ment with, a decrease in the area of the monolayer, measured under a standard compression of 1 dyne/cm.

After the monolayer has been well-aged by an application of pressure, $F = 25$ to 30 dynes/cm., of sufficient duration to drive out all pressure-soluble components, the force–area curves, (F, a), form reproducible closed loops, provided the pressure is raised and lowered according to a standard (F, t) cycle.

At $F > 15$ dynes/cm. sudden increases or decreases in F produce only slight changes in area, but these are followed by gradual changes of larger magnitude. These effects indicate that the apparent large compressibility of protein films results from a squeezing out of certain weakly hydrophobic amino acid residues from positions at the air–water interface into an underfilm where they contribute little to the surface pressure, although they are still attached through the polypeptide chains to the more strongly hydrophobic residues that remain in the overfilm.

This theory of pressure displaceability has led to a correlation of the compressibility curves of proteins to their chemical composition. The amino acids with side chains having a hydrophobicity less than that of $—C_2H_5$ determine the compressibility in the range from $F = 1$ to $F = 3$, while those more hydrophobic determine the areas at $F = 25$.

Films from denatured or partly digested proteins give large proportions of pressure-soluble components. This method of observing time changes in (F, a) curves provides a useful means of studying degradation products of proteins and obtaining information as to their molecular weights.

A general but preliminary theory of pressure solubility is given which is tested by studies of monolayers of Aerosols (dioctyl sodium sulfosuccinates). It indicated that the pressure-soluble components that were detected among the degradation products of proteins have molecular weights ranging from 1000 to 2000.

SCHENECTADY, N. Y. RECEIVED JUNE 15, 1940

[CONTRIBUTION FROM ALLERGEN INVESTIGATIONS, BUREAU OF AGRICULTURAL CHEMISTRY AND ENGINEERING, U. S. DEPARTMENT OF AGRICULTURE, AND THE ALLERGY CLINIC OF PROVIDENCE HOSPITAL, WASHINGTON, D. C.]

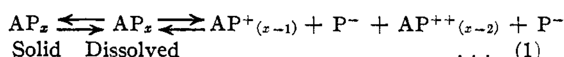
The Chemistry of Allergens. III. The Solubility Behavior of an Active Protein Picrate from Cottonseed[1]

BY JOSEPH R. SPIES, HARRY S. BERNTON AND HENRY STEVENS

Correlated chemical, clinical and immunological investigations[2] have shown that the principal allergenic component of cottonseed embryo is concentrated in the previously described protein picrate fraction CS-5.[2a] The unusual combination of chemical and physiological properties of the active component of CS-5 prompted an exhaustive study to determine whether the allergenic activity of this fraction is inherent or due to and unrecognized contaminant present in minor proportions.

An attempt to test the chromatographic homogeneity of CS-5 by solubility studies led to the development of a new method of fractionating the protein picrate and to the elucidation of some fundamental facts regarding the solubility behavior of a protein picrate. Although solubility measurements have found considerable application in determining homogeneity of proteins, the method has not been previously applied to a protein picrate.

To obtain solubility curves which would provide an evaluation of homogeneity, a weighed sample of CS-5 was equilibrated with successive volumes of solvent at constant temperature. The total nitrogen content of each extract was plotted against total per cent. of nitrogen removed from the original sample. It was assumed that a single component protein picrate, of the type represented by CS-5 or its fractionation products, would behave as a phase in equilibrium with saturated solutions according to equation (1)



$$\underset{\text{Solid}}{AP_x} \rightleftarrows \underset{\text{Dissolved}}{AP_x} \rightleftarrows AP^{+}{}_{(x-1)} + P^{-} + AP^{++}{}_{(x-2)} + P^{-} \quad \ldots (1)$$

(1) Presented in part at the 98th meeting of the American Chemical Society held at Boston, Massachusetts, September, 1939. Original manuscript received January 5, 1940. Not subject to copyright.

(2) (a) Paper II of this series: THIS JOURNAL, **62**, 1420 (1940). (b) Bernton, Spies and Stevens, "The Evidence of Multiplicity of Allergens and Reagins in Cottonseed Sensitiveness," *J. Allergy*, in press. (c) Coulson, Spies and Stevens, "The Immunochemistry of Allergens. I. Antigenic Properties of an Active Protein Component of Cottonseed," to be published.

where A represents active protein and P_x an unknown number of picric acid residues. Accordingly, if the protein picrate were stable, a straight line solubility curve would result from plotting the total nitrogen values of successive saturated extracts of the solid against total per cent. nitrogen dissolved. The ratio of picric acid nitrogen (PN_2) to total nitrogen (TN_2) should also be constant in saturated solutions of a homogeneous picrate. If the solid were not homogeneous, the total nitrogen values of successive saturated solutions would diminish as components of the mixture were progressively removed.

