[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

A Potentiometric Study of the Change in Iodine Binding Capacity of Amylose while Retrograding in Dilute Solution[1]

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The time course, at 3°, of decrease in the iodine binding capacity of dilute, sterile, electrolyte free, molecular dispersions of corn and potato amyloses was determined potentiometrically. The rate of this aging process, *i.e.*, the retrogradation reaction, is characterized by a lag period, followed by a markedly accelerated phase, then by a slow approach to a limit. The influences of kind of amylose, of amylose concentration and the effects of a variety of added salts on the rate and course of this process were measured.

The process of starch retrogradation has been defined as "only those reactions and effects which occur spontaneously during the aseptic aging of a starch sol or gel."[2]

In the present instance, the definition may be limited to the physical changes occurring during the aseptic aging of an amylose sol. This spontaneous association or retrogradation of amylose in aqueous solution, which is attributed to the formation of intermolecular hydrogen bonding, destroys the iodine-complexing ability normally exhibited by freely dispersed amylose molecules. Measurement of the loss of this iodine complexing property constitutes a sensitive method for following the retrogradation process.

Bates, French and Rundle[3] first observed that when freshly dispersed amylose was titrated potentiometrically with I_2–KI solution, the activity of the iodine remained at a low potential until the iodine-complexing ability of the amylose was saturated. It then rose to a value comparable to that of free iodine in solution. A quantitative estimate of the iodine-complexing property of a particular amylose in solution may be obtained from a plot of the free *versus* the bound iodine, which is obtained by potentiometrically titrating equal volumes of solution with iodine in the presence and absence of amylose.[3] This value is referred to, hereafter, as the *iodine binding capacity* (IBC). A plot of the IBC *versus* the age of the amylose solution provides a continuous and quantitative measure of the retrogradation process as it occurs in dilute amylose sols. In addition, this procedure can be employed to study the effects of salts and other additives upon the retrogradation process.

Methods and Materials

Amylose.—Corn amylose was prepared by the method of Lansky, Kooi and Schoch[4] from a highly purified corn starch (Globe brand pearl corn starch No. 144, kindly furnished by Dr. T. J. Schoch of the Corn Products Refining Co.). It was recrystallized four times from 1-butanol to effect the removal of branched molecules. Schoch reported that two such recrystallizations will bring the iodine affinity of the amylose to its maximum value.[4] The final product was repeatedly dispersed in 1-butanol, until free of water, and dried *in vacuo* at 70°.

Potato amylose was prepared in a similar manner from firm Maine potatoes which had been in storage about eight months. The amylose was twice recrystallized from 1-butanol.

Both amyloses were completely soluble in hot water and gave iodine affinity values comparable to those reported in the literature for similar preparations.[4,5]

Procedure Used to Retrograde Amylose.—A weighed amylose sample was placed in a dry flask and carefully moistened with a minimum quantity of 1-butanol. The flask was placed in a boiling water-bath and to it was added boiling distilled water equivalent to about one-third of the desired final volume. The solution was allowed to boil gently (carborundum granules added) until the last traces of butanol had volatilized. Boiling distilled water was added when necessary to maintain approximately the initial volume. The butanol-free solution was transferred quantitatively to a *sterile* volumetric flask, and after rapidly cooling the solution in a cold water bath, it was made up to volume with sterile distilled water.

A sterile technique for handling dilute amylose solutions was essential. Airborne microörganism contamination was capable of reducing the IBC of an 0.08% potato amylose solution (which ordinarily would not retrograde for many days at room temperature) by more than 50% in 24 hr. at room temperature. Figure 1 shows a series of iodine titration curves prepared from aliquots of non-sterilized 0.08% potato amylose. These curves are similar to those described by Dvonch, *et al.*,[5] for a series of mildly acid-hydrolyzed amyloses. Sterile 0.08% amylose solutions prepared at the same time as the non-sterile samples showed no loss of iodine complexing ability even after 322 hr. at room temperature.

The flasks containing the amylose solutions to be retrograded were clamped to a horizontal shaft which could be rotated at a constant rate. With continuous agitation of the solutions, the retrogradation proceeded very much the same as it would have in an undisturbed solution, but the retrograded amylose aggregates that formed usually remained sufficiently small as to yield a stable suspension, which in turn provided for more uniform aliquots, hence, more reproducible results.

