[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Water Absorption of Proteins. I. The Effect of Free Amino Groups in Casein

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Introduction

Many papers have been written on the water-absorbing characteristics of proteins in which the phenomena have been interpreted on the basis of the total protein molecule or the sum of all the polar groups in the molecule. Most of these interpretations have involved the significance of the various sections of the sigmoid curve obtained when the amount of water vapor absorbed is plotted against the relative humidity.

Recently Shaw[2] and Bull[3] by applying the Brunauer–Emmett–Teller[4] (B.E.T.) multilayer theory of adsorption of gases on solid surfaces obtained areas covered in the first layer which appear to be only fractions of the total area of the proteins when they are spread in thin films. Pauling[5] has shown that the number of molecules of water vapor absorbed in the first layer bears a relation to the total number of polar side chains in certain proteins. The various interpretations of the previous data are summarized in the review of McMeekin and Warner.[6] If the initial water absorbed is attached to specific polar groups and not absorbed in general on the surface of the molecule, it would be possible to decrease the amount of water vapor absorbed by converting the polar groups into groups with comparatively less capacity for hydrogen bonding. Such decreases should be proportional to the number of polar groups converted.

Observations on the benzoylation of proteins made by Goldschmidt and Schön[7] indicated that although benzoylation occurred on a number of reactive groups, the benzoyl groups were bound much more firmly to some groups than to others. The benzoyl group seemed to be more firmly attached to polar groups containing nitrogen than to those containing oxygen. Removal of benzoyl groups from the polar groups containing oxygen occurred very readily in weak alkali, leaving a product in which the benzoyl groups were predominantly attached to nitrogen. They also indicated that benzoyl groups attached to nitrogen did not make the protein alkali insoluble, whereas benzoyl groups attached to oxygen made the proteins insoluble in alkali.

It appeared possible that the velocity of reaction of benzoyl chloride with amino groups might be sufficiently greater than that with oxygen-containing groups to allow selective benzoylation. This has been accomplished by using limited amounts of benzoyl chloride in the reaction medium. A series of benzoylated casein samples has been prepared with varying amounts of free amino groups and a minimum of benzoyl groups attached to other polar groups. The free amino content of these benzoylated derivatives was readily determined by the method of Doherty and Ogg[8] for the amino groups of insoluble proteins.

This group of derivatives, in which there has been little change of the protein molecule except the covering of the free amino groups, makes it possible to study the effect of the amino group on the water-absorbing property of casein.

Experimental

Preparation of Benzoylated Caseins.—One liter of water was added to 220 g. of air-dried, high-grade, hydrochloric acid-precipitated casein, and the mixture was stirred for one hour to swell the casein granules. The suspension was diluted with four liters of water, and 1 *N* sodium hydroxide was added slowly with vigorous stirring. The *p*H was not allowed to go above 6.7, and at this *p*H about five hours were required to dissolve the casein. This solution, which was very turbid, was filtered with suction through a 1-cm. layer of paper pulp to remove any suspended fat globules and undissolved calcium phosphates and caseinates. The filtrate showed only a slight trace of turbidity. One normal alkali was added until the *p*H was 9.0. The desired amount of benzoyl chloride (Table I) dissolved in 100 ml. of anhydrous ether, was added slowly from a dropping funnel while the solution was subjected to vigorous agitation. One normal alkali was added continuously as required to keep the *p*H constant. About two hours was taken for the addition of the benzoyl chloride, and the solution was agitated for one half hour after the addition had been completed. The solution of benzoylated casein was then filtered with suction through a 1-cm. layer of paper pulp. The resulting solution, which showed only the faintest trace of turbidity, was diluted with an equal volume of water and precipitated slowly with 0.1 *N* hydrochloric acid while being agitated vigorously. The final *p*H ranged from 4.5 to 4.0, depending on the fraction of the amino groups which was expected to be covered. The finely divided suspended solid was allowed to settle, and the supernatant liquid was decanted. The residue was transferred to 250-ml. centrifuge bottles, and the solids were separated from the remaining liquid by centrifuging. The solid residue, which filled about one third of the bottle, was suspended in distilled water and recentrifuged (about ten times) until the supernatant no longer gave a test for chloride ion. The product was then washed in a similar manner with 50% alcohol and 95% alcohol, and three additional times with water to remove any traces of alcohol. The solid residue remaining after the last centrifuging was frozen in the centrifuge bottle and dried in the frozen state. The products dried to fine, fluffy, white powders, 95% of which passed through a 100-mesh sieve without grinding. The yield in all cases was about 200 g. The products were then spread out in thin layers and allowed to equilibrate with the moisture in the air for four days. All analyses reported in Table I were run on these equilibrated samples. The total nitrogen

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Shaw, *J. Chem. Phys.*, **12**, 391 (1944).

