Discussion

The mixture of sterol glucosides isolated from cottonseed oil resembles that isolated from soybean oil very closely. The melting point of the glucoside mixture from soybean oil is higher than that of the mixture from cottonseed oil. Both glucoside mixtures formed tetraacetates with practically the same melting points but with quite different rotatory powers. Since both glucoside mixtures contain *d*-glucose, it is apparent that these differences must be due to the sterols. The sterols from the soybean glucoside mixture were shown to contain about 25% of stigmasterol,[3] while a number of investigators have reported that this sterol was either entirely absent or present in only small amounts in the free sterols of cottonseed oil.

The sterol glucoside mixture from cottonseed oil differs somewhat from that isolated from the cotton plant by Power and Chesnut. The differences lie chiefly in the higher melting point of the glucoside mixture from the oil and in the lower melting point of the sterols obtained by the hydrolysis of this mixture.

Summary

Mixed sterol glucosides were isolated from cottonseed oil by treatment of the oil with an adsorbent and subsequent extraction of the adsorbed material with acetone.

The sugar obtained by acid hydrolysis of the glucosides was identified as *d*-glucose.

The mixed sterol glucosides isolated from cottonseed oil differ in some respects from those isolated from the cotton plant by Power and Chesnut. These differences are apparent in the higher melting point (248–250°) of the glucosides from the oil and the lower melting point (128–130°) of the sterols which these glucosides yield on hydrolysis.

LAFAYETTE, INDIANA RECEIVED MAY 14, 1941

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Heats of Organic Reactions. XI. The Denaturation of Pepsin by Alkali

BY JOHN B. CONN, DONALD C. GREGG, G. B. KISTIAKOWSKY AND RICHARD M. ROBERTS

In the investigation of the heat of denaturation of methemoglobin by alkali[1] several experimental difficulties were encountered. A comparison of the heats of reaction of native and denatured methemoglobin with alkali could only be made by an extrapolation. Rapid denaturation of methemoglobin occurred at *p*H 11 or greater; at this *p*H considerable disintegration of the protein molecule into fragments is possible. It therefore seemed advisable to choose for further work a protein denaturable at a lower *p*H than methemoglobin. Pepsin is rapidly and completely denatured at *p*H 8.0,[2] and has the further advantage of being soluble in the native and in the denatured condition, in the concentrations used in our experiments, at *p*H 4.3, thus permitting a direct comparison of heats of reaction of native and denatured protein with alkali.

Experimental Procedure

Calorimetric Technique.—The calorimeter used in these experiments has been described in a previous paper of this series.[1] Since performing the experiments described there, the apparatus has been slightly altered to eliminate one source of uncertainty. Addition of alkali changes the viscosity of protein solutions markedly and thus alters the heat of stirring. It is therefore impossible to establish a generally valid relation between the thermal head of the calorimeter and the rate of its temperature change. The result is an uncertainty in the heat of any reaction which does not occur practically instantaneously. A pneumatically operated clutch was installed on the stirrer shaft of the working calorimeter, operated from the outside of the air thermostat surrounding the water thermostat bath. The stirring was repeatedly interrupted for short measured intervals of time (usually five minutes) during the course of a reaction, and the rates of temperature change with and without stirring calculated from systematic readings of the e. m. f.'s of the main and adiabatic thermel. In absence of stirring (and chemical reactions) the rate of temperature change of the calorimeter has been found to be solely determined by its thermal head above the thermostat, as read on the adiabatic thermel. Thus the knowledge of the rates of temperature change with and without stirring in presence of chemical reactions is sufficient to separate for the purpose of calculation the three contributing factors: heat conduction to the outside, heat of stirring and the heat of chemical reaction.

The protein solutions were made up to 840 g. and poured into the outer compartment of the calorimeter. The proper quantity of alkali was diluted to 62 cc. and placed

(1) Conn, Kistiakowsky and Roberts, THIS JOURNAL, **62**, 1895 (1940).

(2) Philpot, *Biochem. J.*, **29**, 2458 (1935), has found that pepsin brought to *p*H 11 remained homogeneous in the ultracentrifuge.

in the inner can. All solutions were made 0.2 in ionic strength by addition of potassium chloride. After adjusting the temperature of the calorimeter to a value near its equilibrium position, the double-plug in the inner can was raised, allowing the alkali to mix with the protein. The major part of the heat evolution was observed within ten minutes after mixing the protein and alkali solutions, and was complete within an hour. An electrical calibration was performed with the calorimeter and contents at the end of the run, for conversion of microvolts to calories.