All retrogradations were conducted at 3 ± 1°. There are distinct advantages in the use of a low temperature. The aging process is accelerated at lower temperatures thereby allowing the use of dilute samples of amylose. Also, problems related to the sterility of the samples and to possible contamination during the removal of aliquots are minimized.

Measurement of Retrogradation.—The course of retrogradation was followed by potentiometrically titrating aliquots of an aging amylose solution with I_2 in KI solution. Measurements were made according to the procedure of Bates, French and Rundle[3] as modified by Wilson, Schoch and Hudson[6] and Lansky, *et al.*,[4] employing a bare platinum wire electrode and a saturated calomel half-cell. Magnetic stirring at a constant rate was provided. The titrations were carried out at a temperature of 30.00 ± 0.02°.

Iodine reagent: A 0.05 *M* KI solution containing 2.0 mg. of I_2 per ml. was standardized against sodium arsenite. It

(1) Paper #3560, Scientific Journal Series, Minnesota Agricultural Experiment Station. A portion of a thesis submitted by Frank A. Loewus to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1952. Presented before the Carbohydrate Division, 126th Meeting of the American Chemical Society, New York City, September 12–17, 1954.

(2) R. L. Whistler in "Starch and Its Derivatives," Vol. I, 3rd Ed., ed. J. A. Radley, 1953.

(3) F. L. Bates, D. French and R. E. Rundle, THIS JOURNAL, **65**, 142 (1943).

(4) S. Lansky, M. Kooi and T. J. Schoch, *ibid.*, **71**, 4066 (1949).

(5) W. Dvonch, H. J. Yearian and R. L. Whistler, *ibid.*, **72**, 1748 (1950).

(6) E. J. Wilson, T. J. Schoch and C. S. Hudson, *ibid.*, **65**, 1380 (1943).

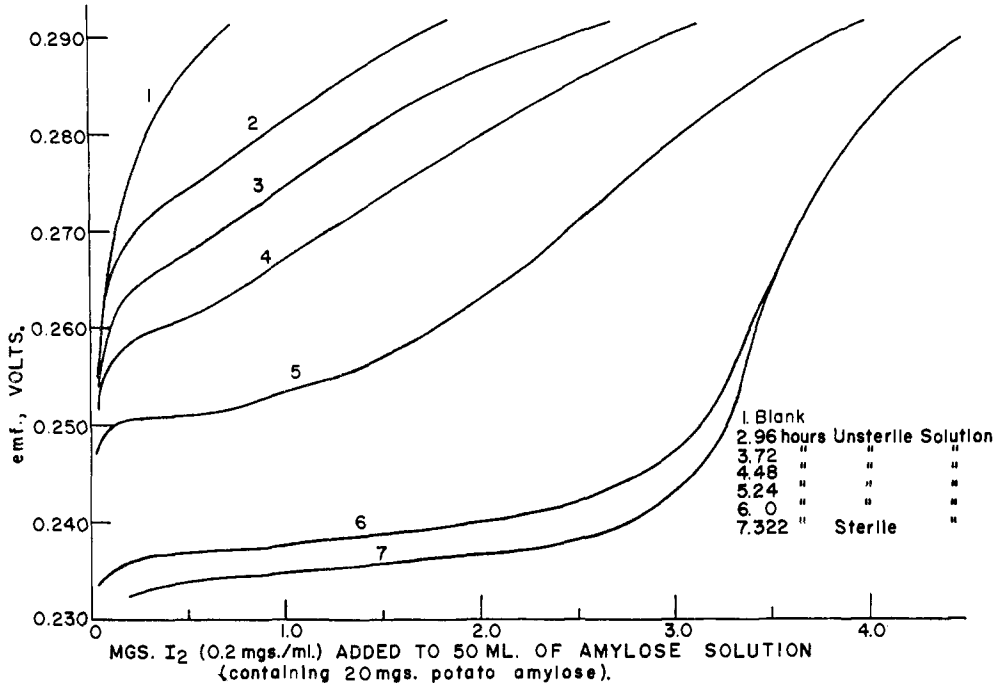

Fig. 1.—Comparison of effects of sterile *versus* non-sterile techniques on changes in potentiometric titration curves for 0.08% potato amylose with aging. (The deviation between curves 6 and 7 probably reflects an increase in perfection, with time, in the helical structure of the starch molecules of the sterile sample.)

