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EFFECT OF NITROGEN SOURCES UPON THE ACTIVITY OF L-GLUTAMATE DEHYDROGENASE OF *LEMNA GIBBA*

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Key Word Index—*Lemna gibba*, Lemnaceae, L-glutamate dehydrogenase, *de novo* synthesis, protein synthesis inhibitors, deuterium oxide labelling, ammonium, L-glutamate, Triton X100, EDTA

Abstract—When *Lemna gibba* cultures, grown on medium containing L-glutamate as the sole nitrogen source are transferred to medium in which ammonium is the only source of nitrogen, the activity of a NAD-dependent L-glutamate dehydrogenase (GDH) increases approximately 5-fold over 3 days Upon re-transfer to glutamate medium the activity declines to its initial value after a further 6 days The rise in activity is independent of the presence of EDTA and is not the result of an increase in the ease with which the enzyme can be extracted *p*-Fluoro-DL-phenylalanine, azetidine-2-carboxylic acid and puromycin but not D-*threo*-chloramphenicol, erythromycin or lincomycin inhibit the increase when included in ammonium medium These observations, together with those obtained from the use of a deuterium oxide-labelling technique, suggest that the increase in GDH activity is due to *de novo* synthesis on 80*S* ribosomes

INTRODUCTION

IT HAS previously been reported[1] that there is an increase in the NAD-dependent GDH activity present in extracts of *Lemna minor* when plants are transferred from a culture medium containing amino acids to one which contain ammonium as the sole nitrogen source Experiments which showed that the increases in GDH activity were proportional to ammonium concentration in the culture medium suggested that the effect might be due to enzyme induction Later, Joy[2] favoured an explanation based upon the observation that the enzyme, extracted from cultures grown on amino acids, was sensitive to inhibition by EDTA, a constituent of the culture medium and solutions used for enzyme extraction This sensitivity was found to be progressively lost when cultures, grown on amino acids, were transferred to ammonium-containing medium, so giving rise to the observed increase in activity

We have re-investigated these observations using *Lemna gibba* in which increases in the activity of GDH have been observed following transfer from a medium containing L-glutamate to one containing ammonium as the sole nitrogen sources The following mechanisms have been investigated as possible causes for the observed increases in GDH activity; (a) toxic effects of ammonium which, as a result of producing tissue degeneration, increase the ease with which the enzyme may be extracted, (b) sensitivity to inhibition by EDTA, and (c) enzyme synthesis *de novo*, investigated with the use of inhibitors and a deuterium oxide labelling technique

[1] JOY, K W (1969) *Plant Physiol* **44**, 849
[2] JOY, K W (1971) *Plant Physiol* **47**, 445

RESULTS

Preliminary Observations

Rather than grow cultures on the complex amino acid medium described by Joy[1] before transfer to ammonium-containing medium, we decided to try to grow our cultures on L-glutamate as sole nitrogen source Accordingly, the ability of *Lemna gibba* to utilize this amino acid as a nitrogen source was first tested (see Table 1) The concentration chosen as a standard for further experiments was 6 8 mM Experiments similar to those described by Joy[1] were now performed in which ammonium (15 mM) was substituted as a nitrogen source after 11 day's growth on medium containing glutamate EDTA ($2\,4 \times 10^{-2}$ mM) was included in both types of culture medium but not in the enzyme extraction medium The increase in GDH activity was measured over 3 days As a control, cultures grown on glutamate were transferred at the same time to fresh glutamate medium Extracts of cultures transferred to ammonium showed increasingly higher levels of GDH activity with increased time of exposure to ammonium medium compared with extracts of cultures transferred to fresh glutamate (see Fig 1) During growth on 15 mM ammonium medium, marked chlorosis and inhibition of respiration occurred 3 75 and 0 15 mM ammonium caused less chlorosis although the degree of respiratory inhibition was similar (see Fig 2) If, after 3 day's growth on 15 mM ammonium, cultures were re-transferred to medium containing glutamate, the GDH activity declined and was back to its original value after 6 days This was accompanied by a recovery from chlorosis and growth of new daughter fronds commenced

TABLE 1 THE EFFECT OF GLUTAMATE UPON GROWTH OF *Lemna gibba* WHEN INCLUDED IN A MODIFIED HILLMAN'S MEDIUM, ALL NITRATE RADICALS REPLACED BY CHLORIDE