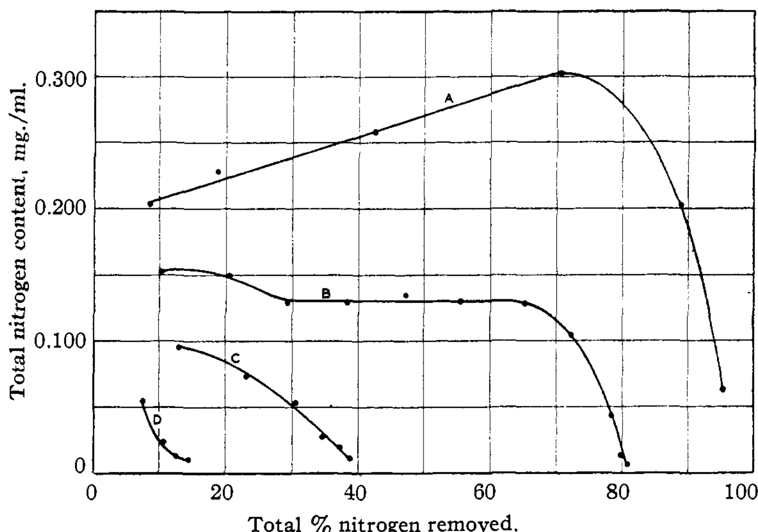


Fig. 1.—Effect of picric acid on the solubility of CS-5: (A) 300 mg. CS-5, solvent contained no picric acid; (B) 200 mg. CS-5, solvent contained 0.25 mg./ml. picric acid; (C) 200 mg. CS-5, solvent contained 0.50 mg./ml. of picric acid; (D) 200 mg. CS-5, solvent contained 1.00 mg./ml. picric acid; each sample equilibrated with successive portions of solvent; temp. 30 ± 0.02°.

When a sample of CS-5 was equilibrated with successive portions of distilled water, an anomalous increase in solubility was observed which was tentatively attributed to progressive removal of inorganic salts or to the rise in *p*H of successive extracts.[3] The same procedure was therefore conducted using 0.1 *N* acetate buffered at *p*H 4.0 as solvent.[4] The even greater rise in this solubility curve, Curve A, Fig. 1, suggested determination of the effect of small concentrations of picric acid on the solubility of CS-5. The solubility depressing effect of 0.25, 0.50, and 1.0 mg. of picric acid per milliliter of solvent is shown in Curves B, C, and D, respectively of Fig. 1.

The depressing effect on the solubility of portions of CS-5 which resulted from varying the concentrations of picric acid in the solvent suggested a means of fractionating the protein picrate. The procedure developed is illustrated graphically in Fig. 2, where the total nitrogen content of successive extracts and the quantities of solid picrate recovered from each extract are shown. Eighty-seven per cent. of the total nitrogen and 74% of the original solid were accounted for in this experiment.

To obtain evidence of significant differences in composition of the fractionation products of CS-5, the three fractions CS-5-1, CS-5-6, and CS-5-10 were selected for comparison by solubility measurements. The solubility curves for these individual fractions, Curves A, B, and C, Fig. 3, indicated that CS-5-1 differed significantly from CS-5-6 and CS-5-10. Difference in composition of CS-5-1 and CS-5-10 was also shown by Curve D, Fig. 3, which was obtained when equal quantities of the two fractions were recombined. The solubility of recombined fractions CS-5-6 and CS-5-10, however, did not differ appreciably from that of the individual fractions.

The solubility depressing effect on CS-5 of small concentrations of picric acid, Fig. 1, and the anomalous decline and rise in the solubility curves of the fractionation products of CS-5, Fig. 3, indicated removal of small quantities of picric acid in successive equilibrations. The picric acid responsible for the anomalous shape of these solubility curves was adsorbed on the protein picrate and was not present as a contaminant. To demonstrate this point, unequal quantities of CS-5-6 were successively equilibrated with equal volumes of solvent. A comparatively large sample of CS-5-6 was used to magnify the effects produced in the initial portion of the curve. The quantity of solvent employed for successive extractions was sufficient to dissolve all picric acid in the initial extract if it were present as contaminant. Figure 4 contains the solubility curves obtained by equilibrating a 330 and a 67-mg.

(3) The *p*H of six successive equal volume distilled water extracts of CS-5, accounting for 93% of the total nitrogen, was 3.7, 3.9, 4.0, 4.1, 4.3 and 4.6, respectively.

(4) Subsequently the term "solvent" will be used to designate 0.1 *N* acetate buffered at *p*H 4.0.

