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Some applications of electrometric methods to the study of the components of starch

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SOME APPLICATIONS OF ELECTROMETRIC METHODS TO THE STUDY OF THE COMPONENTS OF STARCH

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I. INTRODUCTION

Developments have taken place in the field of starch chemistry during the past decade that have resulted in a much more rapid accumulation of facts on basic starch structure than was possible heretofore. Among these innovations, three in particular are outstanding, and investigations contributing to their development will be reviewed in detail elsewhere. However, they must be mentioned here because of the fundamental nature of their relation to the present investigation.

First, in chronological order, was the evolution of the idea that starch chains might, under certain conditions, assume a helical configuration. From it arose a new conception of the nature of the starch-iodine complex. In it the iodine molecules were arranged in a line coinciding with the axis of a long cylinder formed by the convolutions of the starch helix. The fact that starch may assume a helical arrangement under particular conditions need not conflict with evidence that has been found for the existence of other configurations in starch. In naturally occurring starch granules and in retrograded preparations, the starch chains apparently possess a linear formation.

The second development, almost coincident with the first, was the establishment of a branched structure in starch.

Starch was known to consist of glucose units linked together in a straight line. Evidence was found that indicated the probability of branches occurring at intervals, the linkage involved being different than that which held the units together in the chain proper.

The third, and newest, factor in the clarification of starch structure was the fresh support given the two-component theory by efficient methods of fractionating starch. In essence the two-component theory states that most starches are made up of two constituents both of which are basically starch substance in their composition of glucose units; but one is entirely straight chain molecules, the other highly branched molecules. The former has been called amylose, the latter amylopectin. Recent separation procedures leading to two clear-cut fractions lend considerable strength to the two-component hypothesis.

The establishment of branched chain structure, a necessity to the two-component theory in its present state, is also of interest from the viewpoint of the helical concept of starch structure. It offers an explanation for the differences observed in the behavior toward iodine of the two starch fractions. It would be expected that the fraction composed of long, straight-chain amylose molecules would be more efficient in providing the helices for complex formation than would the branched amylopectin fraction. When this probability is combined with the actual fact that amylose does

have a markedly greater affinity for iodine, the starch-iodine complex assumes a position of importance in relation to the entire picture of starch structure.

The present investigation is essentially a study of the starch-iodine complex. It is limited in scope to the formation of the complex in aqueous solution. The emphasis is placed on the behavior toward iodine of the fractions, rather than of whole starch. The methods used depend chiefly upon measurements of iodine activity during the process of complex formation. The iodine activity measurements were made by means of a potentiometric method involving the use of an iodine electrode. Under proper conditions the logarithm of the iodine activity is a linear function of the electrode potential. This method provides a means of estimating quantitatively the amount of iodine in the complex at any stage of its formation, as well as the tenacity with which it is held.

The purpose of the investigation is, in the broadest sense, two-fold. The first, and probably the most basic, objective is the accumulation of knowledge concerning the iodine complex: to compile data on the factors that promote, contribute to, or inhibit complex formation. The second purpose of the investigation is the one that was uppermost in mind when the work was originally planned. It was hoped then to establish an analytical procedure whereby the affinity for iodine of the two starch fractions might be determined

quantitatively. Furthermore, it was realized that such a method would do much toward effecting a clarification of some of the problems of starch structure. It may be said here that this purpose has been fulfilled in an exceedingly satisfactory manner. The applications to even the most general, and seemingly remote, problems have exceeded all earlier expectations.

As a consequence of the work to be described in following sections, it is now possible to evaluate the efficiency of a fractionation procedure and to determine the structure, purity and homogeneity of the fractions. In the case of the amylose component it is possible even to estimate the molecular size. The methods developed permit quantitative analytical determination of the amylose and amylopectin components of natural and modified starches and, to a certain extent, their derivatives.

Some space has been devoted to observations made upon a number of substances whose nature is believed to be analogous to that of the starch-iodine complex.

II. FOUNDATIONS OF THE PRESENT INVESTIGATION

A. New Concepts of the Molecular Structure of Starch

1. The branched-chain theory

The idea of a branched structure for starch was proposed by Staudinger and Husemann (1). They were convinced, on the basis of their viscosity measurements, that starch existed as macro molecules rather than as large aggregations or micelles composed of small molecules. The latter explanation had been offered by Haworth (2). His contention was that the starch molecule could possess only about 25 glucose residues, because methylation studies had discovered one terminal tetramethyl glucose molecule for every 25 trimethyl glucose molecules. However, his views were not only in disagreement with molecular weights determined by physical means, but they could not be reconciled with the low reducing value exhibited by starch. According to Haworth's supposition there should have been an aldehydic glucose unit for each one capable of forming the tetramethyl compound, which would have resulted in a high reducing value. He explained this away by assuming the aldehyde groups were obscured in some manner in the aggregate, perhaps taking part in secondary bonds.

Haworth, Hirst and Isherwood (3) proposed a branched structure for glycogen to explain the presence of dimethyl

glucose among the products obtained upon hydrolysis of the methylated substance. Freudenberg and Boppel (4) found 2,3-dimethyl glucose in the hydrolysate of methylated starch. It was present in quantity approximately equivalent to the tetramethyl derivative. They concluded that the branches were connected to the principal chain by an α-1,6-glucosidic linkage which was responsible for the dimethyl glucose formation.

Further evidence for the branched structure in starch is provided by the action of the enzyme, β-amylase, which attacks the non-reducing end of the starch chain, cleaving off maltose groups until it encounters a linkage other than the α-1,4-glucosidic bond (5). Starch is only partially digested, the enzyme being halted apparently by the branch points. If the α-1,6- linkages involved in the branching are hydrolyzed off by α-glucosidase, the material is digested further by β-amylase, but again the action is halted before complete digestion (6, 7).

2. Fractionation and the two-component concept

The establishment of branching in starch did not necessarily lead to the conclusion that all starch molecules were branched, but rather provided an explanation for the existence of the different fractions or constituents that had been separated by various procedures. Reference may be made to Samec (8), Radley (9), and Walton (10) for reviews of early

attempts to explain the nature of the heterogeneity of starch, and for details of methods used in attempted fractionations. The great variation in properties of the products of these fractionation procedures led to considerable confusion. The presence of many molecular species in starch seemed quite probable until, in recent years, efficient fractionation procedures were developed. The most interesting of such methods is that of Schoch (11) who found that when a hot starch solution was saturated with n-butanol and then allowed to cool slowly, a portion of the starch was precipitated in the form of microscopic, six-lobed rosettes, the exact form of which varied somewhat with different starches. This precipitated material carried with it practically all the iodine-binding ability possessed by the whole starch, giving an intense, pure blue color. On the other hand the unprecipitated fraction gave a relatively weak, purple color with iodine.

The above-mentioned behavior with iodine, together with other properties, permits the classification of Schoch's precipitated and unprecipitated fractions as amylose and amylopectin, respectively, where these two terms have the meanings assigned to them by K. H. Meyer (6, 7). The other properties used by Meyer in classifying starch fractions are quantitative conversion to maltose by β-amylase and strong retrogradation tendencies in the case of amylose, while amylopectin shows little or no disposition to retrograde and is only partially

converted by β-amylase. Meyer's studies were made on fractions prepared by hot water extraction of amylose from the partially swollen starch granule (12). This method is not as efficient as Schoch's procedure in effecting the separation of the fractions. However, by employing various modifications, Meyer was able to prepare relatively pure amylose (13). He was quick to apply the methylation procedure to its characterization (14). He obtained a yield of 2,3,4,6-tetramethyl glucose in the case of amylose, corresponding to one end-group per 300 glucose units. This value corresponded closely to the molecular weight determined by osmotic pressure measurements on the acetylated amylose. His amylopectin fraction produced one terminal glucose unit out of every twenty-eight. Meyer, in this way, identified the amylose component with long straight chains of glucose residues linked by α-1,4-glucosidic bonds and the amylopectin with a branched structure.

A third method of separating the components of starch depends upon adsorption of amylose on cotton fibres. Noted first by Tanret (15) and later by Pacsu and Mullen (16), this phenomenon was used by Baldwin (17) in effecting a fractionation of starch.

The new fractionation procedures have strengthened the position of the two-component concept, but it has not yet gained unanimous acceptance. Only recently Kerr (18) and Kerr and Trubell (19) published procedures for the preparation of gamma-amylose, which they believe is a third component of

starch. However, Schoch's nearly quantitative fractionation showed that if more than two components exist, they must fall into one of two main groups, represented by his butanol-precipitated fraction and his soluble fraction. No one has yet demonstrated that either of these groups contains a sub-fraction of sufficiently individual character to justify classification as a third constituent of starch. On the other hand, it is one of the purposes of this dissertation to submit arguments for the homogeneity of the amylose and amylopectin fractions.

3. The helical configuration and the starch-iodine complex

The idea of a helical configuration for starch chains originated with Hanes (5) and was elaborated upon by Freudenberg and co-workers (20). Hanes had found that the rapid initial digestion of starch by α-amylase produced a large amount of dextrin averaging about six glucose units in length. He proposed the helical configuration to explain this phenomenon. Freudenberg used the same explanation for formation of the cyclic Schardinger dextrins by B. macerans. Caesar and Cushing (21) constructed models of the starch chain with Fisher-Herschfelder atoms and maintained that a helical configuration was a necessity.

Katz (22) discovered an X-ray diffraction pattern in starch pastes and in alcohol-precipitated starch (23) that was quite different from the patterns obtained with natural

starch granules or retrograded starches. Bear (24) suggested that this pattern, which Katz had called the "V" spectrum, might be identified with the helical starch configuration. French (25) then demonstrated that materials having good "V" type diffraction patterns readily took up quantities of iodine vapor to give intensely colored complexes. This was true whether the materials were wet or perfectly dry, but Schoch's butanol-precipitated amyloses gave the clearest patterns. Both Hanes and Freudenberg had suggested the possibility of the iodine molecules being located inside the coils of the helix. The X-ray diagram of the iodine complex was quite similar to the "V" pattern.

Rundle and Baldwin (26) showed that the dichroism of flow exhibited by amylose-iodine complex in solution was compatible with a helical starch chain containing iodine molecules arranged along its axis. Rundle and French (27) studied the optical properties of the small crystals occurring in butanol-precipitated amylose, both before and after treatment with iodine. They concluded that the crystals were made up of closely packed amylose helices. Rundle and French (28) and Rundle and Edwards (29) made X-ray studies of the amylose-iodine complex and of starch in the "V" configuration. They were able to index the patterns using lattices which could be interpreted in terms of a helical configuration for the starch chains.

It must be remembered that the helical configuration is

not postulated as the only form in which starch may exist. In the granule and in retrograded preparations it certainly has an entirely different configuration (30). Recently, Rundle, Daasch and French (31) have prepared films and fibers in which the starch has an extended configuration, as determined by interpretations of X-ray diffraction patterns. These preparations become birefringent when stretched.

The new concept of the starch-iodine complex described in the preceding paragraphs has practically eliminated the older theories. Most workers in the starch field up to recent times subscribed to one of two suppositions concerning the union of starch and iodine. Many claimed that iodine formed a definite chemical compound or, perhaps, a series of compounds with starch, either with or without the inclusion of potassium iodide. Other investigators believed that the starch merely adsorbed the iodine. A few workers proposed other mechanisms for interaction between the two, but most of them were easily disproved. This earlier work has been summarized in reviews by Samec (8), Radley (9), and Barger (32), while Walton (10) has compiled a comprehensive bibliography with abstracts of many articles.

B. Potentiometric Determination of Iodine Activity

1. Application to the study of starch

The work that has been done in the past on the composition

of the starch-iodine complex has served to emphasize its empirical nature and its instability. The composition, and even the constitution, depends on the conditions under which complex formation takes place. Once formed, the complex is unstable and will give up iodine readily if exterior conditions permit. This has always been a source of error in the isolation and accurate analysis of the complex. In aqueous solutions, or even if merely suspended in water, the contents of constituent substances in the complex vary with their concentrations in the solution. This behavior is demonstrated in the work of Lottermoser (33) and Murray (34).

Lottermoser attempted to follow the action of starch in taking up iodine by following the change of iodine concentration in the solution with the aid of the iodine electrode. He was an exponent of the adsorption theory and claimed that his results fit the Freundlich equation, $\frac{x}{m} = \beta C^{1/p}$. In a later report (33a), however, he took a less decided stand on this point and concluded that the iodine in the complex was not in true equilibrium with the iodine in solution, since the same amount of iodine was not held by the amylose when the desired composition was approached from opposite sides. It is difficult to determine why he failed to observe the great departure from true adsorption behavior given by a starch-containing amylose. Since he used a soluble starch prepared by cooking in alkali, it was probably degraded. Lottermoser and Murray used the iodine electrode in combination with partition of iodine between carbon tetrachloride and the aqueous starch phase to

determine the amounts of iodide and tri-iodide taken up by the starch, as well as the amount of iodine. Murray's conclusions were quite different than were Lottermoser's. He plotted the amount of iodine bound against a function of iodine concentration obtaining an end point corresponding to about one iodine molecule for every six glucose residues. The work was done on whole corn starch. The nature of the calculations involved in Murray's method were such as to make the accuracy of the above conclusion questionable.

The methods to be presented in this thesis depend almost entirely on potentiometric measurements of iodine activity in solutions of starch and of its components and derivatives. It will not be out of place, therefore, to devote a few paragraphs to the theory of the electrode system used and related methods.

2. Related methods

Baldwin (35) has made an extensive investigation of the nature of the starch-iodine complex based chiefly on spectrophotometric methods. He made a number of worthwhile observations, among which are the following. First, iodine is taken up more readily by amylose than by amylopectin, and the light absorption is much greater in the case of the amylose-iodine. Second, the complex can be formed in the absence of iodide ions. Third, there is a direct relationship between chain length of starch and the absorption maximum of the corresponding

iodine complex. Fourth, it is possible to estimate quantities of amylose and amylopectin in starches from a consideration of the difference in light absorption qualities of their iodine complexes. Fifth, spectrophotometric titration of amylose with iodine shows an end-point when the ratio of glucose residues to iodine molecules is approximately 6 to 1. All of these conclusions are in accord with and lend support to the helical idea of the starch-iodine complex. The spectrophotometric titration with iodine is of particular interest in view of results obtained in the present investigation.

3. Theory of the iodine electrode

The potential of a bright platinum wire immersed in a solution containing iodine and iodide ions depends on the concentrations of these two substances according to the Nernst equation

$$E = E^{\circ} - \frac{RT}{nF} \ln \frac{[I_2]}{[I^-]^2} , \qquad (I)$$

where E is the observed potential with respect to the normal hydrogen electrode and E° is the potential of the iodine-iodide electrode under the hypothetical conditions of unit activity for the two substances concerned. E° was determined by Murray as -0.6204 volts (34). Substituting this value in equation (I) at 25°C,

$$E = -0.6204 - 0.0295 \log \frac{[I_2]}{[I^-]^2} . \qquad (II)$$

It will be noted that E° pertains here to the electrode Pt, $I^{-}, I_2(aq)$, not to the electrode $Pt, I^{-}, I_2(s)$. E° for the latter is -0.5357 volts. This value was obtained by Lewis and Randall through application of their activity coefficients to data from a number of sources (36). The activity of iodine in the case of the saturated iodine electrode is, for all practical purposes, equal to the solubility. Since the solubility is known, E° for either of the above electrodes may be calculated from a known value of E° for the other electrode. When the normal calomel cell is used as reference electrode, its potential of -0.2822 volts (36) must be subtracted from E°. Equation II then becomes

$$E = -0.3382 - 0.0295 \log \frac{[I_2]}{[I^-]^2} \qquad \text{(III)}$$

In the investigations to be presented in this dissertation the iodide concentrations used are very high compared to the iodine concentration and remain essentially constant at 0.05 normal. (See procedure in experimental section.) The amount of iodide used in forming tri-iodide ion is negligible in view of the small concentrations of iodine. Equation (III) can be simplified for any special iodide concentration with the aid of the activity coefficients of Lewis and Randall. The value for the activity of iodide ion in a solution of ionic strength, μ, equal to 0.05 is 0.84. Therefore, when the normality of potassium iodide is 0.05,

$$E = -0.4194 - .0295 \log [I_2] \qquad \text{(IV)}$$

A similar equation may be set up for any iodide concentration.

The iodine electrode is discussed in some detail in Kolthoff and Furman (37), Lewis and Randall (36), and Murray (34). The conventions regarding sign of potentials in the above discussion are those of Lewis and Randall, but for convenience in preparation of graphs the potential of the electrode is considered as a positive number in subsequent sections.

III. PRESENTATION AND DISCUSSION OF RESULTS

A. Analytical Procedure for Determination of Starch Components

1. Development of the method

The amylose component of starch gives a deep blue color with iodine; the amylopectin component gives a red or purplish-red color that is, relatively speaking, much less intense. It seemed probable that this difference in color might be accompanied by a difference in the amount of iodine bound by these two fractions. Dilute solutions of amylose and amylopectin were titrated with iodine solution. The increase of iodine activity in the solution being titrated was followed by a potentiometric method. This involved simply the measurement of the potential of an iodine electrode in the solution. For full details of the procedures used in the potentiometric iodine titrations, refer to Part IV, Experimental Details.

It was discovered that addition of iodine to an amylopectin solution caused the iodine activity of the solution to increase in a continuous manner. This is illustrated by Table 1 and Figure 1, which is a plot of iodine electrode potential *vs.* milliliters of iodine solution added. On the

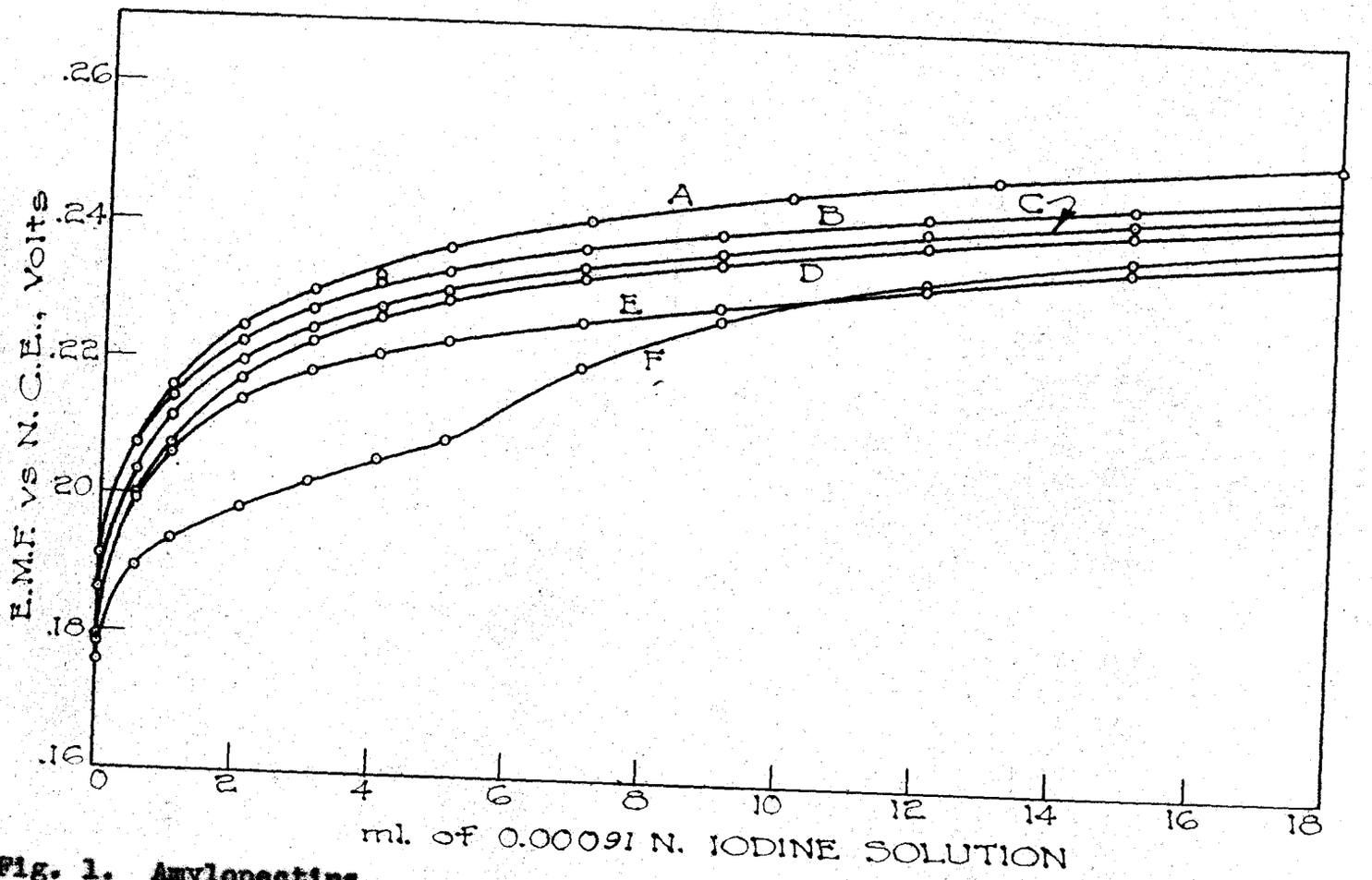

Fig. 1. Amylopectins

A. 0.05 N potassium iodide solution
B. 0.04 grams waxy rice starch
C. 0.04 grams waxy corn starch
D. 0.04 grams tapioca amylopectin
E. 0.04 grams potato amylopectin
F. 0.04 grams corn amylopectin (crude, as obtained by n-butanol fractionation)

Table 1. Titration of Amylopectins

(Iodine added is expressed in milliliters of 0.00091 N solution, E.M.F. as volts with respect to the normal calomel electrode.)

