paralleled by the effects of pressure on the absorption of light by similar solutions.

Summary

We have found that the absorption of visible light by solutions of aromatic amines in nitro or nitroso compounds is pushed very significantly toward the longer wave lengths when the hydrostatic pressure over the solutions is raised at constant temperature, and also when the temperature is raised at constant volume. The absorption of light by these solutions may increase, decrease or remain constant as the temperature is raised at constant pressure. These results have been correlated into a consistent theory which avoids any assumption of the formation of colored compounds by the hypothesis that the colors of the solutions arise from the mutual polarizations of the molecules when appropriate groups are in close proximity, account being taken of the influence of pressure and temperature on the collision frequency, the distance of closest approach and the effect of short-range attractions between groups.

We have also shown from orienting experiments that substituents in the nitro compound and in the amine influence the absorption of light by the solutions in a way that parallels closely their effects on the reducibility of the NO_2 or NO group on the one hand, and the electron mobility in the amino compound on the other. It is suggested that the polarizations which give rise to the colors of the solutions may be regarded as primary steps in possible reactions, such as oxidation and reduction, involving transfer of electrons from the aromatic amine or hydrocarbon to the oxygen of the nitro or nitroso group.

WASHINGTON, D. C. RECEIVED FEBRUARY 21, 1940

[CONTRIBUTION FROM THE MULTIPLE FELLOWSHIP OF THE COTTON RESEARCH FOUNDATION, MELLON INSTITUTE]

Composition of Cottonseeds. III. Solubility of Proteins in Alkaline Solutions of Neutral Salts[1]

BY H. S. OLCOTT AND T. D. FONTAINE

Oil-free cottonseed meats contain approximately 8.5% nitrogen (50–55% protein). Water will extract 25–30% of the nitrogen compounds. Dilute solutions of neutral salts extract 75–85% of the total. The materials not dissolved by water but extracted by salt are the cottonseed globulins. Osborne and Vorhees[2] and Jones and Csonka[3] have recorded some of the properties of these proteins. Their extraction by salt and alkaline solutions has been described more recently.[4]

Methods were sought for isolating the globulin fraction on a large scale. Extraction with salt solutions was efficient but means of recovery therefrom did not appear to be practical. Dilution required relatively large amounts of water, and precipitation with acid, though easily effected, yielded an insoluble product. The cottonseed globulins, like several other vegetable globulins, are very readily denatured in acid solution.

Extraction with alkali at *p*H 10.0 and precipitation by the addition of acid to the isoelectric point (*p*H 6.5–7.0) was found to be a satisfactory procedure but suffered from the disadvantage that cottonseed pigments are easily oxidized in alkaline solution, yielding brown products which affect the color of the protein.

A consideration of the theories of protein solubility suggested that the globulin might be extracted at lower *p*H levels in the presence of low concentrations of salt; that is, that the two methods, salt peptization and alkali solution, would be mutually additive. Experimentally, however, it was found that the opposite was true; in the presence of low concentrations of salt, the solubility of the protein at a *p*H below the maximum for extraction was actually depressed. The phenomena observed were of sufficient interest to invite further investigation.

Experimental

Data were obtained in the following manner. Ether-extracted cottonseed meats[4,5] were used. A sample was

(1) Presented in part before the Division of Biological Chemistry of the American Chemical Society at Boston, Mass., Sept. 11–15, 1939.

(2) Osborne and Vorhees, THIS JOURNAL, **16**, 778 (1894).

(3) Jones and Csonka, *J. Biol. Chem.*, **64**, 673 (1925).

(4) Olcott and Fontaine, THIS JOURNAL, **61**, 2037 (1939).

(5) The amount of nitrogen extracted under a given set of conditions varied for different preparations of cottonseed meal. With the two meals used in the present investigation, alkali at *p*H 9.0 extracted 83% of the nitrogen from one, 77% from the other.

shaken thoroughly with 40 times its weight of water containing sufficient salt and alkali (as indicated by preliminary titrations) to bring the mixture to the required *p*H and salt concentration. Several drops of chloroform were added to prevent spoilage and the mixtures were allowed to stand, with occasional shaking, for sixteen to twenty-four hours. To determine the amount of protein extracted, the suspension was centrifuged, filtered, and aliquots were analyzed for nitrogen by a modified Kjeldahl method. *p*H was determined with the glass electrode.[6]