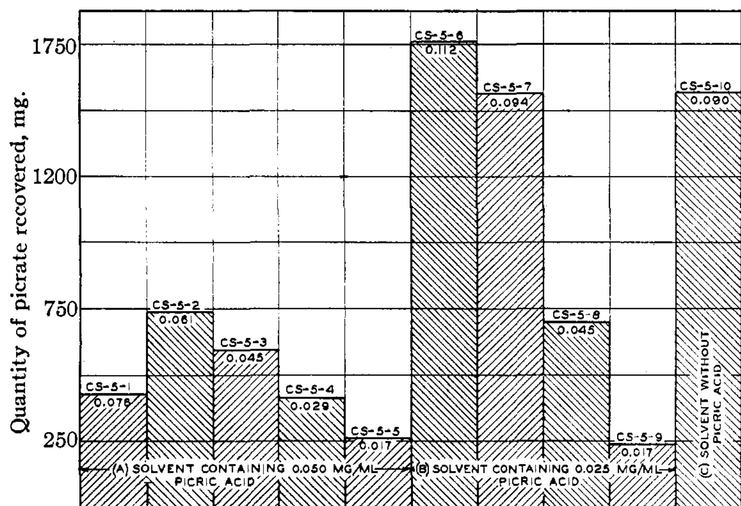


Fig. 2.—Fractionation of CS-5 by control of picric acid concentration of the solvent: (A) 12.0 g. CS-5 equilibrated with 5 successive 3200-ml. portions of solvent containing 0.50 mg./ml. of picric acid; (B) residual CS-5 equilibrated with 4 successive 3200-ml. portions of solvent containing 0.25 mg./ml. picric acid; (C) residue extracted with 3200 ml. of solvent containing no picric acid; total nitrogen content of each saturated solution in mg./ml. is shown by the values in each column; temp. 30 ± 0.02°.

sample of CS-5-6 with successive 11-ml. portions of solvent. The nitrogen content of the first six extracts of the 330-mg. sample, Curve A, progressively decreased and the PN_2/TN_2 ratio indicated that practically all of the nitrogen in these extracts was due to picric acid. With the subsequent six extracts the total nitrogen increased sharply as the ratio PN_2/TN_2 declined until constant. However, the nitrogen content of the 67 mg. sample increased after the second extraction and the ratio PN_2/TN_2 then declined to the same constant level.

Figure 5 shows the solubility curve obtained by making thirteen successive equilibrations of a protein picrate fraction CS-5-6E from which the adsorbed picric acid had been removed. The anomalous initial rise in solubility is absent and the ratio PN_2/TN_2 is constant within experimental error. The gradual departure of the curve from the theoretical straight line indicated possible fractionation.[5] With a more highly purified sample CS-5RE, the theoretical straight line solubility curve and constant PN_2/TN_2 ratio was obtained for the first 75% as shown in Curve A, Fig. 6. Curve B, Fig. 6, shows the solubility depressing effect on CS-5RE of solvent containing 0.1 mg. of picric acid per ml.

A further purified fraction designated CS-5-1RE was also obtained from CS-5-1. Fraction CS-5-1RE was more soluble and had a slightly higher PN_2/TN_2 ratio than fraction CS-5RE as shown in Fig. 7. However, CS-5-1RE appeared to be less homogeneous than CS-5RE.

The clinical activity of fractions CS-5-1, CS-5-6, and CS-5-10 was compared by the cutaneous reaction using gravimetric dilutions ranging from $1:10^5$ to $1:10^6$. Results of an extended series of tests provided evidence indicating that CS-5-1 and CS-5-6 contain at least two different allergenic constituents. The clinical activity of CS-5-6 was not distinguishable from that of CS-5-10.

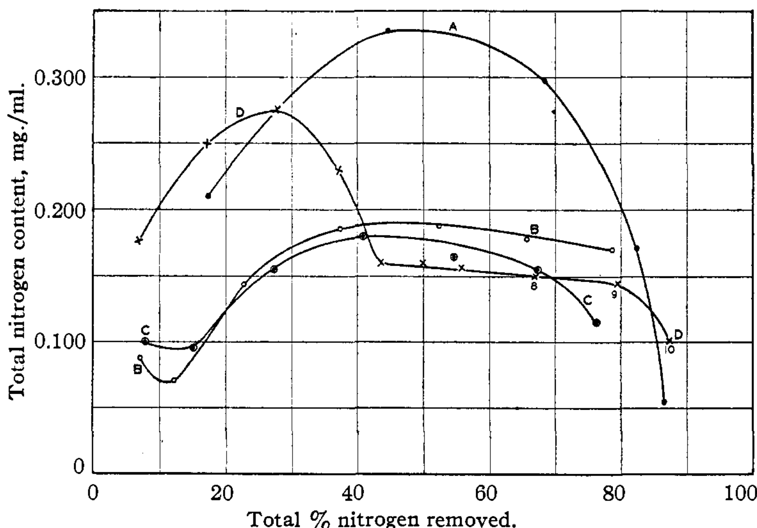
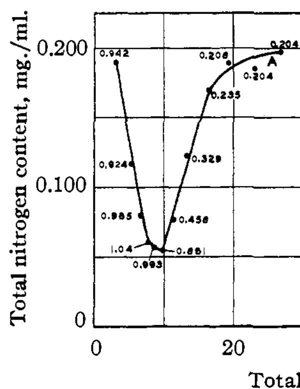


