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EFFECT OF LIGHT AND AGEING ON ENZYMES, PARTICULARLY PHENYLALANINE AMMONIA LYASE, IN DISCS OF STORAGE TISSUE

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Key Word Index—*Solanum tuberosum*; Solanaceae; enzymes of storage tissue; effects of light and ageing; *de novo* synthesis of PAL.

Abstract—The effect of ageing on enzyme levels in potato discs has been examined for 17 enzymes. The activity of most enzymes was found to increase on ageing but of the 17 enzymes only phenylalanine ammonia lyase was stimulated by light. Evidence is presented that the changes in enzyme levels are not due to changes in molecular size. In the case of phenylalanine ammonia lyase, evidence is presented supporting the view that there is a *de novo* synthesis of the enzyme in both light and dark. The results are briefly discussed in relation to changes in metabolic patterns which are associated with the ageing process in discs of storage tissue.

INTRODUCTION

IN AGEING discs or slices of storage tissue such as the potato tuber there is a rapid increase in solute uptake and respiration[1] which is associated with increased synthesis of both protein[2] and RNA.[3] Evidence that the enhanced respiration of aged slices is different both qualitatively and quantitatively from that of fresh slices has been deduced from studies of the effects of inhibitors and the relative activities of both the oxidative pentose pathway[4] and tricarboxylic acid[5] cycle. Consistent with the above findings is the reported increase in enzymes associated with carbohydrate dissimilation in potato slices.[6–10]

The pronounced increase in activity of phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5) which occurs in certain plant tissues on exposure to light, has been the stimulus for a number of studies of the enzyme. This light-stimulated PAL activity was first described by Zucker in potato discs[11] and subsequent studies in which the incorporation of radioactive amino acids into PAL was measured in the presence and absence of inhibitors have been interpreted as indicating *de novo* synthesis of the enzyme.[12] We have examined this question

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by using isopycnic density gradient centrifugation to separate enzymes containing heavy and light isotypes.

This method has been applied successfully to the study of other enzymes by Filner and Varner[13] and others.[14–16] In this method use is made of one or more of the isotopes ^{18}O, ^{15}N or ^{2}H which are administered in suitable form, e.g. $H_2{}^{18}O$, to a tissue believed to be engaged in *de novo* protein synthesis. Incorporation of the heavy isotope into newly synthesized protein increases its buoyant density thus allowing it to be distinguished in gradient ultracentrifugation from the unlabelled species. Deuterium may be expected to be incorporated into amino acids readily because of the lability of the α-hydrogen[17] and the hydrogens bonded to the nitrogen atom and the rapid keto-amino acid shuttle that exists in living cells. ^{18}O, on the other hand, can only be combined in new protein if it is incorporated into amino acids arising from hydrolysis of pre-existing proteins, i.e.

$$\text{protein} + (H_2{}^{18}O)_n \rightarrow (R\text{–}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{–}C\text{–}{}^{18}O_2)_n \rightarrow R\text{–}\underset{\displaystyle {}^{18}O}{\underset{\|}{C}}\text{–}NH\text{–}R$$

new protein

In the course of our studies Schopfer and Hock[18] demonstrated phytochrome-mediated *de novo* synthesis of PAL in *Sinapis alba* seedlings by density labelling with deuterium. Later we learned from Smith, Attridge and Iredale[19] (personal communication) that they also were also using deuterium oxide to demonstrate *de novo* synthesis of PAL in light-exposed potato discs.

The present report includes data on the effects of ageing, deuterium oxide and light on PAL as well as other enzymes in potato and swede discs. Oxidative and hydrolytic enzymes as well as enzymes involved in amino acid metabolism, glycolysis and the pentose and tricarboxylic acid cycles have been examined.

RESULTS AND DISCUSSION

The effect of ageing in light or dark on certain enzymes in discs of Pentland Crown potatoes is shown in Table 1. Three enzymes which show a marked increase on ageing either in light or dark are PAL, shikimate dehydrogenase and ribonuclease. PAL activity is specifically increased in light treated discs. Although the levels of other enzymes involved in the further metabolism of cinnamate are reported to be stimulated by light,[20] we did not examine potato discs for these enzymes.