Concn of L-glutamate in growth medium (mg/l)	Dry wt after 12 days growth (mg)	Concn of L-glutamate in growth medium (mg/l)	Dry wt after 12 days growth (mg)
No nitrogen	11 5 ± 1 5	200	60 4 ± 4 7
Nitrate (15 mM)	44 0 ± 1 2	500	97 5 ± 12 8
10	20 2 ± 3 4	1000 (6 8 mM)	171 6 ± 13 0
50	43 8 ± 1 3		

The Effects of Ammonium Sulphate and Triton X100 upon the Extraction of GDH

Inclusion of ammonium sulphate in the Tris-acetate buffer extractant had no effect upon the amount of GDH activity which could be extracted from cultures grown on L-glutamate Triton X100, however, when included in either extraction medium or in growth medium (see Fig 3) always led to increases in the amounts of GDH activity extracted, except when supplied in growth media at toxic levels Triton X100 at 1 % concentration had no effect upon the enzyme *in vitro*, even after prolonged incubations

Studies with Inhibitors of Protein Synthesis

The effect of these inhibitors (D-*threo*-chloramphenicol, lincomycin, and erythromycin) on the growth of *Lemna* was tested by growing cultures on L-glutamate medium containing a range of concentrations of the former Their effects were then studied upon the levels of GDH activity present in extracts of cultures which had been pre-grown on glutamate

medium for 11 days and then transferred to ammonium medium plus inhibitor for a further 3 days D-*threo*-Chloramphenicol was used at 0 1 mM concentration, lincomycin at 0 67 μM and erythromycin at 1 5 mM concentrations All 3 compounds inhibited growth in the test described above by 99, 25 and 80% respectively. However, none of the compounds inhibited the increase in GDH activity detected in extracts of ammonium-grown cultures In fact, D-*threo*-chloramphenicol after 2 days treatment and lincomycin after three resulted in higher levels of activity being detected than that present in extracts of control cultures (see Fig 4).

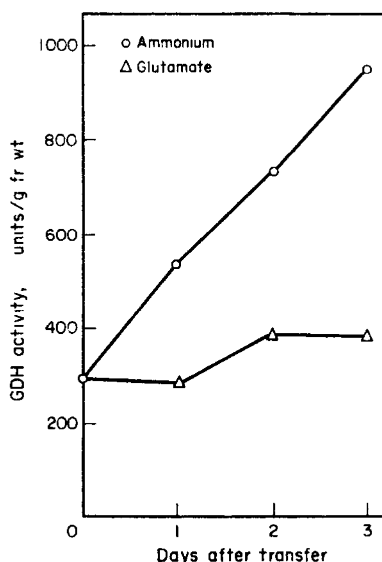

FIG 1 THE INCREASE IN NADH-DEPENDENT GDH ACTIVITIES IN EXTRACTS OF *Lemna gibba* WHEN TRANSFERRED FROM GLUTAMATE TO AMMONIUM MEDIUM

Cultures were pre-grown for 11 days upon glutamate (6 8 mM) medium and then transferred to either ammonium (15 mM) medium or fresh glutamate (6 8 mM) medium

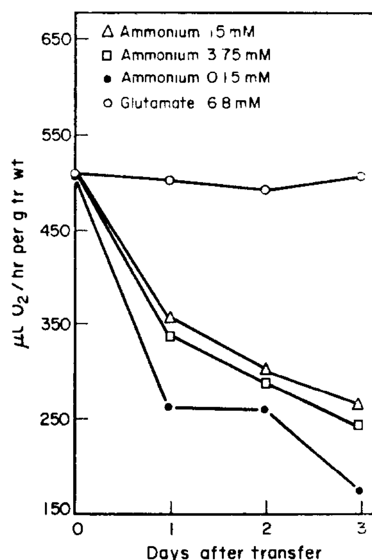

FIG 2 THE EFFECT OF VARIOUS CONCENTRATIONS OF AMMONIUM SULPHATE UPON THE RESPIRATORY RATE OF *Lemna gibba*

Cultures were pre-grown on glutamate for 11 days (6 8 mM) and then transferred to ammonium medium Control transferred to fresh glutamate (6 8 mM) medium

Amino acid analogues and puromycin were used in a similar way to that described for the 70*S* inhibitors, though not enough of these compounds were available to test their effects on growth Azetidine-2-carboxylic acid and *p*-fluoro-DL-phenylalanine, when included in ammonium medium, completely halted the rise in GDH activity and after 3 days the level of activity present in extracts of these cultures was slightly lower than that present in extracts of control cultures on glutamate alone (see Fig 5) Both of these compounds were found to inhibit respiration of *Lemna gibba*, azetidine-2-carboxylic acid at 1 mM by 67% and *p*-fluoro-DL-phenylalanine at 0 55 mM by 75% Puromycin (1 0 mM) produced a slight inhibition of the rise in GDH activity after 2 days growth on ammonium and strong inhibition after 3 days (see Fig 6)

