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CO-FACTOR SPECIFICITY OF PLANT ALCOHOL DEHYDROGENASE

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Key Word Index—*Cucuumis melo*; Cucurbitaceae; Angiosperms; alcohol dehydrogenase; cofactor specificity; enzymic properties.

Abstract—Extracts of seventeen plant tissues show alcohol dehydrogenase activity in the presence of both NADH and NADPH. Using extracts of melon fruits, attempts have been made to separate these two activities by applying a range of chromatographic and electrophoretic techniques but these proved unsuccessful. Evidence from kinetic measurements involving assays of equimolar concentrations of the two co-factors suggests that in the enzyme from the melon there is but a single catalytic site which will accept either co-factor.

INTRODUCTION

THE ENZYME, alcohol dehydrogenase (ADH), is present in a wide range of biological materials and has been shown in most cases to be specific for NAD^+ (i.e. alcohol:NAD^+ oxidoreductase E.C. 1.1.1.1).[1] There are also a number of reports of the corresponding $NADP^+$-dependent alcohol dehydrogenase (alcohol:$NADP^+$ oxidoreductase E.C. 1.1.1.2) which are nearly all related to work with micro-organisms.[2–5] The position is confused by the fact that some of the well characterized NAD^+-dependent alcohol dehydrogenases, e.g. from horse liver[6] and from wheat germ,[7] show some activity towards $NADP^+$, although this is only of the order of 1 % of the activity given by an equimolar concentration of NAD^+.

We have studied acetaldehyde reduction and have found that extracts prepared from a range of higher plant tissues show activity towards both NADH and NADPH. In fact when assays are run at pHs close to 6, with most of the tissues the rate of oxidation of NADPH was greater than that with NADH. This finding suggested the possibility, originally proposed by Stafford and Vennesland,[7] that plants contain two ADH's, one specific for NAD^+ and one for $NADP^+$. The present paper describes unsuccessful attempts to resolve the NADH and NADPH dependent activities and additional kinetic evidence which suggests that in the tissue studied there is only one ADH which will accept either pyridine nucleotide.

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RESULTS

Purification and Fractionation of the Enzymic Activity

Table 1 shows a comparison of the alcohol dehydrogenase activities of crude extracts of a number of plant tissues measured using either NADH or NADPH as co-factor. The table shows that at pH 5·8, normally recommended for the assay of acetaldehyde reduction,[1] the extracts of most of the plants showed greater activity towards NADPH than towards NADH under the experimental conditions. The ratio of activities was as high as 8:1 in the case of the tomato and fell below 1:1 in two cases, namely carrot and parsnip.

TABLE 1. THE ACTIVITY OF ALCOHOL DEHYDROGENASE IN CRUDE EXTRACTS OF SEVERAL PLANT MATERIALS

Plant tissue	E.u.*/10 g Fr. wt. co-factor NADH	NADPH	Plant tissue	E.u.*/10 g Fr. wt. co-factor NADH	NADPH	Plant tissue	E.u.*/10 g Fr. wt co-factor NADH	NADPH
Apple fruit	200	450	Grapefruit flavedo	200	284	Parsnip	1860	820
Avocado pulp	250	1240	Lemon flavedo	264	384	Potato	106	312
Banana	42	132	Lettuce leaf	NM	20	Spinach leaf	32	38
Brussels Sprout	NM	14	Melon flesh	160	765	Swede root	390	1800
Carrot	52	44	Onion	NM	20	Tomato fruit green	193	1600
Cauliflower florets	96	340	Orange flavedo	126	236	Tomato fruit red	320	2480

10 g of each of these tissues was homogenized in 30 ml 0·2 M Tris, 0·001 M EDTA, 0·25 M sucrose, 1% PVP pH 7·5 in an ultra turrax homogenizer for 1 min. The extract was separated by centrifugation and the residue washed with a further 10 ml of extraction buffer. The combined extract and washings were made to 50 ml and used for the ADH assays.

NM—not measurable.

* The enzyme unit is defined as the amount of enzyme which will promote a change in absorbance at 340 nm of 0·1 units per min under the defined assay conditions.