I_2 added	E. M. F.					
	0.05 N KI sol.	Waxy rice	Waxy corn	Tapioca	Potato	Corn
0		0.1913	0.1860	0.1791	0.1787	0.1600
.5		.2075	.2038	.2000	.1996	.1859
1.0	0.2159	.2147	.2115	.2073	.2061	.1912
2	.2250	.2226	.2198	.2171	.2141	.1969
3	.2303	.2275	.2248	.2228	.2186	.2016
4			.2282	.2267	.2212	.2051
5	.2370	.2335	.2309	.2293	.2232	.2097
7	.2413	.2372	.2347	.2331	.2267	.2212
9		.2400	.2373	.2358	.2296	.2278
10	.2458					
12		.2433	.2408	.2391	.2332	.2338
13	.2490					
15		.2458	.2435	.2417	.2365	.2380
18	.2529					
19		.2486	.2465	.2447	.2401	.2419
25	.2567	.2519	.2500	.2486	.2447	.2465

other hand, when iodine was added to an amylose solution, the activity rose slightly at first, but then became almost constant and remained so while considerable iodine was added. Finally, the formation of complex was completed and the iodine activity again increased steadily. Figure 2 and Table 2 contain curves and data for several typical amyloses.

The possibility of using the titration curves as a means of measuring the amounts of amylose in starches or starch fractions was then considered. Before the iodine titration could be used in establishing an analytical method, however, it was necessary to consider the behavior of the amylose fractions more carefully. They varied considerably in the amount of iodine bound and the most logical explanation was that they were impure. It hardly seemed possible that pure amylose could be obtained by the single precipitation used in Schoch's butanol fractionation, the method used in preparing the corn, potato and lily bulb amyloses. Kerr's "crystalline amylose," prepared by butanol precipitation of hot-water-extracted amylose, was more likely to be uncontaminated by amylopectin. By repeated recrystallization he was not able to increase the amylosic properties of this material appreciably, indicating that the originally precipitated material was already quite pure. It was necessary to show, then, that the other amyloses behaved differently because they were impure, not because they were fundamentally different in behavior toward iodine. This was proved to be true by

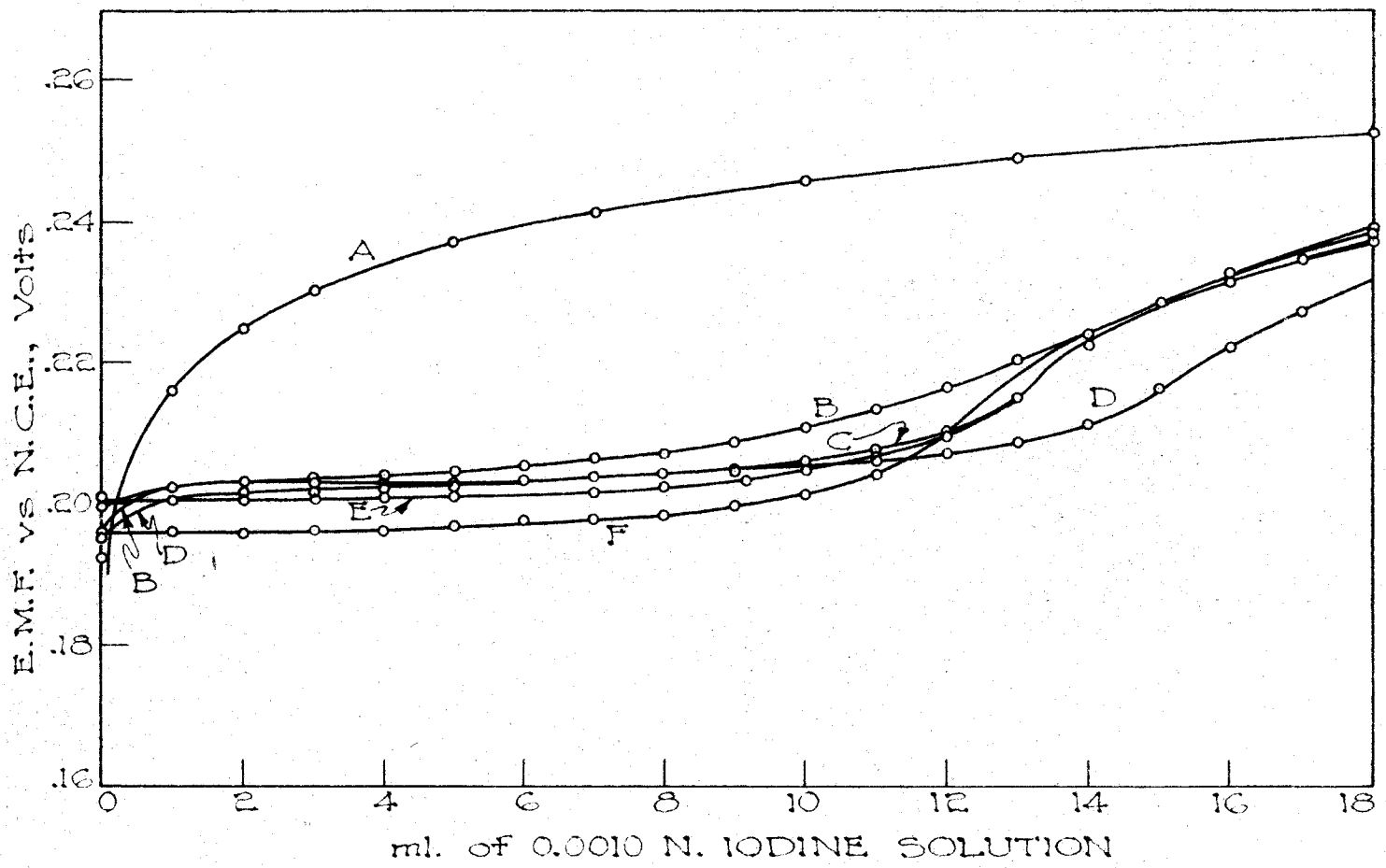

Fig. 2. Amyloses

A. 0.05 N potassium iodide solution (see Table 1)
B. 0.01 grams synthetic starch
C. 0.01 grams corn amylose
D. 0.01 grams "crystalline amylose"
E. 0.01 grams lily bulb amylose
F. 0.01 grams potato amylose

Table 2. Titration of Amyloses

(Iodine added in milliliters of 0.0010 N solution. E.M.F. in volts, referred to the normal calomel electrode.)

Iodine Added	E. M. F.				
	Corn	Potato	Lily bulb	"Crystalline"	Synthetic
0	0.1998	0.1962	0.2010	0.1926	0.1954
1	.2023	.1963	.2006	.2007	.2023
2	.2032	.1961	.2006	.2018	.2032
3	.2032	.1966	.2010	.2021	.2036
4	.2030	.1964	.2011	.2025	.2042
5	.2031	.1971	.2012	.2027	.2047
6	.2034	.1978		.2030	.2056
7	.2039	.1982	.2019	.2036	.2066
8	.2043	.1986	.2026	.2042	.2075
9	.2052	.1999	.2045	.2047	.2090
10	.2062	.2017	.2052	.2054	.2109
11	.2081	.2047	.2070	.2064	.2137
12	.2101	.2106	.2096	.2074	.2168
13	.2154		.2152	.2090	.2205
14	.2226	.2245	.2235	.2116	.2243
15	.2285			.2166	.2281
16	.2329		.2320	.2226	.2318
17	.	.2349		.2279	.2350
18	.2388		.2394		.2379
20					.2426
21	.2446	.2424	.2452		
22					.2464

recrystallizing a butanol-precipitated corn amylose several times. Ultimately this treatment resulted in a material possessing the same iodine-binding ability as "crystalline amylose." It was evident that the crude amyloses were impure, containing 10 to 15 per cent amylopectin. "Crystalline amylose" was chosen as the standard amylose and the amount of iodine it bound in complex formation was carefully established. This value was found to be 0.187 grams per gram of amylose in 0.05 N potassium iodide solution. It has been confirmed by Schoch (38). The necessity of choosing a specific iodide concentration will be discussed, subsequently.

In establishing the figure for the amount of iodine bound by "crystalline amylose," the inflection point of the curve in Figure 2 was taken as the end-point of the titration. A more accurate idea of what was occurring at the inflection point was obtained by plotting the amount of iodine bound per gram of amylose against the concentration of free iodine in the solution, as shown in Figure 3. The curve in Figure 2 for 0.05 N potassium iodide was used to calculate the amount of iodine necessarily present in solution to raise the iodine activity to any desired potential. This amount was then subtracted from the total iodine to get the bound iodine. The actual concentration of free iodine was calculated with the aid of Equation IV. The lower, nearly vertical, section of the curve shown in Figure 3 represents the binding of iodine in complex formation. Along the upper portion of the curve

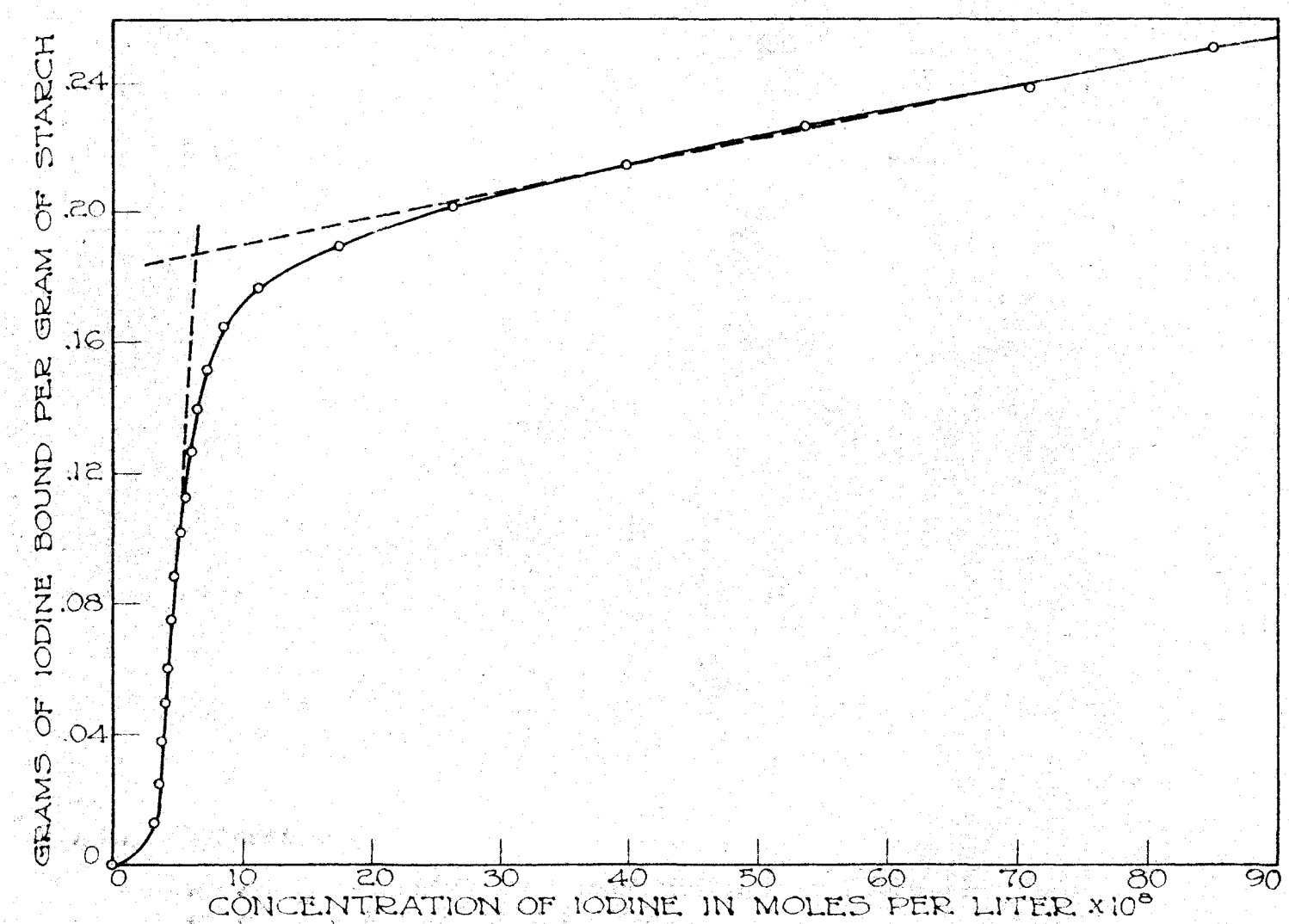

Fig. 3. Amount of Iodine Bound by "Crystalline Amylose" as a Function of Iodine Concentration

increase in bound iodine appears to be proportional to the increase in free iodine concentration. This could be accounted for by an adsorption mechanism, entirely independent of complex formation. The two sections of the curve are nearly linear and extensions may be drawn from them as shown in the figure. The point of intersection corresponds to the inflection point in the curve for "crystalline amylose" in Figure 2. It seemed safe, therefore, to assume that the inflection point corresponds closely to the end of complex formation provided a small correction is made for the "blank," i.e., the amount of iodine necessary to raise a 0.05 N potassium iodide solution to the potential of the inflection point.

The validity of the above assumption was tested by titrating known mixtures of amylose and amylopectin, and calculating their percentage composition with the aid of the resulting curves. Table 4 and Figure 4 contain the data and curves obtained. Table 5 illustrates the procedure used in calculating the composition in per cent amylose and gives a comparison between the actual and calculated values. The correspondence is excellent.

Actually, the results of a large number of titrations have disclosed that the accuracy and duplicability of amylose content determinations can be held within two per cent, with only ordinary precautions regarding iodide concentrations. Relative values of amylose contents calculated from titrations conducted under carefully maintained conditions can be obtained with greater accuracy.

Table 3. Data Used in Calculating Values of Grams of Iodine Bound per Gram of Starch

(1)	(2)	(3)	(4)	(5)	(6)	(7)
0.1910	0	100	1.738	0	0.005	0
.1997	1	101	3.310	1.2694	.008	0.01265
.2007	2	102	3.715	2.538	.010	.0253
.2013	3	103	3.800	3.81	.011	.0380
.2020	4	104	4.07	5.08	.011	.0507
.2026	5	105	4.26	6.345	.012	.0633
.2034	6	106	4.565	7.61	.012	.0760
.2040	7	107	4.785	8.89	.013	.0888
.2048	8	108	5.123	10.16	.014	.1015
.2059	9	109	5.495	11.32	.015	.1130
.2069	10	110	6.02	12.69	.016	.1267
.2081	11	111	6.453	13.96	.018	.1394
.2097	12	112	7.24	15.23	.019	.1521
.2117	13	113	8.505	16.51	.023	.1649
.2150	14	114	11.22	17.77	.028	.1774
.2202	15	115	17.38	19.04	.047	.1899
.2261	16	116	26.28	20.3	.071	.202
.2311	17	117	39.8	21.6	.11	.215
.2350	18	118	53.65	22.85	.15	.227
.2384	19	119	70.8	24.15	.20	.2395
.2411	20	120	85.1	25.4	.23	.252

Column headings:

(1) E.M.F. of iodine electrode in volts, referred to the normal calomel electrode
(2) Milliliters of 0.001 N iodine solution added
(3) Total volume of solution, milliliters
(4) Concentration of free iodine in mols per liter x 10^8, calculated from (1) by means of Equation IV
(5) Total iodine added in grams x 10^4, calculated from (2)
(6) Free iodine in solution in grams x 10^4, calculated from (3) and (4)
(7) Iodine in the complex in grams per gram of starch; (5) minus (6) divided by 0.01

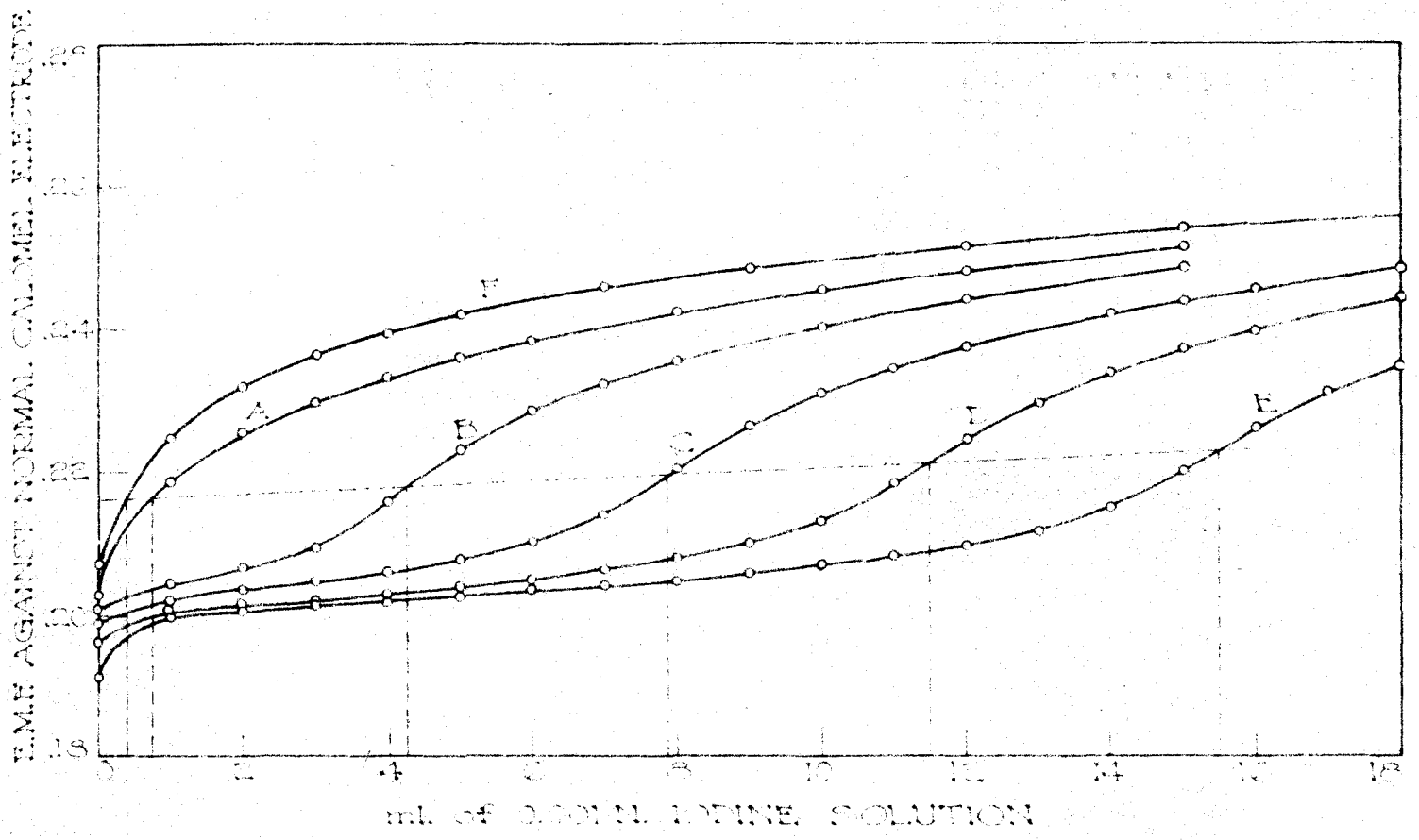

Fig. 4. Titration of Mixtures of Amylose and Amylopectin

A. 0.01 gram of potato amylopectin.
B. 0.0075 gram of potato amylopectin plus 0.0025 gram of "crystalline amylose."
C. 0.0050 gram of potato amylopectin plus 0.0050 gram of "crystalline amylose."
D. 0.0025 gram of potato amylopectin plus 0.0075 gram of "crystalline amylose."
E. 0.01 gram of "crystalline amylose."
F. 0.05 N potassium iodide solution.

