This investigation has shown that direct extraction of cottonseed, using aqueous ethanol as a solvent, is a feasible process in the type of equipment developed previously in this laboratory. The optimum operating conditions for the ethanol extraction of cottonseed have been established.

The pilot plant extractions have shown that in this process a prime quality of crude oil and light-colored meal of good quality, with negligible free gossypol content, are obtained.

Acknowledgments

Thanks are due to the Southern Utilization Research and Development Division, U. S. Department of Agriculture, for the standard gossypol, to Swift and Company for the cottonseed used in the extraction rate studies, and to Southern Cottonseed Oil Company for the cottonseed used in the pilot plant study.

REFERENCES

1. American Oil Chemists' Society, "Official and Tentative Methods," 2nd Revised Ed., Chicago, Ill., 1956.
2. Arnold, L. K., and Juhl, W. G., J. Am. Oil Chemists' Soc., *32*, 25–29, 163–166 (1955).
3. Arnold, L. K., and Patel, D. J., J. Am. Oil Chemists' Soc., *30*, 216–218 (1953).
4. Arnold, L. K., and P'Pool, R. S., J. Am. Oil Chemists' Soc., *30*, 611–613 (1953).
5. Beckel, A. C., Belter, P. A., and Smith, A. K., J. Am. Oil Chemists' Soc., *25*, 7–9, 10–11 (1948).
6. National Cottonseed Products Association, "Trading Rules," 1956–1957, Memphis, Tenn., 1956.
7. Othmer, D. F., and Agarwal, J. C., Chem. Eng. Progress, *51*, 372–378 (1955).
8. Rao, R. K., and Arnold, L. K., J. Am. Oil Chemists' Soc., *33*, 82–84 (1956).
9. Rao, R. K., and Arnold, L. K., J. Am. Oil Chemists' Soc., *33*, 389–391 (1956).
10. Rao, R. K., Krishna, M. G., Zaheer, S. H., and Arnold, L. K., J. Am. Oil Chemists' Soc., *32*, 420–423 (1955).
11. Rao, Y. K. R., and Satyan, B. C., "Alcoholic Extraction of Cottonseed," The Bulletin of Central Food Technological Institute," Mysore, India, vol. II, No. 2, 305–306 (1953).
12. Sato, M., Inaba, T., and Kitagawa, K., J. Soc. Chem. Ind. Japan (Suppl. Bind.), *37*, 618–721 (1934).
13. Sato, J., Inaba, T., and Kitagawa, K., J. Soc. Chem. Ind. Japan. (Suppl. Bind.), *38*, 50B (1935).

[Received May 1, 1957]

A Modified Indophenol-Xylene Extraction Method for the Determination of Ascorbic Acid in Soybeans[1]

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RAPID DECOLORIZATION of the dye, 2,6-dichlorophenolindophenol, to the leuco form is the basis of several methods used to measure ascorbic acid in extracts of food and other materials (6, 15), including germinating soybeans (3, 23, 24). Decolorization of the dye by sulfhydryl groups is generally presumed to occur at a much slower rate than by ascorbic acid (6, 15). However interference of certain sulfhydryl compounds in the indophenol estimation of ascorbic acid appears to depend to a great extent upon the relative concentration of the sulfhydryl components (20) and may possibly be affected by the hydrogen ion concentration (1).

When the indophenol-xylene extraction method, using the formaldehyde treatment (15), was adapted by the authors to measure ascorbic acid in untoasted solvent-extracted soybean oil meal, possible interference resulting from sulfhydryl groups in the meal (11, 13, 20) was suspected. Blocking of these groups was achieved with *p*-chloromercuribenzoic acid (*p*- CMB), previously utilized in estimating the sulfhydryl content of soybean meal (13). Analysis for ascorbic acid in the absence of sulfhydryl groups verified their interference and the absence of ascorbic acid in the meal (13).

Results of studies in which *p*-CMB suppressed completely the ability of hydrogen sulfide, cysteine, and glutathione to decolorize the dye were reported by Owen (18) during the time this reagent was being investigated by the authors to eliminate sulfhydryl interference in ascorbic acid analysis of soybean meals. Owen recommended that *p*-CMB be used in indophenol-ascorbic acid analysis of biological materials containing sulfhydryl compounds and subsequently used *p*-CMB in the ascorbic acid analysis of such materials as human plasma, erythrocytes, and urine (19).