A number of investigators have examined changes in enzyme levels which occur during the ageing of discs. The data of Table 1 adds many more enzymes to the list of those investigated and in Table 2 we present additional data for discs of swedes. Our data on hexokinase, glucose-6-phosphate dehydrogenase and peroxidase are in agreement with those obtained by others[9,10,21] using potato tissue. It is difficult to interpret the changing pattern of enzyme

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TABLE 1. EFFECT OF AGEING ON ENZYMES IN POTATO DISCS

Enzyme	E.C. No.	No. of experiments	Mean activity expressed as % initial activity Light ± S.E.	Dark ± S.E.
Alcohol dehydrogenase	1.1.1.1.	3	225 ± 16	225 ± 16
Lactate dehydrogenase	1.1.1.27	2	115 ± 15	118 ± 6
G-6-P dehydrogenase	1.1.1.49	3	168 ± 18	178 ± 15
Isocitrate dehydrogenase	1.1.1.42	2	200 ± 10	390 ± 40
Malate dehydrogenase	1.1.1.37	2	70 ± 6	86 ± 14
Shikimate dehydrogenase	1.1.1.25	3	565 ± 125	620 ± 115
Hexokinase	2.7.1.1.	1	107	112
Glutamic-OAA transaminase	2.6.1.1.	1	190	186
Aspartic-phenylpyruvic Transaminase	2.6.1.-	1	210	180
PAL	4.3.1.5	4	3500 ± 820	1940 ± 240
Malic enzyme	1.1.1.40	1	80	160
Aldolase	4.1.2.6	1	138 ± 38	137 ± 37
Ribonuclease	2.7.7.16	3	388 ± 33	394 ± 44
Acid phosphatase	3.1.3.2	3	101 ± 14	83 ± 15
Peroxidase	1.11.1.7	3	130 ± 11	130 ± 6
Chlorogenic acid oxidase		1	210	260
Catalase	1.11.1.6	2	61 ± 1	62 ± 5

* Discs maintained in light or dark for 24 hr.

levels. The considerable variation from a 20–30% reduction in malate dehydrogenase to a 35-fold increase in PAL suggests that during the ageing process, the cell is producing enzymes for particular metabolic pathways. Ricardo and ap Rees[22] have suggested a causal relationship between the increased activation of certain enzymes of the pentose phosphate pathway and the increased activity of the pathway in aged tissue. We cannot accept this argument because the activities of most enzymes in a metabolic pathway would appear to exceed the throughput of the pathway. The causal relationship requires that the activity of the enzyme should be rate limiting.

TABLE 2. EFFECTS OF AGEING ON ENZYMES IN SWEDE DISCS

Enzyme	E.C. No.	Mean activity expressed as % initial activity Aged 24 hr ± S.E.	Aged 48 hr ± S.E.	Aged 72 hr ± S.E.
Alcohol dehydrogenase	1.1.1.1	98 ± 8	95 ± 10	90 ± 14
Malate dehydrogenase	1.1.1.37	72 ± 4	70 ± 6	70 ± 10
G-6-P dehydrogenase	1.1.1.49	160 ± 4	290 ± 8	320 ± 24
6-P-gluconate dehydrogenase	1.1.1.44	120 ± 6	150 ± 2	160 ± 15
Glutamate-OAA transaminase	2.6.1.1.	97 ± 12	94 ± 14	91 ± 14

The standard error is based on triplicate determinations.

Despite these reservations it may be noted that our results showing increased activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are in agreement with those of Ricardo and ap Rees—though the large increase in hexokinase reported

[22] C. P. P. RICARDO and T. AP REES, *Phytochem.* **11**, 623 (1972).

by them for carrot slices was not observed in potato slices (see also Refs. 6 and 7). The increased enzyme activities recorded in Table 1 could represent enzyme synthesis or alternatively enzyme modification—for example the aggregation or disaggregation of subunits with a corresponding change in molecular size. To check this possibility protein extracts obtained from initial, light-aged and dark-aged discs were fractionated on Sephadex G200.