Table 4. Titration of Mixtures of Amylose and Amylopectin

(Iodine added in milliliters of 0.001 N solution. E.M.F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.					
	A*	B	C	D	E	F
0	0.2026	0.2006	0.1983	0.1958	0.1910	0.2069
1	.2186	.2042	.2019	.2002	.1997	.2246
2	.2253	.2065	.2033	.2012	.2007	.2318
3	.2297	.2094	.2047	.2018	.2013	.2362
4	.2330	.2157	.2059	.2029	.2020	.2394
5	.2358	.2229	.2076	.2039	.2026	.2419
6	.2382	.2282	.2101	.2049	.2034	
7		.2321	.2139	.2062	.2040	.2458
8	.2422	.2352	.2203	.2079	.2048	
9			.2264	.2099	.2059	.2486
10	.2454	.2402	.2308	.2129	.2069	
11			.2345	.2182	.2081	
12	.2482	.2441	.2374	.2245	.2097	.2518
13				.2296	.2117	
14			.2421	.2338	.2150	
15	.2516	.2486		.2372	.2202	.2542
16			.2457	.2399	.2261	
17					.2311	
18			.2487	.2444	.2350	
19					.2384	.2568
20				.2479	.2411	
22					.2454	

*The letters refer to the mixtures shown in Figure 4, p. 27.

Table 5. Amylose Content of Mixtures Calculated from the Data of Figure 4

(Amounts of iodine expressed in milliliters of 0.001 N solution)

Mixture from Figure 4	Total iodine added	Iodine in 0.05 N KI solution	Iodine taken up by amylopectin	Iodine bound by amylose	Amylose, %	
					Calculated	Actual
A	.9	0.4	0.5		0	0
B	4.45	.5	.4	3.55	24	25
C	8.0	.6	.3	7.1	48	50
D	11.55	.7	.15	10.7	73	75
E	15.5	.85	0	14.65	100	100

Titrations were run on a number of amylose and amylopectin mixtures similar to those just described in the preceding paragraphs. Results indicated that slightly better correlation between actual and observed amylose contents was obtained by assuming that the end-point occurred a little below the inflection of the curve. The difference arose through differences in slope of the various curves in the neighborhood of the inflection point, the slope decreasing with increased amylopectin content.

The iodine titration method is certainly nothing but empirical. There is no stoichiometrical relationship whatsoever involved in the complex formation. The complex continues to take up iodine after the so-called "end-point" has been passed. This end-point serves only to mark approximately the transition from a condition of the complex in which it binds iodine with great facility to one into which the iodine is forced by a greatly increased iodine activity in the solution. In this respect, the spectrophotometric work of Baldwin is of interest (35). Apparently, the light absorption produced by an iodine molecule in the complex is about the same regardless of the iodine activity necessary to hold it in place. Therefore, the end-point in this method does not occur until the complex is actually saturated. For this reason Baldwin's work permits a better estimate of the maximum amount of iodine which amylose can take up. In low iodide concentrations, 0.0025 N, the ratio of glucose residues to iodine

molecules was about 6:1. The iodine titration values approached the ratio of about 7:1 at low iodide concentrations.

2. Analysis of starch fractions

As a result of the work that has been described thus far, it was evident that the potentiometric iodine titration could be used as an analytical procedure for determination of the purity of starch fractions. Its application to the amylose component has already been brought out in the details of development of the method. Curve F of Figure 1 may be used as an example of titration of an amylopectin. Using this curve as a basis for calculation, the amylose content of the crude amylopectin fraction is about six per cent. In practice the accuracy of the determination could be improved by using a larger sample.

As the ratio of amylopectin to amylose increased, the amount of iodine bound by the amylopectin became an appreciable fraction of the total iodine bound. In extreme cases, where only a few per cent of amylose was present, the amylopectin accounted for about one-fourth the total iodine used. In this event the determination of amylose content was subject to considerable error. It was possible only if a pure sample of amylopectin could be prepared. If this could be accomplished, the amount of iodine bound by amylopectin alone could be determined and subtracted from the total iodine bound. A method of effecting the purification of the

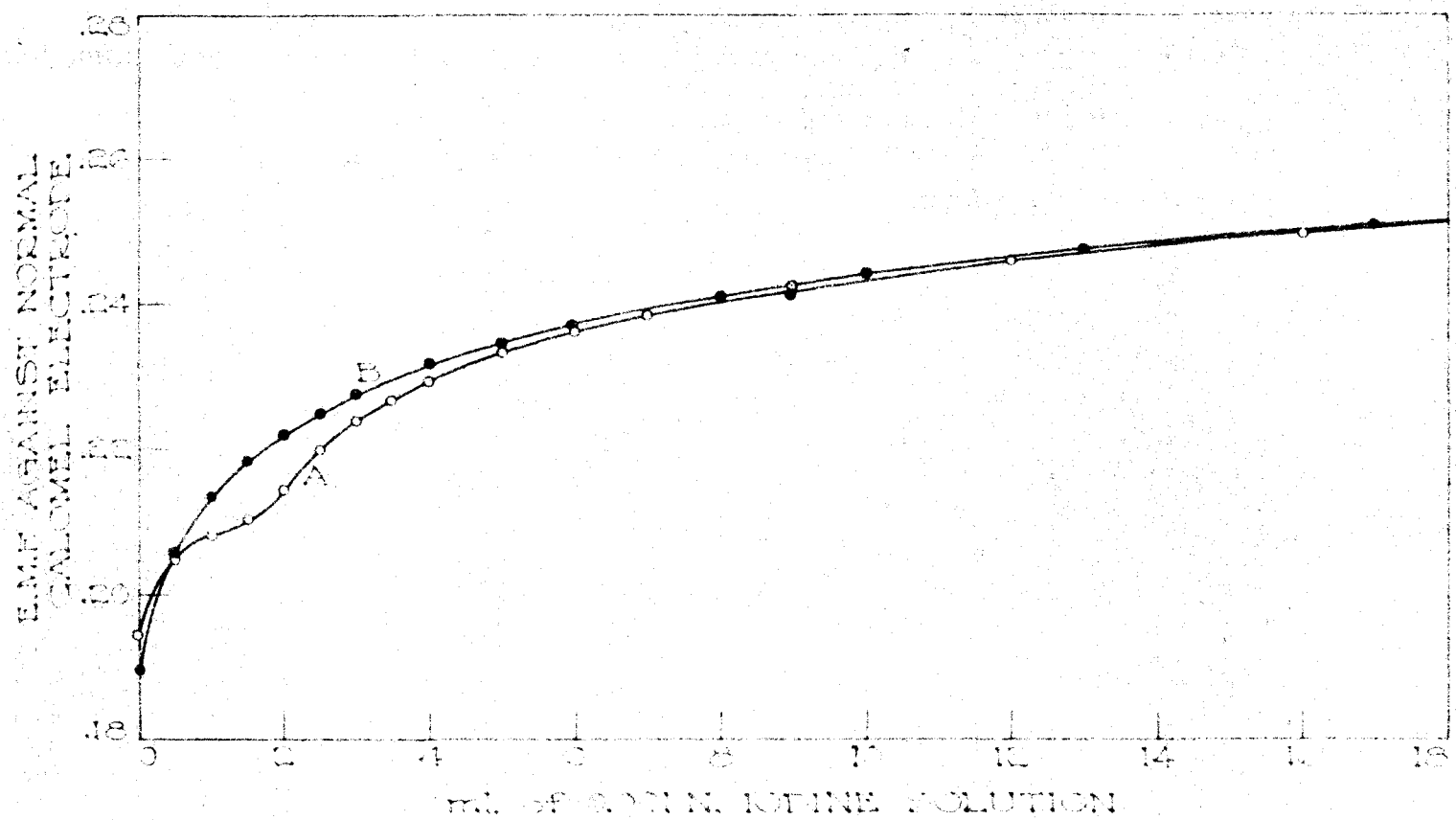

Fig. 5. Purification of Crude Amylopectin from Cornstarch

A. Amylopectin fraction from the butanol separation before treatment with cotton

B. After treatment with cotton

Table 6. Titration of Amylopectin Before and After Cotton Treatment

(Iodine added in milliliters of 0.001 N solution. E.M.F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.		Iodine added	E. M. F.	
	Before	After		Before	After
0	0.1943	0.1895	3.5	0.2269	0.2296
0.25	.2013	.1999	4.0	.2294	.2314
.50	.2050	.2059	4.5	.2314	
.75	.2069	.2097	5	.2331	.2346
1.00	.2081	.2133	6	.2361	.2370
1.25	.2094	.2160	7	.2384	
1.50	.2101	.2183	8		.2410
1.75	.2118	.2201	9	.2420	
2.00	.2144	.2220	10		.2439
2.25	.2172	.2240	12	.2460	
2.50	.2199	.2249	13		.2474
2.75	.2220	.2262	16	.2499	
3.00	.2238	.2274	17		.2508
3.25	.2255		20	.2528	

amylopectins was offered by Tanret's cotton fractionation method. A solution of crude corn amylopectin that had been prepared by butanol fractionation was treated with cotton to remove the small amount of amylose impurity. The results of this treatment are shown by a comparison of the curves in Figure 5.

There have been four amylopectins prepared by the butanol fractionation method, those from corn, tapioca, potato, and lily bulb starches. The first two are obtained with some amylose present as an impurity, the latter two appear to be quite pure. Table 7 contains the amylose contents of the crude amylopectins and values for grams of iodine bound per gram of amylopectin at iodine activities corresponding to the potentials near which the inflection point of amylose titration curves occur. The tapioca amylopectin like the corn component was purified by cotton treatment.

Table 7. Amount of Iodine Bound by Amylopectin

Crude amylopectin from butanol fractionation	Amylose (%)	Grams I_2 bound per gram of pure amylopectin
Corn	7-8	.032
Tapioca	1-2	.013
Potato	trace	.018
Lily bulb	trace	.016
Average		.020
Gm. I_2 bound per gm. of pure amylose		.187

The values for the amounts of iodine bound are quite similar for the four materials. The larger value for corn amylopectin may be due to incomplete removal of amylose by the cotton. The average of 0.02 grams of iodine bound by one gram of amylopectin might be used as an approximation in the analysis of a fraction obtained from a starch whose amylopectin had not yet been isolated in a pure state. The value of the amount of iodine bound by amylose is included in Figure 7 for comparison.

3. Analysis of whole starches

The study of amylose-amylopectin mixtures showed that the latter had no real effect on the amount of iodine taken up by the former. This, of course, was a very fortunate state of affairs, although it didn't prove that amylopectin could not influence the complex formation of amylose in natural starches by means of some mechanism inherent in the granule and not present in artificial mixtures. However, if it was assumed that complete molecular dispersion was achieved in each case before titration, the results in the case of whole starch would be the same as obtained with a mixture. The dispersion was made with alkali. Such a dispersion was complete enough to permit fractionation of starch by the n-butanol separation procedure. This would indicate that the amylose and amylopectin molecules were not associated in any way with one another in the solution. The fractionation of alkali-dispersed starch was carried out on lily bulb starch,

the products being similar in every respect to those obtained from autoclaved solutions of the same starch.

One other possibility to be considered before the potentiometric iodine titration was made applicable to whole starches was that of interference by the minor, non-carbohydrate constituents of natural starches. This was not realized for a time, but it was later found that one of these constituents, namely fatty acid or its salt, had a very great effect on the iodine titration, the nature and magnitude of which will be taken up in another section.

The interference given by fatty acid limits the application of the iodine titration to starches either naturally fat-free or artificially defatted. (See experimental section for details of defatting procedures.)

The results of iodine titration of a number of whole starches are given in Figure 6 and Table 8. With the exception of the pea starch, the starches were either defatted or naturally low in fat content. The potato and lily bulb starches contained less than 0.05 per cent fat and the corn was defatted. Radley (9) gives the fat contents of tapioca, wheat, and sago starches as 0.12, 0.12, and 0.11 per cent, respectively. These amounts are appreciable, but are low enough to introduce only a small error in the titration. For this reason the curve for tapioca was included in Figure 6,

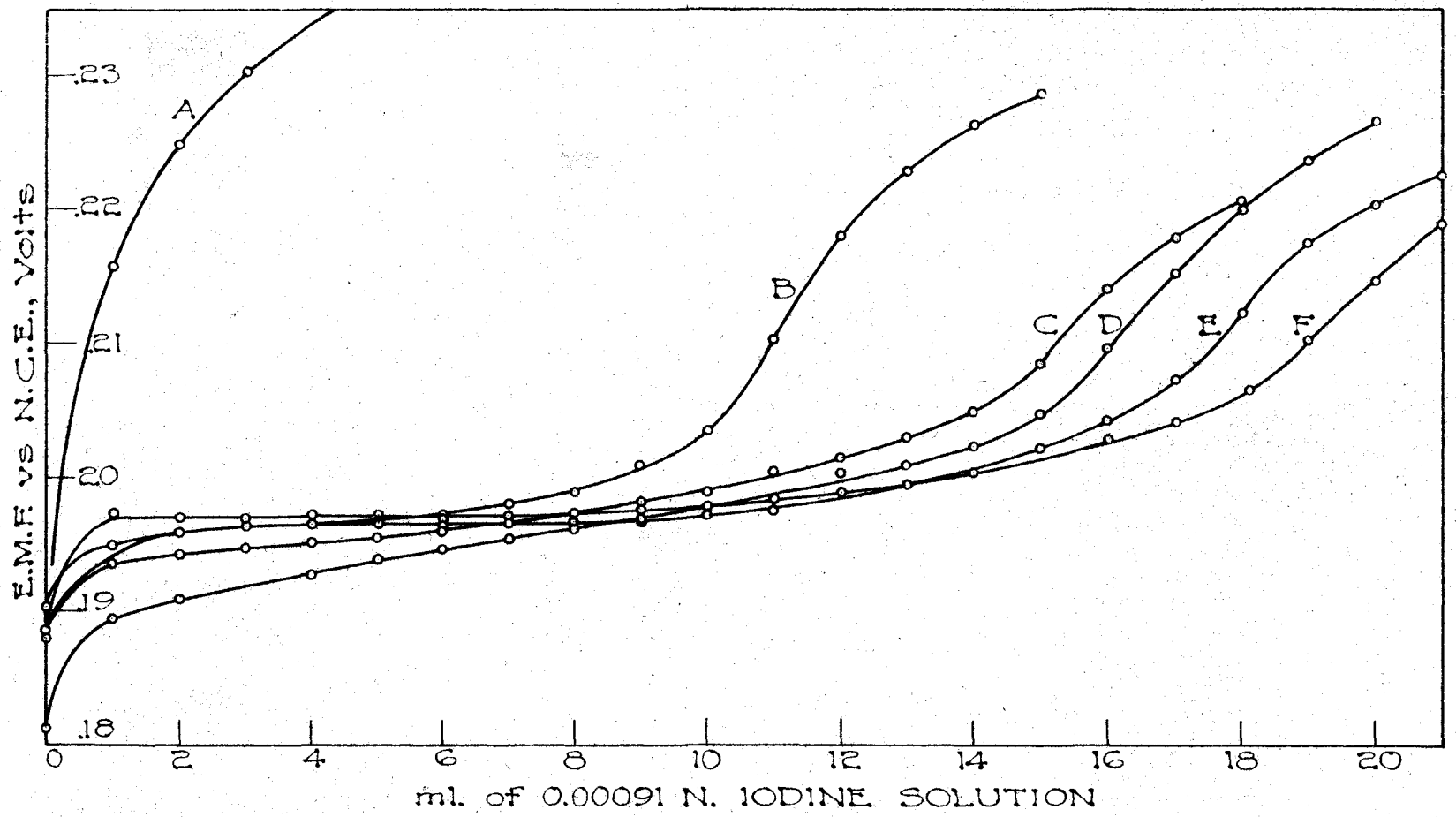

Fig. 6. Whole Starches

A. 0.05 N potassium iodide solution (see Table 1)
B. 0.04 gram tapioca starch
C. 0.04 gram potato starch
D. 0.04 gram corn starch
E. 0.04 gram lily bulb starch
F. 0.04 gram pea starch

Table 8. Titration of Whole Starches

(Iodine added in milliliters of 0.00091 N solution. E.M.F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.						
	Tapioca	Potato	Corn	Lily bulb	Pea	Sago	Wheat
0	0.1903	0.1885	0.1814	0.1892	0.1882	0.1905	0.1857
1	.1950	.1937	.1895	.1873	.1974		.1935
2	.1961	.1943	.1910	.1965	.1971	.2000	.1964
3	.1965	.1948	.1909	.1963	.1971	.2010	.1983
4	.1966	.1952	.1928	.1965	.1972	.2041	.1994
5	.1967	.1956	.1940	.1964	.1973	.2052	.2005
6	.1974	.1961	.1947	.1966	.1971	.2057	.2015
7	.1981	.1968	.1955	.1966	.1972	.2066	.2021
8	.1990	.1975	.1963	.1967	.1974	.2077	.2030
9	.2009	.1983	.1970	.1968	.1976	.2087	.2043
10	.2036	.1991	.1979	.1973	.1979	.2099	.2058
11	.2104	.2005	.1984	.1978	.1986	.2112	.2074
12	.2181	.2015	.2005		.1990	.2125	.2100
13	.2230	.2032	.2011	.1995	.1997	.2151	.2151
14	.2264	.2051	.2026	.2005	.2005	.2182	.2203
15	.2287	.2087	.2048	.2023	.2009		.2246
16		.2142	.2098	.2044	.2030	.2266	.2280
17		.2180	.2154	.2072	.2043		
18		.2207	.2201	.2125	.2067	.2334	.2330
19			.2239	.2177	.2104		
20			.2269	.2205	.2150		
21				.2229	.2192	.2403	.2382

and the data for tapioca, wheat, and sago in Table 8. The amylose contents were calculated from the iodine titration results for the seven starches mentioned above. The results have been collected in Table 9. The corrections for iodine bound by amylopectin were obtained from Table 7. Included in Table 9 is defatted waxy corn starch.

Table 9. Amylose Contents of Starches

Starch	Amylose, %	Starch	Amylose, %
Waxy corn, defatted	0.5-1	Corn, defatted	26
Tapioca	18	Sago	27
Potato	22	Lily bulb	27, 34
Wheat	24	Pea	29

The amylose contents listed here are not compiled only from the data presented in Figure 6 and Table 8, but are the results of numerous titrations. In some of the starch species the amylose content remains much the same even though the origin and variety of the specimens may differ widely. This seems to be true of corn and potato starches. Others give indications of wide variation in composition. The values given in Table 9 for lily bulb starch were found in two samples of that material. A number of oat starches possessed widely varying amylose contents.