Since sulfhydryl groups of the soybean meal interfered in the indophenol method for ascorbic acid, these might be suspected of affecting ascorbic acid determination in the germinating beans. Instead of reported increases in ascorbic acid during germination, as determined by indophenol methods (3, 23), these values may stem from interference of an increasing sulfhydryl content (21).

Data reported here were obtained to emphasize the need for, and the applicability of, a modified indophenol-xylene extraction method to eliminate sulfhydryl interference in the ascorbic acid analysis of soybeans.

Experimental

Hawkeye soybeans, harvested in 1955 and stored at 4°C., were culled, and the choice beans were soaked in a solution of filtered calcium hypochlorite (5 g. in 150 ml. of water) to inhibit mold growth (3, 24). The beans were washed thoroughly with water and placed between multiple layers of moistened cheesecloth in a glass tray. Germination proceeded in the dark at 23°–25°C. Sprinkling the contents of the tray with water several times a day helped to maintain adequate moisture contact with the beans.

After sprouting, the beans were transferred to a Waring Blendor [2] bowl and covered with 2% metaphosphoric acid. Nitrogen was introduced prior to and during the grinding operation by means of a glass tube passing through the bowl cover and extending below the surface of the acid. The slurry was diluted to volume with additional metaphosphoric acid and centrifuged under nitrogen in capped bottles.

The Waring Blendor was not satisfactory for processing whole ungerminated soybeans. Whole beans in 20-g. lots were ground under metaphosphoric acid, using a Vir-Tis "45" homogenizer and a heavy-walled specially constructed flask adapted with a side-arm nitrogen inlet. The beans were first soaked in filtered

[2] Reference to commercial equipment in this publication is not intended to be a recommendation of this equipment by the U. S. Department of Agriculture over others not mentioned.

calcium hypochlorite solution and water-washed to compare with the treatment given the beans to be germinated.

The availability of freshly harvested Kanrich variety vegetable type of soybeans prompted a study of the ascorbic acid content of raw green beans. The extraction technique, using metaphosphoric acid, followed that used for the germinated beans.

Aliquots of the extracts were analyzed by the indophenol-xylene extraction method (15), incorporating the formaldehyde treatment and modified to include the use of *p*-CMB to distinguish between sulfhydryl groups and ascorbic acid. To outline the modified analytical method in its entirety appears repetitious since the modification involves but one change in the normal procedure (15). Thus, to determine total dye-decolorizing substances, buffer is added to an aliquot of the extract, followed by addition of 2.5 ml. of 0.005 M *p*-CMB utilized as the more soluble salt in a slight excess of alkali. After 10 min. the dye and xylene are added, and the residual dye is extracted as usual.

Though losses of ascorbic acid have been reported by Nelson (17) as a result of allowing tomato extracts to stand in the buffer for short periods prior to the addition of the dye and xylene, no such loss was observed with soybean extracts during the time allotted for the *p*-CMB reaction with sulfhydryl groups.

This modified technique parallels the treatment with formaldehyde normally used to measure nonascorbic acid material. It eliminates sulfhydryl groups from among the total dye-decolorizing substances in the aliquot being analyzed for ascorbic acid. Thus the pseudoascorbic acid activity of the sulfhydryl groups (11, 20) is excluded from the calculation of ascorbic acid in the indophenol-xylene extraction method. An indication of sulfhydryl interference is afforded by using the normal method to measure ascorbic acid in soybeans and comparing the values obtained with those of the modified method.

At the time each lot of beans was analyzed for ascorbic acid, a duplicate lot containing a known amount of added reduced ascorbic acid was processed and analyzed to determine recovery of the added ascorbic acid. Hydrogen sulfide was used to convert any dehydroascorbic acid to the reduced form so that a total ascorbic acid value might be obtained. Excess hydrogen sulfide was displaced by a stream of nitrogen. A synthetic mixture of glutathione and ascorbic acid, analyzed for the vitamin by the normal indophenol-xylene extraction method, emphasized the need of eliminating the pseudoascorbic acid interference of the sulfhydryl groups by means of *p*-CMB (Table I).