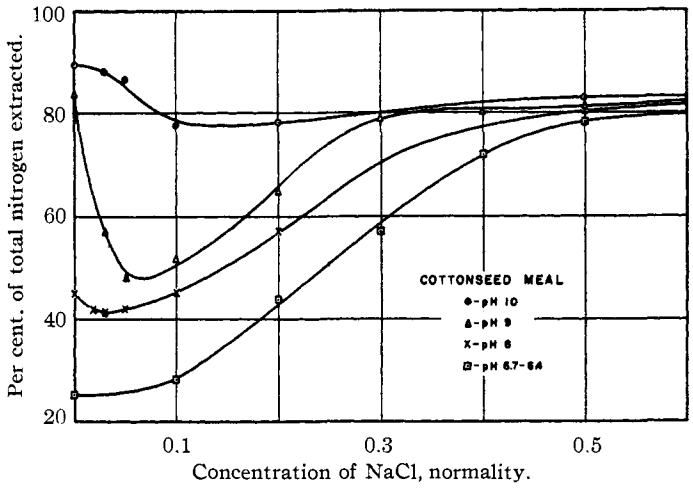


Fig. 1.—Effect of sodium chloride concentration on the extraction of cottonseed proteins from the meal at different *p*H levels. Sodium hydroxide was used to bring the mixtures to the proper *p*H.

The effect of sodium chloride on the extraction of the nitrogen compounds of cottonseed at various *p*H levels is indicated in Fig. 1. The solubility of the protein at *p*H 10 was depressed only slightly by the presence of salt. At *p*H 9, the amount of protein extracted reached a minimum in 0.05–0.07 *N* salt, the solubility again increasing with increasing concentration of salt. At *p*H 8, the antagonistic effect of the salt was only slightly evident. The lowest curve represents the extractability of the cottonseed proteins in solutions of salt to which no alkali had been added. The *p*H of such extracts varied from 6.7 in the absence of salt to 6.4 in 0.5 *N* solution.

That the behavior of cottonseed meal under the conditions described could be attributed specifically to the globulins was demonstrated by duplicating the experimental procedure with the isolated protein (Fig. 2). The material was prepared by leaching cottonseed meal with water to remove water-soluble constituents, then extracting the residue with 0.5 *N* sodium chloride. After centrifugation, the protein was precipitated by dilution with 5 volumes of water. The precipitate was repeatedly washed with distilled water to remove residual salts, separated each time by centrifugation, and finally dried in thin layers on a glass plate in a current of air. Sixty-four % of the product so obtained was soluble at *p*H 9.0, but only 22% could be peptized at the same *p*H in the presence of 0.05 *N* sodium chloride. A solution of the protein at *p*H 9.0 or 9.5 could be partially precipitated by the addition of the correct amount of salt.

The effect of several other inorganic salts on the solubility of the cottonseed proteins was investigated under similar conditions (Fig. 3). In the case of the sodium salts, the important part played by the anion was evident. In contrast to the chloride, the sulfate and iodide caused only a slight depression in the solubility curve. Sodium fluoride is a poor peptizing agent; whereas, in neutral solution, most inorganic salts (0.5 *N*) peptize 75 to 85% of the nitrogen of cottonseed meal, sodium fluoride peptizes only 57%. The results obtained with potassium chloride and sulfate were almost identical with those observed with the corresponding sodium salts (Table I).

The effect of alkaline earth salts was more pronounced. Inasmuch as water alone extracts 25 to 30% of cottonseed nitrogen, the 0.03–0.05 *N* solutions of calcium chloride and magnesium sulfate apparently suppressed entirely the solubility of the globulin. This fact was also demonstrated with the isolated protein. A quantitative method for the separation of the globulin from an alkaline extract of cottonseed meal is suggested, based upon the addition of calcium, magnesium, or barium salts in the correct concentration. The precipitation is not dependent upon a decrease in *p*H.