was diluted 1:10 with 0.05 *M* KI solution just prior to use. In cases where amylose was retrograded in the presence of other solutes, the diluted iodine reagent was made up to contain the same concentration of such solutes as the amylose aliquot in order to eliminate effects at the electrodes other than those due to iodine and amylose.[6]

Amylose aliquot: A 20-mg. amylose aliquot was removed from the retrograding solution by means of a sterile pipet and diluted to exactly 50 ml., including sufficient KI solution (freshly prepared) to make the final solution 0.05 *M* with respect to KI. The solution was transferred to a large mouth 250-ml. erlenmeyer flask. A small glass-enclosed soft iron impeller was placed in the flask, the latter clamped onto the magnetic stirrer in a 30° water-bath and the solution stirred until it reached the temperature of the bath. Then the electrode assembly was introduced and a buret tip lowered to within a few millimeters of the solution surface.

Titration procedure: The potential of the electrode system in the initial solution varied from 0.22 to 0.23 volt, the platinum electrode being positive in sign. The first increment of added iodine always caused a slight rise in the potential even in freshly prepared amylose solutions; however, this rise normally required less than 0.01 mg. of iodine per gram of amylose to bring the reading to a plateau of little change in potential. A similar observation has been reported by Gilbert and Marriott.[7] Subsequent increments of iodine gave only momentary increases in potential which decreased asymptotically with time to the plateau characteristic of the complex. This plateau occurred between 0.235 and 0.245 volt (depending on the presence or absence of salts other than KI). As the amylose approached saturation with iodine, the potential increased more rapidly with added iodine, rising through an inflection point in the region of 0.250 to 0.275 volt and finally tapering off around 0.290 volt beyond which point the course of the titration curve approximated the characteristics of that of the KI control. Throughout the latter portion of the titration, attainment of equilibrium between added increments of iodine reagent and the amylose was rapid. This variation in the time necessary to reach equilibrium between iodine and amylose after addition of each increment of iodine reagent led to a convenient rule which improved considerably the precision and reproducibility of a given titration. The rule was that *for every milliliter of iodine reagent added (0.2 mg. iodine/ml.), a delay of one minute between increments added was allowed before a reading was taken.* Thus, on the plateau of iodine–amylose complexing where equilibrium was reached slowly, there were long time intervals between increments. Conversely, on the steep portion of the titration curve where equilibrium was almost instantaneous, there were only very short time intervals between increments.

All titrations were done in the presence of 0.05 *M* KI, and the amount of iodine complexed or bound by a given amylose aliquot was calculated as the difference between the total iodine added and the free iodine remaining in solution. The latter value was obtained by comparison with a blank titration of iodine reagent into a 0.05 *M* KI[3] solution of equal volume. In experiments where the amylose was retrograded in the presence of a salt, the blank solution also included the salt under consideration in exactly the same concentration as in the amylose solution.

Retrogradation of Amylose in Water Solution.—To conveniently represent the course of the retrogradation process, each titration curve in a retrogradation experiment was used to obtain a single IBC value which was then plotted as a function of the age of the solution at the time the aliquot for titration was taken. The IBC is defined as the maximum number of grams of iodine (in 0.05 *M* KI) that will be bound by one gram (dry weight) of amylose. The grams of iodine calculated as bound per gram of amylose at the inflection point of the titration curve (at about 0.265 m.v.) approximates closely the true IBC and is designated as such in the present experiments.

Figures 2 and 3 show the manner in which the IBC changes with time of retrogradation (at 3°) for a potato and a corn amylose, respectively, when these are present in water alone and at several different concentrations of amyloses. Intrinsic viscosities[8] of these amyloses were determined to be 2.85 for potato and 1.52 for corn, corresponding to "degree of polymerization" values[9] of approximately 1700 and 900, respectively. Examination of the data assembled in Figs. 2 and 3 indicates the following relationships with regard to the retrogradation of the amyloses. (1) The retrogradation process (as reflected by change in IBC) follows the same general pathway for both amyloses and at each amylose concentration, *i.e.*, a phase in which the rate of retro-

(7) G. A. Gilbert and J. V. R. Marriott, *Trans. Faraday Soc.*, **44**, 84 (1948).