TABLE 2. THE PARTIAL PURIFICATION OF ALCOHOL DEHYDROGENASE FROM MELON FRUIT TISSUE

Stage of purification	Total units NADP	NAD	Total* protein content mg N	Specific activity E.u./mg protein N NADP	NAD	Yield NADP	NAD	Purification NADP	NAD
Clarified extract	30 000	—	35·4	850	—	100	—	1	—
	—	4950		—	140	—	100	—	1
55–75% $(NH_4)_2SO_4$ fraction	27 000	—	12·3	2200	—	90	—	2·6	—
	—	5000		—	406	—	100	—	2·9
Bulked fraction after DEAE-cellulose chromatography	21 000	—	2·95	7100	—	70	—	8·3	—
	—	5400		—	1860	—	100	—	13·3
Bulked fraction after G100 chromatography	10 200	—	1·2	8500	—	34	—	10·0	—
	—	4700		—	3900	—	95	—	28

* Protein determinations by measurement of the TCA insoluble nitrogen.[24]

Table 2 shows details of the partial purification of alcohol dehydrogenase from the flesh of the melon fruit. After fractionation with ammonium sulphate, chromatography on DEAE-cellulose and on Sephadex G100, a final purification of about 28-fold for NADH and 10-fold for NADPH was obtained. In general, the NADH activity showed considerable stability while NADPH dependent activity was lost at each stage in the purification. Both the NADH and NADPH dependent activities are precipitated with $(NH_4)_2SO_4$ at 55–75% saturation and do not separate when eluted in a linear gradient from DEAE-cellulose columns or when subsequently run on columns of Sephadex G100 (see Fig. 1). Further chromatography of the eluate from G100 on Sephadex G150 did not resolve the two pyridine nucleotide dependent activities.

Further attempts to resolve the two activities were made by subjecting the active ammonium sulphate fraction (55–75% saturation) to electrophoresis in the continuous electrophoresis apparatus of Hannig.[8] The separation was carried out in 0·1 M Tris-acetate pH 8·3 at 4° and again, although the alcohol dehydrogenase activity separates from the bulk of the protein in the fraction, no resolution between the two pyridine nucleotide specific reactions was obtained. The active fractions after chromatography on DEAE-cellulose and on Sephadex G100 were also subjected to discontinuous gel electrophoresis on rods of polyacrylamide gel at two pHs 7·4 and 8·3. The pattern of protein bands after electrophoresis was displayed by staining with Coomassie blue and the alcohol dehydrogenase located by the standard techniques. The activities in the presence of NAD^+ and $NADP^+$ were found in the same position in the gel under all conditions of electrophoresis employed.

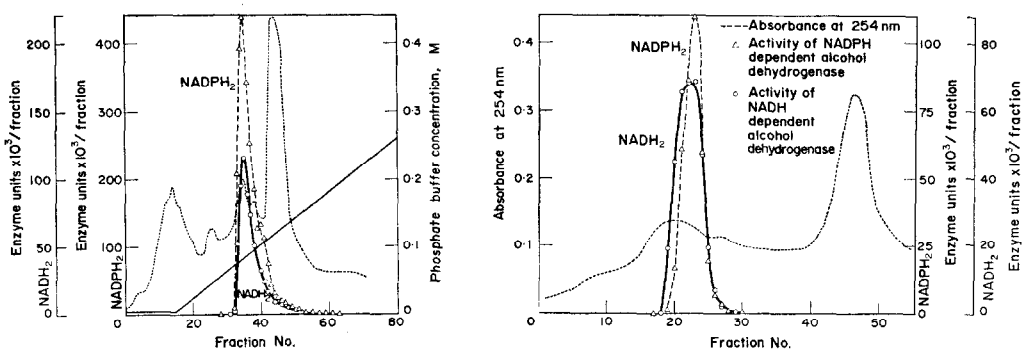

FIG. 1. (a) THE SEPARATION OF THE 55–75% $(NH_4)_2SO_4$ FRACTION ON DEAE-CELLULOSE USING A LINEAR GRADIENT OF POTASSIUM PHOSPHATE, AND (b) THE SUBSEQUENT CHROMATOGRAPHY OF THE ACTIVE FRACTION FROM DEAE-CELLULOSE ON SEPHADEX G100.