Two corrections, apart from that for heat loss, were applied to the calorimetric results. The first, the heat of dilution of pepsin solution with 0.2 *N* potassium chloride, was found to be negligible. The second, the heat of dilution of 0.2 *N* potassium chloride solution with potassium hydroxide, amounted to about 1.2 cal. for the smallest, and 1.5 cal. for the largest amount of alkali used in these experiments. This correction very nearly cancelled in the final calculations.

Preparation of Pepsin.—The crystalline pepsin was obtained by the procedure outlined by Northrop[3] using Parke-Davis U. S. P. 1:10,000 pepsin. Several different lots of purified pepsin were prepared, and stock solutions of various pepsin concentrations were employed, usually containing 20–30 mg. pepsin per cc. Microscopic examination of the purified enzyme showed the characteristic pepsin crystals. The degree of homogeneity of a typical preparation was determined electrophoretically through the courtesy of Dr. John M. Newell of the Harvard Medical School. The electrophoretic examination indicated that not more than 5% of a second component was present in the purified pepsin.[4]

The amount of non-protein nitrogen was determined by precipitating the protein from solution with an equal volume of hot 5% trichloroacetic acid and estimating the total nitrogen in the filtrate.[5]

The non-protein nitrogen content of the stock solutions was never greater than 10% of the total nitrogen, even after standing for two weeks at 5°. This is in accord with the observation of Northrop,[6] that his pepsin preparations contained 5–15% non-protein nitrogen; even after thorough dialysis non-protein nitrogen is produced on standing.

The enzymatic activity of the stock solutions was determined at regular intervals, and was found in all cases to remain constant during the time the several stock solutions were being used in the calorimetric experiments, indicating that no serious decomposition had taken place. The enzymatic activities of three pepsin stock solutions in terms of pepsin units per mg. of nitrogen (by the hemoglobin method[7]) were 0.28, 0.29 and 0.31.

The procedure employed in the preparation of the various stock solutions was as follows: the pepsin crystals were suspended in 0.01 *N* hydrochloric acid and allowed to stand for twelve hours at room temperature. Thorough dialysis in the cold was carried out until chlorides were absent. The suspension was dissolved by addition of 0.05 *N* potassium hydroxide with stirring. The solution was then returned to the desired *p*H with 0.1 *N* hydrochloric acid and made 2 to 3% with respect to protein and 0.2 *N* in potassium chloride. The stock solutions were kept at 5°, and toluene was added to inhibit bacterial growth.

Estimation of Peptic Activity.—The method described by Northrop[7] for the estimation of peptic activity was employed. A dialyzed 2.5% solution of crystalline carboxyhemoglobin was used as the substrate. The digestions were carried out at 30.6°, the temperature at which the calorimetric measurements were made. The enzymatic activity of the pepsin stock solutions was determined before each calorimetric experiment, and in the case of the experiments outlined in Table II, the estimation of the peptic activity at each initial *p*H was made at the same time as the calorimetric measurements.

Since the calorimetric procedure necessitated the use of alkali instead of buffer solutions, the changes in *p*H were made by addition of 0.05 *N* potassium hydroxide. Preliminary experiments showed that when alkali of different concentrations was added in the same manner (through a capillary, with stirring) to bring the pepsin to some definite *p*H, the activity of the pepsin was not altered to the same extent. Using 0.001 *N* potassium hydroxide it was found that at *p*H 7.1 the pepsin had lost 50% of its activity; while by bringing it to *p*H 7.1 with 0.02 *N* alkali no peptic activity remained. In each series of experiments reported here the mode of addition of alkali was the same, and the shape of the *p*H-activity curve was similar to that reported by Northrop,[8] except that the sharp break in the curve was displaced toward lower *p*H. A comparison of enzymatic activity and solubility at *p*H 2.5 of protein in solutions which had been previously treated with various amounts of alkali indicated that the loss of enzymatic activity was paralleled by decrease in solubility at *p*H 2.5; this agrees with the results of Northrop.[9]

Results and Discussion

In the first series of calorimetric experiments sufficient alkali for complete denaturation was added to a solution of native pepsin in the calorimeter. The inner can was then closed, and, after removal of its contents, was filled with an amount of acid equivalent to the alkali remaining in the calorimeter. A run was then made, adding this acid to the denatured protein. Very erratic results were obtained by this method; it was found that an indefinite amount of protein precipitated in the inner can, due to low local *p*H at the point of contact of protein and acid solutions. The precipitate did not entirely redissolve, probably because the rate of stirring was insufficient to give the necessary vigorous mixing. This type of experiment was therefore abandoned.