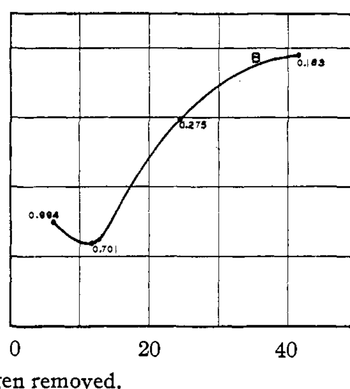
Fig. 3.—Comparative solubility curves of fractions obtained from CS-5: (A) 100 mg. CS-5-1; (B) 100 mg. CS-5-6; (C) 100 mg. CS-5-10; (D) 100 mg. CS-5-1 + 100 mg. CS-5-10; each sample equilibrated with successive 15-ml. portions of solvent except extracts 8, 9, 10 of D which were equilibrated with 30-ml. portions of solvent; temp. 15 ± 0.1°.

Clinical tests also were made with alternate successive extracts of CS-5-6E and CS-5RE which were diluted to equal nitrogen content. The results showed that no detectable fractionation had

(5) J. Steinhardt, *Cold Spring Harbor Symposia Quant. Biol.*, **VI**, 301 (1938), reported that the solubility of crystalline pepsin dropped continuously with successive extractions.

occurred, thus confirming the chemical evidence for essential homogeneity of these fractions.





Total % nitrogen removed.

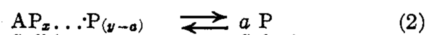
Fig. 4.—Removal of adsorbed picric acid from CS-5-6: (A) 330 mg. CS-5-6; (B) 67 mg. CS-5-6; each sample equilibrated with successive 11-ml. portions of solvent; the values at each point on the curve indicate the PN_2/TN_2 ratio; temp. 15 ± 0.1°.

and the ratio of PN_2/TN_2 decreased to the same constant value obtained with the 330 mg. sample, Curve B, Fig. 4. If the first extract, which might contain free as well as adsorbed picric acid, is disregarded, it may be considered that five times as many equal volume extractions were required to remove adsorbed picric acid from the 330-mg. sample as from the 67-mg. sample. During removal of adsorbed picric acid the solubility of the picrate is largely repressed.

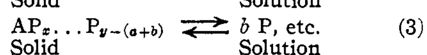
The solubility curves of fractions CS-5-6E and CS-5RE, from which the adsorbed picric acid has been removed, approach the theoretical form of either a single component or solid solution phase. With CS-5RE the total nitrogen content and PN_2/TN_2 ratio of seven successive extracts representing 75% of the total nitrogen is constant. If, as indicated, CS-/5RE were a single component, added picric acid in the solvent would uniformly decrease its solubility and give a solubility curve parallel to Curve A, Fig. 6. Curve B, Fig. 6 shows the solubility depressing effect of 0.1 mg./ml. (4.4×10^{-4} molar) picric acid in the solvent. A decline in TN_2 occurred between the second and third extracts, but the PN_2/TN_2 ratio was constant for the first eight extracts representing 62% of the total nitrogen. With the ninth extract a gradual decrease in total nitrogen content of the extracts occurred and the PN_2/TN_2 ratio became slightly lower. Definite interpretation of this effect is difficult. It should be pointed out, however, that the picrate fraction