(3) Bull, THIS JOURNAL, **66**, 1499 (1944).

(4) Brunauer, Emmett and Teller, *ibid.*, **60**, 309 (1938).

(5) Pauling, *ibid.*, **67**, 555 (1945).

(6) McMeekin and Warner, *Ann. Rev. Biochem.*, **15**, 119 (1946).

(7) Goldschmidt and Schön, *Z. physiol. Chem.*, **165**, 279 (1927).

(8) Doherty and Ogg, *Ind. Eng. Chem., Anal. Ed.*, **15**, 751 (1943).

was determined by a semimicro Kjeldahl method in which copper selenide is used as a catalyst and the digestion period is four hours. The amino nitrogen was determined on the solid materials by the method of Doherty and Ogg.[8] In determining ash calcium acetate[9] was added to the sample, and correction was made for the amount of calcium oxide in the ash. This method retains all the phosphate in the ash as phosphorus pentoxide. Phosphate determinations on the samples digested with sulfuric and nitric acids were made by the method of Fiske and Subbarow.[10] About 1.85% phosphorus pentoxide, on the basis of casein content, was indicated for each of the samples. The true ash value was, therefore, no greater than 0.15% for all the samples. The total benzoyl substitution was determined from the difference in total nitrogen values on the assumption that there was no change in the casein except the addition of benzoyl groups.

TABLE I

CHARACTERIZATION OF BENZOYL CASEINS

Sample	Benzoyl chloride added, ml./220 g.	Kjeldahl nitrogen, %[a]	$Ca(AcO)_2$ ash, %[a]	NH_2-N, %[a]	Benzoyl content mole/100 g. N[a] Total	on NH_2	Residual
1	0	15.50	1.92	0.93	0.000	0.000	0.000
2	8	15.10	1.95	.58	.163	.155	.008
3	14	14.84	1.80	.36	.273	.256	.017
4	20	14.71	1.75	.26	.329	.303	.026
5	22	14.34	1.78	.11	.497	.374	.123

[a] Moisture-free basis.

A control sample (no. 1) was prepared as described above, except that 13.5 g. of sodium benzoate in 100 ml. of water was added instead of the benzoyl chloride. An acid hydrolyzate of this material was extracted with ether to remove any benzoic acid present. The ether was evaporated at low temperature, and the residue was dissolved in water. An ultraviolet absorption spectrum of this water solution showed no trace of the characteristic absorption band of benzoic acid. Therefore, the benzoylated samples should be free of benzoic acid or its salts, which would have been produced by hydrolysis of benzoyl chloride during the preparation.

Absorption Measurements.—Weighing bottles of the "plug in" stopper type, 40 mm. in diameter and 50 mm. high, were carefully washed, rinsed with distilled water, and allowed to dry in air. To prevent contamination, these cleaned bottles as well as those containing samples under equilibration were handled only with chamois finger cots or clean paper strips.

Approximately 2-g. samples of the casein derivatives were placed in weighed, clean bottles. The bottles, with the stoppers resting on edge in the opening, were placed in a vacuum oven and heated at 70° for eighteen hours under a 29-inch vacuum. A small stream of air dried by passing through a magnesium perchlorate tower was used to sweep the liberated moisture from the oven. A preliminary drying experiment had shown that when a dry-air current is used to remove the liberated moisture from the oven the same equilibrium dry weight is obtained at 70, 90 and 105°. Consequently, we believe that this drying procedure gives a true dry weight. When the oven was opened, the bottles were immediately closed to prevent reabsorption of water vapor from the atmosphere, since absorption of moisture is rapid in thoroughly dried proteins. The closed weighing bottles were equilibrated with the atmosphere of the room for thirty minutes before weighing. A second drying period of six hours demonstrated that a stable dry weight had been obtained. An empty weighing bottle was used as a tare for all weighings, and this bottle accompanied the bottles containing the samples through all the manipulations.