Paper chromatography was used to supplement results observed with germinating beans. Chromatograms were made with Whatman No. 1 paper pretreated with 2% oxalic acid and dried. The solvent system consisted of the upper layer from a mixture containing n-butanol and 2% oxalic acid [4:6]. Ammonium molybdate reagent (12) and ammoniacal silver nitrate (2) were used to detect ascorbic acid. Nitroprusside-sodium carbonate (10) was used to detect sulfhydryl groups. Ascorbic acid values for the germinating Hawkeye soybeans were calculated to the dry weight of the starting beans.

TABLE I

Elimination of Glutathione Interference in Ascorbic Acid Analyses by Means of *p*-CMB

Component(s)	Indophenol-xylene method		
	μg. ascorbic acid		Percentage error
	Calc'd	Found	
Ascorbic acid	80	80	0
Ascorbic acid + *p*-CMB [a]	80	80	0
Glutathione (GSH) [b]	0	47	(absolute)
Glutathione + *p*-CMB [a]	0	0	0
Ascorbic acid + (GSH) [b]	80	88	+10
Ascorbic acid + (GSH) [b] + *p*-CMB [a]	80	79	−1

[a] 2.5 ml. of 0.005 M *p*-CMB.
[b] 280 μg. reduced glutathione.

Results and Discussion

Results of analyzing ascorbic acid and/or glutathione in the presence or absence of *p*-CMB are reported in Table I. *p*-CMB has no effect on ascorbic acid. The error in ascorbic acid was 10% when analyzing a 2:1 molar ratio of glutathione and ascorbic acid. Two hundred and eighty μg. of glutathione alone gave on erroneous ascorbic acid value of 47 μg. even when utmost speed was used in extracting excess dye in the indophenol-xylene procedure. *p*-CMB is capable of eliminating sulfhydryl interference and of providing quantitative determination of ascorbic acid in thiol mixtures by using the indophenol-xylene extraction method.

A metaphosphoric acid extract of whole ungerminated soybeans, assayed by the usual formaldehyde treatment of indophenol-xylene extraction method, indicated a reduced ascorbic acid content of 96 μg. per gram. Following treatment of the extract with hydrogen sulfide to reduce dehydroascorbic acid, the total reduced ascorbic acid was increased to 120 μg. per gram. However the use of *p*-CMB, which does not interfere in the assay of known ascorbic acid (Table I), completely eliminated the values for reduced and total ascorbic acid in whole ungerminated soybeans. Recovery of added ascorbic acid was 93% in the direct reduced ascorbic acid analysis and 100% following hydrogen sulfide reduction. Since the dye-reducing activity of the pseudoascorbic acid material was equally and respectively eliminated by formaldehyde and by *p*-CMB, it is assumed that *p*-CMB removed sulfhydryl groups as the only interfering substance in the ungerminated beans.

Results of the present investigation indicate that ascorbic acid appears shortly after the start of germination and increases rapidly, especially between the first and second days of germination (column C,

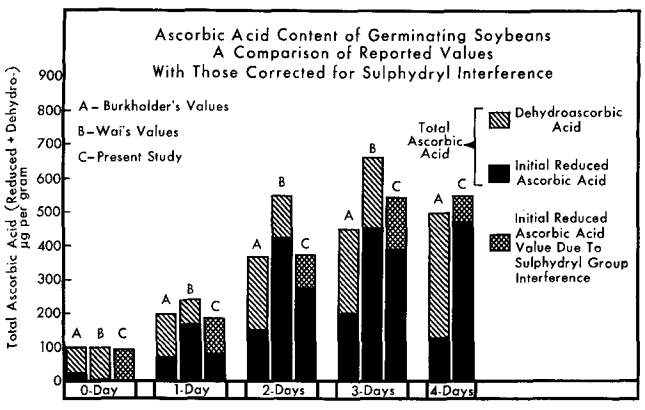

FIG. 1. High values previously reported for the ascorbic acid content of nongerminated and germinated soybeans correlated with interference by sulfhydryl groups.

Figure 1). After four days of germination the ascorbic acid had increased to 470 μg. per gram.

The usual formaldehyde treatment of the indophenol-xylene extraction method indicated an ascorbic acid content of 0.29 mg. per gram (as is basis) for freshly harvested Kanrich variety green soybeans (68% moisture). A decrease in the value to 0.23 mg. per gram resulted from use of *p*-CMB by the modified method. Ascorbic acid added to the extract was quantitatively recovered. Without the aid of formaldehyde and *p*-CMB in increasing ascorbic acid specificity, the dye-decolorizing activity indicated an ascorbic acid content of 0.31 mg. per gram. This value is in very close agreement with other freshly harvested vegetable variety soybeans that have comparable moisture content (22).