A number of experiments were then made with a fresh stock solution of pepsin. The protein was

(3) Northrop, "Crystalline Enzymes," Columbia Univ. Press, 1939, p. 129.

(4) Crystalline pepsin, although homogeneous in the Tiselius apparatus, consists of two components (see footnote 14).

(5) All estimations of total nitrogen were made by the semi-micro Kjeldahl technique.

(6) Ref. 3, p. 29.

(7) Ref. 3, p. 153.

(8) Ref. 3, p. 34.

(9) Ref. 3, p. 33.

TABLE I

HEATS OF REACTION OF PEPSIN AND POTASSIUM HYDROXIDE AT 30.6°, *p*H 5 → 9

Run	N, g.	Mmoles KOH/g. N added outside calorimeter	Mmoles KOH/g. N added in calorimeter	*p*H initial	*p*H final	$-\Delta H_1$, cal./g. N.	$-\Delta H_2$, cal./g. N.	$+\Delta H = \Delta H_1 - \Delta H_2$
1a	1.48	2.33	5.94	5.00	9.10	32.8		
2a	1.70	2.31	5.94	5.00	9.15	31.6		
b	1.48		5.94	5.00	9.10		47.3	
c	1.28		5.95	4.95	9.15		48.8	
3a	1.28	2.31	5.95	5.00	9.05	31.4		
b	1.11		5.94	5.00	9.05		48.1	
4a	1.58	2.31	0.00	4.95	4.95	(0.0)		
b	1.48		5.94	4.95	9.10	31.8		
c	1.28		5.95	4.95	9.05		48.0	
					Average	31.9	48.1	16.2

brought to *p*H 5.0 outside the calorimeter by addition of dilute potassium hydroxide (about 0.02 *N*) through a capillary with vigorous stirring. The solution was then placed in the calorimeter and sufficient alkali was added to bring the solution to *p*H 9.1, the heat of this reaction being measured. The solution was subsequently removed from the calorimeter and an amount of acid equivalent to the alkali added in the calorimeter was allowed to run into the protein solution with stirring. The acid returned the denatured protein to the neighborhood of *p*H 5.0; 840 g. of this protein solution was again placed in the calorimeter and its heat of reaction with the equivalent quantity of alkali was determined.

The results of experiments performed in the manner described above are shown in Table I. The same quantity of alkali per gram nitrogen was added in each case, and the initial and final *p*H values were the same in all runs. The heat of dilution of the protein solution (Run 4a) was negligible, so that the effect of protein concentration may be disregarded. The resulting values for the heat evolution per gram nitrogen both for the native and denatured protein are in good agreement. That subsequent treatment with alkali does not substantially alter the heat of reaction of the already denatured protein can be seen from Run 2c. In this experiment the protein solution at the end of 2b was acidified and alkali was again added to it in the calorimeter. The resulting heat evolution was only slightly higher than for other runs with protein which had been treated only once with alkali. The data of Table I show that pepsin denatured at *p*H 9 has a very markedly increased heat of neutralization. The difference $\Delta H_1 - \Delta H_2$ in heats of reaction between native and denatured pepsin is the "heat of denaturation," ΔH. The thermochemical equations involved here are identical with those previously written for the case of methemoglobin.[1]

The remaining experiments were made with two ideas in mind—first, to examine the effect of *p*H upon the heat of denaturation; and second, to compare the change of heat of denaturation and inactivation of the enzyme at increasing initial *p*H. Northrop[10] has found that extent of denaturation (as estimated by insolubility at low *p*H after treatment with alkali) and inactivation of pepsin are identical functions of *p*H. We wished to determine whether the change in heat of denaturation and inactivation of the enzyme are also the same function of *p*H.

Runs 7 and 9 in Table II show the results of exploratory experiments comparing heat of denaturation with activity at two different initial *p*H values where partial denaturation had taken place. In Run 9 ΔH was small (about 19% of the value at *p*H 5.0), yet 45% peptic activity remained in the solution at the start of 9a. Run 7, with a higher initial *p*H, gave a negligible difference in heat evolution between the native protein brought to *p*H 6.95 before the run, and the fully denatured protein; 10% of the original enzymatic activity nevertheless remained at *p*H 6.95.