The data for experiments typical of those which have been plotted on the graphs are shown in Table I. Preliminary runs were used to determine how much alkali was necessary to bring the mixture to the required *p*H. It will be noted that increasing amounts of alkali were needed with increasing concentrations of salt.[7]

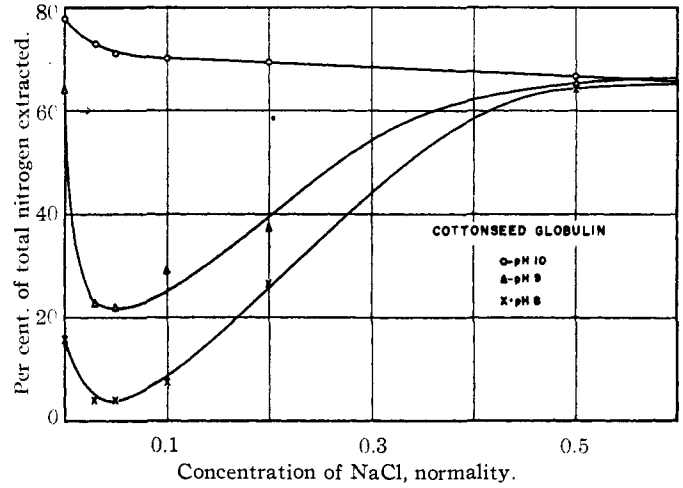


Fig. 2.—Effect of sodium chloride on the solubility of a cottonseed globulin preparation at different *p*H levels.

The effect of sodium and calcium chlorides, in the concentrations resulting in minimum solubility, on the extrac-

(6) Coleman electrometer, model 3D.

(7) This result was observed in every series of experiments. The complete data have been omitted for the sake of brevity.

TABLE I

EXTRACTION OF NITROGENOUS SUBSTANCES FROM ETHER-EXTRACTED COTTONSEED[a]

Salt concn.,[b] N	NaCl NaOH Milli-equiv.	NaCl pH	NaCl % N extd.	KCl NaOH Milli-equiv.	KCl pH	KCl % N extd.	Na_2SO_4 NaOH Milli-equiv.	Na_2SO_4 pH	Na_2SO_4 % N extd.	K_2SO_4 NaOH Milli-equiv.	K_2SO_4 pH	K_2SO_4 % N extd.	$MgSO_4$ NaOH Milli-equiv.	$MgSO_4$ pH	$MgSO_4$ % N extd.
..	1.35	9.00	83.6	1.40	8.95	77.4	1.40	8.95	77.4	1.40	8.95	77.4	1.45	9.00	83.6
0.01	..	..	..	1.41	8.88	71.4	1.51	8.92	74.1	1.41	8.90	74.8	1.55	8.95	30.8
.02	..	..	..	1.45	8.88	58.8	1.56	9.00	73.5	1.45	8.93	71.6	1.60	8.90	28.2
.03	1.40	9.10	57.3	1.47	8.90	57.6	1.61	9.00	73.3	1.47	8.88	68.6	1.65	8.92	27.2
.05	1.45	9.05	48.7	1.51	8.88	50.5	1.66	8.97	69.5	1.51	8.90	67.2	1.75	8.90	31.8
.10	1.52	8.95	52.0	1.55	8.85	52.8	1.75	9.05	75.6	1.55	8.90	67.2	1.90	8.88	47.2
.20	1.62	8.90	65.2	1.60	8.85	62.3	1.82	9.05	78.5	1.60	8.88	75.5	2.05	8.90	67.5
.30	1.65	8.95	79.6	1.61	8.95	74.2	..	..	..	1.61	9.00	76.0	2.20	8.95	71.5
.40	1.66	8.88	80.5	1.62	8.95	75.4	..	..	..	1.62	9.00	76.3	..	..	..
.50	1.67	8.90	81.5	1.63	8.82	75.5	1.90	9.00	79.0	1.63	8.85	76.0	2.25	8.92	82.8

[a] Five g. of meal was extracted with 200 cc. of solution for sixteen to twenty-four hours. The amount of nitrogen contained in a filtered aliquot of the extract was calculated to 200 cc. to obtain the data. [b] Cottonseed meal contains 8% ash, more than half of which is soluble in water. The extractability of the protein is probably influenced somewhat by the presence of these salts of unknown nature.

tion of the nitrogenous compounds from cottonseed meal throughout the *p*H range 3 to 11 is shown in Fig. 4. For purposes of comparison, the curves showing the efficiency of extraction with 0.5 *N* sodium chloride and in the absence of salt are repeated from the earlier publication.[4] The specific antagonistic effect of 0.07 *N* sodium chloride is marked in the *p*H range between 8 and 10, while that of calcium chloride does not disappear until a *p*H of 11 is attained.

