CCVIII. PRUNE AS AN OXIDATION-REDUCTION INDICATOR. ITS SUITABILITY FOR TITRATION OF ASCORBIC ACID.

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(Received June 28th, 1934.)

THE use of indophenols for oxidative titration of ascorbic acid [Tillmans *et al.*, 1932; Birch *et al.*, 1933; Zilva, 1934] stimulates interest in other positive oxidation-reduction indicators. In particular, the character of the somewhat elusive potentials of ascorbic acid [Green, 1933] suggests that indicators with a range between the indophenols and methylene blue may have practical significance. The oxazine "prune" was found to have such a range and seemed to be a substance of general utility for three reasons: (a) prune is readily available as a commercial dyestuff [No. 893, Rowe, 1924], or is easily synthesised from methyl gallate and *p*-nitrosodimethylaniline, (b) its potential range is such as to minimise fortuitous oxidation of phenols, *etc.*, by the reagent and to provide the greatest specificity consistent with titration of the somewhat weakly reducing ascorbic acid, and (c) its acid-solubility, stability and considerable tinctorial power, as compared with the acid-instability and "rather pale reddish colour" of indophenols [Gibbs *et al.*, 1925] offer considerable advantages where the desired degree of specificity requires the use of acid solutions [Birch *et al.*, 1933]. As other likely indicators (indamines such as toluylene blue [Phillips *et al.*, 1927], amino-indophenols [Cohen and Phillips, 1929], or oxazines such as cresyl blue [Cohen and Preisler, 1931] were chemically unstable in solution, it became desirable to submit the system, prune—reduced prune, to potentiometric investigation.

General properties.

Prune is an intense magenta red in acid solution, a deep blue between p_H 4 and 8 and purple beyond p_H 8. The red material is readily soluble in water, somewhat soluble in aqueous alcohols and insoluble in other solvents. The blue material aggregates slowly in water and precipitates at a rate varying with concentration; it is soluble in chloroform, ethyl acetate, alcohols and benzene, slightly soluble in ether and carbon tetrachloride, insoluble in light petroleum. The purple material is rather unstable but is most soluble in water. From its aqueous solution, prune is readily extractable as the blue material by chloroform or ethyl acetate at any p_H , or by benzene at p_H 4–7, yet satisfactory crystallisation from these solvents is hindered by a low temperature coefficient of solubility. The blue material separates from water (after treating the acid solution with sodium acetate) by slow precipitation of an aggregate of clustered needles and amorphous residues, readily soluble in acids. This aggregate is crystallisable from xylene [Kehrmann and Beyer, 1912] as clustered prisms. The red material separates more satisfactorily from 0.1 *M* HCl solution, as prisms of steely lustre, either by salting out or by continuous extraction with butyl alcohol.

It appears that the red material is the cation (hydrochloride), the blue is unionised, and the purple the anion (phenoxide), or in other words that the dissociation constants found at p_H 3.6 and 8.0 represent, respectively, the dissociations of basic and acid groups. This is confirmed by a colour change from red to blue when acetone is added to a solution of prune in phthalate buffer of p_H 3 (evidence of a basic group), and by the reverse change from purple to blue in an ammonia buffer of p_H 9.3 (evidence of an acid group [Richardson, 1934]); in assessing the purple colour, care is needed because of pronounced dichroism in the purple shade.

The magnitudes of these constants are such as are usually associated with anilines (p_K 3 to 5) and phenols (p_K 8 to 10), respectively. Consequently, the following formulae best fit the known properties of prune (including acylation and methylation reactions [Gnehm and Bauer, 1905; Kehrmann and Beyer, 1912]):

It will be observed that the usual oxonium salt formulation is discarded since the basic dissociation common to pyrones is very weak (p_K about 0), and would be responsible only for the third colour change (red-blue) found to occur in prune in approximately 8*M* acid solutions. Nevertheless, in certain chemical properties the influence of its structural analogy with pyrones is observed. These are the colloidal tendencies of the blue solution, the formation with iodine of a brown sediment decomposable by thiosulphate and the precipitation of acid solutions by ferrocyanides. The influence of its phenolic group is apparent in the formation with ferric chloride in both acid and neutral solution of a blue complex insoluble in butyl alcohol.