Discussion

The protein picrate components of CS-5 may be represented by the formula, $AP_x \ldots P_y$, where P_y represents adsorbed picric acid. P_y is constant if the protein picrate has been precipitated with a sufficient excess of picric acid to satisfy the adsorption affinity of the salt AP_x. Successive equilibrations of this adsorption complex would remove P_y in steps according to the equations



$$\underset{\text{Solid}}{AP_x \ldots P_{(y-a)}} \rightleftharpoons \underset{\text{Solution}}{a\,P} \quad (2)$$



$$\underset{\text{Solid}}{AP_x \ldots P_{y-(a+b)}} \rightleftharpoons \underset{\text{Solution}}{b\,P, \text{ etc.}} \quad (3)$$

where a, b, etc., are concentrations of picric acid which are constant for a given extraction only if the ratio of solid picrate to solvent is constant. The amount of adsorbed picric acid removable by successive equal volume extractions progressively decreased as the firmness of adsorption of the last traces of picric acid increased. Thus, in equations 2 and 3, $a > b, \ldots$ etc., as is experimentally shown in Curve A, Fig. 4. After removal of most of the adsorbed picric acid from CS-5-6 (Fig. 4) the total nitrogen content rapidly increased and the PN_2/TN_2 ratio diminished to a constant value at the tenth extract. With the 67 mg.-sample, however, the total nitrogen content increased after the second extraction

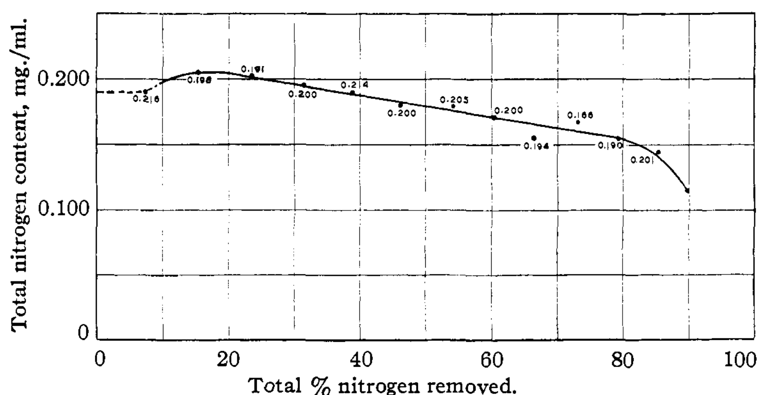


Fig. 5.—Solubility curve of CS-5-6E: 200 mg. of CS-5-6E equilibrated with successive 15-ml. portions of solvent; the values at each point on the curve indicate the PN_2/TN_2 ratio; temp. 15 ± 0.1°.

CS-5RE is considered a true salt (AP_x) in the solid phase only when the extracting solvent contains no excess picric acid. Concentrations of picric acid

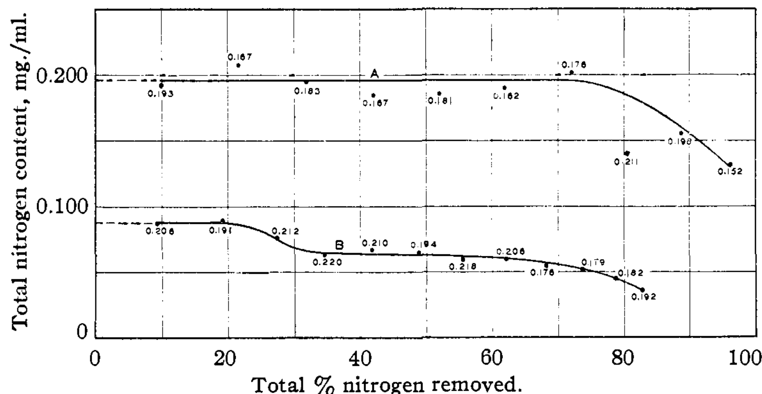


Fig. 6.—Solubility curves of CS-5RE: (A) 200 mg. CS-5RE, solvent contained no picric acid; (B) 100 mg. CS-5RE, solvent contained 0.1 mg./ml. picric acid; each sample equilibrated with successive 20-ml. portions of solvent; the values at each point on the curve indicate the PN_2/TN_2 ratio; temp. 15 ± 0.1°.

in the extracting solvent (Curve B, Fig. 6) lower than those required to satisfy the adsorption affinity of AP_x would convert the solid to $AP_x \ldots P_z$, where z represents a quantity of adsorbed picric less than y. The changes in slope in Curve B, Fig. 6, may therefore be due to the continuously diminishing ratio of solid picrate to picric acid concentration of the solvent as a result of successive extractions. Thus, as the quantity of solid diminished and the picric acid content of the solvent remained constant, z would progressively increase and the solubility of AP_x would thus gradually decrease. CS-5RE may still, therefore, represent a single component phase. If CS-5RE is a mixture or solid solution, the components must be closely related structurally.

The solubility curves of individual and recombined picrate fractions were compared in Fig. 3 to obtain an indication of the extent of fractionation effected. Quantitative additive solubility of two different protein picrates, however, could not occur because the concentration of picrate ion formed by each protein picrate in the system would have to equal the sum of the concentrations in independent systems. This would shift the equilibrium to the left (equation 1) thereby exerting a mutual solubility depressing effect. Thus the maximum value in the solubility curve for recombined fractions CS-5-1 and CS-5-10, Curve D, Fig. 3, does not equal the maximum value in the solubility curve of CS-5-1 alone, Curve A, Fig. 3. The modification in shape of the curve of these recombined fractions, however, indicates significant difference in composition of CS-5-1 and CS-5-10.