(8) R. H. Wagner, *J. Polymer Sci.*, **2**, 21 (1947).

(9) A. L. Potter and W. Z. Hassid, THIS JOURNAL, **73**, 593 (1951).

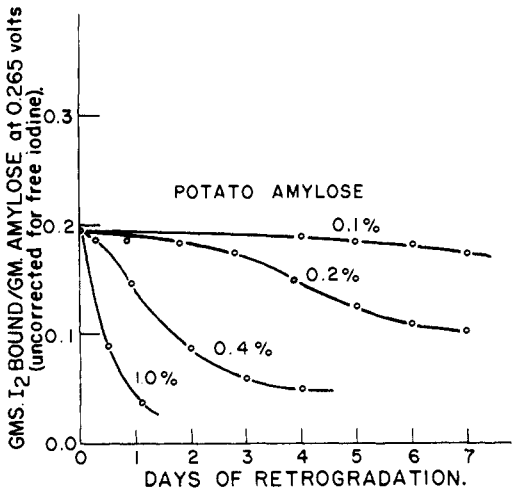

Fig. 2.—Change in iodine binding capacity, with time, of potato amylose in solution at different concentrations.

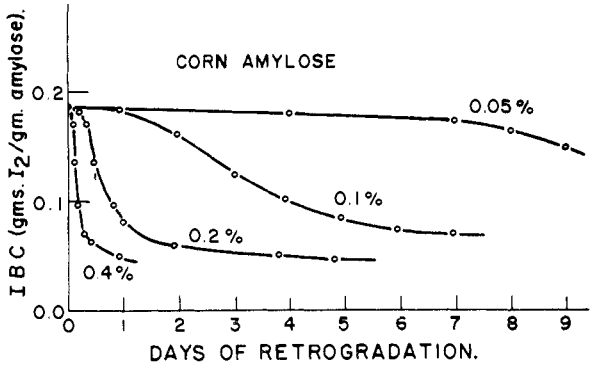

Fig. 3.—Change in the IBC, with time, of corn amylose in solution at different concentrations.

gradation accelerates rapidly with time (autocatalytic) is followed by a phase of deceleration as the process approaches the final limiting value. (2) The time required to reach any stage in the process is a constant fraction of the time required to reach any later stage, independent of concentration or type of amylose. (3) For a given type of amylose, the time required to reach any chosen stage in the process (for example, the time at which the inflection point on the IBC-age curve occurs) varies, roughly, inversely with the square of the original amylose concentration. (4) At equal weight concentrations of amylose, a doubling of the degree of polymerization value increases the time required to reach a corresponding stage in the process by a factor of six to eight.

Further observations of significance were made. (1) Corn amylose, in the concentration range studied, when retrograded to the point of no further change in IBC, formed no gross precipitate. The retrograded particles remained microscopically so small as to form a stable suspension as long as the solution was kept in motion. With potato amylose, when the IBC had decreased to about one-half the original value, the retrograded material aggregated to a ball of fluffy precipitate. (2) Corn amylose, at all concentrations, reached a limiting value of IBC greater than zero (approximately equal to one-fifth of the original IBC) while potato amylose, although the retrogradation proceeded at a slower rate, eventually exhibited a limiting IBC very close to zero. This is a consequence not of the difference in molecular sizes of the two types of amylose but rather of the circumstance that the corn amylose contains a low degree of branching of the molecules while the potato amylose consists entirely of linear molecules.[9]

Retrogradation of Amylose in the Presence of Salts and Other Additives.—A limited study of the effects of salts and other additives on the course of the IBC-age curve of corn amylose illustrates the applicability of the method to such studies and serves to indicate some broad generalizations with respect to these effects.