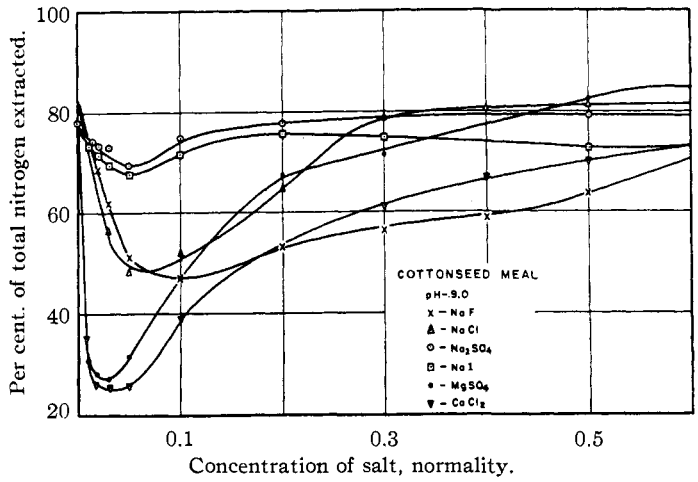


Fig. 3.—A comparison of the effect of several inorganic salts on the extraction of cottonseed proteins from the meal at *p*H 9.0.

The results shown graphically in Fig. 5 were obtained with ether-extracted hempseed meal under experimental conditions similar to those used with cottonseed meal. The absolute amounts of nitrogenous substances peptized differ from those observed with cottonseed meal but the shapes of the curves are somewhat similar. The antagonistic effect of sodium sulfate was more marked than in the case of cottonseed meal but was still less than that of sodium chloride.

Discussion

The phenomena of protein solubility have recently been reviewed by Edsall.[8] According to the investigators cited, neutral salts invariably *increase* the solubility of globulins on the alkaline side of the isoelectric point.

Sörensen and Sladek[9] concluded, from their carefully controlled experiments, that the solubility of casein varied directly with the concentration of sodium chloride in solutions alkaline to the isoelectric point. However, in the series in which the largest amount of alkali was used, a depression of solubility is recorded at a concentration of 0.025 *N* sodium chloride, as shown in Table II. This discrepancy is not commented upon in the original paper nor by Green[10] in her interpretation of the results. If the data are significant, they would indicate that, within a limited *p*H range, low concentrations of salt may antagonize rather than increase the solubility of casein in alkali. There is no suggestion in Green's[10] solubility determinations of hemoglobin in alkaline salt solutions of any parallelism with the phenomena described in this paper. The *p*H, how-

TABLE II

EFFECT OF SALT ON THE SOLUBILITY OF CASEIN IN ALKALI[a]
(Data of Sörensen and Sladek)[9]

Concn. of NaCl, N	pH (cor.)	% of total casein in solution
0.01	5.50	69.3
.025	5.40	63.5
.05	5.31	65.4
.10	5.24	82.2
.25	5.17	97.5

[a] In each case 91.6 milligram equivalents of casein nitrogen and 0.85 milligram equivalent of surplus NaOH were present in 1000 cc. of the mixture.

(8) Schmidt, "The Chemistry of the Amino Acids and Proteins," Charles C. Thomas, Springfield, Ill., Chapter XVI, 1938.

(9) Sörensen and Sladek, *Compt. rend. trav. lab. Carlsberg*, **17**, No. 14 (1927–1929).

(10) Green, *J. Biol. Chem.*, **93**, 517 (1931).

ever, did not vary by more than 1 *p*H unit from the isoelectric point, nor were salts other than phosphate buffers investigated. In the case of the cottonseed globulin, the region of maximum