Reduced prune is pale yellow in acid or neutral solution, or somewhat brownish alkaline to p_H 9. The acid leuco-compound is most soluble in water and alcohols, the neutral compound in chloroform, alcohols and ether. Aerobic oxidation proceeds slowly in acid solution, much more rapidly in neutral or alkaline solution.

EXPERIMENTAL.

Materials. Prune was prepared as the hydrochloride on account of its ready solubility. Specimens are obtained easily only by salting out, so that purification of commercial materials controlled by analyses was not attempted. Instead, methyl gallate (m.p. 199°) and *p*-nitrosodimethylaniline hydrochloride, both freshly recrystallised, were boiled with methyl alcohol until a spot on filter-paper gave no yellow fringe. The crystalline precipitate of prune hydrochloride was washed with methyl alcohol, dried and thrice salted out of its warm 1 % solution

in 0.05 *M* HCl by bringing the HCl concentration to 0.25 *M* and cooling; it was dried in a vacuum in the presence of NaOH. The resulting prisms gave a positive chloride test after incineration with HNO₃. By titration at a quinhydrone electrode, they required one equivalent of NaOH for neutralisation and were thus crystals of a monohydrochloride. A stock solution of 0.004 *M* prune HCl (mol. wt. 350.6) in 0.05 *M* HCl proved admirably stable.

Buffer solutions were of standard type [Clark, 1928; Britton and Robinson, 1931], freshly prepared from recrystallised materials and CO₂-free NaOH. As reducing agents, sodium hydrosulphite or titanous salts were unsuitable, and sodium anthrahydroquinone- β -sulphonate was used. This was prepared by reducing a fresh 0.004 *M* solution of recrystallised sodium anthraquinone- β -sulphonate with hydrogen and platinised asbestos; the solution was filtered through sintered glass into 4 volumes of deaerated buffer and stored under nitrogen for delivery from a burette. As oxidising agent, recrystallised freshly-dissolved 0.004 *M* benzoquinone, or 0.002 *M* iodine-KI, was employed. Nitrogen and hydrogen were commercial supplies suitably purified by conc. NaOH and hot copper gauze, and conveyed to the titration vessel through copper leads.

Apparatus. Titrations were conducted at $30 \pm 0.02^\circ$ in an electrically controlled kerosene thermostat. The titration vessel, of standard Clark pattern [1928, p. 301], was in electrical circuit with a saturated calomel half-cell through a saturated KCl bridge of the bulb and goose-neck capillary type. Duplicate gold-plated platinum wire electrodes were used, or, for p_H determination, freshly palladised gold-plated wire electrodes. Calomel electrodes were maintained constantly at 30° and frequently standardised against a hydrogen electrode in 0.05 *M* potassium hydrogen phthalate, of an assumed potential of $+0.2386$ v. at 30° [Clark, 1928].

Procedure. For reduction experiments, the p_H of 25 ml. of the appropriate 0.025 *M* buffer was determined in the titration vessel, and then 1 ml. of 0.004 *M* prune in 0.05 *M* HCl, with sufficient 0.01 *M* NaOH for neutralisation to the buffer p_H , was added. Titration from a micro-burette was carried out under nitrogen with approximately 0.0008 *M* anthrahydroquinone- β -sulphonate in the same buffer.

For oxidation experiments, the stock 0.004 *M* prune was suitably diluted with 0.05 *M* HCl, an aliquot reduced by hydrogen and platinised asbestos and discarded, the main bulk added to the same catalyst, reduced colourless (15 min.), and immediately withdrawn through sintered glass for storage under hydrogen. To 25 ml. of deaerated 0.025 *M* buffer, about 3 ml. of approximately 0.001 *M* reduced prune were usually added, and the p_H was determined. The solution was then titrated under nitrogen either with iodine (avoiding excess), or with a dilution of benzoquinone in the desired buffer, all solutions being deaerated.

In general, oxidising and reducing agents with metallic ions were avoided, because of a marked tendency to co-ordination, exemplified in the behaviour of prune as a mordant dyestuff. Since this has necessitated the use of catalytically reduced reagents, their concentration has been known only approximately. Consequently, E_0' values have been separately determined at each p_H by a complete titration, and each value represents the mean constant for the curve. During the titration, duplicate electrodes rarely disagreed by more than 0.0001 v.