In order to investigate this behavior more fully, a series of experiments (11–16 in Table II) was made with a fresh preparation of pepsin. In this series the total number of moles of alkali added to the protein (outside + inside the calorimeter) was the same in each run. For the final *p*H in this series was chosen the lowest *p*H at which rapid and complete denaturation was found to take place. Runs 15 and 16 were identical check runs.

A plot of ΔH per g. of nitrogen against quan-

(10) Ref. 3, p. 33.

TABLE II

HEATS OF REACTION OF PEPSIN AND POTASSIUM HYDROXIDE AT 30.6°

Run	N, g.	Mmoles KOH/g. N added outside calorimeter	% activity remaining	Mmoles KOH/g. N added in calorimeter	pH initial	pH final	$-\Delta H_1$ cal./g. N	$-\Delta H_2$ cal./g. N	$+\Delta H = \Delta H_1 - \Delta H_2$
9a	0.900		45	4.76	6.60	8.95	17.7		
b	0.797		0	4.75	6.00	8.80		20.7	3.0
7a	1.07		10	4.00	6.95	9.15	14.5		
b	0.930		0	4.01	6.75	9.15		14.2	−0.3
11a	1.08	0.00	100	6.95	4.30	8.20	51.9		
b	0.955		0	6.95	4.40	8.20		68.0	16.1
12a	1.08	1.56	100	5.39	4.95	8.20	32.9		
b	0.955		0	5.39	4.90	8.15		47.8	14.9
13a	1.08	2.97	100	3.98	5.60	8.10	16.3		
b	0.981		0	3.98	5.15	7.85		30.4	14.1
14a	1.08	3.67	100	3.28	6.20	8.10	10.0		
b	0.981		0	3.28	5.45	7.95		22.6	12.6
15a	1.08	5.03	29	1.92	6.80	8.05	8.3		
b	0.981		0	1.92	6.45	8.00		8.5	0.2
16a	1.08	5.03	30	1.92	6.80	8.00	7.9		
b	0.981		0	1.92	6.45	8.00		8.7	0.8

tity of alkali added in the calorimeter (Fig. 1) shows a marked dependence of ΔH upon that quantity, that is, upon the *p*H to which the native protein was initially brought outside the calorimeter. As this *p*H is raised, the value of ΔH

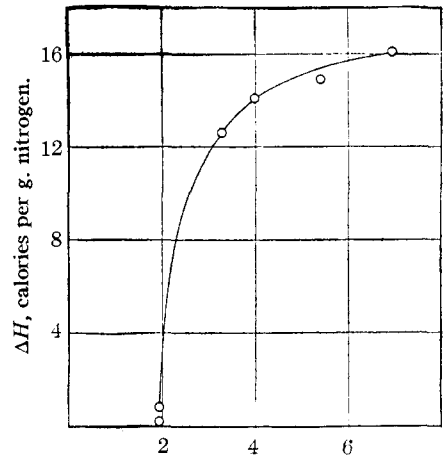

Fig. 1.

falls off, the decrease becoming very abrupt above *p*H 6.2. At *p*H 6.8 ΔH is negligible, indicating that native protein brought to *p*H 6.8 by our method of adding alkali[11] has at least by the criterion of heat of reaction with alkali been fully denatured. ΔH also depends upon final *p*H; in Run 12 of Table II, from *p*H 4.95 to 8.20, ΔH = 14.95, while the average result of the runs of Table I, from *p*H 5.0 to 9.1, is ΔH = 16.2.

(11) Complete retention of enzymatic activity by pepsin brought to *p*H as high as 6.2 by the same method of addition shows that by ordinary standards our treatment was not drastic.

It is possible to give a partial explanation of dependence of ΔH upon *p*H from a consideration of the titration curves of native and denatured pepsin. The denaturation of pepsin by alkali is almost completely irreversible, and the titration curves of native and denatured pepsin (Fig. 2) are evidence of this irreversibility.[12] The points on the upper curve show the *p*H immediately reached by adding various amounts of potassium hydroxide to 2 cc. of pepsin solution (about 25 mg./cc.) and diluting to 10 cc. with water. The points on the lower curve were ob-

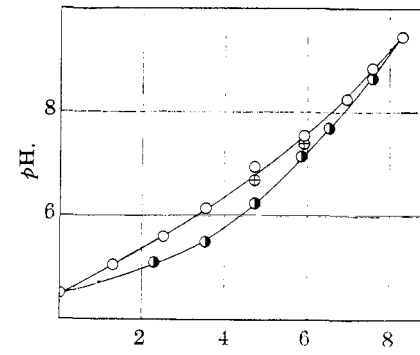

Fig. 2.