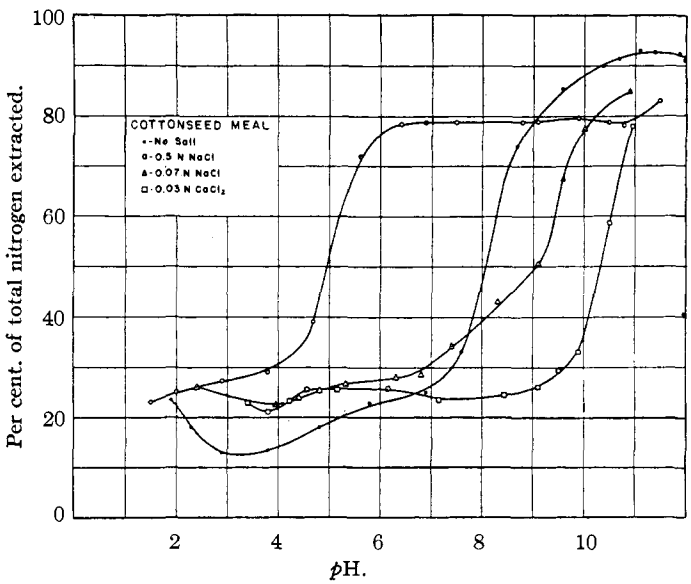


Fig. 4.—Extraction of proteins from cottonseed meal. Sulfuric acid and sodium hydroxide were used to adjust *p*H.

antagonism appears to be 2 *p*H units higher than the isoelectric point of the protein.

Edsall[11] observed that a clear gel of myosin in alkaline solution was precipitated by the addition of a small amount of salt. Upon the addition of further salt the precipitate dissolved. No quantitative data were recorded.

The data of Smith, Brother and Circle[12] can be interpreted as indicating a direct parallelism between soybean meal and cottonseed meal proteins (Fig. 6). More protein can be extracted from soybean meal with distilled water than with dilute salt solutions. Minimum solubility occurs at a concentration of 0.1 *N* sodium chloride (1 part meal:40 parts water). The isoelectric point of the principal soybean protein lies in the *p*H range 4.2–4.7; therefore the solubility behavior in neutral salt solutions (*p*H 6.7–6.0) was measured at approximately 2 *p*H units above the isoelectric point. While there was no attempt to maintain a constant *p*H level, the variations were probably not large enough to change essentially the character of the solubility curves.

(11) Edsall, *J. Biol. Chem.*, **89**, 289 (1930).

(12) Smith, Circle and Brother, THIS JOURNAL, **60**, 1316 (1938).

That the results obtained with soybean meal are a reflection of the behavior of the soybean globulin is indicated by the data shown graphically in Fig. 7. In this case the equilibrium was approached from the solution in dilute alkali.

A fresh precipitate of purified soybean globulin[13] was washed thoroughly with water, then dissolved by the careful addition of sodium hydroxide to *p*H 6.7. Upon the addition of different amounts of sodium chloride to aliquot portions, some cloudiness appeared but no precipitate could be removed by centrifugation. Quantities of acid sufficient to lower the *p*H to approximately 5.7 were then added to each aliquot, the several samples were centrifuged, and portions of the supernatant solution were analyzed for nitrogen. The sodium chloride concentration for minimum solubility appeared to be 0.1 to 0.15 *N*[14] or about twice that indicated by Smith, *et al.*,[12] for the meal. However, the solution contained approximately 3% protein, whereas with the meal, at maximum solubility, the protein was present to the extent of only 1.25%. The data for calcium chloride were obtained after the salt had been added to the solution at *p*H 6.7. In this case, no further addition of acid was necessary. The concentration of calcium chloride

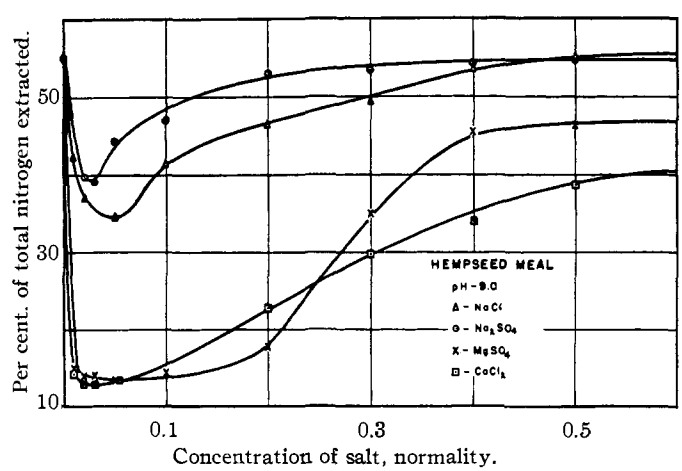


Fig. 5.—Effect of several inorganic salts on the extraction at *p*H 9.0 of the proteins from ether-extracted hempseed meal.

(13) Kindly supplied by Dr. N. J. Beaber, Mellon Institute.

(14) The salt concentrations are approximations. It was not determined how much of the sodium hydroxide used to bring the protein into solution had been required to neutralize adsorbed sulfuric acid.