Potentiometric results.

Admirable potentiometric data were obtained on the acid side of p_H 4, and indeed were satisfactory as far as p_H 10, since the prune solutions employed (0.00015 *M*), though richly coloured, were sufficiently dilute to avoid any

considerable aggregation during titration. In presence of borates and glycine, however, reproducibility of E_0' values was hindered by co-ordination and aggregation effects. Decomposition of prune to dimethylaniline [Kehrmann and Beyer, 1912] begins above p_H 10.

In Table I data for two oxidations of reduced prune by benzoquinone are given, while Table II presents two reductions of prune by anthrahydroquinone- β -sulphonate. It seems likely from Table II that our thrice crystallised material still contained some 5 % of an impurity, 0.08 v. more negative than prune at p_H 2.4. Possibly this was the demethylated dyestuff, gallocyanine [Michaelis and Eagle, 1930], or an oxazone [cf. cresyl blue, Cohen and Preisler, 1931]. In any event its amount was not increased above that for a fresh (2 hours) solution by keeping for 10 days in 0.05 M HCl, and its influence on Tables I and II is negligible except at the negative extremes.

The relation of E_0' to p_H is given in Table III, adopted values of the dissociation constants being confirmed by hydrogen electrode titration of reduced prune, and by colorimetric examination of prune. The electrode equation from which calculation is made [cf. Cohen and Preisler, 1931] is as follows:

$$E_h = E_0 - 0.03006 \log \frac{S_r}{S_0} - 0.03006 \log \frac{K_{ob}K_{oa} + K_{ob}[H^+] + [H^+]^2}{K_{rb}K_{ra}[H^+]^2 + K_{rb}[H^+]^3 + [H^+]^4}$$

where $E_h + 0.03006 \log \frac{S_r}{S_0} = E_0'$. It is based on the assumption of the following hydrion dissociations:

of which K_{ras} is negligible, and K_{ra2} is also inoperative in the range described

Table I. *Titration of reduced prune with benzoquinone.*

(a) p_H 2.886	35 ml. 0.025 M phthalate 0.0008 M benzoquinone	7 ml. 0.001 M reduced prune
(b) p_H 6.938	25 ml. 0.025 M phosphate 0.0008 M benzoquinone	3 ml. 0.001 M reduced prune

	Titre (ml.)	$0.03006 \log \frac{S_r}{S_0}$	E_h	E_0'
(a)	0.47	+0.038	+0.3172	+0.355
	0.71	0.0318	0.3220	0.3538
	1.03	0.0264	0.3272	0.3536
	1.31	0.0228	0.3310	0.3538
	1.85	0.0173	0.3360	0.3533
	2.47	0.0123	0.3410	0.3533
	3.08	0.0080	0.3454	0.3534
	3.86	+0.0032	0.3503	0.3535
	4.58	-0.0011	0.3546	0.3534
	5.37	0.0059	0.3593	0.3534
	6.17	0.0112	0.3649	0.3537
	6.90	0.0169	0.3706	0.3537
	7.57	0.0238	0.3771	0.3533
	7.91	0.0286	0.3813	0.3527
	8.24	0.035	0.3864	0.351
	8.79 (end-point)			
(b)	0.50	+0.028	+0.0283	+0.056
	1.20	0.0140	0.0462	0.0602
	2.00	+0.0039	0.0560	0.0599
	2.70	-0.0039	0.0637	0.0598
	3.20	0.0099	0.0695	0.0596
	3.70	0.0171	0.0770	0.0599
	4.30	0.031	0.0870	0.056
	4.70 (end-point)			

Table II. *Titration of prune with anthrahydroquinone- β -sulphonate.*

(a) p_H 2.402. 25 ml. 0.025 *M* phthalate. 1 ml. 0.004 *M* prune in 0.05 *M* HCl. 2.55 ml. 0.014 *M* NaOH. 0.0008 *M* (approx.) anthrahydroquinone- β -sulphonate buffered at p_H 2.4.