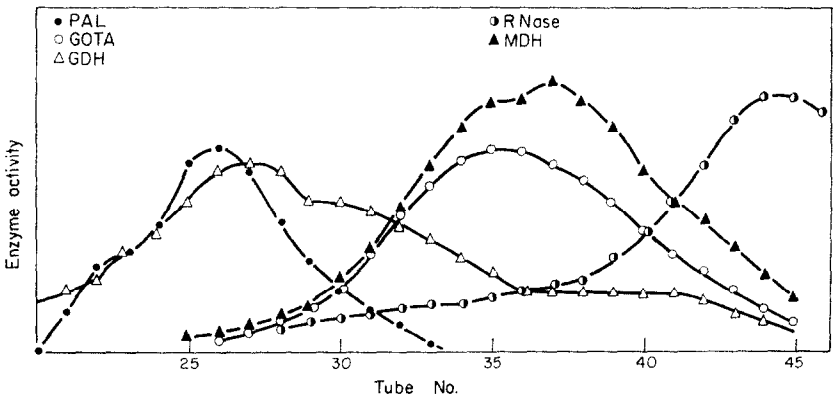

FIG. 1. FRACTIONATION OF POTATO ENZYMES ON SEPHADEX G200.

The elution patterns showed that during the ageing of the discs there was no significant difference in the molecular size of a number of enzymes and specifically this was checked for PAL, shikimate dehydrogenase, alcohol dehydrogenase, ribonuclease and chlorogenic acid oxidase. A typical elution pattern of some potato enzymes is shown in Fig. 1—for clarity

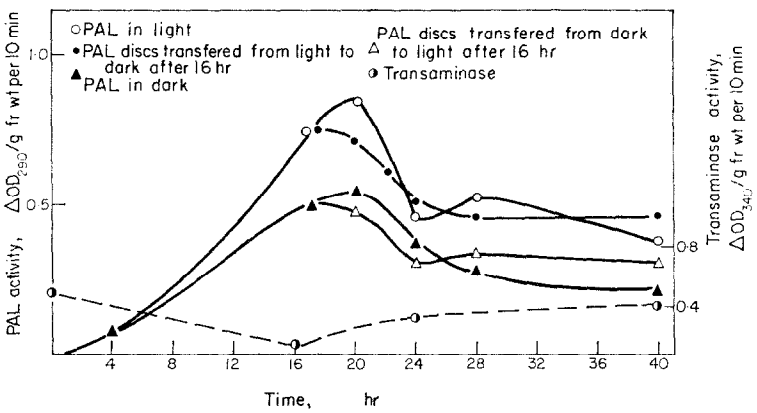

FIG. 2. TIME-COURSE OF PAL AND PHENYLPYRUVIC-ASPARTIC TRANSAMINASE IN POTATO DISCS.

many of the enzymes studied have been omitted from the figure. Time-course studies in the development of PAL in slices of potatoes established that the peak of activity was around 20 hr under the conditions used (Fig. 2). PAL activity increases on ageing in light or dark but there is significant stimulation by light. However, it should be noted that the light stimulation does not occur if given some 16 hr after cutting—in fact a slight decrease in PAL activity

was recorded, though this was reversed after a further 12 hr. Determinations of aspartic-phenylpyruvic transaminase activity in light grown discs showed relatively minor fluctuations in activity. Studies of the development of ribonuclease activity in slices of both Pent- and Crown and Idaho Russet potatoes (Fig. 3) show that activity increases 3- to 4-fold in 11–24 hr and then begins to decline. This parallels the general pattern of development or enzymes associated with carbohydrate catabolism in potato slices during ageing,[7,10] as well as increases in ribonuclease which occur upon cutting and ageing of sections of *Rhoeo* leaves[23] or bean endocarp tissue.[24] In the latter two tissues the increase in ribonuclease is associated with tissue senescence, while the metabolic behaviour of potato slices during 'ageing' is commonly viewed as a rejuvenation phenomenon.[25]

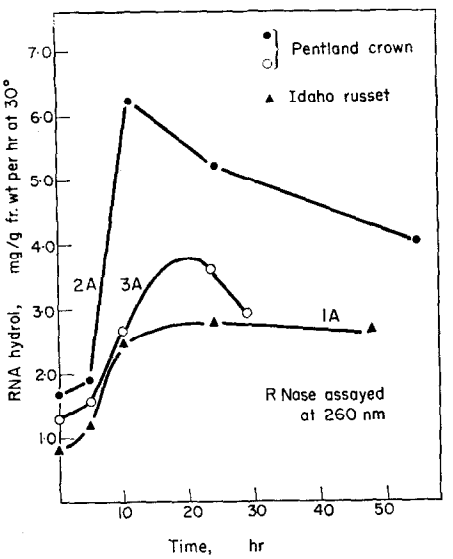

FIG. 3. TIME-COURSE OF RNASE ACTIVITY IN POTATO DISCS.