The Properties of the Enzyme Activity

The pH optimum (Fig. 2) for the alcohol dehydrogenase from melon measured in the direction of aldehyde reduction is 5·70 when either NADH or NADPH is used as the co-factor. The NADH activity has a much broader range of activity than the NADP dependent activity which occurs only at pHs below 7. When ethanol oxidation is studied with NAD^+ as the co-factor the optimum is close to pH 9·5 while with $NADP^+$ the activity is low and the pH optimum was not accurately determined.

It has been shown that both the NADH and NADPH dependent activities are inhibited

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by *p*-chloromercuribenzozte at a concentration of 5×10^{-4} M. *o*-Phenanthroline, a potent inhibitor of horse liver alcohol dehydrogenase,[1] is only a weak inhibitor of the ADH from melon. At a concentration of 10^{-3} M inhibition was observed only after relatively long periods of pre-incubation of the enzymes with inhibitor and even with a 3-hr pre-incubation inhibition only reached 30%. The activity with either pyridine nucleotide was inhibited to a similar extent.

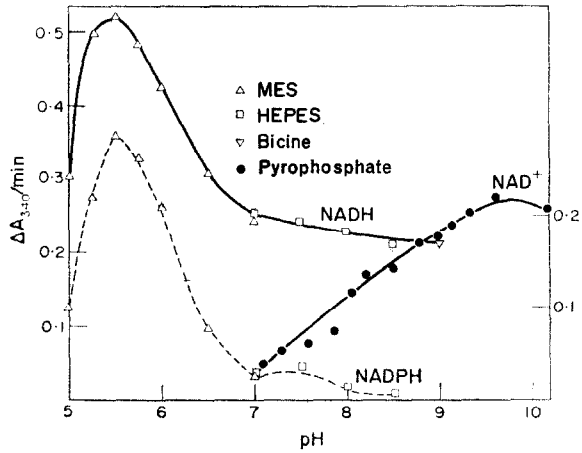

FIG. 2. THE pH CURVES FOR ACETALDEHYDE REDUCTION USING EITHER NADH OR NADPH AS CO-FACTOR AND FOR ETHANOL OXIDATION USING NAD^+ AS CO-FACTOR. BUFFERS USED MES, HEPES, BICINE AND PYROPHOSPHATE.

Kinetic Evidence

The failure of the attempts to separate the two ADH activities suggests that there is only one ADH protein accepting either co-factor but this evidence is inconclusive. We have used kinetic measurements in an attempt to resolve this problem and, in doing so, have encountered the problem, noted by other workers,[9,10] of the presence of inhibitors of dehydrogenase activity in many commercial samples of NADH. Dalziel[10] showed that the decomposition of NADH under moist conditions led to the formation of a compound which inhibited horse liver but not yeast ADH and which was particularly inhibitory at about pH 6·0. The presence of inhibitor in an NADH preparation is indicated by a high ratio of E_{260}/E_{340} (greater than 2·6) and a high residual absorption at 340 nm after complete enzymic oxidation of the co-factor (greater than 2% of the initial E_{340}). We found that our melon ADH preparation was sensitive to the presence of inhibitors in either old or 'maltreated' samples of NADH. Two effects were noted with a sample of NADH which had been stored in a refrigerator for about 18 months since purchase and was slightly yellow in colour. This gave a lower V_{max} at 5·70 compared with a freshly purchased sample and also gave an apparent shift in the pH optimum of the enzyme to pH 8·0. This can be explained by the presence of an inhibitor particularly active at acid pHs. The old NADH sample had an E_{260}/E_{340} ratio of 2·84 and a residual absorption at 340 nm of 7·5%. It is now possible

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to purchase samples of NADH which are claimed to be free of inhibitors. This material gives an optimum pH value for both co-factors at 5·70, but the material on storage under moist conditions for 2 days at 0° forms some inhibitor since there is a reduction in the reaction rate at the pH optimum and the appearance of a second pH optimum at about 8·0 (see Fig. 3). For all the subsequent kinetic studies only inhibitor free co-factors were used. These were stored in a desiccator at 0–4° and used within 1 week of purchase.

Table 3 shows the K_m values for the enzyme purified from melon tissue for both pyridine nucleotides and for acetaldehyde. The affinity of the enzyme for NADH is more than 10 times that for NADPH even though the maximum velocity is 3 times greater when NADPH is the co-factor. The affinity of the enzyme for the substrate acetaldehyde is 6 times greater when NADH is the co-factor compared with NADPH.