Pyrex vacuum desiccators of 150 mm. inside diameter with sleeve-type stopcocks were used as the humidity chambers. These were well washed, rinsed with distilled water, and dried without wiping. About 150 g. of the desired analytical reagent-grade solid salt (Table III) was placed in the bottom of the desiccator, and distilled water was added until about one-tenth of an inch of solution was obtained above the salt. The salt solution was thoroughly stirred with a glass rod every time that samples were placed in the desiccator. The salt solution was covered with a solid-glass plate supported on a square of glass rod so that there was a space of about 3 mm. between the edge of the plate and the desiccator wall for the passage of water vapor from the salt solutions to the samples. This solid plate was necessary to prevent contamination of the weighing bottles by small droplets of salt solution sprayed into the air by the breaking of bubbles produced as the desiccator was evacuated.

The weighing bottles to be equilibrated were opened and placed immediately in the desiccator over the proper salt solution. The desiccator was evacuated to a pressure of 40 mm. of mercury and completely submerged in a water bath the temperature of which was controlled by a thermostat to ±0.1°. After four days, the desiccators were removed from the bath, filled with dry air and opened. The lids were closed on the bottles immediately. The closed bottles were allowed to equilibrate for thirty minutes with the atmosphere of the room before weighing. The equilibration at each humidity was repeated until the change in weight from the previous weighing was less than 2 mg.

With only a few exceptions the samples were started at the lowest humidity and, on reaching equilibrium there, were moved into the next higher humidity. This continued through all the humidities shown in Table III. After equilibration at 93% humidity, the samples were dried at 70° to check the original weight.

The room in which the water-bath was placed was kept at 30.0 ± 2° to minimize temperature changes in the salt solutions and desiccators when they were removed from the 30° water-bath.

Results and Discussion

In all but a few cases three water-absorption values were obtained at different times for each of the casein derivatives at each of the relative humidities studied. The average of these three values is presented in Table II. In all cases the standard error of this mean was less than 0.1 g., and in two-

TABLE II

WATER ABSORPTION OF BENZOYL CASEINS

Relative humidity, %	Water absorbed, g./100 g. dry material Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
		30.0°			
5.9	2.46	2.12	1.87	1.82	1.62
11.8	3.51	3.08	2.77	2.69	2.41
31.4	7.21	6.39	5.85	5.68	5.26
50.9	10.18	9.11	8.29	8.04	7.56
75.1	15.20	13.47	12.48	12.06	11.10
83.6	17.82	15.70	14.51	13.87	12.67
93.3	22.34	19.45	17.71	16.85	15.35
		40.0°			
11.0	3.32	2.97	2.69	2.55	2.40
31.3	6.61	5.89	5.36	5.21	4.85
48.3	9.01	8.13	7.38	7.08	6.69
74.7	14.21	12.55	11.57	11.16	10.40

(9) Sutermeister and Browne, "Casein and its Industrial Applications," Reinhold Publishing Corporation, New York, N. Y., 1939, p. 157.

(10) Fiske and Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

thirds of the cases it was less than 0.04 g., indicating good reproducibility in the experimental results.

In order to compare the water-absorbing properties of these benzoylated caseins containing various amounts of benzoyl substitution, it was assumed that the benzoyl group and the amino group substituted with benzoyl do not absorb water. If these assumptions are valid, the water absorbed per gram of total nitrogen is the significant variable to be considered, since it relates the water absorbed to the casein content of the material. These absorption figures for samples 1 and 4 are plotted as curves 1 and 2, respectively, against relative humidity in Fig. 1. The typical sigmoid-shaped absorption curve is obtained.

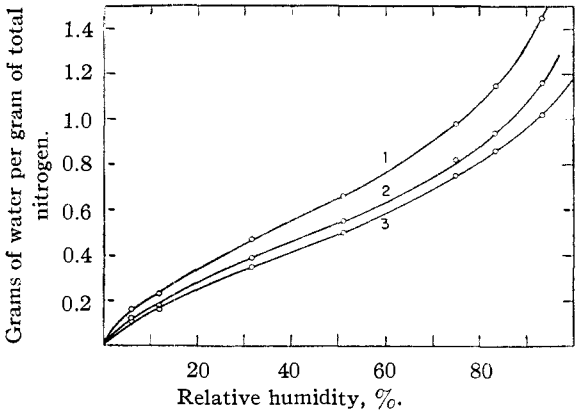


Fig. 1.—Absorption isotherms for casein (1), a benzoylated casein (2) and a hypothetical casein having no free amino groups (3).