(b) p_H 7.698. 25 ml. 0.025 *M* barbitone. 1 ml. 0.004 *M* prune in 0.05 *M* HCl. 3.9 ml. 0.014 *M* NaOH. 0.0008 *M* (approx.) anthrahydroquinone- β -sulphonate buffered at p_H 7.7. Correction, 0.08 ml.*

	Titre ml.	Titre (corr.) ml.	$0.03006 \log \frac{S_r}{S_o}$	E_h	E'_0
(a)	0.25	—	-0.0408	+0.4253	+0.3845
	0.50	—	-0.0312	0.4162	0.3850
	1.00	—	0.0208	0.4062	0.3854
	1.70	—	0.0119	0.3973	0.3854
	2.52	—	-0.0040	0.3891	0.3851
	3.31	—	+0.0030	0.3820	0.3850
	4.30	—	0.0127	0.3723	0.3850
	5.01	—	0.0222	0.3629	0.3851
	5.50	—	0.033	0.3532	0.386
	5.93 (end-point)	—	—	—	—
	5.96	—	—	0.331	—
	6.06 (colourless)	—	—	0.318	—
	6.15	—	—	0.302	—
	6.27	—	—	0.234	—
(b)	0.25	0.17	-0.0448	+0.0669	+0.0221
	0.50	0.42	0.0324	0.0543	0.0219
	1.00	0.92	0.0200	0.0427	0.0219
	1.72	1.64	0.0109	0.0330	0.0221
	2.50	2.42	-0.0028	0.0249	0.0221
	3.50	3.42	+0.0070	0.0153	0.0223
	4.30	4.22	0.0164	+0.0057	0.0221
	4.80	4.72	0.0249	-0.0025	0.0224
	5.19	5.11	0.0366	0.0116	0.025
	—	5.42 (end-point)	—	—	—
	5.60	—	—	0.024	—
	5.91	—	—	0.033	—

* An artefact due to aggregation of oxidant in the early stage of titration in neutral solution.

Fig. 1. E'_0 - p_H curves for prune and adjacent dyes. Data of Table III ○; with borate ×; with glycine •.

Inset. E'_0 - $\log$ (concentration) curves for prune.

(E'_0 in 0.00025 *M* solution adopted as zero potential.)

p_H 4.43 ○; p_H 1.76 ×; Nile blue at p_H 4.88 and 1.44 - - -.

($p_{Kra_2}=10$). The oxidation-reduction is the ordinary two-electron transfer, $Ox^+ \rightleftharpoons Red^-$. In Fig. 1, the $E_0'-p_H$ curve for prune is compared with adjacent indicator systems.

Obviously, prune provides a valuable indicator intermediate between Lauth's violet and the indophenols over the p_H range 2-7, for which region there has hitherto been no indicator chemically stable in solution. At ordinary biological

Table III. *Relation of E_0' to p_H .*

Approx. 0.00015 *M* prune. $E_0 = +0.528$ v. $p_{Kob}=3.60$, $p_{Krb}=5.35$, $p_{Kra}=7.45$, $p_{Koa}=8.05$.