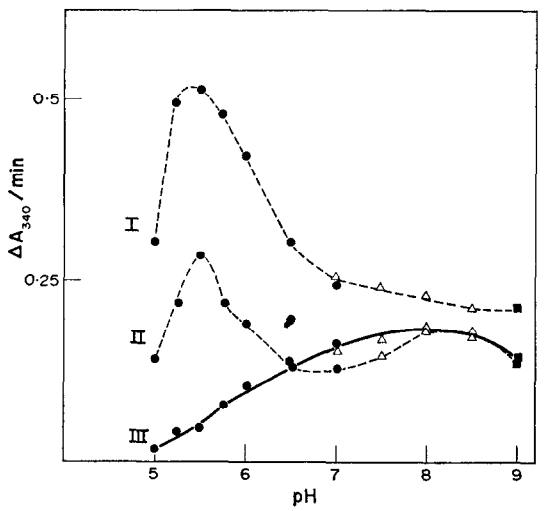

FIG. 3. THE EFFECT OF pH ON THE ACTIVITY OF MELON ADH MEASURED USING I, GRADE I NADH; II, GRADE I NADH WHICH WAS STORED UNDER MOIST CONDITIONS FOR 2 DAYS AT 0° AND III, GRADE II NADH WHICH HAD BEEN STORED FOR 18 MONTHS AT 0° PRIOR TO USE. BUFFERS AS SHOWN IN FIG. 2.

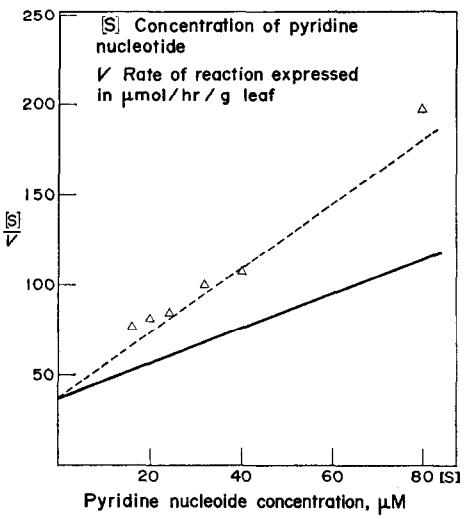

FIG. 4. THE KINETICS OF ADH ASSAYS EMPLOYING A RANGE OF CONCENTRATIONS OF EQUIMOLAR MIXTURES OF NADH AND NADPH (△). THE CONTINUOUS LINE REPRESENTS THE KINETICS PREDICTED ASSUMING THAT THE NADH AND NADPH ACT AT SEPARATE INDEPENDENT CATALYTIC SITES WHILE THE DISCONTINUOUS LINE REPRESENTS THE KINETICS ASSUMING A SINGLE CATALYTIC SITE FOR WHICH NADH AND NADPH COMPETE.[11] K_m AND V_{max} AS SHOWN IN TABLE 3.

Both the NADH and NADPH dependent activities follow the Michaelis–Menten type of kinetics when assayed separately. In the presence of a wide range of equimolar mixtures of NADH and NADPH, the rates of reaction observed are considerably lower than the sum of the rates with the two acceptors assayed separately. The experimental data are, however, in good agreement with the model described by Dixon and Webb[11,12] assuming mutual competition between the two co-enzymes for the same active enzymic site. Figure 4 shows the experimental values obtained in the assay of ADH activity with a range of equimolar concentrations of NADH and NADPH. The same concentrations of NADH and NADPH were also used separately in similar assays and the values of K_m and V_{max} determined. These

data were used to calculate the expected kinetics if, (1) there were two separate and independent catalytic sites, one accepting NADH and the other NADPH, i.e. the rates given by equimolar mixtures of co-factors should be the sum of the rates given by the two co-factors assayed separately. This datum is shown as a continuous line in Fig. 4, and (2) there was a single catalytic site for which the two co-factors competed. The theoretical treatment of the situation is given by Dixon and Webb[11] and the predicted kinetics for this mutual competition between the two co-factors is shown as a discontinuous line. The experimental points clearly fit closely to this model.