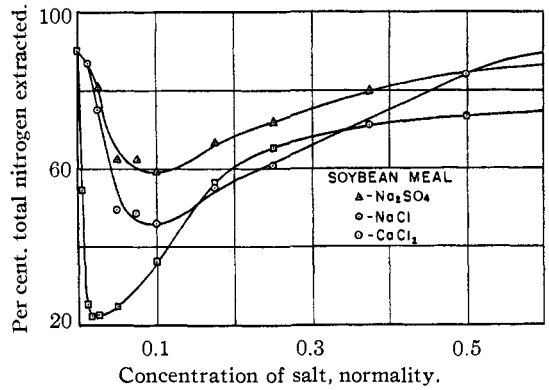


Fig. 6.—Data of Smith, Brother and Circle[12] on the extraction of soybean proteins by aqueous salt solutions. The *p*H was not held constant but probably varied from 6.7 to 6.3 with sodium chloride and from 6.7 to 5.2 with calcium chloride.

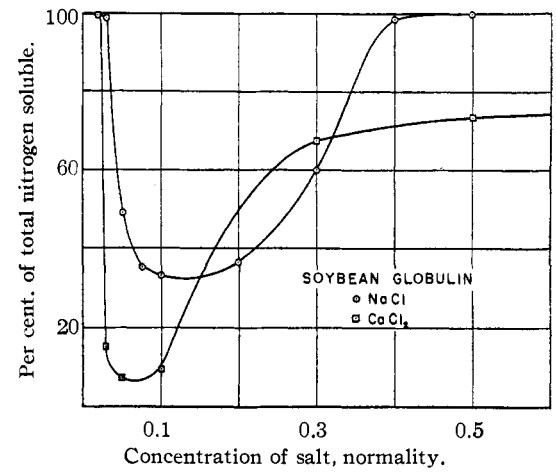


Fig. 7.—Effects of salts on the solubility of a soybean globulin preparation. The *p*H varied from 5.8 to 5.63 in the sodium chloride series and from 6.48 to 6.25 in the calcium chloride series.

necessary to ensure minimum solubility was also approximately twice that required for the meal.

The solubility of cystine in water is increased in the presence of salt. Cystine is also soluble in dilute alkali. This resemblance to the behavior of globulins, which has been discussed by Cohn,[15] suggested that the antagonism might be observable with the amino acid. However, in a short series of determinations, in which the sodium chloride concentration was varied from 0.1 to 2.0 *N* and the *p*H maintained at 8.0, the solubility was found to vary directly with the salt concentration.

A complete interpretation of the physical and chemical reactions involved in the antagonism of small amounts of salts to alkaline extraction of the several globulins does not seem possible at present. In the case of the alkaline earth salts, the inference that metallic proteinates are precipitated appears justified. The insoluble proteinate either may again be peptized in the presence of higher concentrations of salt or may react to form more soluble compounds containing larger amounts of calcium, barium, or magnesium or consisting of double salts of the metallic proteinate and the salt.

In the case of the sodium and potassium salts, the anion plays an important role.[16] Thus the sodium proteinate which is insoluble in sodium chloride solution (0.05–0.1 *N* at *p*H 9.0), is soluble in sodium sulfate or sodium iodide when these salts are present at the same concentration.

(15) Cohn, *Chem. Rev.*, **19**, 241 (1936).

(16) It has been suggested that the results may be interpreted in terms of electrokinetic potential changes. However, in the simplest application of such an explanation, it would be expected that a divalent anion (sulfate) would be a more effective precipitant than a monovalent ion (chloride), which is contrary to the observations.

As the proteins discussed in this communication, except myosin, are known to be mixtures of several individual entities, the possibility that the solubility phenomena observed with sodium chloride may be ascribed to a fractionation requires further investigation. A comparison of the physical and chemical properties of the precipitate and the soluble portion would be of interest. The data obtained with hempseed meal suggest that edestin would be a suitable purified protein for a more critical investigation of the phenomenon.

Inasmuch as it is not possible at present to continue this work, the attention of other investigators is invited to the possibilities for study of protein behavior suggested by these observations.