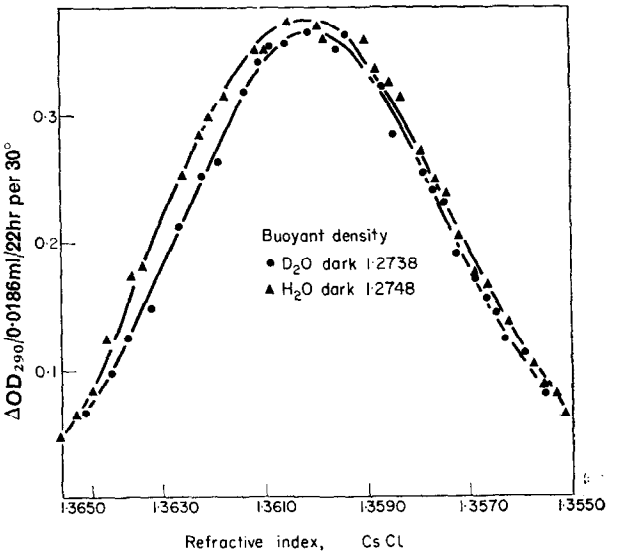

FIG. 4. DISTRIBUTION OF MALIC DEHYDROGENASE IN A CsCl GRADIENT OBTAINED BY ULTRACENTRIFUGATION.

In order to perform the density labelling experiments it was necessary to determine the effect of deuterium on the development of enzymes in ageing discs. Deuterium oxide produced a significant reduction in the level of a number of enzymes present in both light-aged and dark-aged discs (Table 3). However, the maximum inhibition of PAL activity observed (70% inhibition in 94% D_2O) did not preclude the use of density labelling. Table 4 shows the effect of deuterium oxide on a number of enzymes present in dark-aged discs. Enzymes from each sample were assayed at three concentrations and each concentration was assayed in triplicate. The observed inhibition by deuterium oxide could be interpreted as evidence for *de novo* synthesis of enzyme. Accepting this it follows that there is little or no synthesis of malate dehydrogenase during ageing of potato discs—a conclusion which is supported by the density labelling experiments (Fig. 4).

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TABLE 3. EFFECT OF DEUTERIUM OXIDE ON ENZYME LEVELS IN POTATO DISCS

Enzyme	Treatment	Approx.* D_2 conc. in the tissue	% Inhibition
Alcohol dehydrogenase	Light 18 hr	50	0
Malate dehydrogenase	Light 18 hr	50	0
Shikimate dehydrogenase	Light 18 hr	50	0
Shikimate dehydrogenase	Light 18 hr	75	80
Peroxidase	Light 18 hr	50	39
Acid phosphatase	Light 18 hr	50	33
Ribonuclease	Light 18 hr	50	71
Ribonuclease	Light 17 hr	50	71
Chlorogenic acid oxidase	Light 18 hr	75	81
PAL	Light 18 hr	50	12
PAL	Light 18 hr	50	37
PAL	Light 18 hr	50	31
PAL	Light 19 hr	50	52
PAL	Light 18 hr	50	55
PAL	Light 16½ hr	50	0
PAL	Light 18 hr	50	0
PAL	Light 18 hr	75	25
PAL	Dark 18 hr	75	50
PAL	Light 18 hr	94	70

* A final D_2O concentration of 50% in the tissue results from ageing 5 g of discs directly in 5 ml of 99·7% D_2O. Endogenous concentrations of *ca.* 75 and 94% were obtained by washing the tissue once or 3 times respectively for 30 min in 99·7% D_2O before transferring to the 99·7% D_2O ageing solution (see Experimental).

The results of the density labelling experiments presented in Table 5 show that in every experiment there was an increase in the buoyant density of PAL. An example of the separation obtained is shown in Fig. 5. In the particular experiment which is graphically represented here the buoyant density increased as a result of the treatment either in light or dark. In the same experiment malate dehydrogenase did not show an increase in buoyant density during the incubation (Fig. 4) and served, therefore, as an internal marker.