The only effective check on the accuracy of the potentiometric iodine titration as a measure of the amylose

contents of whole starches lies in an actual quantitative separation of the two components. Such a separation has never been carried out with anywhere near the accuracy of which the iodine titration is capable. However, it is the only available way of checking and, as carried out by Schoch's method, is fairly efficient. In many ways the two methods are complementary since, in spite of alkali labile values, extent of digestion by β-amylase and other methods of characterization, the effectiveness of Schoch's fractionation is best demonstrated by the iodine titration of the fractions produced. It shows the crude fractions to be generally impure, and it can also be used to follow the progress of their purification by recrystallization in the case of the amylose, and by treatment with cotton in the case of the amylopectin. A good insight into the relationship between the two methods is provided in the case of tapioca starch. Schoch obtained an average yield of 21.3 per cent precipitated by butanol (38). On the other hand, the iodine titration indicated an amylose content of only 18 per cent. The iodine titration also showed the crude butanol precipitate to be only 84 per cent pure, while the amylopectin had a trace of amylose in it. When the amount of pure amylose present in the two fractions was totaled, it amounted to 19 per cent, only slightly more than the 18 per cent found by iodine titration.

B. Factors Affecting the Iodine Titration

1. Iodide concentration

The concentration of potassium iodide in a solution of starch affects the results of the potentiometric iodine titration in two ways. First, the amount of iodine bound by starch in complex formation varies inversely with the concentration of iodide in the solution. This was shown experimentally by titrating aliquot portions of an amylose solution, different amounts of iodide being added to each portion. The results are contained in Figure 7 and Table 10. It was apparent that the iodide concentration had a great effect on the amount of iodine bound by the amylose. This made it necessary to establish a certain definite concentration for the titration procedure. It will be recalled that the amount of iodine taken up by "crystalline amylose" was measured in a 0.05 N potassium iodide solution. This value of 0.187 grams per gram is the standard upon which the entire method is based, and it can be used only when the proper iodide concentration is maintained. If it becomes necessary to use a different iodide concentration, the iodine-binding ability of the standard amylose must be measured under the new conditions.

In order to produce the effect just discussed, the iodine must exert some influence on the amylose-iodine complex formation. The most logical explanation is that iodide or triiodide ions as well as iodine enter the amylose helix. Their

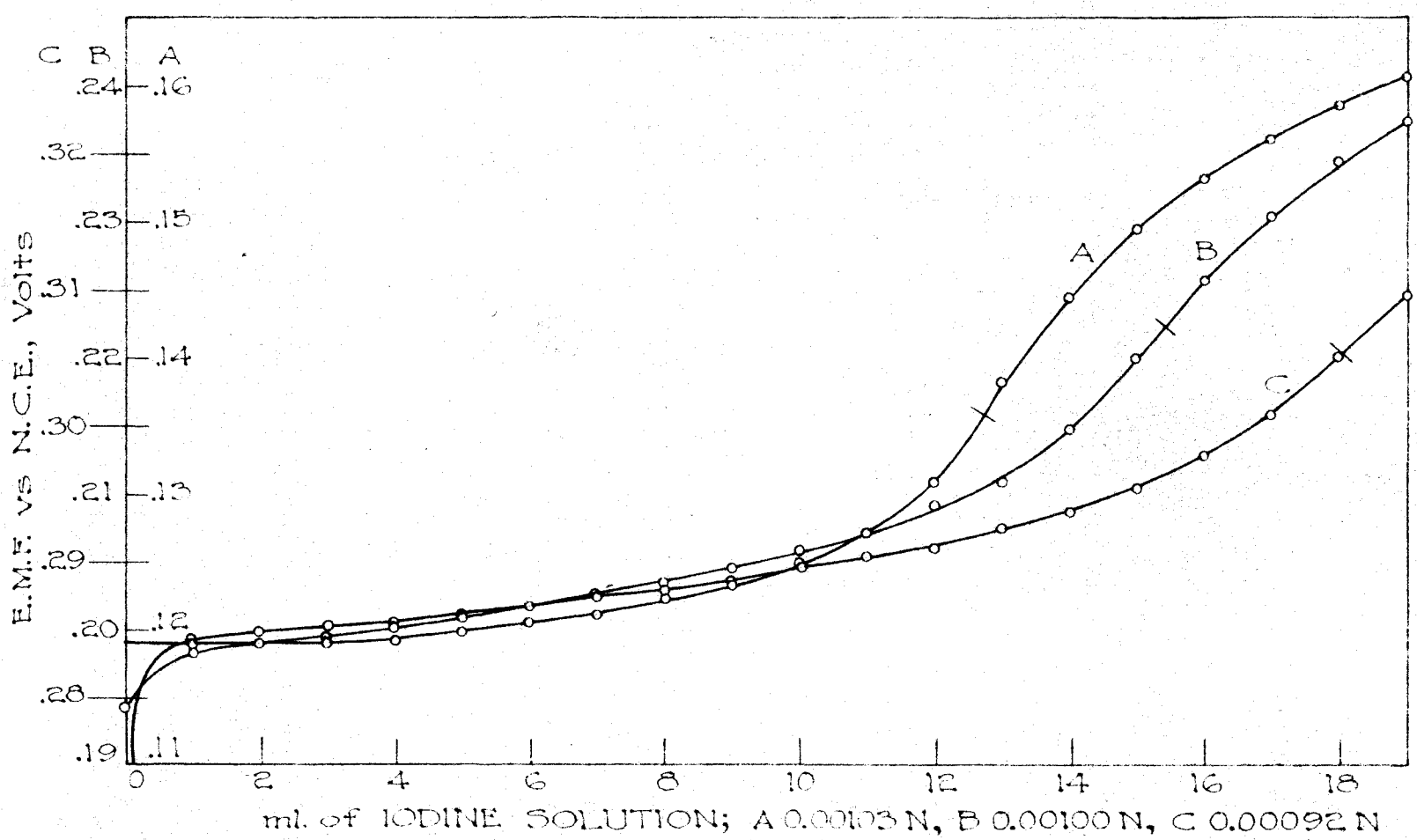

Fig. 7. Effect of Potassium Iodide Concentration on the Amount of Iodine Bound by Amylose

A. 0.5 N potassium iodide
B. 0.05 N potassium iodide
C. 0.005 N potassium iodide

Table 10. Titration of "Crystalline Amylose" in Various Iodide Concentrations

(Iodine added in milliliters of solution. E.M.F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.		
	0.5 N (0.00103 N I_2)	0.05 N (0.00100 N I_2)	0.005 N (0.00092 N I_2)
0	0.1203	0.1941	0.2488
1	.1189	.1982	.2842
2	.1188	.1988	.2849
3	.1189	.1995	.2853
4	.1190	.2003	.2857
5	.1199	.2010	.2863
6	.1207	.2018	.2867
7	.1212	.2026	.2875
8	.1223	.2036	.2880
9	.1233	.2045	.2885
10	.1248	.2058	.2895
11	.1271	.2072	.2904
12	.1310	.2089	.2911
13	.1381	.2110	.2924
14	.1444	.2146	.2936
15	.1493	.2199	.2954
16	.1531	.2256	.2978
17	.1562	.2304	.3008
18	.1586	.2342	.3050
19		.2373	.3094
20	.1626	.2400	.3133

number and consequently the amount of space they occupy depends on their concentration in the solution.

An attempt was made to extrapolate to zero iodide concentration, using the data of Figure 7. When the amount of iodine bound was plotted against log of iodide concentration, a straight line was obtained. Extrapolation of such a curve to zero concentration would indicate an infinite amount of bound iodine. Obviously, this cannot be true. The only logical conclusion is that the curve which is apparently linear in the observed range actually has a much different curvature in the region of very dilute iodide solutions.

The second way in which the iodide concentration affects the titration may be most easily explained by reference to Equation I in Part II. It will be noted that the potential corresponding to any given iodine activity depends on the log of the iodide activity. The result of changes in iodide activity is to shift the entire titration curve up or down the potential scale. The form of the curve remains practically unchanged, and within reasonable limits any iodide concentration may be used in the titration, subject to the conditions just discussed in the preceding paragraphs. However, it is convenient, and in some applications of the method necessary, to maintain the iodide concentration at some chosen value. Needless to say, the iodide concentration of the iodine solution used in the titration must be the same as that of the solution to be titrated.

In making iodine titrations in which the potential level of complex formation is to be the important factor, it is obvious that iodide concentration must be controlled with the utmost care. In fact, more accurate results might be obtained in some applications if the iodide concentration in the solution to be titrated was determined by an analytical method. A correction could be applied if it was found to be necessary. The extent of errors introduced by small variations in iodide activity can be calculated easily with the aid of a few assumptions. Differentiation of equation II, holding $[I_2]$ constant, gives

$$dE = 0.0256 \frac{d[I^-]}{[I^-]} \qquad (V)$$

and changing to finite, but small increments,

$$\Delta E = 0.0256 \frac{\Delta[I^-]}{[I^-]} \qquad (VI)$$

Now, if we assume that the change in activity, $\Delta[I^-]$, is very small compared to the total activity, $[I^-]$, then $[I^-]$ may be looked upon as a constant. If the concentration of iodide is 0.05 N, at which value the activity coefficient is 0.84, then $[I^-] = 0.05 \times 0.84 = 0.042$, and we have

$$\Delta E = 0.61 \, \Delta[I^-] \qquad (VII)$$

A similar equation may be developed to correct for small differences at any iodide concentration.

Equation VII was verified experimentally. A number of

potassium iodide solutions with normalities ranging from 0.04 to 0.06 were prepared. These were titrated with iodine solution and the potentials were plotted vs. log of milliliters of iodine solution. This function produces linear curves as shown in Figure 8. The small graph inserted in the lower righthand corner of the figure is self-explanatory. The slope of the line plotted in it corresponds to the constant of Equation VII. The value of 0.70 is a little higher than the calculated quantity in the equation, 0.61. The average of the two, 0.65, is probably near the correct value and may be used in calculating the errors in potential due to small differences in iodide concentration.

2. Other electrolytes

Schoch (40) has proposed a modification of the titration method wherein the potassium hydroxide used to disperse the starch is neutralized with hydrochloric acid instead of hydriodic acid, the necessary iodide being added as a solution of the potassium salt. This introduces an amount of chloride equivalent to the amount of iodide. The modification was proposed to eliminate the necessity of preparing and preserving an iodine-free solution of hydriodic acid. Solutions of amylose were prepared for titration with and without the addition of potassium chloride. The amount of chloride added was equivalent to the iodide present, i.e., 0.05 N. The results revealed only a small change in the

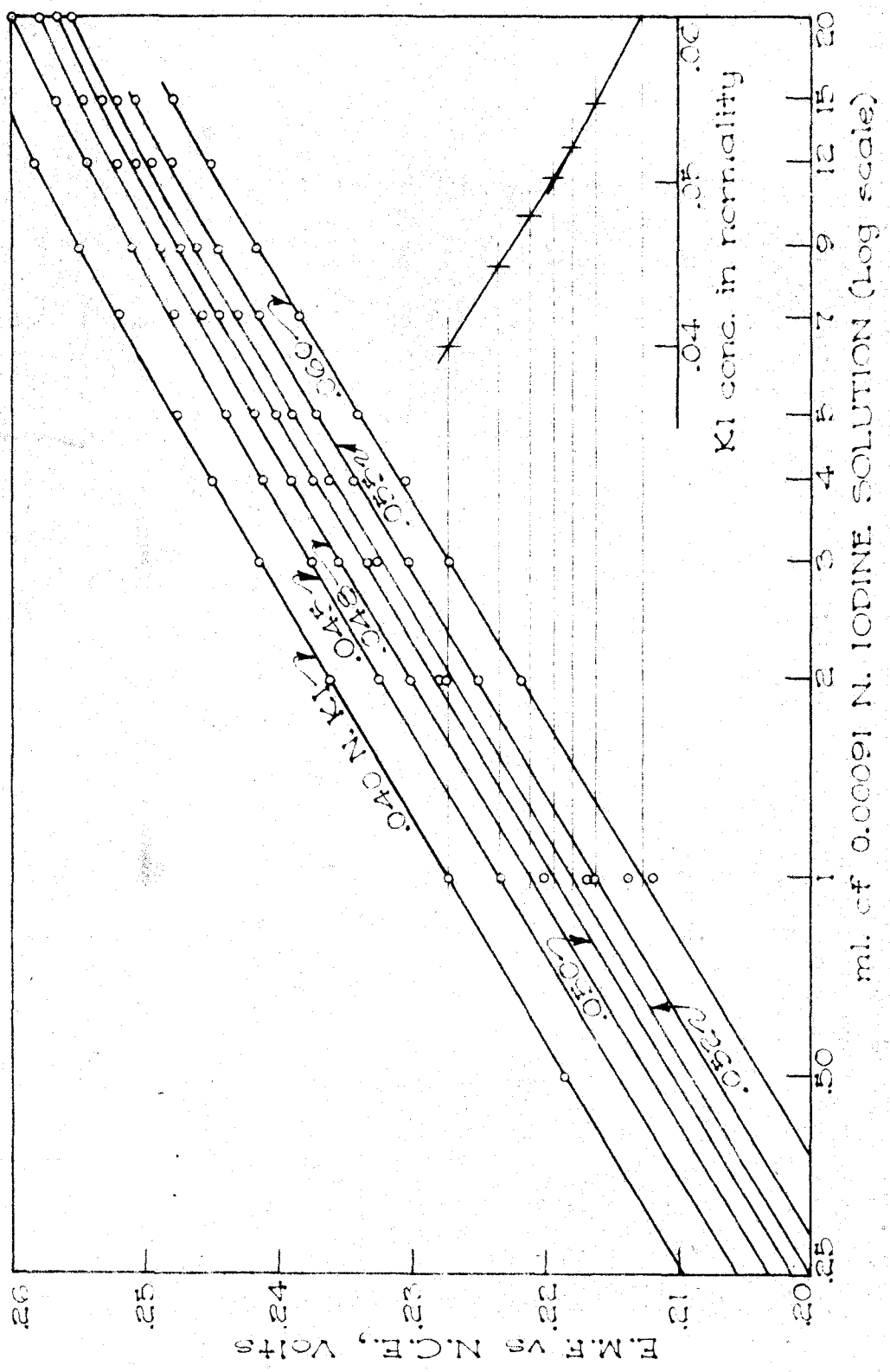

Fig. 8. Effect of Iodide Concentration on the Potential of the Iodine Electrode

Table 11. Titration of Potassium Iodide Solutions of Various Concentrations

(Iodine added in milliliters of 0.00091 N solution. E. M. F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.						
	0.040 N	0.045 N	0.048 N	0.050 N	0.052 N	0.055 N	0.060 N
1	0.2273	0.2236	0.2200	0.2164	0.2169	0.2137	0.2118
2	.2363	.2326	.2301	.2278	.2275	.2249	.2217
3	.2415	.2377	.2356	.2333	.2326	.2304	.2272
4	.2451	.2413	.2391	.2374	.2363	.2344	.2311
5	.2478	.2440	.2418	.2402	.2389	.2372	.2341
7	.2518	.2480	.2457	.2444	.2431	.2414	.2384
9	.2548	.2510	.2487	.2473	.2460	.2445	.2415
12	.2582	.2543	.2521	.2507	.2494	.2480	.2451
15	.2605	.2568	.2546	.2532	.2520	.2507	.2478
20	.2634	.2599	.2578	.2565	.2554		

position and shape of the curve. The amylose in the solution containing added chloride took up slightly less iodine, as may be seen by referring to Figure 9. In this respect the chloride probably acts in a manner analogous to that of iodide, actually becoming a minor constituent of the complex.

According to Lewis and Randall (36) the activity of the iodide ion should have decreased upon increase of the ionic strength of the solution. Using the values for iodide activities, calculations show that the entire curve should be shifted about one millivolt higher on the potential scale. This effect would likely have been more noticeable in Figure 9, had the titrating solution also contained chloride.

In the case of whole starches, there are small amounts of inorganic impurities that contribute to the electrolyte content of solutions prepared for iodine titration. The amounts present are so small, in most cases, as to be negligible. However, certain starches contain a considerable amount of phosphate. Addition of similar amounts of phosphate to starch solutions before titration was found to have no effect on the results obtained.

3. Fatty substances

Schoch found that fatty acids had an inhibitory influence on the formation of the starch-iodine complex and, therefore, on the amount of iodine bound by starch (38). Many starches contain a considerable amount of fat present in the granules

as a natural constituent in the form of free fatty acids, their soaps, or as phospholipid. Cereal starches in particular may have as much as one per cent fat content. For this reason the application of the potentiometric iodine titration procedure to many whole starches is considered to be of doubtful value unless steps are taken to remove the fat first. Methods of removing fatty material from granular starch have been published, as well as procedures for impregnating fat-free starch with fatty acid (40). Starch fatted by the latter process will retain the fat even when extracted with carbon tetrachloride.

Using the methods mentioned in the preceding paragraph, corn starches of various fat contents were prepared. Furthermore, a sample of potato starch, naturally fat-free, was impregnated with oleic acid. Its fat content after this treatment was found to be 0.37 per cent. For details of procedures see Part IV, Experimental Details. The following figures are the fat contents of various corn starch preparations with the respective amounts of iodine bound per gram of starch: 0.55, 0.038 grams; 0.20, 0.041 grams; 0.12, 0.048 grams; and 0.03, 0.051 grams. Some idea of the magnitudes of the effect may be gained from Figure 10, which contains the titration curves for the original and fatted potato starches. The fat-free starch contains about 22 per cent amylose. The curve for the starch containing only 0.37 per cent fat shows an <u>apparent</u> amylose content of 15 per cent. Certainly, this is enough to show the necessity of defatting

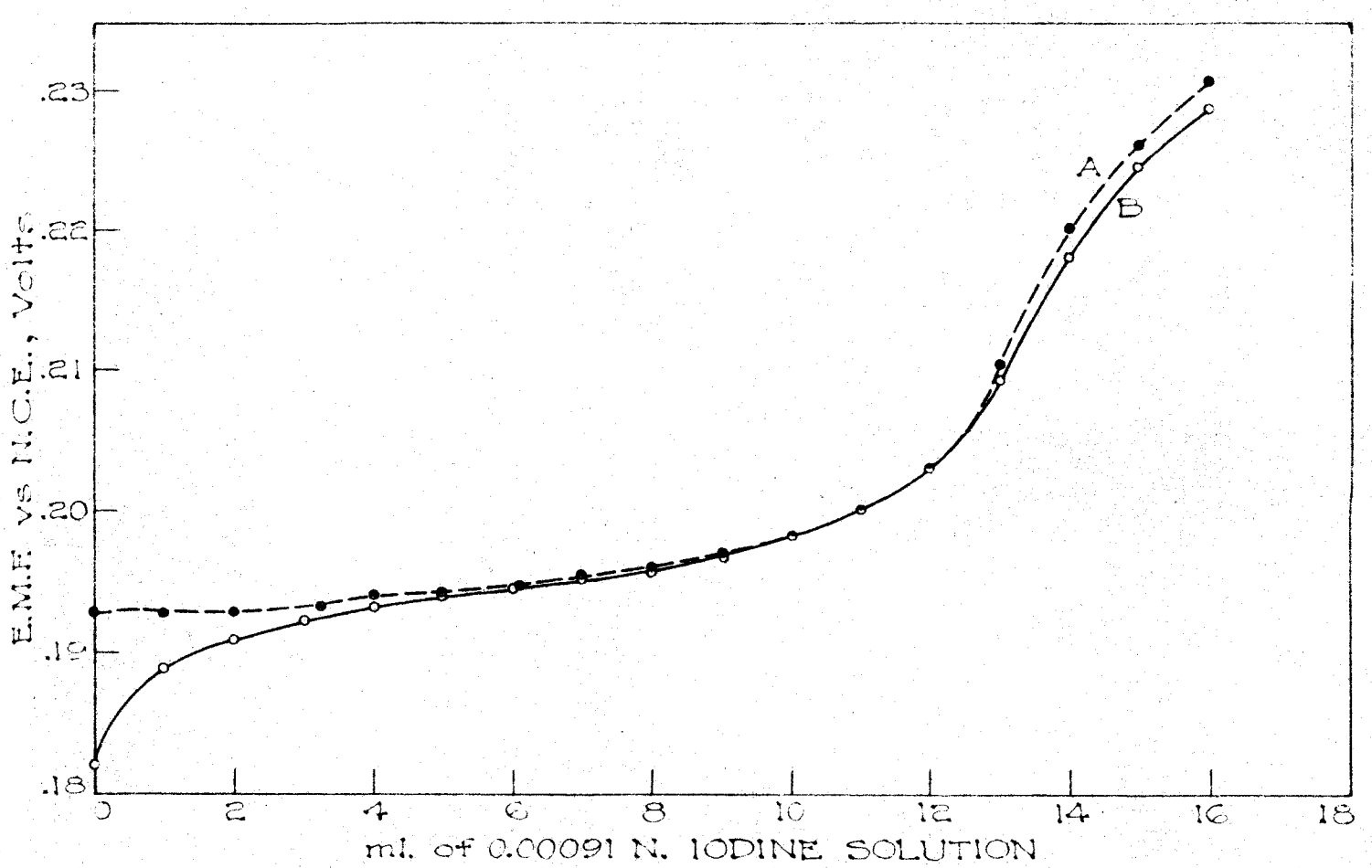

Fig. 9. Effect of Added Chloride on Titration of Amylose

A. 0.05 M potassium iodide plus 0.05 M potassium chloride
B. 0.05 M potassium iodide

Table 12. Titration of Amylose in the Presence of Chloride.