The increase in cysteine content of germinating soybeans, as reported by Sugimoto (21), is indicated in a comparison of the sulfhydryl interference data of Table I and Figure 1. Table I shows that the specific interference of glutathione, calculated as μg. ascorbic acid, decreased appreciably in the presence of known ascorbic acid. Figure 1 shows that, even in the presence of ever increasing ascorbic acid in the germinating soybeans, no appreciable decrease in sulfhydryl interference, calculated as μg. ascorbic acid, was apparent until the fourth day of germination. Thus an analysis of data in Table I and Figure 1 suggests an increase in the sulfhydryl content in the germinating soybeans. Sugimoto (21) reported an increase of 122% in the cysteine content of soybeans during five days of germination.

There was little or no change in the reduced ascorbic acid value, following use of hydrogen sulfide to reduce possible dehydroascorbic acid. Figure 1 shows that this observation was in contrast with the results of Burkholder (3) and Wai (23). The present study reveals that the ascorbic acid in the germinating soybean is practically, if not all, in the reduced form. Burkholder indicates a predominance of dehydroascorbic acid and Wai, except for the nongerminated bean, reports the opposite. By comparison dry pea seeds and legumes contain practically no ascorbic acid, but, when germinated, this vitamin appears within a few hours (14, 25). In plants, fresh foods, and vegetables ascorbic acid is nearly always found in the reduced form to the extent of 90% or more of the total ascorbic acid (7, 9, 16, 25). Harris and Olliver (8) attribute the large amount of dehydroascorbic acid, sometimes reported in the literature, to an artifact generally resulting from failure to guard against oxidation.

A comparison of total ascorbic acid values in Figure 1 indicates sulfhydryl interference in values reported in previous studies. This interference is best indicated by all values for the nongerminated beans and in the one-day germinated beans. The subsequent increased variation in total ascorbic acid by Wai for succeeding days probably results from germination carried out at a higher temperature, which is conducive to more rapid sprouting (5).

Evidently the sprouted soybeans should be analyzed immediately and not stored even in refrigerated units protected from light. Duplicate results for three- and four-day germinated beans which had been stored whole and in dark bottles in a refrigerator for approximately five days showed much less reduced ascorbic acid than those analyzed immediately. Interference was observed in total ascorbic acid estimation, following the use of hydrogen sulfide. Even though excess hydrogen sulfide was removed with a stream of nitrogen, unexplainable results were encountered in the form of low total ascorbic acid values, poor recovery of added ascorbic acid, and appreciable dye-decolorization not eliminated by formaldehyde condensation or by using *p*-CMB.

Further evidence of the presence of sulfhydryl interference in acid extracts of germinated beans was obtained by paper chromatography. Sulfhydryl groups were detected on paper chromatograms from 72-hour beans. Since the metaphosphoric acid extracts of the beans interfered with the uniform movement of ascorbic acid and the use of the molybdate reagent, oxalic acid extracts (4), clarified by protein precipitation with excess trichloroacetic acid, were used instead. The presence of ascorbic acid was identified. Positive evidence of the sulfhydryl group was obtained by using a combination of chromatographic reagents. A paper strip sprayed with nitroprusside-sodium carbonate reagent clearly showed a nitroprusside-positive spot. When a duplicate was sprayed first with *p*-CMB reagent and, after drying, with nitroprusside-sodium carbonate reagent, no nitroprusside positive spot was observed.

Summary

The indophenol-xylene extraction method used to estimate ascorbic acid was modified to eliminate appreciable sulfhydryl interference encountered when extracts of soybeans are assayed for this vitamin. The sulfhydryl interference was eliminated by means of *p*-chloromercuribenzoic acid (*p*-CMB). This reagent did not affect the indophenol color nor did it interfere in the estimation of true ascorbic acid. The ascorbic acid values for soybeans germinated up to four days, as determined by the modified method, were 15% to 54% lower than the values obtained by the standard procedure (15), in which sulfhydryl groups are not masked. Ungerminated soybeans assayed by the modified method contained no ascorbic acid. The vitamin appeared shortly after the start of germination and increased rapidly to 470 p.p.m. in four days. The ascorbic acid found was predominantly, if not all, in the reduced form. The ascorbic acid content of freshly harvested Kanrich green soybeans as determined by the modified method was 20–25% lower than that obtained by the normal method. Present data indicate possible sulfhydryl interference in ascorbic acid values previously reported in soybean germination studies that employed indophenol methods of assay. Paper chromatography was used to establish the presence of ascorbic acid and sulfhydryl groups in acid extracts of the germinated soybeans.