In these experiments, corn amylose at 0.1% concentration was used as the standard amylose solution because, as shown in Fig. 3, the time required to span the retrogradation process at this concentration was neither too short nor too long for convenient and accurate estimation of the influences of added solutes. Figure 4, a and b, illustrates the IBC-time

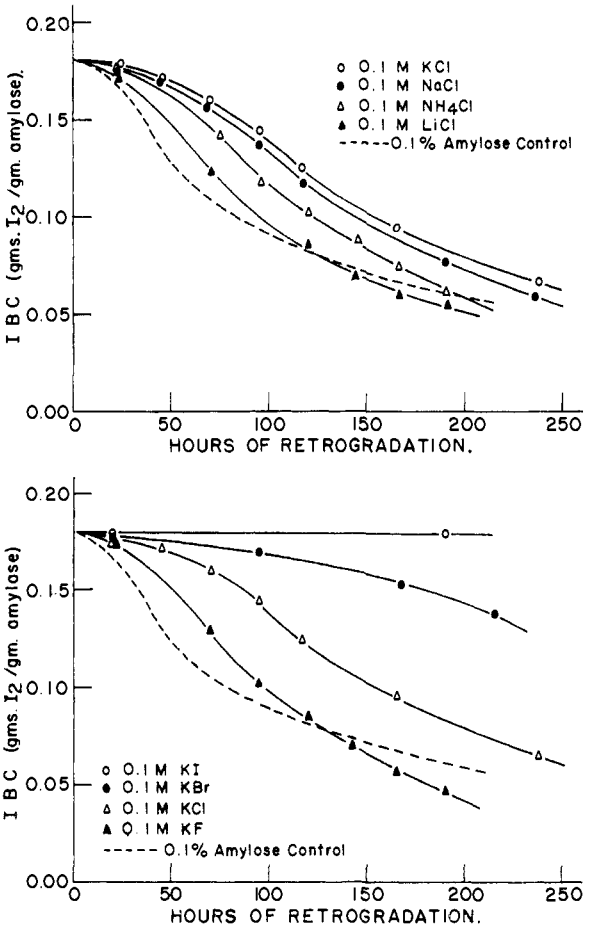

Fig. 4.—Showing effects (a) of alkali chlorides and (b) of potassium halides on the IBC *versus* time of aging relationship for 0.1% corn amylose in solution.

curves for such standard amylose solutions where retrogradation was allowed to proceed (at 3°) in the presence of various 0.1 *M* alkali chlorides and potassium halides, respectively. The general form of these IBC-time curves is not noticeably different from that obtained with the amylose alone; any influence that the salts have on the retrogradation process appears to be proportionate at all stages of the process. It is possible therefore to compare the effects of such additives in terms of the times required for the process to reach any comparable stage, in the absence and presence of the additive. This is done in Table I for a number of salts and a few other additives, where the times required to reach the inflection point on the IBC-time curves in the presence of the additives are compared with the time at which the inflection point is reached by the standard amylose dissolved in water alone.

In contrast to the observation that corn amylose failed to form a macro precipitate when allowed to retrograde in water alone, in the presence of certain salts the retrograded particles became unstable and collected as a film on the walls of the containing vessel. The onset of film formation was always detectable when the IBC was reduced to the order of one-half that of the original. This was well beyond the point of inflection of the IBC-age curve at which the comparisons

TABLE I

COMPARISON OF THE EFFECTS OF ADDITIVES ON THE RETROGRADATION RATE OF CORN AMYLOSE. BASIS OF COMPARISON IS THE TIME REQUIRED TO REACH THE INFLECTION POINT ON THE IBC–AGE CURVE

Amylose concentration = 0.1%; temperature = 3 + 1°

Additive	Concn., *M*	Time, hours	Additive	Concn., *M*	Time, hours
None	0.1	45	K_2SO_4	0.1	85
KI	.1	>>500	K_2SO_4	.05	61
KBr	.1	230	$MgSO_4$	.1	38
KCl	.1	105	$MgSO_4$	.05	42
KF	.1	70	$LaCl_3$	.01	58
NaI	.1	>>500	$LaCl_3$	.001	52
NaBr	.1	230	$ThCl_4$	.001	18
NaCl	.1	100	$ThCl_4$	.0001	42
NaF	.1	60	NaCl	0.1	100
LiBr	.1	155	Plus $ThCl_4$	0.5×10^{-4}	70
LiCl	.1	60	Plus $ThCl_4$	1.0×10^{-4}	60
NH_4I	.1	>>500	Plus $ThCl_4$	2.0×10^{-4}	43
NH_4Cl	.1	80	Plus $ThCl_4$	4.0×10^{-4}	32
$BaCl_2$	.1	230	Plus $ThCl_4$	10.0×10^{-4}	23
$CaCl_2$	.1	110	Plus $ThCl_4$	25.0×10^{-4}	15
$MgCl_2$	.1	85	Glucose	0.1	45
$BaCl_2$	.01	50	Sucrose	.1	61
$Ca(NO_3)$	.1	>>500	Urea	.1	100
$Ca(NO_3)$	.02	70	Urea	.4	200

in Table I are made so that film formation does not affect these comparisons. However, in Fig. 4, the IBC values shown are uncorrected for the removal of a part of the amylose to the films at the times the later aliquots were taken. The apparent approach to a lower limiting IBC, than was characteristic of amylose alone, was due entirely to loss of amylose from the suspension to the film.