(Iodine added in milliliters of 0.00091 N solution. E.M.F. in volts, referred to the normal calomel electrode.)

Iodine added	E.M.F.	
	0.05 N KI -0.05 N KCl	0.05 N KI
0	0.1929	0.1820
1	.1929	.1890
2	.1929	.1910
3		.1923
3.25	.1935	
4	.1939	.1933
5	.1944	.1932
6	.1948	.1947
7	.1957	.1953
8	.1963	.1959
9	.1972	.1969
10	.1983	.1983
11	.2001	.2002
12	.2030	.2032
13	.2107	.2095
14	.2202	.2182
15	.2263	.2246
16	.2307	.2290

Table 13. Titration of Natural and Fatted Potato Starch

(Iodine added in milliliters of 0.00091 N solution. E.M.F. in volts, referred to the normal calomel electrode.)

Iodine added	E.M.F.	
	0.016% fat	0.37% fat
0	0.1885	0.1797
1	.1937	.1880
2	.1943	.1918
3	.1948	.1940
4	.1952	.1956
5	.1956	.1972
6	.1961	.1988
7	.1968	.2004
8	.1975	.2017
9	.1983	.2040
10	.1991	.2067
11	.2005	.2111
12	.2015	.2152
13	.2032	.2181
14	.2051	.2208
15	.2087	
16	.2142	
17	.2180	
18	.2207	

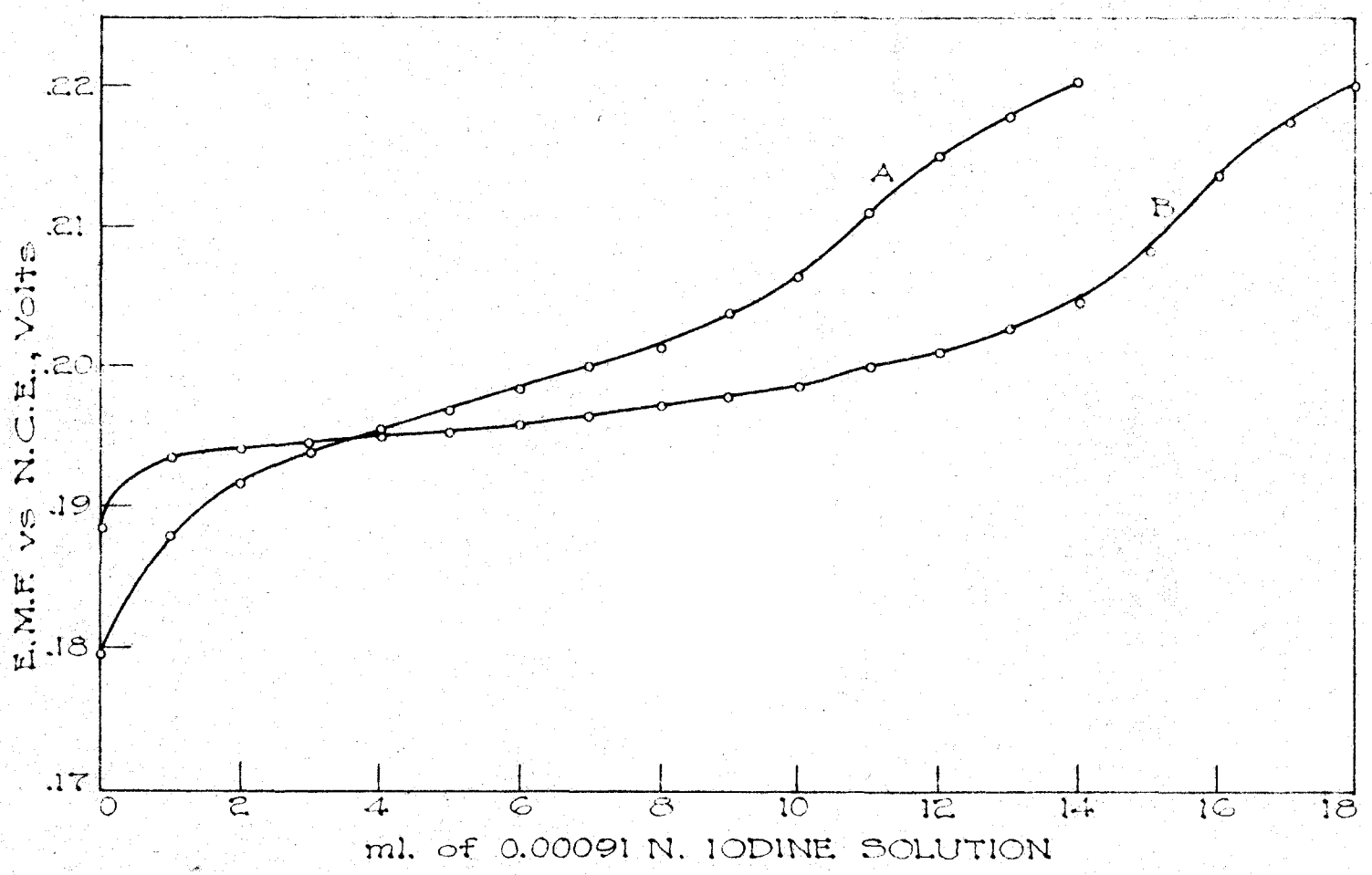

Fig. 10. Effect of Fatty Acid on Titration of Potato Starch

A. 0.37 per cent oleic acid added
B. 0.016 per cent natural fat content

starches before applying the iodine titration.

Little work was done on the study of fat interference other than to show its effect on the iodine titration. Some experiments were carried out that disclosed a number of interesting facts. Potassium oleate was found to be practically as effective as the free oleic acid. The approximate amount of oleate necessary to fill the amylose helices of a solution was calculated. Addition of this amount caused the amylose to lose almost all of its ability to bind iodine. A more soluble soap, potassium caproate, was not nearly as effective as the oleate. Caproate, in amounts thirty times greater than the amount of oleate necessary to prevent iodine complex formation, has only a small effect. Addition of fatty acid to a solution of amylose-iodine complex caused an increase in the activity of free iodine in the solution. This indicated that iodine was released from the complex. The viscosity of an amylose solution containing a small amount of potassium oleate was much less than that of a similar solution containing no oleate. All the above facts point to formation of a complex between starch and the fatty material, with the starch very probably in the helical configuration.

By reference to Figure 10 it will be seen that the curve for the starch containing fat has considerable slope throughout. Nowhere does it approach the horizontal as does the curve for fat-free starch. This was found to be true in the case of corn starch, too. It may be characteristic of

starches containing fat. If such is the case, it can be predicted that pea starch (see Figure 6) has a low fat content. Others, for example bean and arrowroot starches, also appear to be almost fat-free. Since these starches among others are not available in the quantities necessary for defatting and for fat analysis, it will probably be some time before sufficient data will be accumulated to test the above predictions.

4. Rate of complex formation

In setting the potentiometer to measure the potential of the iodine electrode during the course of a titration, it was noted that the potential drifted considerably immediately after addition of iodine solution. Presumably this was due to a measurably slow rate of reaction between the starch and the iodine. The change of potential was fairly rapid immediately after addition of a little iodine, but it quickly tapered off and became practically constant after a few minutes' time. This phenomenon is of considerable importance, since too rapid addition of iodine would result in serious changes in the shapes of the titration curves. Under such conditions the titration method would be of little or no value. Experiments were run that showed intervals of two to five minutes to be sufficient between addition of a milliliter of 0.001 N iodine solution and the reading of the potential. The longer interval is recommended for accurate work. Generally speaking, a drift of potential of more than a few tenths of a millivolt

per minute after five minutes, can be considered a sign of poor dispersion of the starch.

No further work was done on rate of complex formation, but there is a distinct possibility of throwing some light on the mechanism involved by conducting a thorough kinetic study.

C. The Amylose Component

1. Relative chain lengths of different amyloses

When an amylose is titrated with iodine, the iodine enters the complex so readily that the activity of the iodine in solution remains almost unchanged until the amylose is nearly saturated. In such a case, when the potential is plotted against milliliters of iodine solution added, the curve has a long, almost horizontal, portion. It was observed that the potential and therefore the activity of iodine at which this portion of the curve occurred varied with different amyloses. At first this was thought to be due to variation in iodide concentration, but it was soon found that a real difference existed. Reference to Figure 2 will disclose the magnitude of the variations observed. This phenomenon seemed to be caused by differences in the amyloses themselves. Amylopectin impurity apparently had little effect on the potential level of the curves. The amyloses were assumed to be composed entirely of straight-chain molecules. The only possible explanation seemed to be a difference in

molecular size, in other words, chain length. A clue was found in the comparison of the butanol-precipitated fraction from corn starch with Kerr's "crystalline amylose." The average chain length of the latter was considered to be shorter, being prepared from the hot-water-soluble portion of the corn starch. Its potential level always fell above that of corn amylose. The inference was drawn that long amylose chains took up iodine at a lower electrode potential. This meant that the activity of iodine in a complex prepared with long amylose molecules was less than in one prepared with short molecules.

Considering the above assumptions to be true, it seemed possible to arrange the available amyloses in a list according to their relative sizes as determined from the potential levels. By this procedure the amyloses fall in the following order of increasing molecular weights: "crystalline," synthetic, corn, lily bulb, tapioca, and potato. In securing this arrangement the point on the titration curve corresponding to the addition of exactly half the amount of iodine required by the amylose for complex formation was determined. The potential of this point was designated as the "characteristic potential" of the amylose.

Foster and Hixon (41) have investigated the solution viscosities of the various amyloses. Their measurements place the relative molecular weights in the same order as that obtained from the iodine potential measurements. An exception

was found in the case of synthetic amylose whose molecular weight was lower on the scale when determined by the viscosity method. This discrepancy was believed due to considerable heterogeneity of chain length in the synthetic amylose. Lack of homogeneity is indicated by the slope of the titration curve, a phenomenon which will be discussed in another section. It was pointed out that different average molecular weights are obtained by different methods of determination in the case of heterogeneous materials. Tentative values for the absolute molecular weights of the amyloses were listed by the above-mentioned investigators based upon osmometric measurements made on acetylated corn amylose. Their data are reproduced here to facilitate discussion.

Table 14. Molecular Weights and Characteristic Iodine Potentials of the Amyloses

Material	Molecular size (Glucose units)	Characteristic potential, volts
Potato amylose	500	0.197
Tapioca amylose	450	.200
Lily bulb amylose	310	.202
Corn amylose	250	.203
"Crystalline amylose"	175	.205
Synthetic starch	85	.204
Amylodextrin fraction #3	44	.218

Table 14 may be used to estimate the accuracy attainable upon application of the iodine titration to the determination

of molecular size. For molecules above 100 glucose units in length the maximum change in length required to produce a change of one millivolt was 70 units. The instruments used are capable of measuring changes of 0.1 millivolt with great accuracy. Changes of E. M. F. are directly proportional to changes in iodide concentration as shown by Equation VII, so that the error introduced here is dependent on the sensitivity of the method employed in establishing the iodide concentration. Using a volumetric method, errors of about three- or four-tenths of a millivolt may be expected. Actually, differences of this order of magnitude are observed experimentally between duplicate titrations run in quick succession. This of course is the ideal case. Even day to day changes in the platinum electrode surface, the condition of the salt bridge, the potential of the reference electrode, temperature, etc., will introduce much greater errors. In general, it may be said that in establishing the "characteristic potentials" of the amyloses the iodine titration method is sensitive to differences of perhaps 30 to 40 glucose units. The idea of absolute accuracy is practically meaningless when applied to the iodine titration method. The comparative accuracy depends on what advantage is taken of the sensitivity. It appears that the most logical procedure would be to set up potato amylose, the longest one yet encountered, as a standard. Other amyloses could then be compared to the standard under carefully controlled conditions.

2. Low molecular weight amyloses

The increase in iodine activity necessary to bring about complex formation with the shorter, naturally occurring amyloses led to speculation upon the behavior of very short amylosic material. Means were sought for obtaining straight-chain molecules shorter than those present in the natural amyloses. An amylodextrin prepared by hydrolysis in cold sulphuric acid was available, and certain of its properties led to the belief that it contained a great deal of straight-chain molecules. Some of this material was fractionated on the basis of solubility in mixed butanol and methanol, after being subjected to a regular butanol fractionation to remove all the long amylose molecules. The details of the fractionation and the yields as well as the procedures used to characterize the various fractions obtained are contained in the experimental section.

The iodine titration curves of the original amylodextrin and the fractions obtained from it are shown in Figure 11. For convenience in comparing the results, the yields and properties of the fractions are recorded in Table 15. The first fraction obtained by butanol precipitation contained some of the original material that did not go into solution. This insoluble material was probably retrograded amylose. From the yield of this fraction it is plain that little of the dextrin is long enough to be precipitated by the butanol.

Table 15. Properties of the Amylodextrin Fractions

	Fraction				
	1	2	3	4	5
Yield, per cent	2.5	1.6	4.4	13.2	60*
Solubility in hot water	insoluble	insoluble	partly soluble	soluble (cloudy)	soluble
Retrogradation from neutralized alkali dispersion	rapid	rapid	rapid	slow	nil
Specific rotation, $[\alpha]_D^{25^\circ}$					193°
Molecular weight (approx.):					
by R_{Cu}				6400	3200
by iodine reducing value**				9600	4850
by optical rotation					3500
Conversion by β-amylase, %				94	94

*Fraction 5 is the residual material precipitable in 60 per cent methanol. About 15 per cent remains in solution.

**Kline and Acree, Ind. Eng. Chem., Anal. Ed., 2, 413 (1930).

The second and third fractions are obviously straight-chain materials from the amount of iodine they take up. The fourth fraction and the fifth fraction, composed of the residual material, give titration curves somewhat similar to amylopectin curves. However, close inspection of the curve for the fourth fraction reveals a "dip" in the curve with two broad inflections. Both of these last two fractions are essentially straight-chain material as shown by β-amylase conversion. Their average molecular weights were

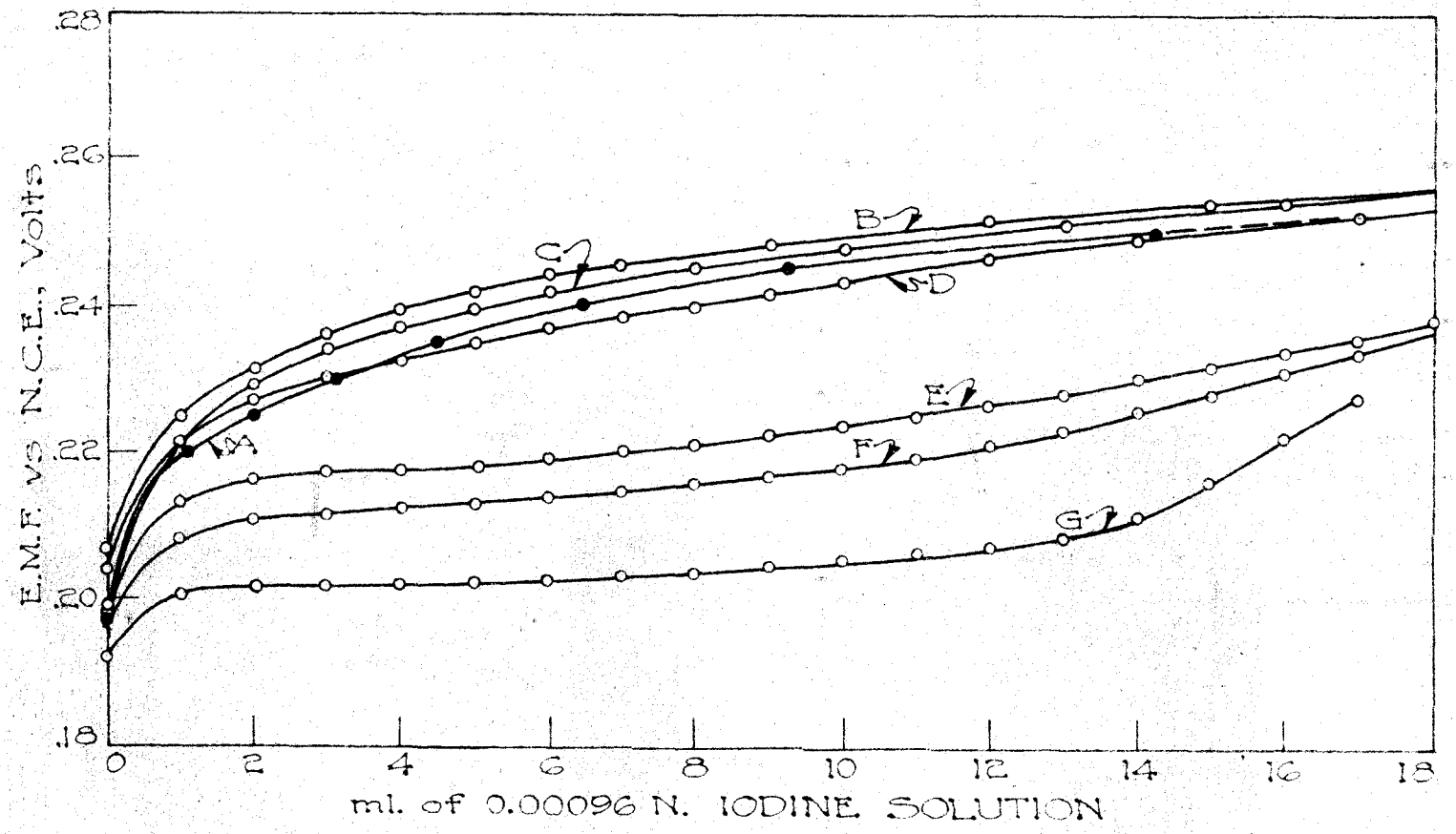

Fig. 11. Corn Amylodextrin and Its Fractions

A. 0.01 gram corn amylodextrin
B. 0.05 N potassium iodide solution (see Table 1)
C. 0.01 gram of fifth fraction (residual material)
D. 0.01 gram of fourth fraction
E. 0.01 gram of third fraction
F. 0.01 gram of second fraction
G. 0.01 gram of "crystalline amylose" (see Table 2)

Table 16. Titration of Corn Amylodextrin and Its Fractions

(Iodine added in milliliters of 0.00096 N solution. E. M. F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.				
	A*	C	D	E	F
0	0.1969	0.1996	0.2040	0.1990	0.1969
1	.2137	.2216	.2218	.2135	.2083
2	.2183	.2293	.2273	.2164	.2108
3	.2210	.2340	.2305	.2172	.2119
4	.2232	.2372	.2327	.2179	.2127
5	.2253	.2398	.2351	.2185	.2134
6	.2274	.2419	.2370	.2193	.2140
7	.2295		.2387	.2203	.2148
8		.2453	.2403	.2213	.2159
9	.2338		.2419	.2226	.2169
10		.2481	.2435	.2239	.2181
11	.2371			.2252	.2196
12			.2465	.2267	.2212
13		.2515		.2285	.2234
14	.2414		.2491	.2302	.2258
15				.2320	.2286
16		.2543		.2340	.2312
17	.2446		.2524	.2360	.2339
18				.2380	
19				.2399	.2394
20		.2574	.2552	.2419	

*Letters refer to the curves of Figure 11.

estimated by ferricyanide and iodine-reducing values and by optical rotation. The residual material, representing the major portion of the original amylodextrin, has a molecular weight of 3200-4800, which corresponds to a chain length of 20-30 glucose units. The fourth fraction has amylose chains averaging about twice this size, or about 50±10 glucose units. The available methods of determining reducing values become more insensitive with increasing molecular weights and this latter fraction is near the limit of their effectiveness. Even though the methods were sufficiently accurate, their use would be limited by the increasing insolubility of the amylose as its molecular weight increases. The iodine method gave somewhat higher values for molecular weight of both the fourth and fifth fractions. This method requires more concentrated solutions, and it is possible that some retrogradation took place during the determination. The second and third fractions both exhibited a very strong tendency to retrograde. When their dispersions in KOH were diluted to about one per cent amylose content and then neutralized with HCl, they began to retrograde almost immediately, and their solutions became opaque in the space of a few minutes. This made it very difficult to work with any except very dilute solutions of these amyloses. The exaggerated tendency toward retrogradation is probably due to the homogeneity of the fractions and the fact that they are short enough to be easily oriented but yet long enough to be insoluble once crystallized. As a consequence,

there is little information on fractions 2 and 3 other than the potentiometric iodine curves. These curves put them on a relative molecular weight scale that is consistent with the order in which they were precipitated and indicate that they are composed substantially of linear molecules.