When the average water absorption per gram of total nitrogen is plotted against the average amino nitrogen content per gram of total nitrogen (Fig. 2) a straight line is obtained. The straight lines were calculated by the method of least squares. The individual measurements of samples 1, 2, 3 and 4 were used; sample 5 was not used because it showed (Table I) a significant amount of non-amino benzoylation which may be on other hygroscopic groups. The slopes and intercepts of these lines are given in Table III. The intercepts when the amino content approaches zero should correspond to the water absorption due to the other groups of the protein. We have thus been able to distinguish between the water absorption occurring on the amino groups and that occurring on the other groups of the casein molecule. The intercepts for the 30.0° curves are plotted (curve 3) in Fig. 1. The sigmoid shape is still apparent.

TABLE III

WATER ABSORPTION BY AMINO AND NON-AMINO GROUPS OF CASEIN

Salt soln.	Relative humidity,[a] %	Figure II constants: Curve no.	Slope, g. H_2O / g. NH_2-N	Intercept, g. H_2O / g. N	Absorption on NH_2 groups in casein, % of total
		30.0°			
LiBr	5.9	1	0.87	0.106	33
LiCl	11.8	2	1.07	.162	29
$MgCl_2$	31.4	3	1.90	.350	25
$Mg(NO_3)_2$	50.9	4	2.73	.497	25
NaCl	75.1	5	3.82	.749	24
KCl	83.6	6	4.84	.858	25
KNO_3	93.3	7	7.29	1.011	30
		40.0°			
LiCl	11.0	8	0.97	0.157	27
$MgCl_2$	31.3	9	1.75	.321	25
$Mg(NO_3)_2$	48.3	10	2.38	.441	24
NaCl	74.7	11	3.75	.690	25

[a] Obtained from the linear plot of p *vs.* $1/T$ for all the available vapor pressure data of reasonable accuracy.

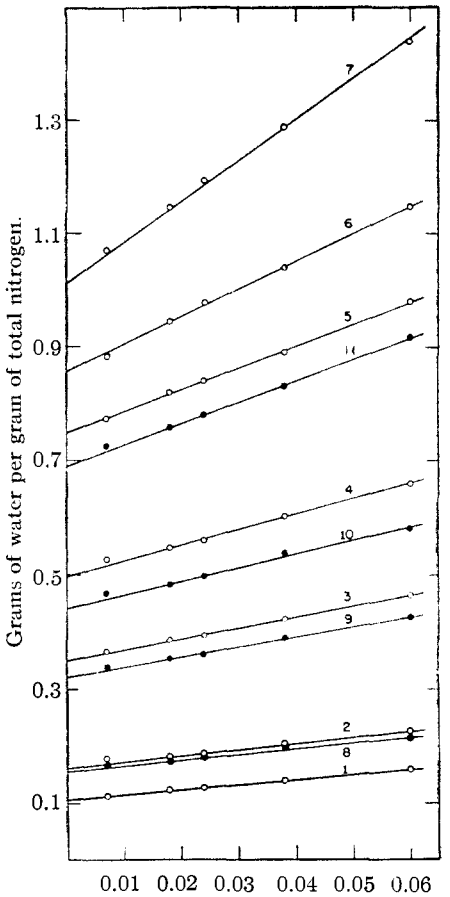


Fig. 2.—Water absorption by free amino groups in casein. The curves are identified by number in Table III.

The slope of the lines in Fig. 2 represents the grams of water absorbed per gram of amino nitrogen. These values have been plotted against relative humidity in Fig. 3 and may be interpreted as follows: Up to about 70% relative hu-

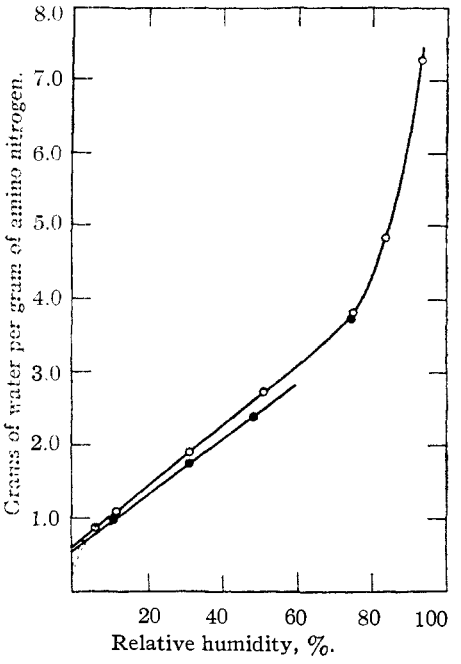
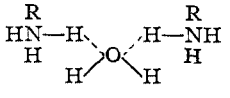