tained by adding sufficient alkali to 2 cc. of pepsin solution to bring the *p*H to 9.5, then adding various amounts of hydrochloric acid of the same

(12) Northrop, *J. Gen. Physiol.*, **14**, 713 (1931), has shown that a very small fraction of denatured pepsin can be converted to native pepsin after long standing. Irreversibility of titration curves of proteins which have been carried beyond their range of stability with respect to *p*H has often been observed—for example, the observations of Cohn and Berggren, *ibid.*, **7**, 45 (1924).

strength as the base used, and diluting the resulting solution to 10 cc. The lower curve was identically reproduced when alkali was added a second time to the acidified denatured pepsin. In the *p*H range 6.5–7.5 the *p*H of solutions represented by points on the upper curve fell slightly on standing. The crossed circles show the drop in *p*H in two solutions after two hours. Denaturation is probably slow in this *p*H range. The divergence between the two curves explains the observation in the runs of Table II above *p*H 5.0, that the *p*H reached upon acidifying the fully denatured protein was always much lower than the initial *p*H in part *a* of these runs.

The difference between the titration curves of native and denatured pepsin points to a liberation of a number of acidic groups. This difference may be due in part to hydrolysis of amide linkages, a view which is sustained by our observation of an increase of about 5% in non-protein nitrogen on exposure of the protein to *p*H 9. Of the total nitrogen of pepsin (15.4%) 8.8% is said to be amide nitrogen,[13] corresponding to about thirty amide groups per molecule. It is possible that the difference in titration curves is the result of side-chain carboxyl groups released by amide hydrolysis.

When enzymatic activity is compared with the percentage decrease of ΔH at increasing initial

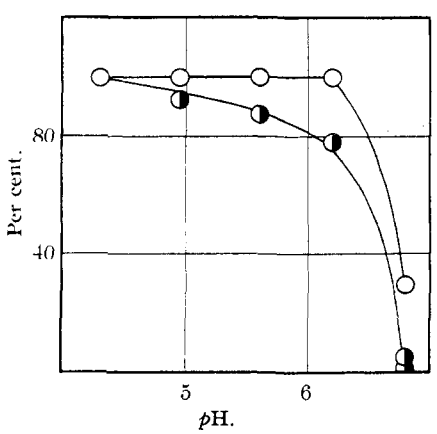

Fig. 3.—This figure represents part of the data on Runs 11–16 in Table II. Open circles show the percentage of peptic activity remaining in solutions brought to various *p*H outside the calorimeter. Runs then made with each of these solutions gave values of ΔH, the heat of denaturation, corresponding to these initial *p*H. Shaded circles show $(\Delta H_{p\mathrm{H}4.3} - \Delta H_{p\mathrm{Hx}}) \times 100/\Delta H_{p\mathrm{H}4.3}$, the percentage decrease of ΔH with increasing initial *p*H from its value at *p*H 4.3.

(13) Calvery, Herriott and Northrop, *J. Biol. Chem.*, **113**, 11 (1936).

*p*H (Fig. 3), it is seen that these two quantities change in a different manner. Between *p*H 4.3 and 6.2 no loss of activity has occurred, ΔH having decreased by 22%. More striking still, in going from *p*H 6.2 to 6.8 only 70% of the peptic activity was lost, yet ΔH decreased by 96%.

It has recently been shown[14] that crystalline pepsin can be separated into two fractions differing slightly in peptic activity, each fraction having a solubility independent of the quantity of solid phase present. The possibility that the presence of two components in our preparations may be responsible for the discrepancy between the two curves of Fig. 3 must be considered. If this explanation were correct, it would be necessary to assume that the more active of the two fractions is also more resistant to denaturation by alkali, and that its heat of denaturation is very much smaller than that of the other fraction. These assumptions taken together seem to us very improbable.

It is much simpler to suppose that alkali affects the protein molecule in various ways, one of which is the disturbance of the configuration of groups responsible for enzymatic activity. These effects will certainly not be expected to occur to the same extent. The ΔH of our experiments, which has been designated the "heat of denaturation," must be regarded as the sum of changes in heat content due to several chemical processes, including the heat of inactivation, although the present experiments do not permit its calculation. It is therefore not surprising that ΔH and peptic activity are different functions of the *p*H.