TABLE 3. KINETIC PROPERTIES OF ALCOHOL DEHYDROGENASE PURIFIED FROM MELON FRUITS

K_m	Co-factor	K_m (M)	V_{max} (μmol co-factor oxidized/ min/g fr. wt.)
Co-factor	NADH	$2{\cdot}4 \times 10^{-5}$	5·2
	NADPH	$3{\cdot}2 \times 10^{-4}$	14·9
Acetaldehyde	NADH	$1{\cdot}3 \times 10^{-3}$	—
	NADPH	$8{\cdot}8 \times 10^{-3}$	—

DISCUSSION

The physical and enzymic properties of the alcohol dehydrogenase from yeast and horse liver have been extensively studied.[1,13,14] Similar detailed studies on the enzyme isolated from higher plant tissues have yet to be made. With a number of plant tissues, instability of the enzyme has led to difficulty in its purification. However, partially successful attempts to purify the enzyme from pea tissue,[15–17] maize[18] and peanut kernels[19,20] have been made and the results of these studies suggest that the plant ADH resembles in general terms its counterpart in mammalian and yeast cells in being a metalloprotein (probably containing Zn^{2+})[20] and requiring -SH groups for its catalytic activity. It shows a low specificity for substrate[16] acting on a wide range of alcohols and aldehydes but a much higher specificity towards its co-factor. The enzymes from peanut kernels[20] and pea pods[16] are specific for NAD^+ while those from barley embryos[21] and wheat germ[7] accept both co-factors. However, the rates of $NADP^+$ reduction with the enzyme from these sources is only 1 % of that given for NAD^+ reduction. The enzyme from barley embryos[21] shows a very low affinity for $NADP^+$ in the oxidation of ethanol (K_m 0·19 M) compared with NAD (K_m $3{\cdot}1 \times 10^{-3}$ M). The yeast enzyme is inactive with $NADP^+$ while the horse liver enzyme will accept either co-factor but gives only very low rates with NADPH.[6]

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Very few studies on the co-factor specificity of plant ADHs in aldehyde reduction have been made but Stafford and Vennesland,[7] working with extracts of wheat germ, showed that the enzyme is capable of using both NADH and NADPH for the oxidation of aldehydes and suggested that there might be two enzymes in wheat germ, one NAD^+ and the other $NADP^+$ dependent. Our finding that a number of plant tissues contain alcohol dehydrogenase activity with high activity towards NADPH at about pH 6·0 suggested that there might, in fact, be two separate specific ADH's in these tissues. Although there appears to be a difference in the stability of the two activities, our exhaustive attempts to separate the activities using various chromatographic and electrophoretic techniques have failed, but this does leave open the possibility that the active sites for NAD^+ and $NADP^+$ are contained in protein moieties of very similar chemical and physical properties. The fact that the $NADP^+$ activity is more labile than that for NAD^+ does not necessarily indicate the presence of two separate enzymes. It could be that during the isolation there is a change in the physical environment of a single active centre which hinders the access of $NADP^+$ to the catalytic site. The properties of the enzyme in relation to inhibitors and to pH are very similar for each co-factor. However, the reaction with $NADP^+$ is active for aldehyde reduction at acid pH but proceeds only very slowly for ethanol oxidation which suggests that the NADP catalysed reaction is not easily reversible. The rate of reaction catalysed by the pyridine nucleotides present in equimolar concentrations is lower than the sum of the rates catalysed by the pyridine nucleotides individually. In fact, the rate of the combined assay is between the rates given by the two pyridine nucleotides individually and this clearly suggests the presence of a single enzymic site having affinity for both NAD^+ and $NADP^+$. Quantitative analysis of the experimental data obtained for mixed nucleotide assays fits well with the model for an enzyme having more than one substrate or co-factor. These kinetic data strongly suggest that in the melon there is a single alcohol dehydrogenase which will preferentially accept $NAD^+(H)$ but under certain conditions will also accept $NADP^+(H)$.