The set of curves for the titration of the amylodextrin fractions provide an excellent picture of the complex-forming ability of very short amylose molecules. The actual amount of iodine bound by the third fraction whose chain of glucose residues numbers 100 or less is practically as great as that bound by potato amylose with chains of 500 or more glucose residues. However, as the number of glucose residues decreases from 100 to 25, the change in the amount of iodine taken up is tremendous. The iodine color, of course, is red for the short molecules in the fifth fraction. The titration curve of a very short amylose is indistinguishable from that of an amylopectin.

3. Homogeneity

Examination of the iodine titration curves of the amyloses (Figure 2) revealed that most of the lower portion of the curves had some slope and that the truly horizontal section was not very long. This phenomenon, coupled with the relationship between iodine potential and chain length discussed in the previous section, caused speculation on the possibility of correlating the slope of a curve with the homogeneity of

the corresponding amylose. There was only one other explanation that seemed at all probable. It might be that the iodine was being added faster than the complex formation was taking place. This idea was disproved by varying the rate of addition without obtaining a difference in the observed slope.

The problem was solved by mixing amyloses of different average chain lengths, thus producing an artificial material possessing a wider range of molecular weights. Such mixtures always resulted in titration curves having greater slopes than the natural amyloses. When two amyloses of widely different molecular weights were mixed, two horizontal sections separated by a short sloping portion were observed. This is illustrated by the curves of Figure 12. Calculations show that the first end-point in curve B corresponds almost exactly to the amount of iodine necessary for titration of the 0.005 grams of long-chain amylose present in the solution. This means that practically all the long-chain complex must be formed before any appreciable amount of short-chain complex appears in the solution. There is no doubt that in the process of adding the iodine to a solution of the mixture local centers of high concentration were built up momentarily. These were very likely of sufficient iodine activity to result in formation of complex with the short-chain material. This short-chain complex must decompose when the iodine activity is lowered in the solution with which it is in direct contact. This is necessary in order that the iodine be made available for the

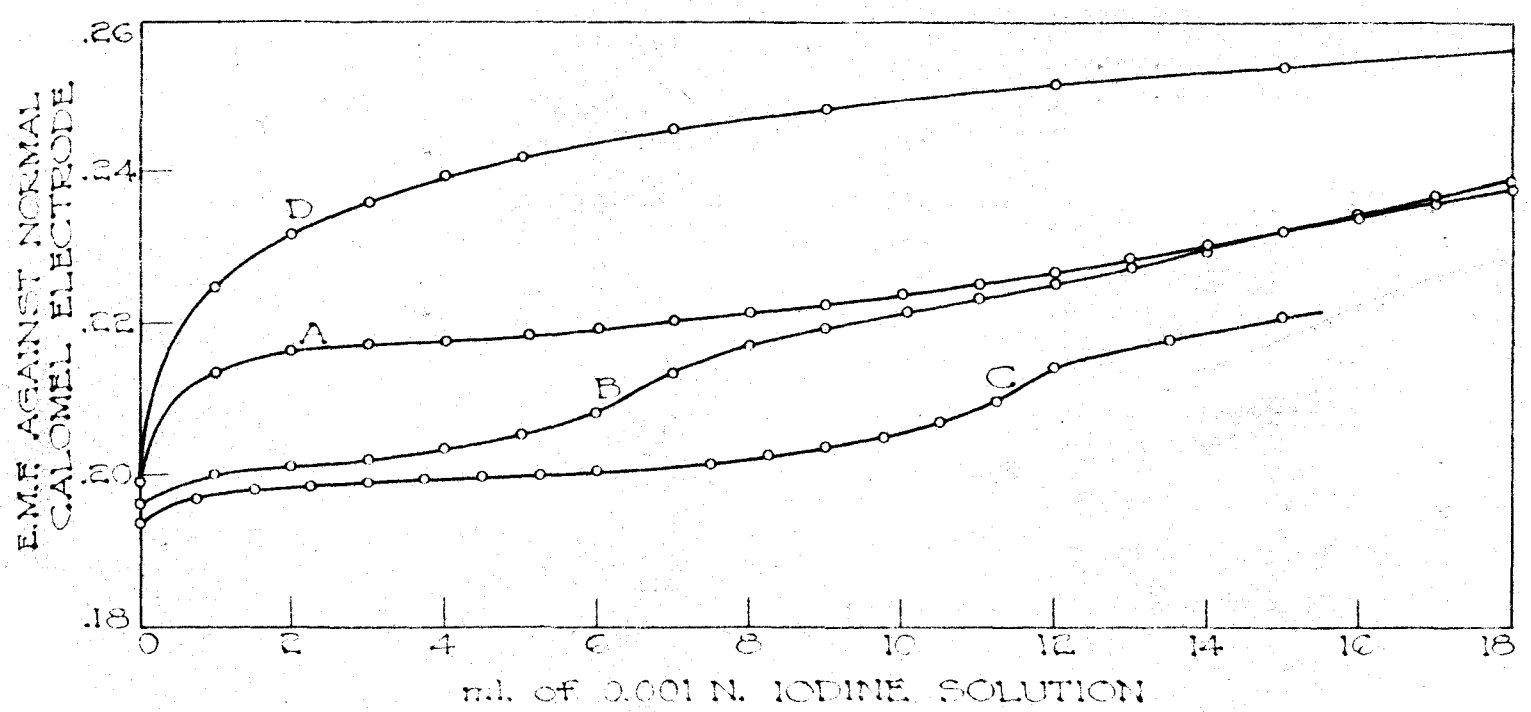

Fig. 12. Mixture of Long- and Short-Chain Amyloses

A. 0.010 grams of amylodextrin fraction 3 (see Table 15)

B. 0.005 grams of amylodextrin fraction 3 plus 0.005 grams of potato amylose

C. 0.010 grams of potato amylose (see Table 2)

D. 0.05 N potassium iodide solution (see Table 1)

Table 17. Titration of a Mixture of Amylodextrin Fraction 3 and Potato Amylose

(Iodine added in milliliters of 0.001 N solution. E. M. F. in volts, referred to the normal calomel electrode.)

Iodine added	E.M.F.	Iodine added	E.M.F.
0	0.1961	12	0.2253
1	.2000	13	.2274
2	.2012	14	.2298
3	.2021	15	.2321
4	.2034	16	.2345
5	.2055	17	.2369
6	.2083	18	.2390
7	.2136	19	.2411
8	.2172	20	.2430
9	.2194	21	.2448
10	.2214	22	.2465
11	.2233	23	.2479

long-chain complex formation. The existence of reversible equilibrium between the iodine in solution and that in the complex seems to be unquestionable.

4. Fractionation of amylose

The selective nature of the complex formation with regard to the size of amylose molecules suggested an interesting method of fractionating any given amylose. Potassium iodide and potassium chloride up to 0.1 N can be added to solutions of amylose without causing precipitation. On the other hand, amylose-iodine complex is quickly coagulated and precipitated by such concentrations of these salts. It seemed that addition of iodine in successive measured portions would result in precipitation of a series of fractions of decreasing molecular weight.

To test the possibility of an iodine complex fractionation a mixture of two amyloses was prepared. Equal weights of corn and tapioca amyloses were used. These were dispersed to form a moderately dilute solution and half the amylose was precipitated by addition of the calculated amount of iodine. This first fraction was separated by centrifuging and the remainder precipitated in like manner. For details see Part IV. The two fractions and the two original amyloses were then titrated with iodine potentiometrically, and the results were compared. They may be seen by referring to Figure 13. The curves show that the fractionation is not as effective as

was desired. The slope of the curves for the two fractions indicate a greater heterogeneity than do those for the original amyloses. However, there was a fractionation, and it is possible that much more favorable conditions could have been chosen. For example, in order to take full advantage of the reversibility of the complex formation, the coagulating electrolyte should not be added until after the complex for each fraction has been formed. This would require that the residual material be purified by dialysis, or by recovery and redispersion, after each successive fraction is precipitated. Such a procedure, though tedious, might well repay the effort, since a knowledge of the distribution function of the chain lengths in an amylose would be of considerable value. The practicability of plotting such a function with data taken off the iodine titration curve was considered. However, it was not found to be feasible because of the uncertain nature of the curve in those regions corresponding to complex formation of the very long and very short molecules of the mixture.

One interesting outcome of the iodine fractionation experiment is found in the "characteristic potentials" of the four curves shown in Figure 13. The curves for both the fractions fall above those of the original materials. If the average molecular weights represented by the "characteristic potentials" are true ones, this situation would not occur. In order to bring about the proper relationship the characteristic point would have to be taken somewhat to the left of the present choice.

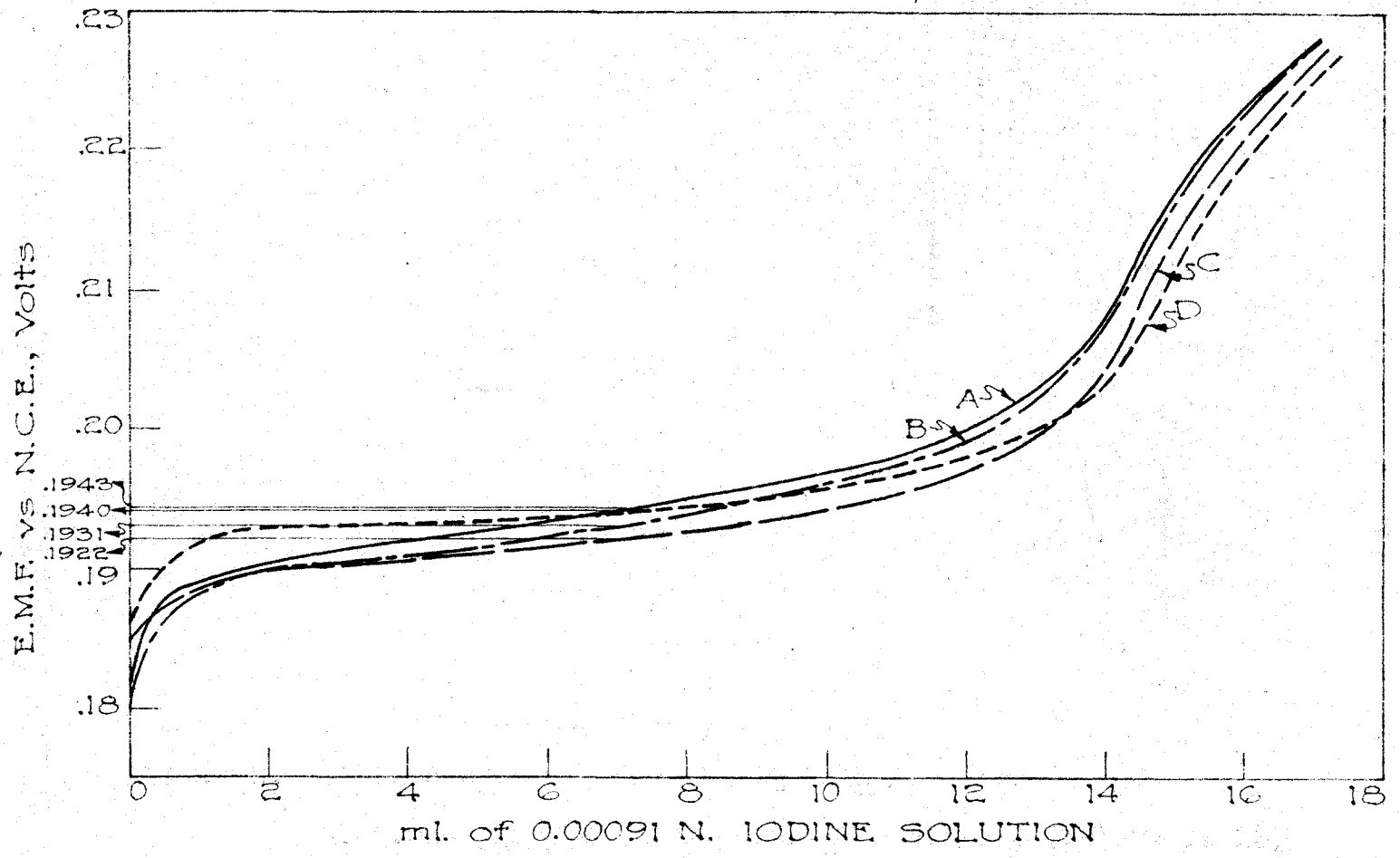

Fig. 13. Fractionation of a Mixture of Amyloses

A. 0.01 gram of fraction 2
B. 0.01 gram of fraction 1
C. 0.01 gram of tapioca amylose
D. 0.01 gram of corn amylose

Table 18. Titration of Amyloses and Fractions

(Iodine added in milliliters of 0.00091 N solution.
E. M. F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.			
	Corn amylose	Tapioca amylose	First fraction	Second fraction
0	0.1850	0.1847	0.1796	0.1813
1	.1917	.1886	.1882	.1890
2	.1927	.1895	.1896	.1902
3	.1930	.1901	.1903	.1913
4	.1931	.1907	.1910	.1922
5	.1934	.1912	.1917	.1930
6	.1936	.1918	.1923	.1936
7	.1940	.1922	.1932	.1942
8	.1944	.1927	.1940	.1950
9	.1950	.1934	.1950	.1959
10	.1958	.1943	.1962	.1971
11	.1968	.1955	.1971	.1983
12	.1982	.1972	.1991	.2002
13	.2003	.1997	.2022	.2028
14	.2036	.2046	.2078	.2078
15	.2115	.2145	.2167	.2171
16	.2196	.2215	.2231	.2234
17	.2252	.2266	.2276	.2278

5. Effect of amylose concentration on complex formation

When amylose solutions of various concentrations are titrated with iodine, it is found that the horizontal portion of the curve occurs at a higher iodine activity with lower amylose contents. See Figure 14. This effect was first noticed in mixtures of amylose and amylopectin and was thought to be due to the presence of the latter component, but further experiment showed this idea to be false. It should be noted in Figure 14 that the experimental points fall closer together on the curves for the solutions of lower amylose concentration. Since the same interval of time was allowed between each successive addition of iodine and the taking of the potentiometer reading, the complex was formed at a rate proportional to the total amount of available amylose present at each concentration. This change of rate was used to eliminate any influence that rate of formation might have on the curves. The general phenomenon is analogous to the mass effect in a chemical reaction. Thus one might write the equation, Amylose + $xI_2 \rightleftharpoons$ Amylose $(I_2)_x$, and imagine that increase in concentration of amylose would force the reaction to the right, thus reducing the iodine concentration. The equation, of course, represents a physical equilibrium rather than a chemical one, although in some respects the complex formation takes on the aspects of a chemical union. If, as seems to be true, there is considerable interaction

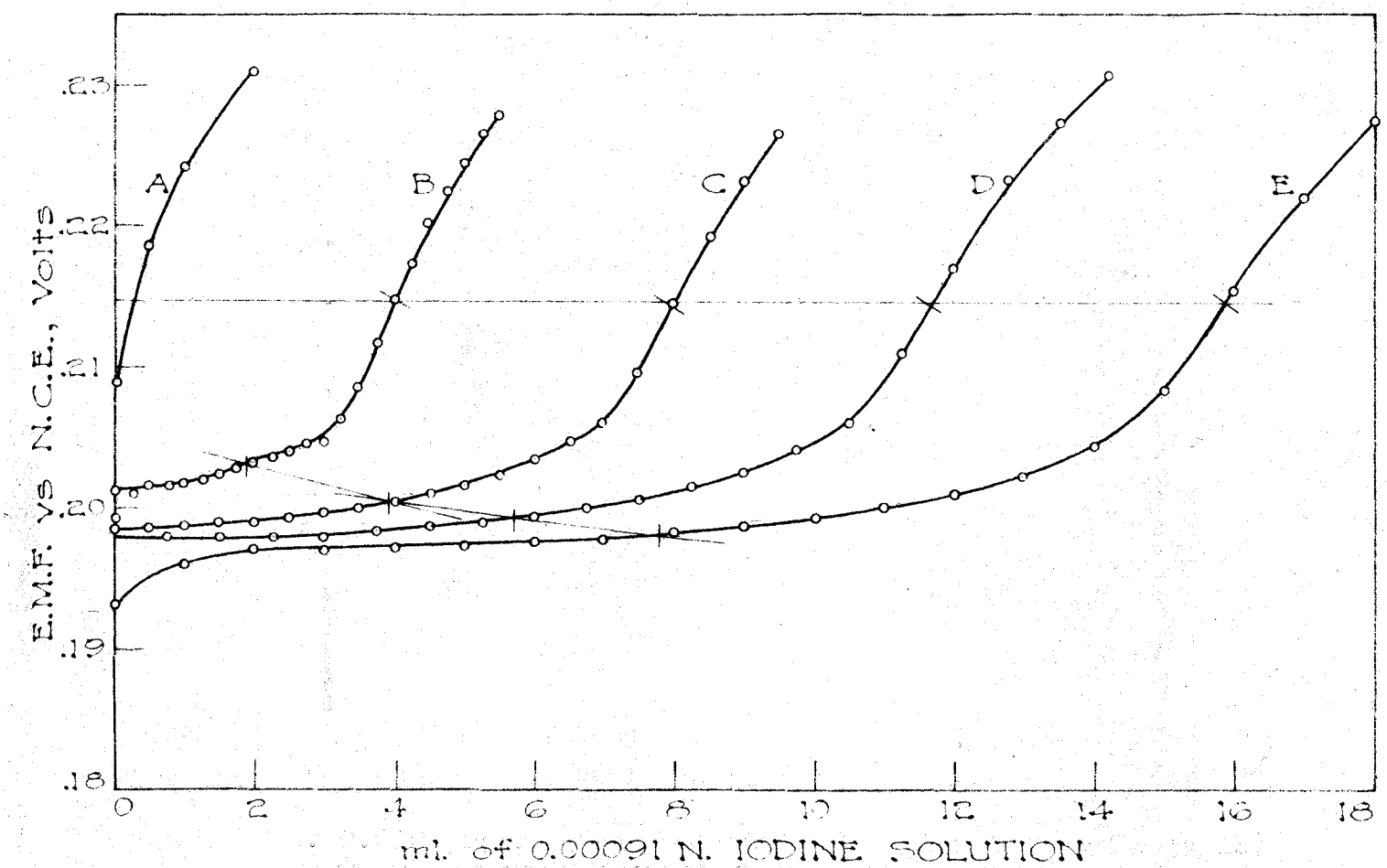

Fig. 14. Effect of Amylose Concentration on the Characteristic Potential

A. 0.0000 per cent solution of "crystalline amylose"
B. 0.0025 per cent solution of "crystalline amylose"
C. 0.0050 per cent solution of "crystalline amylose"
D. 0.0075 per cent solution of "crystalline amylose"
E. 0.0100 per cent solution of "crystalline amylose"

Table 19. Titration of Amylose Solutions of Various Concentrations

(Iodine added in milliliters of 0.00091 N solution. E. M. F. in volts, referred to the normal calomel electrode.)