p_H	Reagent	Buffer	E_0' (observed)	E_0' (calculated)	Difference
0.200	Anthr.- β -sulph.	HCl	+0.515	+0.516	-0.001
1.046	"	"	0.465	0.465	0.000
1.417	Iodine "	HCl-phosphate	0.442	0.443	-0.001
1.980	"	"	0.408	0.409	-0.001
2.402	Anthr.- β -sulph.	0.025 <i>M</i> phthalate	0.385	0.383	+0.002
2.683	"	"	0.363	0.365	-0.002
2.886	Quinone	"	0.353	0.352	+0.001
3.097	Anthr.- β -sulph.	"	0.340	0.338	+0.002
3.488	Iodine	"	0.310	0.311	-0.001
3.870	Anthr.- β -sulph.	"	0.283	0.282	+0.001
4.195	"	"	0.255	0.256	-0.001
4.590	"	"	0.225	0.223	+0.002
4.727	Iodine	0.036 <i>M</i> phthalate	0.210	0.212	-0.002
5.290	Anthr.- β -sulph.	0.025 <i>M</i> phthalate	0.168	0.167	+0.001
5.595	"	"	0.146	0.145	+0.001
6.108	"	0.025 <i>M</i> phosphate	0.110	0.111	-0.001
6.492	"	"	0.088	0.087	+0.001
6.938	Quinone	"	0.060	0.061	-0.001
7.032	Anthr.- β -sulph.	"	0.056	0.056	0.000
7.298	"	0.025 <i>M</i> barbitone	0.042	0.042	0.000
7.550	"	0.025 <i>M</i> phosphate	0.029	0.029	0.000
7.698	"	0.025 <i>M</i> barbitone	0.022	0.021	+0.001
7.852	"	0.025 <i>M</i> phosphate	0.012	0.013	-0.001
8.058	"	0.025 <i>M</i> barbitone	+0.004	+0.003	+0.001
8.418	"	"	-0.017	-0.016	-0.001
8.795	"	"	0.037	0.037	0.000
9.261	"	0.025 <i>M</i> carbonate	0.064	0.064	0.000
9.792	"	"	0.099	—	—
9.931	"	"	0.104	—	—
10.265	"	"	0.115	—	—

values of p_H it exists as a neutral molecule as distinct from Lauth's violet, the salt of a base. It has therefore considerable value, at least until replaced by a more soluble indicator of similar range. Its disadvantages arise from its colloidal tendencies and are apparent (a) in the dependence of E_0' on the concentration of dyestuff (inset in Fig. 1) which is of the same magnitude as for the oxazine, Nile blue [Cohen and Preisler, 1931], and (b) in the eventual aggregation of the neutral solution to a precipitate, which is not serious as it is delayed 12-24 hours in concentrations less than 0.0001 *M*. Prune (blue) has a slightly greater tinctorial power than either methylene blue or 2:6-dichlorophenolindophenol (blue).

It is interesting to note that methylation of the free carboxyl group of gallocyanine [Michaelis and Eagle, 1930] to produce prune, gives a more positive indicator, in which the dissociation of phenolic groups is, as expected, considerably enhanced.

Titrimetric estimation of ascorbic acid.

Oxidative titration as an approximate measure of ascorbic acid assumes that only ascorbic acid in its reduced condition (or capable of reduction [Tillmans *et al.*, 1932; Zilva, 1934]) is possessed of antiscorbutic activity. Likewise, it is also

necessary in biological fluids that only ascorbic acid, among reducing substances present, shall be titrated; examples of this difficulty are multiplying. Now, should substances more strongly reducing than ascorbic acid be present, it is clearly impossible for direct oxidative titration to give reliable results. On the other hand, if these substances are weakly reducing, then increase of specificity will follow the use of as negative an oxidising agent as possible, a development hitherto hindered by lack of any suitable reagent [Birch *et al.*, 1933].

It was therefore desirable to discover how far the use of 2:6-dichlorophenol-indophenol could now be bettered, or how far oxidative methods were incapable of direct improvement. A feature of the oxidation of ascorbic acid is its lack of thermodynamic reversibility [Green, 1933], whence there may be expected a somewhat empirical behaviour with oxidising agents or a considerable time lag in the occurrence of possible reactions (see note, p. 1574). For example, the reduction of methylene blue by ascorbic acid is possible anaerobically at p_H 7 [Green, 1933], but is insufficiently facile for adaptation to titrimetric ends.

In a study therefore of the reaction of lemon juice with selected dyes, results were obtained as follows:

(a) The degree of reduction at given p_H in a test-tube at room temperature was estimated by adding 1 ml. 0.001 *M* dye (0.4 equiv.) to a mixture of 3 ml. 0.05 *M* buffer + 1 ml. lemon juice + requisite NaOH + water to 9 ml., and comparing with control colour dilutions. *Methylene blue* under these conditions became slowly reduced to about 50 % at p_H 1, but only slightly reduced at p_H 7. *Lauth's violet*, acid to p_H 4, reached a steady state in 20 mins. at about 70 to 80 % reduction, but alkaline to p_H 5 showed only slight visible reduction. *Gallocyanine* showed optimum reduction (70 to 80 %) at p_H 3 and 4 in 15 mins.; at p_H 2 reduction appeared steady at 50 % in 30 mins., but alkaline to p_H 5 reduction was only slight. *Prune* was fully reduced between p_H 2 and 6, the maximum rate (10 mins.) being at p_H 3 and 4; at p_H 1 and p_H 9 reduction was incomplete or else very slow. *1-Naphthol-2-sulphonic acid indophenol*, like *prune*, was reduced only slowly. *Thymolindophenol* and *2:6-dichlorophenolindophenol* were reduced almost immediately.