The following observations may be made regarding the effects of salts or other additives on the retrogradation process of corn amylose. (1) Any influence that additives have on the retrogradation process is proportionate at *all* stages of the process. (2) Salts of monovalent anions and monovalent cations all retard retrogradation, the anions demonstrating a wider lyotropic spread than the cations. Anions are effective in the order $F^- < Cl^- < Br^- < I^-$ while the order for the cations is $Li^+ < Na^+ < MH_4^+ < K^+$. Films were formed only in the solutions containing Cl^- and F^-. (3) Those salts that act as cold gelatinizing agents for starch (*e.g.*, $Ca(NO_3)_2$ and alkali iodides) exhibit a very marked retardation effect on retrogradation. (4) Some cations of high valency (*e.g.*, Th^{++++}) cause an acceleration of the retrogradation process. (5) Increase in concentration of a given salt increases the effect of that salt on the retrogradation process. (6) Urea, a strong hydrogen bonding reagent, retards the retrogradation process.

Discussion

While the salient features of the course of the retrogradation process are apparent from the results reported, the scope of the study is too limited as yet to attempt any hypothetical interpretations. It is to be emphasized that the change in IBC which is followed in this study reflects only the progress of the process through which the "helical" form of the amylose molecule is converted to the intermolecular hydrogen bonded "crystal" form as retrogradation proceeds. The initial high degree of aggregation of amylose when dispersed with KOH, while still in the helical form, as reported by Paschall and Foster[10] would not be detected by the method employed in the present study.

(10) E. F. Paschall and J. F. Foster, *J. Polymer Sci.*, **9**, 73, 85 (1952); THIS JOURNAL, **75**, 1177, 1181 (1953).

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[CONTRIBUTION FROM THE NAVAL STORES STATION, U. S. DEPARTMENT OF AGRICULTURE[1]]

The Thermal Isomerization of Neoabietic Acid

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A chromatographic study of the products formed by the thermal isomerization of neoabietic acid at 200° has shown that they consist almost entirely of palustric acid and *l*-abietic acid. The concentration of the various acids present in the isomerized products indicated that the formation of *l*-abietic acid was favored in this isomerization. A new crystalline derivative, neoabietenol, was prepared and characterized. The rate of isomerization of this alcohol and of methyl neoabietate were found to be much slower than the rate of isomerization of the free acid, indicating that the isomerization is catalyzed by the H^+ of the carboxyl group.

Neoabietic acid is one of the major constituents of pine oleoresin and rosin.[2] It is an abietic-type resin acid and thus subject to isomerization by heat and acids to *l*-abietic acid. This factor of isomerization is largely responsible for the major changes in rosin during processing and the preparation of commercial derivatives.

For this reason, a series of thermal and acid isomerization studies have been initiated in this Laboratory on the abietic-type acids in oleoresin and rosin—namely, levopimaric acid,[3,4] neoabietic acid and the most recently isolated palustric acid.[5]

The present paper describes a study of the thermal isomerization of pure neoabietic acid. A temperature of 200° was found to give a measurable rate of isomerization. Eight samples were heated over a period of 72 hours and the progress of the isomerization was followed by obtaining the specific

(1) One of the laboratories of the Southern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) G. C. Harris and T. F. Sanderson, THIS JOURNAL, **70**, 334 (1948); **70**, 339 (1948).

(3) V. M. Loeblich, D. E. Baldwin, R. T. O'Connor and R. V. Lawrence, *ibid.*, **77**, 6311 (1955).

(4) D. E. Baldwin, V. M. Loeblich and R. V. Lawrence, *ibid.*, **78**, 2015 (1956).

(5) V. M. Loeblich, D. E. Baldwin and R. V. Lawrence, *ibid.*, **77**, 2823 (1955).