Iodine added	E.M.F.	
	0.0025%	0.0075%
0	0.2012	0.1993
.25	.2010	
.50	.2015	
.75		.1980
1.00	.2017	
1.25	.2019	
1.50	.2025	.1979
1.75	.2027	
2.00	.2033	
2.25	.2038	.1980
2.50	.2040	
2.75		
3.00	.2046	.1980
3.25	.2064	
3.50	.2088	
3.75	.2118	.1984
4.00	.2146	
4.25	.2174	
4.50	.2203	.1987
4.75	.2225	
5.00	.2244	
5.25		.1991
5.50	.2280	
6.00		.1994
6.75		.2000
7.50		.2006
8.25		.2015
9.00		.2025
9.75		.2038
10.50		.2060
11.25		.2110
12.00		.2170
12.75		.2229
13.50		.2273
14.25		.2308

Iodine added	E.M.F.		
	0.0100%	0.0050%	0.0000%
0	0.1931	0.1986	0.2089
.5		.1984	.2187
1.0	.1961	.1988	.2244
1.5		.1989	
2.0	.1969	.1991	.2311
2.5		.1993	
3.0	.1969	.1997	
3.5		.2001	
4.0	.1971	.2004	
4.5		.2011	
5.0	.1975	.2016	
5.5		.2023	
6.0	.1976	.2034	
6.5		.2045	
7.0	.1978	.2060	
7.5		.2097	
8.0	.1983	.2145	
8.5		.2193	
9.0	.1987	.2231	
9.5		.2265	
10	.1993		
11	.2002		
12	.2011		
13	.2023		
14	.2044		
15	.2085		
16	.2155		
17	.2221		
18	.2275		

between iodine molecules within the complex, its formation or decomposition may involve the making or disruption of attractive forces between the iodine atoms that are strong enough to be classified as bonds. At any rate, too little is known of the nature of the complex to attempt to set up an equation representing the equilibrium, even if it were firmly established that such an equilibrium existed.

The chief conclusion to be drawn from these findings in a practical way is that similar amylose concentrations must be used if the titration curves are to be made the basis of chain length comparison.

D. The Amylopectin Component

1. Iodine complex with amylopectin

It was shown that amylopectins bind some iodine although the amounts are small compared to those taken up by amyloses. When the amylopectin titration curves are plotted as they are in Figure 1 it is difficult to draw any conclusions concerning the amounts of iodine they take up or the range of iodine activities within which they bind the iodine. However, the potential of the iodine electrode is a linear function of the log of the iodine activity. Therefore, the titration curve of a solution of potassium iodide can be put in the form of a straight line by plotting the log of the milliliters of iodine solution against the potential. Since the iodine activity

will not be exactly proportional to the milliliters of iodine solution added, this linearity will not hold strictly. Figure 15 shows the curves for 0.05 N potassium iodide solution and for a number of amylopectins and waxy starches. Bound iodine now shows up as a deviation from a straight line and the amount is more easily noted. More important, however, the potential at which the maximum deviation occurs can be seen, as well as the breadth of the maximum. This enables a much better analysis of the behavior of amylopectin with iodine. This is rather important if the iodine titration is to be used as a method for determining the amylose contents of whole starches, since the majority, about 75 per cent, of such starches is amylopectin. There is little reason to doubt that some of the iodine bound by amylopectin is held by much the same mechanism as in the case of amylose. Most of the amylopectins probably have some lengths of free chain, particularly in the terminal branches, and it is generally the case that substances that give indications of longer average free chain length by other methods also bind more iodine. On the other hand, there are signs that point to a second mechanism for binding iodine in the case of amylopectins. Thus, glycogen with practically no straight chain capable of a single loop still binds some iodine. More striking, however, is the comparison of an amylodextrin of average chain length of about 25 glucose residues with any amylopectin having an average free chain of 25 or less. In

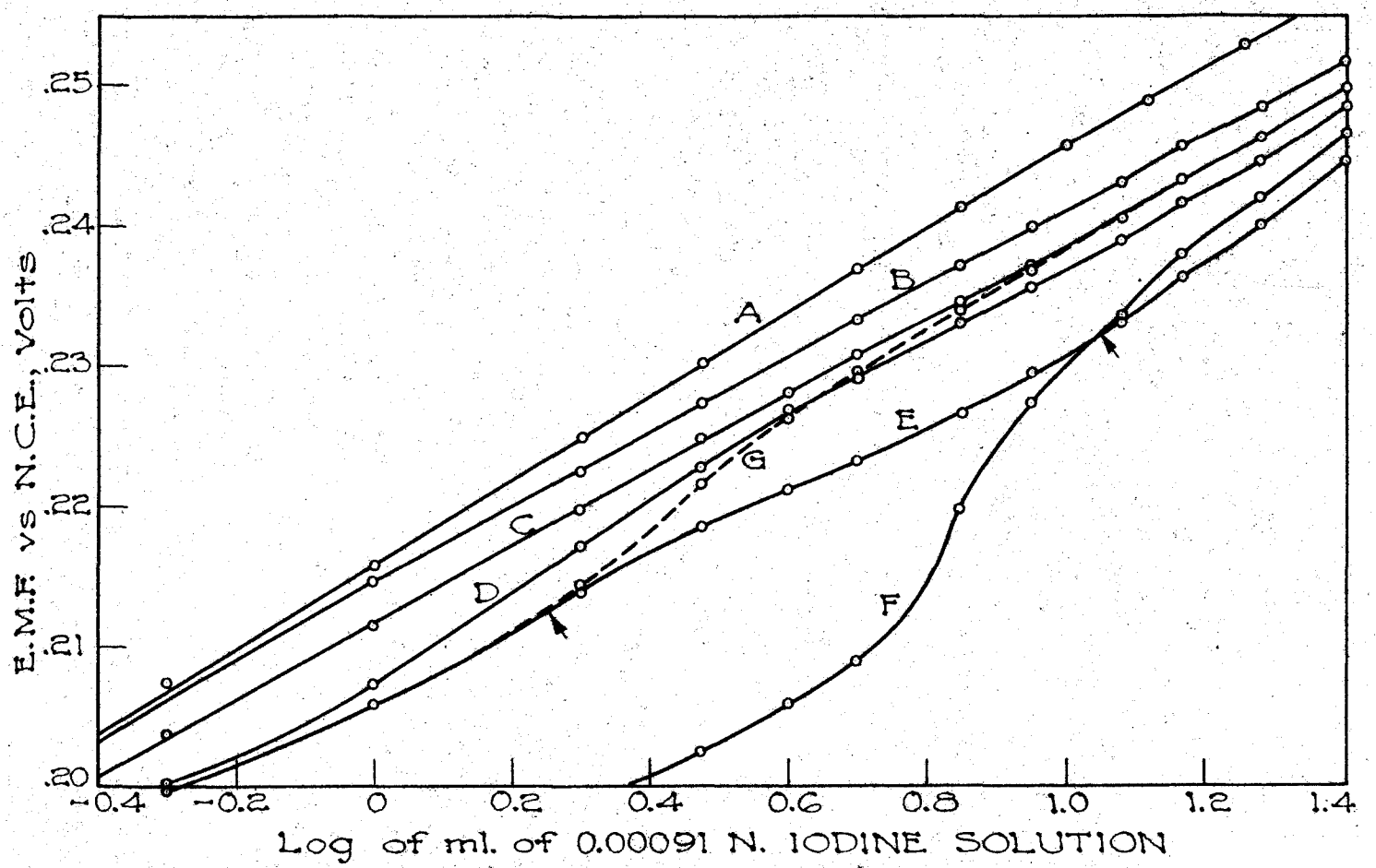

Fig. 15. Amylopectins (see Table 1 for data)

A. 0.05 N potassium iodide solution
B. 0.04 grams waxy rice starch
C. 0.04 grams waxy corn starch
D. 0.04 grams tapioca amylopectin
E. 0.04 grams potato amylopectin
F. 0.04 grams corn amylopectin
G. 0.04 grams waxy corn starch, defatted.

spite of interference to be expected from interlacing and entangling of the branches the amylopectin binds more iodine than does the amylodextrin. This could be due to a number of particularly long terminal branches on the amylopectin, since relatively few of these would increase the iodine-binding ability considerably. However, if such were the case, there would be quite a marked difference between the amount of iodine taken up by an amylopectin and that bound by its limit dextrin. These materials have been compared in an experiment that will be described presently, and little difference was observed. It is difficult to believe that there can be any great difference in degree of branching within the limit dextrin. It seems probable that some iodine is adsorbed by the large colloidal particles of the amylopectin. The curves of Figure 15 show that iodine is bound by amylopectin materials in a slowly but continuously increasing amount as the iodine concentration is increased. It will be noted, however, that all the curves turn up toward Curve A at their upper ends. A number of the curves were carried out to much greater iodine concentrations and it was found that beyond the range shown in the figure they were practically linear and tended to approach Curve A as an asymptote. This produces in each curve at least one point of maximum deviation from the "blank," i.e., Curve A. This maximum represents a definite change in the proportion of added iodine that is bound by the amylopectin. Furthermore, some of the curves exhibit two of these

maxima, indicating a subordinate variability in the proportion of added to bound iodine.

The various maxima observed in the amylopectin curves may be explained very nicely by an extension of the relationship between chain length and iodine activity or potential. Curve E which was obtained by titration of potato amylopectin may be taken as an example. It has two maxima indicated by the two small arrowheads. The lowest one is produced by a trace of amylose. That this maximum occurs in the potential range of amylose may be seen by reference to Curve F, produced by crude corn amylopectin. The large maximum in this curve was caused by the presence of 6 to 8 per cent amylose. The second maximum in Curve E presumably is caused by complex formation with the amylopectin itself and the potential at which it occurs is probably related to the average chain length of the amylopectin. On this basis it may be predicted that potato amylopectin has the longest average chain length and that the degree of branching in the amylopectins increases in the order: corn, tapioca, waxy corn, and waxy rice.

Comparison of Curves C and G produced by waxy corn before and after defatting shows that the presence of small amounts of amylose may be effectively hidden by fat. It is probable that other waxy starches which are apparently amylose-free, actually contain small amounts of this component, as do cotton-purified amylopectin preparations. These traces of iodine-binding material are really amylose and not merely

extra long branches, otherwise there would not be two distinct maxima; only a single broad one would be observable.

2. Limit dextrins

Evidence was sought to confirm the relationship between the average chain length of amylopectins and the potential of maximum deviation of the iodine titration curve, as described in the preceding section. Meyer's plan for the branching of amylopectin (6, 7) postulates terminal branches containing about twice as many glucose units as are in the segments of chain between branch-points. Limit dextrins prepared from amylopectins by β-amylase digestion would accordingly have shorter average chain lengths.

The limit dextrins of waxy rice, waxy corn, tapioca amylopectin, and potato amylopectin, together with samples of the original materials were titrated with iodine. The results are contained in Figure 16. For convenience in comparing the materials, the potentials of maximum deviation have been placed together in Table 20. They are indicated in the figure by small arrow-heads. In every case the limit dextrin potential is higher than the potential of the corresponding amylopectin, as was expected. The four limit dextrins fall in the same relative order as do the four amylopectins. The differences between the potentials of the related pairs are all of the same order of magnitude. In general, the difference between an amylopectin and its limit dextrin is less

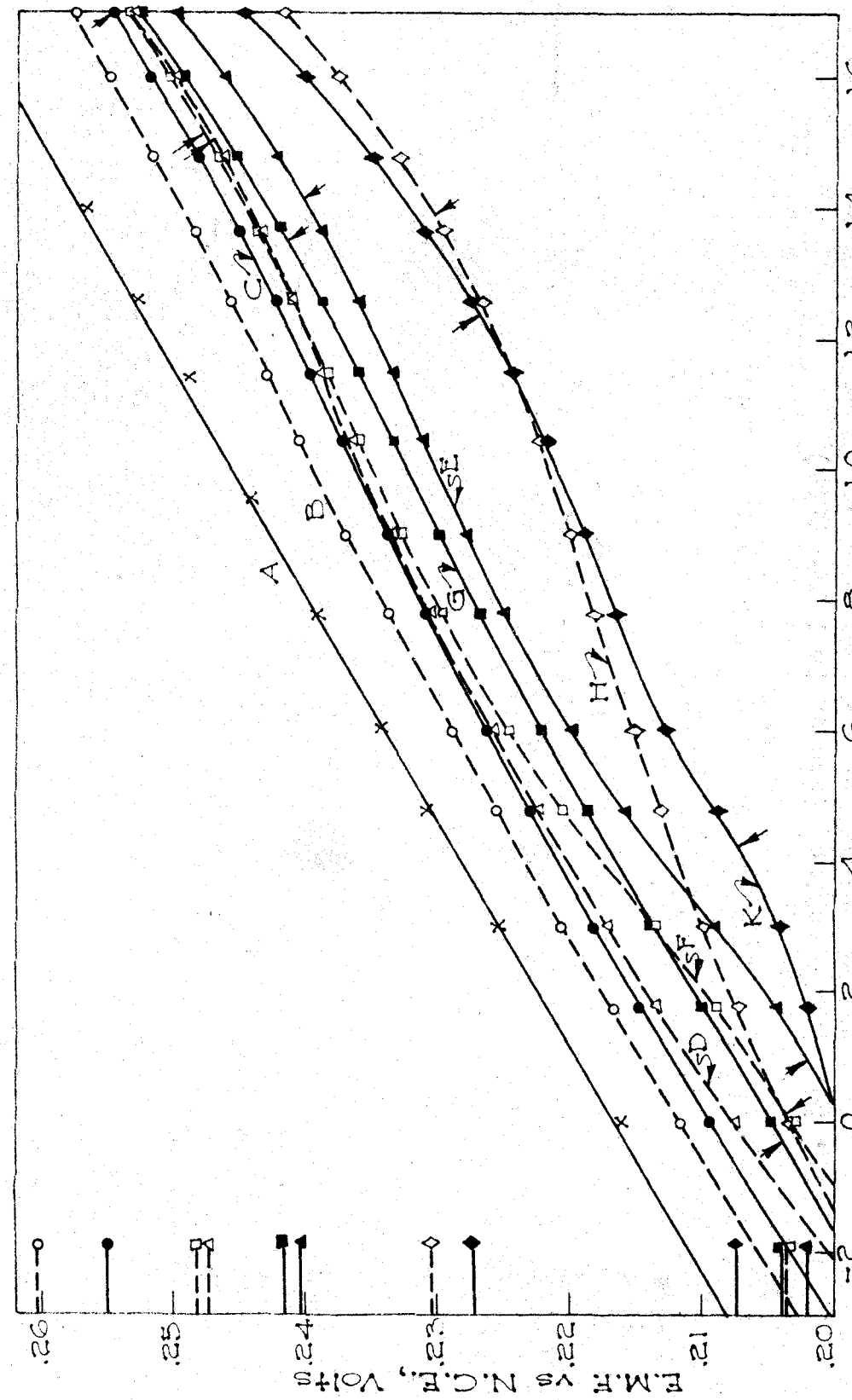

Fig. 16. Amylopectins and Their Limit Dextrins

A. 0.05 M potassium iodide solution

Amylopectins, 0.04 gram: Limit dextrins, 0.04 gram:

B. Waxy rice starch D. Tapioca C. Waxy rice E. Tapioca

F. Waxy corn starch H. Potato G. Waxy corn K. Potato

Table 20. Iodine Potentials of Amylopectins and Their Limit Dextrins

(In volts, referred to the normal calomel electrode)

Material	Potential		
	Amylopectin	Limit dextrin	Difference
Waxy rice	0.2553	0.2600	0.0047
Waxy corn	0.2418	0.2482	0.0064
Tapioca	0.2406	0.2477	0.0071
Potato	0.2272	0.2307	0.0035

than the difference between neighboring amylopectins. Obviously the determination of the iodine potentials involved is so uncertain that no quantitative estimates of chain lengths are possible. However, some idea of the probable values may be gained from the titration curve of an amylodextrin fraction with an average chain length of 20-25 glucose residues. The potential at which it deviated most from the 0.05 N potassium iodide curve was in the neighborhood of 0.2400 volts. Contributing to the uncertainty is the doubt which exists regarding the possibility of iodine adsorption by the amylopectins.

E. Other Starch Complexes

The material that is precipitated in the course of Schoch's starch fractionation procedure (11) is probably an amylose-butanol complex analogous in structure to the amylose-iodine complex (27). Apparently it is the superior

Table 21. Titration of Amylopectins and Their Limit Dextrins

(Iodine added in milliliters of 0.00091 N solution.
E. M. F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.				
	Waxy rice	Tapioca	Waxy corn	Potato	0.05 N KI sol.
			Amylopectins		
.25	0.1902	0.1811	0.1896	0.1869	
.50	.2000	.1907	.1966	.1928	
1.0	.2093	.1993	.2048	.1995	0.2166
1.5	.2148	.2043	.2101	.2022	
2	.2184	.2091	.2138	.2043	.2255
3	.2232	.2158	.2189	.2091	
4	.2266	.2201	.2225	.2130	.2345
6	.2312	.2252	.2372	.2168	.2396
8	.2343	.2282	.2304	.2193	
9					.2447
11	.2376	.2315	.2339	.2224	
13					.2493
14	.2401	.2338	.2365	.2248	
18	.2427	.2365	.2394	.2280	.2532
23	.2455	.2393	.2423	.2314	
25					.2571
30	.2487	.2427	.2458	.2356	
40	.2524	.2469	.2498	.2408	
50	.2553	.2504	.2531	.2454	
			Limit Dextrins		
.25	.1934	.1826	.1811	.1865	
.50	.2030	.1961	.1930	.1955	
1.0	.2117	.2072	.2032	.2035	
1.5	.2169	.2133	.2090	.2071	
2	.2206	.2171	.2141	.2093	
3	.2257	.2227	.2206	.2133	
4	.2292	.2263	.2248	.2155	
6	.2340	.2309	.2299	.2184	
8	.2373	.2339	.2331	.2203	
11	.2408	.2369	.2365	.2223	
14	.2434	.2392	.2390	.2248	
18	.2462	.2416	.2415	.2273	
23	.2489	.2442	.2442	.2300	
30	.2520	.2469	.2473	.2335	
40	.2554	.2505	.2510	.2381	
50	.2582	.2536	.2542	.2422	

crystallizing tendency of the butanol-filled helices that results in the formation of the characteristic "rosettes" and the subsequent separation of the two components.

1. The pyridine complex

Much thought and considerable effort was given to the problem of analysis of the butanol complex, but with little success. The possibility of preparing a similar complex with a substance that would lend itself more readily to analysis was considered. Reschke and Hartman (42) describe a crystalline pyridine-amylose complex which was formed in an aqueous pyridine solution of potato starch. Their experiment was duplicated and a material was obtained that closely resembled the potato amylose-butanol complex, being composed of double tufts and clusters of very fine needles. These were stained blue by iodine, but no dichroism could be detected. The complex did not lend itself to an efficient fractionation. In the mass it was so voluminous and jelly-like that it could not be separated cleanly from the liquid phase either by filtration or centrifugation.

2. The phenol and aniline complexes

By analogy with pyridine and butanol the next most likely substances for complex formation were aniline and phenol. Accordingly, they were used in attempted fractionation of

defatted corn starch. The procedure was similar to that used in Schoch's butanol fractionation; a two per cent starch paste was autoclaved, saturated with the precipitant and allowed to cool slowly. The details of procedure are described fully in Part IV.