TABLE 4. EFFECT OF AGEING AND DEUTERIUM OXIDE* ON ENZYMES IN POTATO DISCS

Enzyme	Mean activity expressed as† % initial activity: Light 24 hr	Dark 24 hr	Dark deuterium 24 hr	% Inhibition
Alcohol dehydrogenase	188 ± 2	207 ± 5	126 ± 6	39
Malate dehydrogenase	77 ± 2	72 ± 0	77 ± 4	—
Glutamic-OAA transaminase	128 ± 3	135 ± 4	110 ± 0	7
G-6-P dehydrogenase	160 ± 3	163 ± 0	129 ± 2	21
Isocitrate dehydrogenase	228 ± 28	250 ± 11	194 ± 6	22
Malic enzyme	112 ± 4	111 ± 2	93 ± 1	16
Shikimate dehydrogenase	538 ± 32	510 ± 24	487 ± 16	5
Aldolase	247 ± 4	277 ± 4	366 ± 10	—

* Final concentration of D_2O in tissues estimated to be 50%.
† Means and standard errors for triplicate samples of tissue.

We therefore conclude that there is *de novo* synthesis of PAL during the ageing process, and, moreover, light is not essential for *de novo* synthesis. It remains to be established whether *de novo* synthesis also proceeds during the phase following the initial rise in PAL activity in light, i.e. when PAL activity declines.

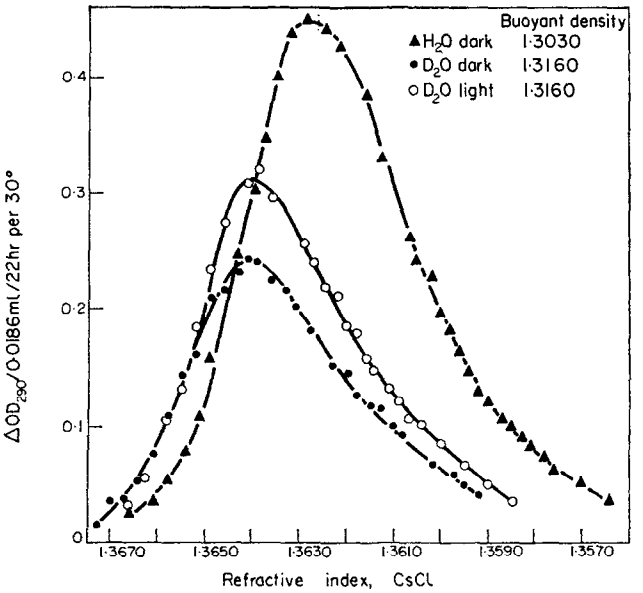

FIG. 5. DISTRIBUTION OF PAL IN A CsCl GRADIENT OBTAINED BY ULTRACENTRIFUGATION (SAME RUN AS IN FIG. 4).

The variation in buoyant densities of the enzyme extracted from discs maintained on water is not easy to understand unless the enzyme is associated with variable amounts of non protein material, e.g. carbohydrate. The average buoyant density reported for PAL in this paper is 1·312 kg/l. the mean of two determinations reported by Smith *et al.* is 1·307 kg/l. whereas the buoyant density of PAL isolated from *Sinapis alba* by Schopfer and Hock is 1·257 kg/l.

TABLE 5. EFFECT OF DEUTERIUM OXIDE ON ENZYME LEVEL AND BUOYANT DENSITY OF PAL IN POTATO DISCS

Duration of treatment (hr)	% Inhibition	Density H enzyme	Density D enzyme	Increase in density (%)	Potato variety
18	51	1·3115	1·3154	0·30	Pentland Crown
19	52	1·3125	1·3167	0·32	Pentland Crown
18	55	1·3030	1·3140	0·84	Pentland Crown
16·5	0	1·3205	1·3314	0·68	New Potato
18	67	1·3140	1·3285	1·1	South African

The results reported in this paper are consistent with the occurrence of a rather general increase in protein synthesis during ageing. Such a general increase could be due to a derepression phenomenon producing a wide range of *m*RNAs. The fact that certain enzymes do not

increase during the 'ageing' process could be due to selectivity in the production of *m*RNAs alternatively the enzyme level may be lowered by protein degradation. The lack of increase in activity of malate dehydrogenase may be due to a failure to produce the specific *m*RNA. In the case of PAL there is clear evidence of enzyme induction but protein degradation also plays a role in controlling the level of the enzyme. The mechanism whereby synthesis is balanced against degradation remains a major problem for further investigation.

EXPERIMENTAL

Preparation of potato discs. Discs were cut from cylinders of tissue obtained with a No. 7 cork borer using washed and peeled tubers. Discs were washed in running water for 15 min, weighed and placed in Petri dishes containing a solution of streptomycin (25 mg/l.) and $CaSO_4$ (30 ml/l.). Two 5 g lots of discs were used per treatment.