The method of fractionation developed in this study based on an application of the common ion effect in the law of mass action could undoubtedly be applied to other protein picrates or analogous protein salts. The solubility method also appears to have promise as a means of determining homogeneity of such salts. A suitable choice of solvent and pH would, however, have to be determined for each system.

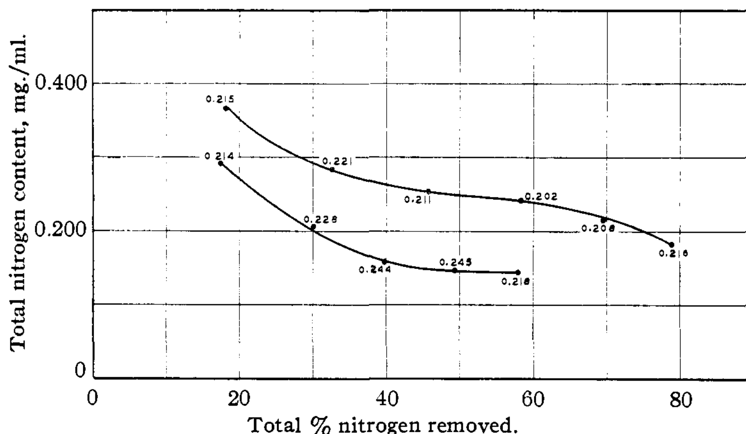


Fig. 7.—Solubility curve of CS-5-1RE: (A) 120 mg. CS-5-1RE, solvent contained no picric acid; (B) 100 mg. CS-5-1RE, solvent contained 0.1 mg./ml. of picric acid; each sample equilibrated with successive 11-ml. portions of solvent; the values at each point on the curve indicate the PN_2/TN_2 ratio; temp. 15 ± 0.1°.

Experimental[6]

Preparation of the Protein Picrate CS-5.—All solubility studies described in this paper were carried out using the protein picrate fraction CS-5 or fractions prepared from it. A detailed account of the preparation and properties of CS-5 is given in Paper II of this series. Residual picric acid was removed from CS-5 by one washing with a ten-fold weight of water. The sample was then dried in a vacuum over

(6) Microanalyses were carried out by Ernest J. Umberger and Thomas H. Harris. Dorris C. Chambers assisted in clinical studies.

phosphorus pentoxide. The dried sample was ground to pass a 100-mesh sieve and spread on a watch glass to become equilibrated with atmospheric moisture. *Anal.* Found: N, 17.9, 18.1; H_2O, 6.4 (dried to constant weight at 110 degrees in a vacuum Abderhalden); ash, 2.45.

Method of Fractionating CS-5.—CS-5 (100-mesh) in 12-g. lots, was equilibrated with 3200 ml. of solvent containing 0.5 mg. of picric acid per ml. at 30 ± 0.02°, by stirring at least twenty hours. The undissolved picrate was allowed to settle and the saturated solution siphoned off and filtered. The residual picrate was similarly extracted with four more 3200 ml. volumes of solvent containing 0.5 mg. picric acid per ml. The undissolved residue from the fifth extraction was equilibrated in the same manner four times with solvent containing 0.25 mg. of picric acid per ml. Finally, the undissolved residue was extracted with one 3200 ml. volume of solvent without picric acid. To each 3.2 liters of saturated solution was added 800 ml. of saturated aqueous picric acid solution. The picrate suspensions so obtained were cooled overnight at 5°. The solid, obtained by centrifuging, was washed with 100 ml. of water and dried overnight in a vacuum over phosphorus pentoxide. The samples were ground to pass a 100-mesh sieve and then exposed to air to constant weight (two days). The quantity of each fraction obtained is shown graphically in Fig. 2. The total nitrogen content of the solid fractions varied from 18 to 19.1%. The nitrogen content of these and all subsequent picrate fractions was determined on samples which had been equilibrated with air after drying in a vacuum over phosphorus pentoxide. Exposure to air caused approximately 2% increase in weight. The ash content of the fractions was negligible. Fractions CS-5-6 through CS-5-9 contained the higher percentages of nitrogen.

Preparation of CS-5-6E.—Two grams of CS-5-6 (picric acid content, 27.3%) was extracted at 15° by stirring at least twenty hours with 300 ml. of solvent. The solution was removed and the residue was similarly extracted with two more 300-ml. portions of solvent. The insoluble picrate was washed with 50 ml. of water, dried in a vacuum over phosphorus pentoxide and prepared for solubility tests by grinding to 100 mesh and equilibration with air; yield, 1.31 g. *Anal.* Found: N, 18.7; picric acid, 19.2, 19.3.