(b) In developing these findings for titrimetric adaptation, they were repeated in hot solution under CO_2 , using 1 ml. 0.001 *M* dye added to 1 ml. lemon juice suitably neutralised. *Lauth's violet* now reacted in 2 mins., reaching (in absence of oxygen) 90 % reduction at p_H 1, and 95 to 98 % at p_H 3 and 4. *Gallocyanine* at p_H 4 became rapidly 80 to 90 % reduced. *Prune* was almost immediately reduced at p_H 3, and reduced in 5 mins. at p_H 1. At p_H 4, reduction was immediate but not quite complete. In general, the dyes appeared to reach an equilibrium with the lemon juice, the equilibrium being more slowly attained but favouring greater reduction the cooler the solution. Using ascorbic acid itself in various instances, no essential difference was found. It seems therefore that the behaviour of the dyes examined is mutually quite consistent with their positions in Fig. 1, but that the reactivity of ascorbic acid is such that *prune* is as weak an oxidising agent as can suitably be used (see note, p. 1574).

Procedure. The following procedure should therefore provide optimum specificity in titration.

Place an aliquot of the extract for examination in a short test-tube under an atmosphere of CO_2 for titration by 0.0004 *M* *prune*—from the stock 0.004 *M* solution in 0.05 *M* HCl. After a small addition of *prune*, adjust the p_H to about 3 (a bluish red) with acid or alkali, heat almost to boiling, and continue the titration under a stream of CO_2 . The *prune* solution of course must be substantially free of all more negative coloured impurities if sharp titration is to be attained.

Under these conditions, prune was not reduced by quinol or pyrogallol, and only slowly by cysteine (especially in the presence of cyanide—cation). The reaction of lemon juice with positive dyes was not significantly catalysed by iron or inhibited by cyanide. Yet it was interesting that well-boiled lemon juice (which did not reduce prune) was found to give immediate reduction on addition of ferrous iron; this however was due to enhancement of the reducing intensity of the iron consequent upon co-ordination. Potassium ferricyanide may be used to titrate lemon juice at p_H 4 with prune as indicator, though titration is tedious since the solution may not be warmed.

Comparison of titration methods. Although the above technique is thus less straightforward than for 2:6-dichlorophenolindophenol, the use of prune has the practical advantages (a) that its acid tinctorial power is roughly three times that of acid indophenol, and (b) that it is satisfactorily stable under the conditions of test. Now, even though Birch *et al.* [1933] note that their titration in 5 % trichloroacetic acid must be accomplished in 2 mins., the point still needs strong emphasis. It was found, using dilutions of 2:6-dichlorophenolindophenol at p_H 4 as colour controls, that 0.0002 *M* indophenol in 4 % trichloroacetic acid was half decolorised in 50 seconds, 75 % in 100 seconds and fully in 5 mins., simply as a result of acid decomposition of the dye. Similar results were obtained for other strong acids and other indophenols. There was however considerable improvement with more dilute or weaker acids. The rate of fading grew insignificant only when acidity was less than p_H 2.5 (*e.g.* using acetic acid). In contrast, dilute solutions of prune are stable for many hours under the experimental conditions at p_H 3 and p_H adjustment is easy because prune is its own indicator.

In Table IV, comparison of various titration methods is made on fresh, centrifuged lemon juice (kept in CO_2), using titrants standardised against each other and ferric iron *via* a solution of anthrahydroquinone- β -sulphonate. Prune gives titres which are consistently smaller than those by other reagents. The disparity is not due to the presence of citrates, nor is it the result of hot titration

Table IV. *Comparison of titration methods on lemon juice.*

(a) Titres as ml./ml. lemon juice.

