. R. Buc, THIS JOURNAL, **69**, 254 (1947).