In the previous work on methemoglobin a substantially constant heat of denaturation of the protein by alkali was found, equal to 12.6 cal. per g. of nitrogen, or 142 kcal. per mole. The nature of those experiments was such that a slow change of the heat of denaturation with changing initial *p*H would have escaped observation. In the case of pepsin, however, the results definitely indicate that the heat of denaturation changes with initial *p*H in the range where, according to the peptic activity measurements, no denaturation occurs. The final *p*H also affects the heat of denaturation, although in this range the peptic activity has been already completely destroyed. It is therefore impossible to speak of a definite heat of denaturation, but one may take 15 cal.

(14) Desreux, Herriott and Northrop, *J. Gen. Physiol.*, **24**, 213 (1940).

per g. nitrogen as a "representative" value. Since pepsin has a molecular weight of 35,000, this amounts to 85 kcal. per mole of protein, roughly half of that observed for methemoglobin. Thus the heat of denaturation per gram of protein material is about the same for both compounds, which may be interpreted to mean that alkali has a similar effect on molecular structure in both cases.

We wish to thank the Rockefeller Foundation for its grant in support of this work.

Summary

The heats of reaction of native and denatured pepsin with potassium hydroxide have been measured at 30°. The difference between these heats, which may be called the heat of denaturation, was found to depend on the initial and final *p*H of the experiment. Between *p*H 4.3 and 8.2 the heat of denaturation is +16.06 cal. per g. of nitrogen, or 85 kcal. per mole of pepsin. The heat of denaturation was found to decrease with increasing *p*H more rapidly than the enzymatic activity. It is concluded that the effects of previous treatment with alkali on the heat of denaturation and on the enzymatic activity are to some extent independent.

CAMBRIDGE, MASS. RECEIVED APRIL 28, 1941

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

An Application of the Ilkovic Equation to the Analysis of Mixtures of Reducible Substances with the Dropping Mercury Electrode

BY I. M. KOLTHOFF AND E. F. ORLEMANN

The current–voltage curve of a solution of two substances which are reducible at the dropping mercury electrode at different potentials has the general appearance of that given in Fig. 1. When the plotted values of the current are corrected for the residual current the following relations are found.

$$i_{d_1} = k_1 c_1 \quad (1)$$

$$i_{d_2} = k_2 c_2 \quad (2)$$

The subscript (1) refers to the first component reduced and subscript (2) to the second component. i_d is the diffusion current, k is the diffusion current constant and c is the concentration of the substance. The total current i_t can be measured at a certain potential π_t and is equal to the sum of i_{d_1} and i_{d_2} at this same potential π_t. We may therefore write

$$i_t = (i_{d_1})_{\pi_t} + t(i_{d_2})_{\pi_t} \quad (3)$$

Obviously i_{d_1} cannot be measured directly at the potential π_t but can be measured only at potentials between π_a and π_b as shown in Fig. 1. It is necessary therefore to find the value of i_{d_1} at the potential π_t from the value of i_{d_1} obtained at some more positive potential in order to use equation (3) for the determination of the diffusion current of component two.

Various empirical procedures have been proposed to meet the above problem which in effect either assume that i_{d_1} is independent of the potential or assume an extrapolation of i_{d_1} which is not fundamentally correct.[1] For a given empirical procedure the error involved may be very small or very large dependent upon the ratio of i_{d_1}/i_{d_2} and the potential region involved in the measurements of i_{d_1} and i_{d_2}. In the present paper a general discussion of the problem based on the Ilkovic equation is given and a procedure based on fundamental principles is described.

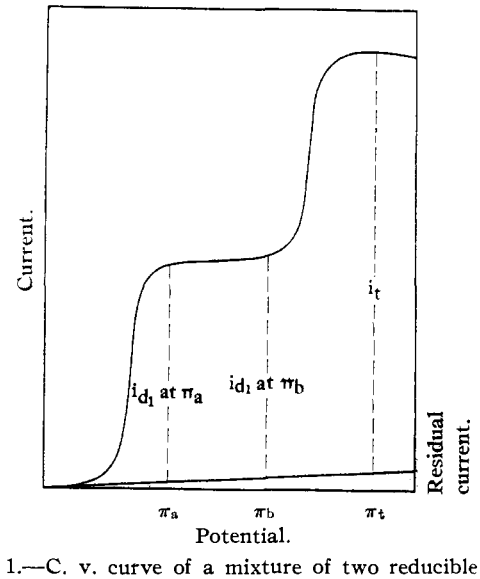

Fig. 1.—C. v. curve of a mixture of two reducible substances.

(1) I. M. Kolthoff, *Chem. Rev.*, **24**, 1 (1939).