REFERENCES

1. Basford, R. E., and Huennekens, F. M., J. Am. Chem. Soc., *77*, 3873–3877 (1955).
2. Block, R. J., Durrum, E. L., and Zweig, G., "A Manual of Paper Chromatography and Paper Electrophoresis," pp. 301–302, New York, Academic Press, 1955.
3. Burkholder, P. R., and McVeigh, I., Plant Physiol., *20*, 301–306 (1945).
4. Chen, Yu-Tuan, Isherwood, F. A., and Mapson, L. W., Biochem. J., 55, 821–823 (1953).
5. Edwards, T. I., Plant Physiol., *9*, 1–30 (1934).
6. Glick, D., "Methods of Biochemical Analysis," vol. I, pp. 118–126, New York, Interscience Publishers Inc., 1954.
7. Goldblith, S. A., and Harris, R. S., Anal Chem., *20*, 649–651 (1948).
8. Harris, L. J., and Olliver, M., Biochem. J., *36*, 155–182 (1942).
9. Hellström, V., Vår föda, *5*, 37–40 (1953); C. A., *48*, 6614h.
10. MacDonnell, L. R., Silva, R. B., and Feeney, R. E., Arch. Biochem. Biophys., *32*, 288–299 (1951).
11. Mapson, L. W., J. Soc. Chem. Ind. (London), *62*, 223–232 (1943).

12. Matt, F., Angew. Chem., *66*, 485 (1954).
13. McKinney, L. L., Weakley, F. B., Campbell, R. E., Eldridge, A. C., Cowan, J. C., Picken, J. C. Jr., and Jacobson, N. L., J. Am. Oil Chemists' Soc., *34*, 461–466 (1957).
14. Merck Service Bulletin, Ascorbic Acid (Vitamin C), p. 14, August, 1941.
15. "Methods of Vitamin Assay," The Association of Vitamin Chemists Inc., 2nd ed., pp. 71–106, New York, Interscience Publishers Inc., 1951.
16. Mills, M. B., Damron, C. M., and Roe, J. H., Anal. Chem., *21*, 707–709 (1949).
17. Nelson, W. L., and Somers, G. F., Anal. Ed., *17*, 754–756 (1945).
18. Owen, J. A., Iggo, B., and Horn, D. B., Nature, *174*, 701 (1954).
19. Owen, J. A., and Iggo, B., Biochem. J., *62*, 675–680 (1956).
20. Robinson, W. B., and Stotz, E., J. Biol. Chem., *160*, 217–225 (1945).
21. Sugimoto, K., Bull. Osaka Med. Sch., *1*, 1–16 (1954).
22. Van Duyne, F. O., Charles, V. R., Batcher, O. M., and Hivon, K. J., The Soybean Digest, *17*, 18–20 (1957).
23. Wai, K. N. T., Bishop, J. C., Mack, P. B., and Cotton, R. H., Plant Physiol., *22*, 117–126 (1947).
24. Wu, C. H., and Fenton, F., Food Res., *18*, 640–645 (1953).
25. Yamaguchi, M., and Joslyn, M. A., Plant Physiol., *26*, 757–772 (1951).

[Received November 14, 1957]

Factors in the Decolorizing of Tallow. II. Oxidation

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THIS IS THE SECOND of a series of articles describing the various factors involved in the commercial decolorizing of tallow. The first of the series appeared in the September 1954 issue of the Journal (7) and described the effect of adding water with the decolorizing adsorbent. Oxidation, the factor covered in the current article, plays an important role in decolorizing tallow. Unfortunately the reactions of oxygen with the fat are complex, and the resulting effect on bleached color, keeping quality, and color stability of the fat is often confusing and unpredictable. A striking parallel on vegetable oils illustrates this. King and Wharton (6) report that in decolorizing oils there are four reactions, two working in favor of color reduction and two working against it. The two factors working in favor of it are the decolorizing action of the adsorbent and the reduction in color by oxidation of some of the color bodies, such as the carotenoid pigments. Working against it are the "setting" of other color bodies, so they cannot be removed by the adsorbent, and formation of colored compounds from colorless precursors, both as the result of oxidation.