Fig. 3.—Water absorption isotherms for amino groups in casein: O, 30°; ●, 40°.

midity the graph is a straight line plot for which the equation by the method of least squares is

30.0° g. H_2O/g. NH_2—N = 0.0417 (% R. H.) + 0.60 (1)
40.0° g. H_2O/g. NH_2—N = 0.0378 (% R. H.) + 0.56 (2)

The intercepts correspond to 0.47 and 0.44 mole of water per mole of amino group, and appear to be identical within the limits of experimental error. We believe that this indicates the absorption of one molecule of water by two amino groups within the region between 0 and 6% relative humidity. Pauling[5] has postulated that there is a similar attachment of one molecule of water between two arginine side chain groups in salmin, and Bull[3] has indicated "a layer of water molecules between two coherent hydrophilic planes of protein molecules." He has suggested this as an interpretation of the a_1 term in the B.E.T. adsorption equation. Since the amino groups of casein are in the $-NH_3+$ form at the isoelectric point, it seems that the attachment of the water molecule must be between the positive hydrogen of the amino group and the oxygen of the water molecule.



The lysine content of casein accounts for more than 80% of the total free amino content. Consequently, if this assumption is correct, there must be a restriction on the position of the lysine residues within the casein molecule. One lysine epsilon amino group can approach another closely enough to hold a molecule of water between them only if the lysines are nearly opposite in adjacent peptide chains or laminae; or they are within the same chain either adjacent or separated by no more than two other amino acid residues which must have short side groups.

It is obvious that our data do not show the manner in which the curves of Fig. 3 approach zero absorption at zero relative humidity. It is, therefore, of value to present the alternative treatment where the graphs are assumed to follow a curved path in this region. These curves would be sigmoid in shape and the data could be treated by B.E.T. multilayer adsorption theory. According to this treatment, 1.05 moles of water per mole of amino group are required to form the first layer on the amino group, and the amino group accounts for 22% of the water absorbed in the monolayer on pure casein (sample 1), as calculated by the B.E.T. equation. This percentage agrees closely with the fraction of the total absorption due to the amino groups reported in Table III. The fact that the polar amino group, which constitutes less than 1% of the total weight of the protein, can account for about one quarter of the total water absorption indicates strongly that the water absorption of proteins occurs on specific sites (the polar groups) and that general surface adsorption plays a less important role. Cassie[11] has recently shown that such local sites for absorption do not necessarily have to be in the surface layer of the material; they can be distributed through the solid as in a hygroscopic gel.

The data can be further analyzed in terms of the B.E.T. treatment as follows. The statistical monolayer is complete at 18% R.H. The net heat of adsorption for this monolayer is 1.7 kcal. per mole. Similar analysis of curve 1, Fig. 1, gives 1.4 kcal. at 30° and 1.5 kcal. at 40° for the net heat of adsorption of the monolayer upon the pure casein sample. The heat of adsorption in the linear regoin of Fig. 3 can be directly calculated from the Clausius–Clapeyron equation. If we omit the intercepts (to eliminate the initial half mole of water) and assume that ΔH is independent of temperature, the heat of absorption for the straight line portion of Fig. 3 is 12.3 kcal. per mole of water absorbed from the vapor state. A similar calculation gives 1.85 kcal. per mole of water absorbed from the liquid state (the net heat of absorption). This heat of absorption is constant between 6 and 60% relative humidity and is of about the same magnitude as the heat of adsorption calculated by the B.E.T. theory for the first monolayer on the amino groups.

The linear relationship of Fig. 3 permits several explanations. The simplest is that there is a straight Henry's law solubility of water in the amino groups on the casein molecule. A more complicated explanation in terms of a series of equilibria wherein water molecules are added one at a time to the various hydrates is possible by using the mathematical treatment of Klotz.[12]

(11) Cassie, *Trans. Faraday Soc.*, **41**, 450 (1945).
(12) Klotz, *Arch. Biochem.*, **9**, 109 (1946).

These equilibria and their equilibrium constants have not been presented here because there appears to be a hysteresis phenomenon over part of the region concerned. A detailed study of this hysteresis is being made, and the effects which it may have on the mechanism of this linear adsorption will be presented in a later publication.

Above 70% relative humidity there is a rapid increase in the water absorbed on the amino groups. This is believed to be a condensation of water molecules on water molecules previously absorbed on the amino groups. This seems plausible because approximately 2.5 moles of water per amino group are absorbed at 60% relative humidity. This amount would be sufficient to saturate the hydrogen-bonding capacity of the amino group if the first molecule absorbed remains associated with two amino groups.