Preparation of CS-5RE.—Allergenic picrate consisting of 1.1 g. CS-5-7, 1.8 g. CS-5-8, and 0.5 g. CS-5-9 was dissolved in 270 ml. of 0.05 *N* sodium hydroxide. Absolute ethanol, 270 ml., was added with stirring. To the clear yellow solution was added 50% acetic acid until the *p*H was 6.3. A 540 ml. volume of alcohol was then added to the suspension and the *p*H adjusted to the coagulation point (6.1–6.3). After cooling overnight to −7°, the yellowish solid was centrifuged off and dissolved in 70 ml. of warm water. The solution was boiled twice with carbon, centrifuged, and filtered through a Seitz sterilizing pad. The solution was poured into 300 ml. of cold ethanol and cooled to −7°. The coagulated solid was recovered by centrifuging and dried in vacuum over phosphorus pentoxide. A yield of 1.67 g. of white solid containing 18.9% nitrogen (moisture-free basis) was obtained.

One gram of this recovered picric acid-free solid was dissolved in water and reprecipitated by picric acid. A yield of 1.19 g. of picrate was obtained, from which 516 mg. of protein, containing 18.9% nitrogen (moisture-free basis) was isolated as before. Results of protein color tests on a 1% aqueous solution of this purified picric acid-free compound were: pinkish biuret, deep bluish-purple ninhydrin, and positive Millon. This compound corresponds to the picric acid-free fractions CS-13 and CS-13A. Chemical and immunological evidence for the protein nature of CS-13 and CS-13A is discussed in Paper II of this series. Further evidence of the protein nature of CS-13 awaits determination of its amino acid composition. This study is in progress. From 450 mg. of this recovered protein 566 mg. of picrate was again formed. The adsorbed picric acid was removed from this picrate by the same method used in preparing CS-5-6E. A yield of 362 mg. of picrate, designated CS-5-RE was obtained. The sample, dried in a vacuum over phosphorus pentoxide, was ground to pass a 100-mesh sieve and was equilibrated with air. *Anal.* Found: N, 18.5; H, 5.82; C, 43.08; ash, neg.

Preparation of CS-5-1RE.—From 1.0 g. of CS-5-1 was obtained 480 mg. of a white picric acid-free protein containing 16.8% nitrogen (moisture-free basis) by the methods used before. A 1% aqueous solution of this picric acid-free compound gave the following protein color tests: purple ninhydrin, positive Millon, pinkish blue biuret. These tests were, however, not as pronounced as those obtained with the active component of CS-5-RE (above). From 450 mg. of this protein was obtained 522 mg. of picrate. The adsorbed picric acid was removed as described before, and the dried sample was ground to 100 mesh and equilibrated with air. A yield of 284 mg. of picrate (CS-5-1RE) was thus obtained. *Anal.* Found: N, 18.0.

Preparation of Saturated Solutions.—A weighed quantity of picrate fraction (100-mesh) was moderately stirred mechanically in a 100-ml. Pyrex centrifuge tube for at least twenty hours at constant temperature. Experiment showed that four to six hours of stirring was insufficient for the attainment of equilibrium. However, stirring up to ninety-six hours would dissolve no more than twenty hours stirring. Attempts were not made to attain saturation by cooling a solution saturated at higher temperature because this technique yielded unfilterable colloidal suspensions. A few drops of toluene were added to each suspension as a preservative.

After reaching equilibrium the suspended picrate was allowed to settle or was centrifuged for one-half to one minute at controlled temperature. The saturated supernatant solution was kept at constant temperature and filtered off, using a 9-cm. (B.2) porcelain filterstick. The residual picrate was then equilibrated with solvent in a similar manner. Using this technique, up to 96% of the total nitrogen could be accounted for.

Determination of Total Nitrogen in Saturated Solutions.—Total nitrogen content of picrate solutions was determined by the Kjeldahl micromethod after preliminary reduction with hydriodic acid according to a modification of Friedrich's method.[7] Where picric acid was employed in the solvent, dissolved nitrogen was obtained by difference.

Picric Acid.—Reagent grade picric acid was further purified by recrystallization from water and dried in a vacuum over phosphorus pentoxide.

(7) Friedrich, *Z. physiol. Chem.*, **216**, 68 (1933).