It was the purpose of the work involved in this article to study the results of oxidation in a number of phases of tallow decolorizing and, if possible, achieve a better understanding of its basic mechanics as well as its effects. Included among the phases studied herein are storage of fat before decolorizing, amount of fat decolorized, agitation rate during decolorizing, vacuum *vs.* atmospheric decolorizing, and plant *vs.* laboratory decolorizing.

Storage of Fat Before Decolorizing

Work published on vegetable oils indicates that the oil, particularly after neutralizing, deteriorates in decolorizing response when stored (4). To express it differently, when oil is stored, its bleached color progressively darkens; the bleach test is assumed to be carried out each time under the same conditions and with the same dosage of adsorbent.

The effect of storage upon animal fat was studied first by drying a freshly rendered lot of Fancy Tallow A, clarifying it by mixing 0.5% diatomaceous earth with 100-lb. batches at 125°F. under vacuum (7 mm.) for one hour, then filtering. A portion of the tallow was stored in a closed 5-gal. can at 120°F., and samples were withdrawn from the can at the start and after 1 and 5 days, respectively. These were decolorized with varying dosages of A.O.C.S. Official Activated Bleaching Earth (hereafter referred to as A.O.C.S. Activated Clay) to obtain complete bleached color-dosage curves.

The following modification of A.O.C.S. Official Method Cc 8c for bleaching tallows, greases, and other animal fats was used.

a) First 300 g. of melted tallow at 120°–130°F. are added to the refining cup specified in Method Cc 8c, followed by the adsorbent.
b) Employing the equipment designated in the A.O.C.S. method, the fat is agitated at 250 ± 10 r.p.m. throughout the bleach.
c) Heat is applied, and the temperature is increased to 250°F. in 5–7 min.
d) Temperature is maintained at 250°F. for 15 min.
e) The fat is filtered immediately through a dry Whatman No. 2 filter paper, and Lovibond color is determined.

The above modified method henceforth will be referred to as the standard method. The results are given in Figure 1 and show that deterioration occurred in the decolorizing response of the fat in the five-day period of storage.

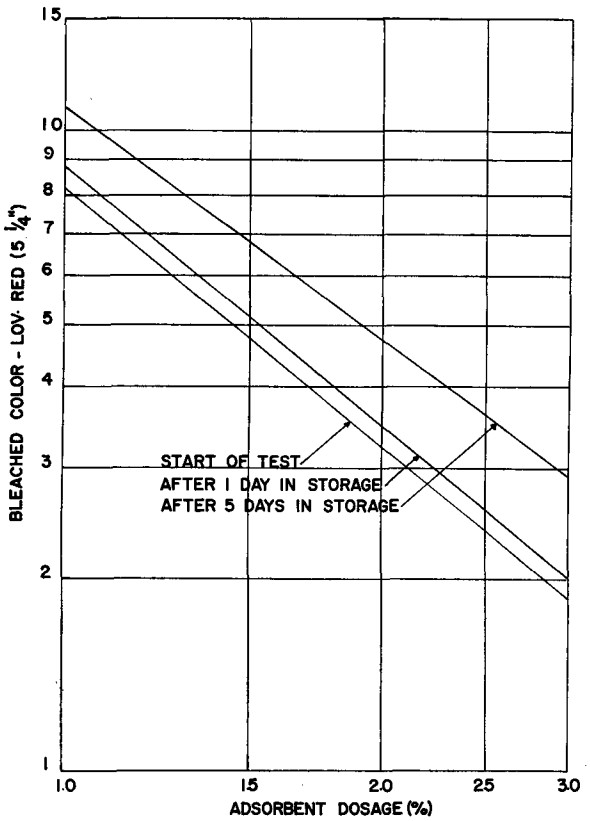

FIG. 1. Bleached color of Fancy Tallow *vs.* dosage of A.O.C.S. Activated Clay on unbleached fat stored at 120°F.