Deuterium Labelling Experiments

Lemna cultures were pre-grown on L-glutamate medium and then transferred to aqueous ammonium medium or to 50 % deuterium oxide (99 8 % purity, tritium-free)–ammonium medium for 3 days Though deuterium produced some chlorosis at this concentration, the rises in GDH were of a similar order to those detected on aqueous ammonium medium Control cultures were pre-grown on glutamate medium and then transferred to either fresh aqueous glutamate medium or to 50 % deuterium oxide-glutamate medium

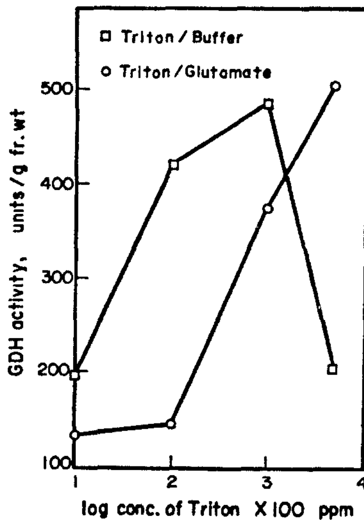

FIG 3 THE EFFECT OF INCLUDING TRITON X100 IN EITHER EXTRACTANT OR GLUTAMATE MEDIUM UPON THE NADH-DEPENDENT GDH ACTIVITIES DETECTED IN EXTRACTS OF *Lemna gibba*

Cultures were pre-grown on glutamate (6 8 mM) medium and then transferred either to fresh 'glutamate (6 8 mM) medium and extracted after 3 days with buffer containing Triton X100 (1000 ppm) or to glutamate (6 8 mM) medium containing Triton X100 and extracted without Triton X100 in the buffer

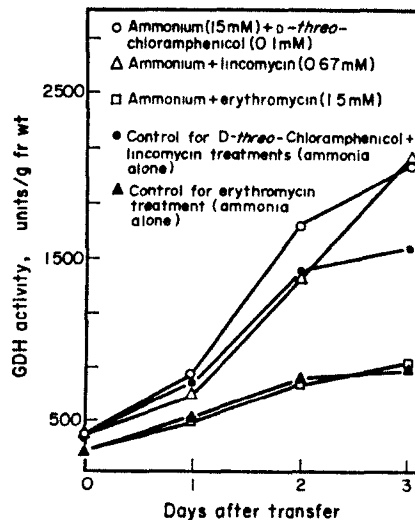

FIG 4 THE EFFECT OF VARIOUS ANTIBIOTICS UPON THE NADH-DEPENDENT GDH ACTIVITIES DETECTED IN EXTRACTS OF *Lemna gibba* GROWN IN AMMONIUM MEDIUM

Cultures were pre-grown on glutamate (6 8 mM) medium for 11 days and then transferred to ammonium (15 mM) medium containing D-*threo*-chloramphenicol (0 1 mM), lincomycin (0 67 mM) erythromycin (1 5 mM), and fresh ammonium medium

The density of GDH present in extracts of these cultures was then determined, as described in the Experimental, by centrifugation on caesium chloride gradients The distribution of GDH activity on such gradients is shown in Fig 7 for the enzyme present in extracts of cultures grown on aqueous ammonium medium and on deuterium oxide-ammonium medium Significant differences were detected between the densities of the enzyme extracted from these two sources within any one experiment indicated in Table 2 It was also observed that the greater the increase in the enzyme activity, the greater was the density shift Table 2 also shows the densities of the GDH determined, in two separate experiments, for the enzyme present in extracts of aqueous glutamate cultures and deuterium oxide-glutamate cultures

The density shifts observed were small compared with those obtained on ammonium medium In both experiments, a slight increase in GDH activity was detected upon transfer from aqueous glutamate to deuterium oxide-glutamate medium When GDH density shifts were compared with reference to the densities of glucose-6-phosphate dehydrogenase or alcohol dehydrogenase which were included as markers during some experiments (Table 3), significant density shifts for GDH indicative of *de novo* synthesis could not be reproducibly demonstrated This was thought to be caused by the large variability in the density values recorded for these two marker enzymes