Summary

At *p*H 9.0 small amounts of sodium chloride markedly depress the solubility of cottonseed protein, as determined by extraction studies with cottonseed meal. Greater or smaller concentrations of salt permit the peptization of larger amounts of the total nitrogen. The alkaline earth salts are particularly effective precipitants; under the conditions used, 0.03–0.05 *N* calcium or magnesium salt solutions suppress the solubility of the globulin at *p*H 9.0 completely. The effects of several other salts on the solubility of the nitrogenous constituents of cottonseed meal have been determined. That the observed phenomena can be ascribed to the globulins and not to other constituents of the meal is shown in experiments with isolated globulin preparations.

Analogous results have been obtained with hempseed meal.

The data of Smith and co-workers[11] have been interpreted to indicate that the influence of salts on the solubility of soybean proteins in the neutral *p*H range is a parallel phenomenon.

The observations cited are exceptions to the hitherto-accepted generalization that the solvent action on proteins of salts and alkali is additive.

PITTSBURGH, PA. RECEIVED JANUARY 22, 1940

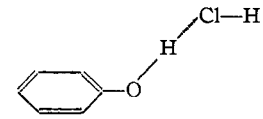
[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

Increases in the Acid Strength of Hydrogen Chloride in Dioxane Brought about by Phenols and Alcohols

BY PAUL D. BARTLETT AND HYP J. DAUBEN, JR.

Some years ago Meerwein and his co-workers[1] showed that the metallic chlorides which catalyze the rearrangement of camphene hydrochloride into isobornyl chloride are all capable of forming complexes with hydrogen chloride which are stronger acids than hydrogen chloride itself. This property was demonstrated by a method introduced by Hantzsch.[2] A solution of hydrogen chloride in dry ether was made just too dilute (0.02 *N*) to redden the indicator butter yellow (*p*-dimethylaminoazobenzene). The addition of equivalent stannic chloride (which alone at this concentration did not redden the indicator) now turned the indicator red, and a dilution of 120-fold was necessary to restore the yellow color. This was clearly due to the fact that in ether an acid of the type H_2SnCl_6 is stronger than hydrochloric acid. Meerwein concluded that the catalytic activity of the chloride and its acid-strengthening power were closely related manifestations of its complex-forming ability.

By Hantzsch's method it can be shown that the phenols, which also catalyze the rearrangement of camphene hydrochloride, conform to this generalization in that they, too, increase the acid strength of hydrogen chloride in ethereal solution. The effect is much less than with stannic chloride, but quite definite. We are forced to the conclusion that phenols, like the complex-forming inorganic chlorides, can form complexes with hydrogen chloride in which the phenol is attached to the chlorine and encourages the separation of this as an ion. The only probable manner in which a phenol can form such an attachment is through hydrogen-bond formation at its hydroxyl group



and we thus have a method of making some quantitative measurements on the relative tendencies to hydrogen bonding by a series of hydroxyl compounds.

This subject is of particular interest at the present time in view of the attention directed by G. N. Lewis[3,4] to the general aspects of the acid–base relationship. Any molecular species X which can form a coördinate link with the chloride ion, $X:\ddot{Cl}:^-$, is functioning as an "acceptor" center, in the terminology of Sidgwick,[5] while the chloride ion functions as a "donor." Lewis prefers to substitute the terms "acid" and "base" for "acceptor" and "donor," and says of the Sidgwick notation: "This expressive nomenclature I should be glad to adopt if I did not hope to show that his classification coincides absolutely with the classification of acids and bases, so that the need for new names disappears."[3,4]

Now in the present instance we have a substance, a phenol, which can function as an acid in the Brönsted sense by donating a proton to some acceptor; it can also function as an acid in the Lewis sense, as an acceptor, by forming a complex with the chloride ion or with hydrogen chloride through its chlorine. If these two kinds of acidity run completely parallel in a series of phenols, being governed by exactly the same structural features, then we have no need to differentiate between them. However, as we shall show, the Lewis

(1) Meerwein, Hammel, Serini and Vorster, *Ann.*, **453**, 16 (1927); Meerwein, *ibid.*, **455**, 227 (1927).

(2) Hantzsch, *Z. Elektrochem.*, **29**, 226 (1923); *Ber.*, **58**, 627, 631 (1925).

(3) G. N. Lewis, *J. Franklin Inst.*, **226**, 293 (1938).

(4) Lewis and Seaborg, THIS JOURNAL, **61**, 1886, 1894 (1939).

(5) Sidgwick, "The Electronic Theory of Valency," Oxford University Press, New York, N. Y., 1929.