Determination of Picric Acid.—Picric acid in solutions was determined with nitron[8] reagent by the following micromethod. Three to five ml. of picrate solution was pipetted into a porcelain crucible and diluted to 10 ml. One drop of 2:3 sulfuric acid was added and solution warmed in an oven to nearly boiling. One ml. of 10% nitron reagent[8] was added and the crucible heated at near the boiling point for fifteen to twenty minutes. The suspension was cooled for two hours and the solution removed from the crystalline precipitate with a porcelain filterstick. The precipitate was washed with 5 ml. of ice water and dried one hour at 105°. After cooling overnight the crucible and filterstick were weighed on a microbalance.

Summary

1. A new method of fractionating the allergenic protein picrate from cottonseed has been developed. The method is an application of the common ion effect of the mass action law.

2. Picric acid has been shown to combine with the active protein in true salt combination and to be adsorbed on the picrate.

3. A protein picrate fraction (CS-5RE) has been obtained which behaves essentially as a single component phase in solubility studies.

4. Chemical and clinical evidence is presented showing that the active picrate fraction CS-5 contains more than one allergenic component, probably very closely related structurally.

(8) Cope and Barab, THIS JOURNAL, **39**, 504 (1917).

WASHINGTON, D. C. RECEIVED JUNE 17, 1940

[CONTRIBUTION FROM THE ABBOTT LABORATORIES]

Esters of Brominated Aminobenzoic Acids

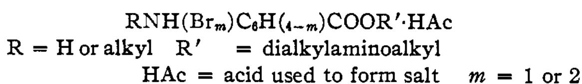
BY M. B. MOORE AND E. H. VOLWILER

The great majority of commercial synthetic local anesthetics belong to the class of esters of benzoic or aminobenzoic acids, and many such compounds have been described in the scientific literature. In contrast to this wealth of information is the scanty reference to alkamine esters of halogen-substituted benzoic acids, especially those containing exclusively bromine and amino groups in the ring. Only five such compounds were found described in the literature.

β-Diethylaminoethyl and β-piperidinoethyl 3,5-dibromo-4-aminobenzoates are described in a British patent[1] and the former is probably identical with the compound obtained by Morel, Leulier and Denoyel[2] by direct bromination of procaine in aqueous solution.

β-Diethylaminoethyl 2-bromo-4-aminobenzoate was prepared by Frejka and Vitha.[3] Two isomers of this, the β-diethylaminoethyl esters of 4-bromo-2-aminobenzoic acid and of 3-bromo-4-aminobenzoic acid, have been described,[4,5] the latter being formed by direct bromination of procaine in ether solution in sunlight.

The purpose of this communication is to report a number of brominated amino-benzoates and brominated alkylamino-benzoates which have been prepared in our laboratories during the past few years. The general formula for these compounds may be expressed as:



$$RNH(Br_m)C_6H_{(4-m)}COOR'\cdot HAc$$

R = H or alkyl R′ = dialkylaminoalkyl
HAc = acid used to form salt m = 1 or 2

Their properties are summarized in Table I.

These compounds were tested for local anesthetic efficiency and toxicity by Dr. C. C. Pfeiffer[6] and Dr. R. Kohn-Richards. All the monobrominated derivatives are quite efficient anesthetics and some have favorable efficiency/toxicity ratios. The water solubility of their common salts, however, is not very great, the hydrochlorides being so insoluble that a precipitate is formed when sulfates or acetates are used to produce corneal anesthesia. The dibrominated derivatives are, in general, less anesthetic and more convulsant.

Experimental Part

γ-Bromopropyl 2-Bromo-3-nitrobenzoate.—The potassium salt of 2-bromo-3-nitrobenzoic acid was prepared by the reactions: 3-nitrophthalic acid ⟶ anhydro-2-hydroxymercuri-3-nitrobenzoic acid ⟶ 2-bromo-3-nitrobenzoic acid as described in "Organic Syntheses."

The potassium salt of 2-bromo-3-nitrobenzoic acid (13.6 g.) was mixed with dry trimethylene bromide (47 g.) and a few drops of diethylamine and heated in a paraffin-bath at about 140° for twenty-four hours. The product was

(1) Schering-Kahlbaum A.-G., British Patent 321,968 (1928).

(2) Morel, Leulier and Denoyel, *Bull. soc. chim.*, [4] **45**, 457–463 (1929).

(3) Frejka and Vitha, *Pub. faculté sci. univ. Masaryk*, No. 48, 1–22 (1925); *C. A.*, **19**, 2332 (1925).

(4) Frejka and Vymetal, *Collection Czechoslov. Chem. Commun.*, **7**, 436–443 (1935); *C. A.*, **30**, 1370–1371 (1936).

(5) Frejka and Čižmář, *Chem. Listy*, **31**, 460–464 (1937); *C. A.*, **32**, 4967 (1938).

(6) University of Chicago.