Preparation of crude enzymes. Discs were ground in a chilled mortar with 5 ml 0·1 M borate buffer pH 8·8 and 2 drops mercaptoethanol. The Brei was filtered through nylon after the addition of another 5 ml buffer and the filtrate centrifuged at 27 000 *g* for 10 min. The supernatant was passed through a column of Sephadex G25, coarse and the eluate used directly as a source of enzymes,

Enzyme assay. PAL activity was measured by reading the increase in A at 290 nm in the presence of L-phenylalanine. Dehydroshikimic reductase activity was determined by the method of Balinsky and Davies,[26] chlorogenic acid oxidase by the method of Alberghina,[27] Glutamic-oxaloacetic-transaminase by the method of Ellis and Davies.[28] Malic enzyme, alcohol-, lactate, malate, isocitrate, glucose-6-phosphate and 6-phosphogluconate dehydrogenases as described in Ref. 29. The method of Sacher[30] was used for the determination of ribonuclease, the method of DeLeo and Sacher[23] for the determination of acid phosphatase, the method of Kawashima and Uritani[31] for the determination of peroxidase, the method described by Worthington[32] for the determination of aldolase, and the method of Brown and Wray[33] for the determination of hexokinase, and for some experiments (Tables 1 and 3), the combined activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Catalase was determined according to the procedure of Luck.[34] Aspartic phenylpyruvic acid transaminase activity was determined spectrophotometrically by following the oxidation of NADH which occurred as a result of reduction of oxaloacetate produced in the reaction by malic dehydrogenase. The latter enzyme was highly active in all preparations.

Deuterium labelling and isopycnic density gradient ultracentrifugation of enzymes. Discs (5 g) were aged in a solution containing 25 mg l^{-1} streptomycin sulfate in 5 ml of H_2O or 99·7% D_2O for 17 hr in light or dark. This D_2O treatment would yield an endogenous concentration of *ca.* 50% D_2O. In later experiments the procedure was altered by bathing the discs for 30 min in one change of 5 ml of 99·7% D_2O, followed by blotting before placing them in a petri dish with 99·7% D_2O for the ageing period. The latter method provided an endogenous concentration of *ca.* 75% D_2O, without causing undue inhibition of enzyme activity. Using three 30-min washings in 99·7% D_2O, to give an endogenous concn of *ca.* 94% before the 18-hr ageing in D_2O resulted in severe (70%) inhibition of PAL activity. A comparison of duplicate samples of *Rhoeo discolor* leaf sections aged for 15 hr in a final endogenous concn of 50% vs. 75% D_2O yielded increases in buoyant density of ribonuclease of 0·52% and 1·10% respectively as compared with the water-controls (Sacher and Davies, unpublished).

Preparation of gradient tubes was essentially according to the method of Schopfer and Hock.[18] The gradient was made with 2 layers, the lower of 1·8 ml of CsCl solution (0·825 g/ml), onto which 1·8 ml of a CsCl solution containing 0·22 g/ml was carefully pipetted. On top of this was layered 0·72 ml of enzyme solution, followed by 0·5 ml of paraffin oil. The tubes were centrifuged in a SW 50 L rotor at 39 000 rpm in a Spinco Model L2-65B ultracentrifuge for 42–48 hr at 2°. After the run 1-drop fractions (*ca.* 24 μl vol.) of the gradient were collected using a peristaltic pump. To each tube 1 ml of 20 mM L-phenylalanine in 0·05 M, pH 8·8

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[34] H. LUCK, in *Methoden der enzymatischen Analyse* (edited by U. BERGMEYER), p. 885, Verlag Chemie, Weinheim (1961).

borate buffer was added. After incubation of the reaction mixtures at 35° for 18–30 hr PAL activity was assayed by measuring the increase in absorbance at 290 nm. Every tenth fraction was used for a refractive index determination using a Bausch and Lomb Abbe-3L refractometer. A standard curve for obtaining buoyant densities from refractive indices was prepared using the equation $Q^{25\,\circ} = (10{\cdot}8601 \times \text{refractive index}) - 13{\cdot}4974$ given in the *Handbook of Biochemistry*.[35]

Acknowledgement—J. A. Sacher expresses his appreciation for support provided by the National Science Foundation (U.S.).

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