Table III shows the fraction of the total water absorption of casein which occurs on the free amino groups at each humidity studied. Previous treatments of the water-absorption of proteins have associated the effect of the various polar groups with some particular portion of the curve.[6] Our data show that the strongly polar amino group has its effect in all portions of the absorption isotherm.

Acknowledgment.—The authors gratefully acknowledge the suggestions of C. Roland Eddy regarding the free energy and heat of surface adsorption.

Summary

A series of benzoylated caseins has been prepared with varying amounts of free amino groups. Water-absorption studies on these samples have made it possible to distinguish between water absorbed on amino groups and water absorbed on the remaining groups of the casein.

The first step in the binding of water by the amino groups of casein seems to be a sharing of one molecule of water between two amino groups below 6% relative humidity. The B.E.T. treatment of the data, however, indicates a monolayer of one water molecule per amino group.

The second step is a linear increase in absorbed water with increase in relative humidity. Equations are presented for this increase between 0 and 60% relative humidity.

The third step is a rapidly increasing amount of absorption with increase of relative humidity and appears to be a condensation of water on water molecules already attached to the amino groups.

From 24 to 33% (depending on the relative humidity) of the water absorbed by casein is absorbed by the amino groups.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF WAYNE UNIVERSITY]

"Nitro Captax"—6-Nitro-2-mercaptobenzothiazole—as a Reagent for the Identification of Alkyl Halides[1]

BY HAROLD B. CUTTER AND HAROLD R. GOLDEN[1a]

In spite of the importance of the alkyl halides, there are few satisfactory reagents for their identification. Therefore, it seemed desirable to attempt to find a compound that would react with alkyl halides to yield crystalline derivatives. 6-Nitro-mercaptobenzothiazole is such a substance. It reacts readily with primary and secondary but not with tertiary-alkyl halides to yield 6-nitrobenzothiazole sulfides. These may be oxidized to the corresponding sulfones, thus affording two crystalline derivatives for the original alkyl halide.

Experimental

Preparation of 6-Nitro-2-mercaptobenzothiazole.—This compound has been prepared by Teppema and Sebrell[1b] by the direct nitration of Captax (2-mercaptobenzothiazole) by a mixture of sulfuric acid and fuming nitric acid. We have found that a somewhat better method is by the nitration of "Altax" (benzothiazole disulfide) by means of nitric–sulfuric acid mixture, and reduction of the dinitrobenzothiazole disulfide by means of alkaline sodium sulfite.

50 g. of Altax (benzothiazole disulfide)[2] is dissolved in 250 g. (135 ml.) of concentrated sulfuric acid. The lumps are broken up and the mixture well stirred to assist solution. Since considerable heat is evolved by the dissolving of the Altax, the solution must be cooled before beginning the nitration. A mixture of 40 g. (25 ml.) of fuming nitric acid and 55 g. (30 ml.) of concentrated sulfuric acid is slowly added with constant stirring to the cooled reaction mixture. The temperature is not allowed to rise above 50°. After all the acid is added, the solution is stirred at room temperature for thirty minutes or more. The solution is then poured with stirring into 3 liters of ice water, filtered, washed well with water, and the wet cake of dinitrobenzothiazole disulfide suspended in 500 ml. of water. By stirring this suspension with a solution of 40 g. of sodium sulfite (anhydrous) and 20 g. of sodium hydroxide in 450 ml. of water, the disulfide is reduced and brought into solution as the sodium mercaptide. The deep red solution thus obtained is filtered, and poured into 3000 ml. of boiling 5% hydrochloric acid to precipitate the free mercaptan. It is necessary to acidify the solution in this manner in order to obtain a filterable precipitate. The mercaptan is then filtered, washed, and dried as thoroughly as possible on the filter. The nitromercaptobenzothiazole

(1) Extract from a thesis presented by Harold R. Golden to the Graduate School of Wayne University in partial fulfilment of the requirements for the degree of Master of Science.

(1a) Present address: U. S. Rubber Company, Detroit, Mich.

(1b) Teppema and Sebrell, THIS JOURNAL, **49**, 1779–1785 (1927).

(2) The Altax used in these experiments was the Naugatuck Chemical Company product. Different batches varied slightly in melting points, but most samples melted in the range 173–175°. The Captax was prepared by the same company; it was about 95% pure and melted 170–173°,