	0.0040 <i>M</i> $K_3Fe(CN)_6$	0.0020 <i>M</i> 2:6-Indo.*		0.0018 <i>M</i> prune (hot, in CO_2)
		(in air)	(in CO_2)	
Sample A, p_H 4	1.38–1.41	1.37	1.43	1.31
	1.38–1.40	1.39	1.43	1.33
	—	1.38	—	—
	0.001 <i>M</i> iodine	0.00043 <i>M</i> 2:6-Indo.		0.00041 <i>M</i> prune
Sample B, p_H 4	—	5.60	5.76 5.74 5.82 (hot)	4.8–5.0
p_H 3	6.60	—	5.76 (hot)	5.00 4.96
0.005 <i>M</i> cysteine in sample B	—	5.84	—	5.6 (fades) 5.5 (fades)

(b) Titratable "ascorbic acid" in lemon juice.

	Iodine	$K_3Fe(CN)_6$	2:6-Indo.	Prune
Sample A	—	0.0028 <i>M</i>	0.0028 <i>M</i>	0.0024 <i>M</i>
Sample B	0.0033 <i>M</i>	—	0.0025	0.0020
Data of Johnson [1933]	0.0034	—	0.0024	—
"	0.0051	—	0.0041	—

* 2:6-Dichlorophenolindophenol.

(*cf.* indophenol titres). Consequently it seems certain that prune, by reason of its more negative potentials, is able to eliminate from titration certain substances to which the other reagents are sensitive.

On the other hand, a disadvantage of prune titration is that its longer duration makes it more susceptible to interference by cysteine in bulk. Moreover, prune, even though less easily reduced than indophenols, will still be reducible by dialuric acid, lactoflavine, various natural pigments *etc.* [Bierich *et al.*, 1934; Barron and Hastings, 1934; Friedheim, 1934, Table VI]. Likewise, it is reducible by the brain extract of Young and Mitolo [1934] (to whom we are indebted for a sample). Unfortunately, then even though prune is as negative a titrant as it is convenient to use, it cannot offer really emphatic advantages in specificity.

In conclusion therefore we find in prune a deeply coloured, comparatively stable, readily procured titrating agent, which allows the maximum possible specificity in the direct oxidative titration of ascorbic acid. Its rather negative potential range necessitates its use in an inert atmosphere and in hot solution to accelerate the reaction. Although it thus represents the limit to which oxidising agents may be weakened in titrating ascorbic acid, it is not weak enough to eliminate various other biological reducing agents from the titration. In consequence, it offers a slight though not emphatic advantage over 2:6-dichlorophenolindophenol, but further emphasises that direct oxidative titration of "ascorbic acid" in biological fluids must be regarded with suspicion.

SUMMARY.

The electrode equation for the oxazine dye, prune [Rowe, 1924, No. 893], has been determined for the p_H range 0 to 10. As an oxidation-reduction indicator, it usefully occupies the region intermediate between Lauth's violet and the indophenols ($E_0 = +0.528$ v.). It is most useful in acid ranges, since neutral solutions show colloidal tendencies and become aggregated on keeping.

As a reagent for the titration of ascorbic acid (warmed at p_H 3 under CO_2), it represents the limit to which oxidising agents may be weakened without hindering successful titration. It is however not weak enough to eliminate various other biological reducing agents from the titration. Oxidative titration methods must continue to be regarded as having only tentative significance.

The authors wish to thank Prof. A. C. Chibnall for providing every facility for their work. One of us (J. M.) is indebted to the University of New Zealand for a scholarship, the other (G. M. R.) to the Department of Scientific and Industrial Research for a research grant.

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Note added July 26th, 1934. Borsook and Keighley [1933] hold that the oxidation of ascorbic acid is thermodynamically reversible, measurements being confused by negative drifts due to instability of dehydroascorbic acid. Their potential values (positive of course to those of Fig. 1) overlie, alkaline to p_H 5, those for Lauth's violet. This view accords well with our indicator studies and still accounts for the delayed reduction of more negative indicators (2 to 18 days [Fruton, 1934]). Interference with the indophenol titration by cysteine has been considered by Emmerie [1934], and with special reference to p_H control, by Martius and Euler [1934].