EXPERIMENTAL

Assay of enzymic activity. The reduction of acetaldehyde to EtOH was followed spectrophotometrically by measuring changes in absorbancy at 340 nm of a reaction mixture containing 200 μmol MES (2-[N-morpholino]ethane sulphonic acid) buffer pH 5·75, 250 μg NADH or NADPH, 170 μmol acetaldehyde, enzyme and H_2O in a final vol. of 3·0 ml. The reaction was run at 25° and was initiated by the addition of acetaldehyde. Freshly re-distilled AnalaR acetaldehyde was used throughout and in the experiments in which the enzyme kinetics were studied Grade 1 reduced pyridine nucleotides (Boehringer, Mannheim) which are free of dehydrogenase inhibitors were used in freshly prepared solutions. For the work on the localization of the enzyme activity during chromatography or electrophoresis freshly purchased Grade II nucleotides were used. The spectrophotometric method of Bonnichsen and Brink[22] was used for the measurement of the NAD or NADP dependent oxidation of EtOH.

Purification of the enzyme activity from melon fruits. 400 g of melon fruit flesh were homogenized in 500 ml of a medium containing 0·2 M Tris, 0·001 M EDTA, 0·25 M sucrose, 10 mM mercaptoethanol pH 8·0. The homogenate was filtered through muslin and clarified by centrifugation at 25 000 *g* for 20 min. The supernatant was fractionated with $(NH_4)_2SO_4$ and the fraction between 55 and 75% saturation was resuspended in 20 ml 0·005 M potassium phosphate, 10 mM mercaptoethanol pH 7·5 and dialysed overnight against the same buffer. The dialysed fraction was applied to a column (25 × 1·5 cm) of DEAE cellulose and eluted with a linear gradient between 0·005 and 0·4 M potassium phosphate pH 7·5. The fractions of eluate were assayed for alcohol dehydrogenase activity and for absorption at 254 nm. The active fractions were bulked and an aliquot applied to a column (36·5 × 2·5 cm) of Sephadex G100 (superfine) equilibrated with 0·1 M potassium phosphate, 0·1 M KCl pH 7·5. The column was eluted with the same buffer and the activity of the resulting fractions assayed in the usual way. Subsequently an aliquot of the active fraction

[22] R. K. Bonnischsen and N. G. Brink, *The Enzymes* (edited by S. P. Colowick and N. D. Kaplan), Vol. 1, p. 495, Academic Press, New York (1955).

from the separation on Sephadex G100 was further fractionated on a similar column of Sephadex G150 using the same elution system.

Electrophoretic study of the isolated enzyme. A sample of dialysed 55–75% saturation $(NH_4)_2SO_4$ fraction from the melon extract was subjected to electrophoresis in the continuous electrophoretic apparatus of Hannig.[8] The electrophoresis was carried out in 0·1 M Tris-acetate pH 8·3 at 1700 V, 170 mA at 0°. The fractions from the separation were assayed for alcohol dehydrogenase activity and protein content using measurement of their UV absorption at 280 nm. The standard methods of polyacrylamide gel electrophoresis developed by Ornstein and Davis[23] were used in the further study of the active fractions after DEAE-cellulose and Sephadex G100 chromatography. 7% gel rods were prepared in the standard fashion using Tris-HCl as the gel buffer and Tris-glycine as the electrophoresis buffer. Electrophoresis was carried out at either pH 7·4 or 8·3 at room temp. for up to 2·5 hr at 2–4 mA/gel. The gels after electrophoresis were stained with 0·25% Coomassie blue in 7% HOAc in 25% MeOH to show the localization of the protein bands. For the localization of the alcohol dehydrogenase activity, gels were incubated at room temp. for 5 min in a reaction mixture containing 300 μmol HEPES pH 8·0, 1·25 mg nitroblue tetrazonium, 1·33 mg phenazine methosulphate, 0·17 ml EtOH, 2 mg NAD^+ and H_2O to 4 ml. A deep purple band of formazan was deposited on the gel in the position of the alcohol dehydrogenase. Owing to the lower activity of the $NADP^+$ dependent activity, incubation times of 30–45 min were generally necessary for the formation of sufficient formazan to localize the enzymic activity with $NADP^+$ as co-factor.

Acknowledgements—I wish to acknowledge the excellent technical assistance during the course of this work by Mr. L. S. C. Wooltorton and Miss S. Edwards.

[23] L. ORNSTEIN and B. J. DAVIES, *Disc Electrophoresis*, Eastman Kodak Co. London (1959).
[24] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).