The results of the phenol and aniline fractionations were similar to those obtained with butanol. Solid precipitates were formed that could be centrifuged out. These were washed and dehydrated with ethanol to rid them of excess phenol or aniline, and were then dried. The unprecipitated material was recovered as usual by adding excess methanol.

The effectiveness of the fractionation in each case was determined by running potentiometric iodine titrations on the fractions to determine their amylose contents. The titration curves are shown in Figure 17. The phenol effects a fractionation that is practically as good as that produced by butanol. The amylose obtained is 80 per cent pure and the amylopectin fraction contains about six per cent of amylose. The butanol-precipitated amylose is only slightly purer. The aniline fractionation is not as effective. The amylose produced is only 60 per cent pure. It is probable that the fractionation procedures could be improved in the case of phenol and aniline. There is no reason to believe that the conditions used in the butanol fractionation are also desirable when other precipitants are employed. This is particularly true in the case of aniline whose solubility is only half that of butanol and phenol.

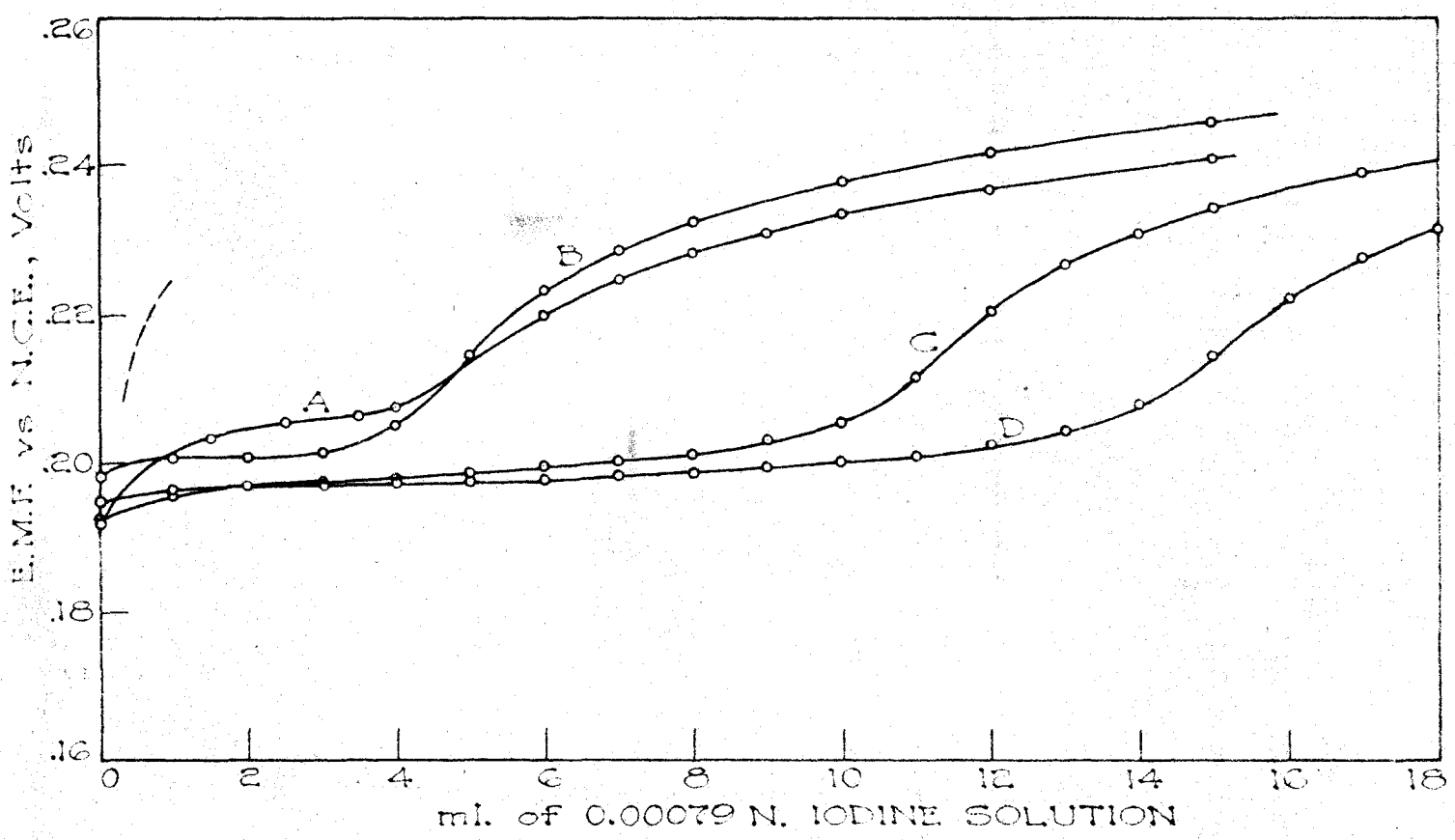

Fig. 17. Results of Phenol, Aniline and p-Aminophenol Fractionations

A. 0.04 gram phenol unprecipitated fraction
B. 0.01 gram p-aminophenol precipitated fraction
C. 0.01 gram aniline precipitated fraction
D. 0.01 gram phenol precipitated fraction

Table 22. Titration of Phenol, Aniline, and p-Aminophenol Fractions

(Iodine added in milliliters of 0.00079 N solution. E. M. F. in volts, referred to the normal calomel electrode.)

Iodine added	E. M. F.			
	A*	B	C	D
0	0.1917	0.1983	0.1922	0.1948
1	.2016	.2010	.1957	.1965
1.5	.2035			
2	.2045	.2008	.1968	.1970
2.5	.2054			
3		.2016	.1974	.1974
3.5	.2062			
4	.2076	.2052	.1980	.1975
4.5	.2098			
5	.2135	.2145	.1986	.1976
6	.2201	.2232	.1993	.1980
7	.2249	.2287	.2001	.1984
8	.2285	.2324	.2011	.1987
9	.2312		.2029	.1993
10	.2334	.2379	.2053	.2002
11			.2115	.2010
12	.2369	.2416	.2205	.2024
13			.2267	.2044
14			.2308	.2075
15	.2406	.2459	.2342	.2143
16				.2220
17			.2391	.2275
18				.2317
20			.2442	.2374

*Letters refer to the curves of Figure 17.

The appearances of the phenol and aniline complexes were quite similar except that the phenol complex particles were a little larger. The particles looked like very fat ellipsoids, being almost spherical in some cases. They generally had a faint band or belt around the smaller diameter. Some partially formed particles appeared to be made up of sheaves of needles bound tightly at the middle. The two ends of the sheaf seemed to branch out and become very "bushy" so that each one tended to assume a hemispherical aspect and the sheaf as a whole became almost spherical. Particles of the phenol complex are shown in Figure 18. They are stained with iodine which accentuates the appearance of the tufts of needles. A particularly good example of a particle that nearly reached a spherical shape is encircled in the figure. The particles that closely approached a spherical shape produced perfect polarization crosses, resembling tiny starch granules in this respect. The sign of the birefringence is positive as in the case of the starch granule.

An attempt was made to use p-aminophenol as an amylose precipitant without success. It is not very soluble and hot aqueous solutions are oxidized quite rapidly by air. A small amount of precipitate was obtained, but it contained only 25 per cent amylose and was dark colored because of contamination with oxidation products of the p-aminophenol.

There are no doubt other compounds that will form complexes with amylose and produce effective separation of the starch components.

Fig. 18. Phenol-Amylose Complex
Stained with Iodine
(400x)

IV. EXPERIMENTAL DETAILS

A. Materials Used

1. Starches

The corn, waxy corn, and waxy rice starches as well as one sample of lily bulb starch were milled under the direction of R. M. Hixon. The lily bulbs and another sample of lily bulb starch were provided by S. L. Emsweller of the U. S. D. A. Experiment Station, Beltsville, Maryland. Baker and Adamson's potato starch was obtained from the General Chemical Company. The bean, pea, oat, and arrowroot starches were from the Eli Lilly and Company assortment of "Authentic Starches." A sample of oat starch was also supplied by the Quaker Oats Company.

2. Starch fractions

Fractions of corn, potato, and tapioca starches prepared by the n-butanol separation procedure (11) were supplied by T. J. Schoch.

The butanol fractions of lily bulb starch were prepared by following Schoch's procedure.

The amylopectin fractions of corn and tapioca starch contained a few per cent of amylose. This was removed by

treatment with cotton (15, 16). The amylose fractions were about 85-90 per cent pure.

Samples of "crystalline amylose" were supplied by R. W. Kerr who has described the method of preparation (43). One of these samples had been recrystallized three times and was apparently pure amylose.

3. Recrystallized corn amylose

Crude corn amylose, freshly precipitated with n-butanol, was recrystallized six times as follows: The wet amylose was redispersed in boiling water and a small amount of insoluble material was removed by hot filtration. The hot solution was then saturated with butanol, wrapped in towels, and allowed to cool slowly. The precipitate was centrifuged out, redispersed and the cycle repeated. After the final crystallization the material was dehydrated with butanol and dried in a vacuum oven.

4. Synthetic starch

Synthetic starch prepared by the action of phosphorylase on glucose-1-phosphate was supplied by W. Z. Hassid. From methylation studies he concluded that it was essentially long, straight-chain starch (44).

5. Defatted corn starch

Corn starches containing various amounts of fat were

prepared by J. F. Foster and F. F. Mikus by extracting with 80 per cent dioxane or refluxing with methanol for different lengths of time.

6. Fatted potato starch

Potato starch containing only 0.016 per cent fat was impregnated with oleic acid according to the procedure described by Schoch (40). After treatment the starch contained 0.37 per cent fat. Schoch's preparation had a 0.77 per cent fat content.

7. Glycogen

The glycogen used was the C. P. grade produced by Pfanstiehl Chemical Company.

8. Limit dextrins

Limit dextrins of waxy rice starch, waxy corn starch, tapioca amylopectin, and potato amylopectin were prepared by B. Brimhall. The materials were digested twice with β-amylase.

9. Amylodextrin fractions

Fractionation. The corn amylodextrin used was prepared by D. French by a procedure involving three months' hydrolysis of granular starch in 10 per cent sulphuric acid with subsequent purification.

Fifty grams of the amylodextrin was dissolved in boiling water to make a liter of opalescent solution. About 100

milliliters of n-butanol and 40 milliliters of iso-amyl alcohol were stirred in and the flask containing the hot solution was wrapped in towels and allowed to cool slowly to room temperature. The precipitate was centrifuged out, washed with a saturated solution of butanol, dehydrated with butanol, and finally dried in a vacuum oven at 60°C. For yields of this and subsequent fractions, see Table 15.

A second fraction was obtained by adding 100 milliliters of methanol to the supernatant liquid from the butanol precipitation. Additional butanol was added to saturate the solution and it was carried through the same sequence of operations as described above for the butanol precipitation.

The third fraction was prepared by following the same procedure after another addition of 100 milliliters of methanol. In this case the saturation with butanol must be carried out in the cold solution, since the butanol is perfectly miscible with aqueous methanol solution of this strength at elevated temperatures.

To obtain the fourth fraction another 200 milliliters of methanol was added, but no more butanol. Again the same procedure was applied in the precipitation.

The supernatant liquid from the fourth fraction was evaporated to half its original volume and enough methanol was added to bring the percentage up to about 60 per cent. Most of the residual material was precipitated and designated as the fifth fraction. Approximately 15 per cent of the

original material remained dissolved in the 60 per cent methanol solution.

Characterization of the fractions. Since the object of the amylodextrin fractionation was the preparation of short-chain amyloses, it was necessary to show that the products were of this nature. Fractions one, two, and three were obviously almost pure amyloses since they took up nearly as much iodine as "crystalline amylose" according to the potentiometric iodine titration. They also gave deep blue iodine colors, were insoluble in boiling water, and retrograded readily when their solutions in alkali were neutralized.

It was necessary to use other criteria in establishing the nature of the fourth and fifth fractions. In their behavior toward iodine they were practically indistinguishable from amylopectins, their colors being purple and purplish red, respectively. Both were soluble in hot water. That they were essentially straight-chain material was demonstrated by subjecting them to the action of β-amylase. Ninety-four per cent conversion to maltose was obtained in each case. The β-amylase digestions were run by the procedure of Newton, Farley and Naylor (45). The extent of conversion was measured in terms of the reducing power of the material as determined by the ferricyanide method of Farley and Hixon (46). Expressed in terms of copper numbers, R_{Cu}, the reducing powers of the fourth fraction before and after digestion were 93 and 1790; of the fifth fraction, 161 and 1790. The first value in each

case may be used to calculate the chain length of the fraction, assuming straight chains. Accordingly, the length of the fourth fraction is about 40 glucose units; of the fifth, 20 glucose units. Solutions of the fifth fraction are clear enough for measurement of the optical rotation and $[\alpha]_D^{25^\circ} = 193^\circ$. This corresponds to a chain length of about 23 glucose units.

The iodine-reducing values of the fourth and fifth fractions were also determined, using the procedure of Kline and Acree (47). According to this method the chain lengths were 60±3 and 32±2 glucose units, respectively. There is not much basis for choice between the molecular weights as determined by this method and those calculated from R_{Cu} values, except that the latter is confirmed by the rotatory power in the case of the fifth fraction.

10. Starch complexes

Pyridine complex. Amylose-pyridine complex was prepared just as described by Reschke and Hartmenn (42), except that the use of exceptionally dry starch was found to be unnecessary.

Phenol complex. A paste composed of five grams of defatted corn starch in 250 milliliters of water was autoclaved for four hours at 15 pounds pressure. Thirty grams of phenol was then added to the hot solution. This amount was somewhat more than enough for saturation. The flask containing the solution was wrapped in towels and allowed to cool slowly.

The resulting precipitate was centrifuged out, washed with alcohol to free it of phenol and water, and then dried *in vacuo*. For microscopic examination the complex was taken as it was first precipitated. It was suspended in saturated phenol-water and only enough alcohol was added to dissolve globules of excess phenol.

The unprecipitated material was recovered by adding an equal volume of methanol to the supernatant liquid according to the usual procedure for precipitating amylopectin.

Aniline complex. The procedure followed in preparing the aniline complex was similar to that employed in the case of phenol except that only 15 grams of the aniline were used, the solubility being lower.

p-Aminophenol complex. The futile attempt to prepare the p-aminophenol complex followed the same procedure as the phenol preparation. In this case the solubility required that only six grams of the precipitant be added. Even freshly purified p-aminophenol colored rapidly when the solution was hot.

11. Fractionation of amylose with iodine

A mixture of 0.1 gram each of corn amylose and tapioca amylose was dispersed in 20 milliliters of 0.5 N potassium hydroxide. The purities of the amyloses were 89 and 85 per cent, respectively. The solution was neutralized to methyl orange with hydrochloric acid and water was added to make a

volume of 200 milliliters.

The amount of iodine required to saturate exactly half the amylose was calculated. This was added in solution, 6.65 milliliters of 0.0188 N iodine in 0.07 N potassium iodide. It was added very slowly with constant stirring. The mixture was then centrifuged, but the complex was not entirely coagulated and the supernatant liquid was quite blue. About 0.8 gram of solid potassium iodide was stirred in to aid coagulation, and the precipitate was centrifuged out. The supernatant liquid was decanted and 6.96 milliliters of the iodine solution was added to it as before. The second precipitate was then centrifuged out.

Each of the two fractions was stirred into about 100 milliliters of water and a slight excess of sodium thiosulphate solution was added. Upon standing a short time, the complex was decomposed, the amylose going back into solution. The excess thiosulphate was then back titrated with iodine. There was a little excess of iodine found in the supernatant liquid from the second fraction.

Each amylose fraction was precipitated from solution by addition of an equal volume of ethanol, centrifuged out, dehydrated with ethanol, and, finally, dried in a vacuum dessicator over phosphoric anhydride.

B. Details and Variations of the Potentiometric Iodine Titration Procedures

In practically all cases the material to be titrated was dispersed in a small volume of 0.5 N potassium hydroxide and subsequently diluted. Some dextrins and freshly precipitated starch fractions could be dissolved in hot water. For alkali dispersions the starch was dried in a vacuum oven at about 60°C. The concentration of starch generally used was 0.01 to 0.04 per cent, depending on the anticipated amylose content of the sample.

In a typical procedure 0.01 to 0.04 grams of starch was dispersed in 10 milliliters of 0.5 N potassium hydroxide. When dispersal was complete, the alkali was neutralized and distilled water was added to make the total volume 100 milliliters.

There were two choices available in neutralizing the alkali. In one case hydriodic acid was used, thus providing the necessary iodide concentration directly. In the second case hydrochloric acid was used and a measured amount of potassium iodide solution was added. This latter modification was proposed by Schoch (39) to avoid the preparation and preservation of iodine-free hydriodic acid. It had a second advantage in that it permitted a better control of iodide concentration. However, the use of hydriodic acid was necessary in applications where the presence of other ions was undesirable.

When prepared in the manner described above, the iodide concentration of the solution was 0.05 N. The reasons for choosing this value are discussed at great length in another section.

The solution was made neutral to methyl orange because it was desirable to have the system slightly on the acid side where the iodine electrode is practically independent of pH.

The properly prepared solution was titrated with 0.001 N iodine solution which also had a potassium iodide concentration of 0.05 N. After each addition of one milliliter, an interval of two to five minutes was allowed before reading the potential of the iodine electrode, which was immersed in the solution. If the change of potential near the end of the interval took place at a rate exceeding a few tenths of a millivolt per minute, more time was allowed between successive additions of titrating solution.

Apparatus. A Leeds and Northrup type K potentiometer was used to measure potentials. The electrode employed in the iodine-iodide half-cell was simply a bright platinum wire sealed in the end of a piece of soft glass tubing. A mercury connection was made with the potentiometer lead. The reference cell was a normal calomel electrode. The only requirement of the galvanometer used, a Leeds and Northrup instrument, was that it permit adjustment of the potentiometer within 0.1 millivolt.

V. SUMMARY AND CONCLUSIONS

1. It has been shown that starch contains two components that are distinctly different in their behavior toward iodine. One component is capable of binding 1/5 to 1/4 its weight of iodine in complex formation. The other component is capable of binding very little iodine.

2. The component with the strong affinity for iodine can be completely separated from the second component. It has been identified with long, straight-chain starch containing no branches, and has been termed "amylose." The second component has been identified with highly branched starch containing no long straight branches, and has been called "amylopectin."

3. The amount of iodine bound by pure amylose under definite and reproducible conditions has been measured by a potentiometric method.

4. The potentiometric method has been applied to the analytical determination of the amylose content of starches and starch fractions. By this method it has been found that fractionation of starch by the n-butanol precipitation procedure does not produce complete separation of the two components; hot water extraction of amylose results in very poor separation; and the cotton fractionation method gives almost

complete separation, although recovery of the amylose component is almost impossible and the method is very unsatisfactory in other respects. It was found that waxy starches consist almost entirely of amylopectin.

5. The amount of iodine bound by amylose varies inversely with the iodide concentration. The binding of iodine is inhibited by the presence of fatty acids and their alkali metal salts.

6. Affinity for iodine varies directly with the length of the amylose chain. Amyloses from different sources have different chain lengths. Very short amyloses behave toward iodine in a manner similar to amylopectin. An individual amylose prepared from a natural starch is quite homogeneous in chain-length as compared to the wide range represented by all the amyloses.

7. An equilibrium exists between the free iodine in an aqueous solution and the iodine in the amylose-iodine complex present in the same solution. Long-chain amylose molecules having greater affinity for iodine bind all the available iodine before the short chain molecules are able to do so.

8. Fractionation of an amylose containing a range of chain lengths can be accomplished by selective precipitation of the longest molecules in the form of iodine complex.

9. Affinity for iodine varies inversely with the degree of branching of amylopectins. Amylopectins prepared from ordinary starches are less branched than waxy starches.

Limit dextrins exhibit a higher degree of branching than the amylopectins from which they are prepared.

10. Amylose forms complexes resembling the n-butanol complex with pyridine, aniline and phenol. The complexes with phenol and aniline can be employed in effecting fractionation of starch. In the case of phenol the fractions obtained are similar in purity to those produced by the n-butanol method.

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