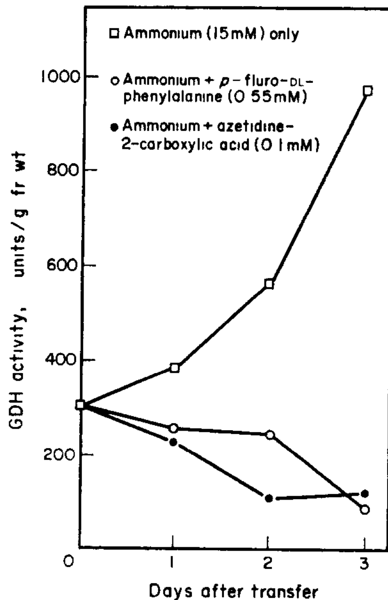

FIG 5 THE EFFECT OF AMINO ACID ANALOGUES UPON THE NADH-DEPENDENT GDH ACTIVITIES IN EXTRACTS OF *Lemna gibba* GROWN IN AMMONIUM MEDIUM
Cultures were pre-grown for 11 days on glutamate (6 8 mM) medium and then transferred to ammonium (15 mM) medium containing *p*-fluoro-DL-phenylalanine, (0 55 mM), azetidine-2-carboxylic acid, (0 1 mM) or fresh ammonium (15 mM) medium alone

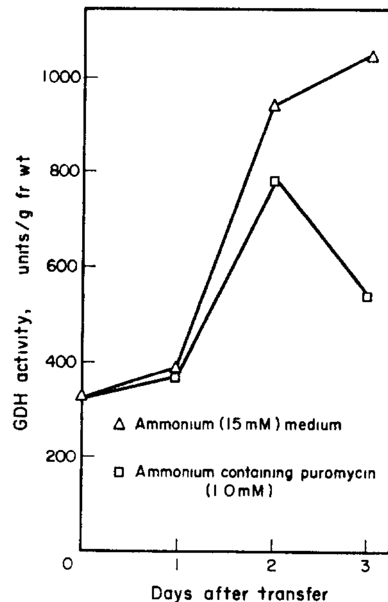

FIG 6 THE EFFECT OF PUROMYCIN UPON NADH-DEPENDENT GDH ACTIVITIES DETECTED IN EXTRACTS OF *Lemna gibba* GROWN IN AMMONIUM MEDIUM
Cultures were pre-grown on glutamate (6 8 mM) medium for 11 days and then transferred to ammonium (15 mM) medium or to ammonium medium (15 mM) containing puromycin (1 0 mM)

Electrophoresis of Lemna gibba *GDH*

Disc electrophoresis of extracts of L-glutamate-grown cultures upon 7 5% polyacrylamide gels followed by the detection of GDH activity using a tetrazolium staining procedure revealed the presence of a single formazan band with a R_p value of 0 36 (mobility relative to bromophenol blue marker) Electrophoresis of extracts of cultures grown on ammonium for 3 days also showed this formazan band and in addition, immediately behind it, another much broader formazan band (stretching to R_p 0 30) This formazan band was never detected on formazan-stained gels of glutamate-grown cultures

TABLE 2 BUOYANT DENSITIES OF *Lemna* GDH

Experiment	Buoyant density 'light' enzyme	Buoyant density 'heavy' enzyme	Buoyant density increase of enzyme in D_2O	Fold increase in GDH activity over 3 days
Glutamate to ammonium transfer				
1	1 3359	1 3638	0 0279	6 3
		1 3569	0 0210	
2	1 3159	1 3359	0 0200	4 5
		1 3317	0 0158	
3	1 3379	1 3442	0 0063	4 1
		1 3493	0 0114	
Glutamate to glutamate transfer				
4	1 3291	1 3338	0 0047	1 7
		1 3333	0 0042	
5	1 3253	1 3295	0 0042	1 2

Calculated from the distribution of the enzyme on caesium chloride gradients, extracted from cultures pre-grown on glutamate (6 8 mM) medium for 11 days and then transferred (experiments 1–3) for a further 3 days to either 50% D_2O (99 8%—tritium-free)-ammonium (15 mM) medium, designated 'heavy enzyme', or to aq ammonium (15 mM) medium designated 'light enzyme' In experiments 4–5 plants were pre-grown on glutamate (6 8 mM) for 11 days and then transferred for a further 3 days to either 50% D_2O (99 8%—tritium free)—glutamate (6 8 mM) or to aq glutamate medium (6 8 mM)

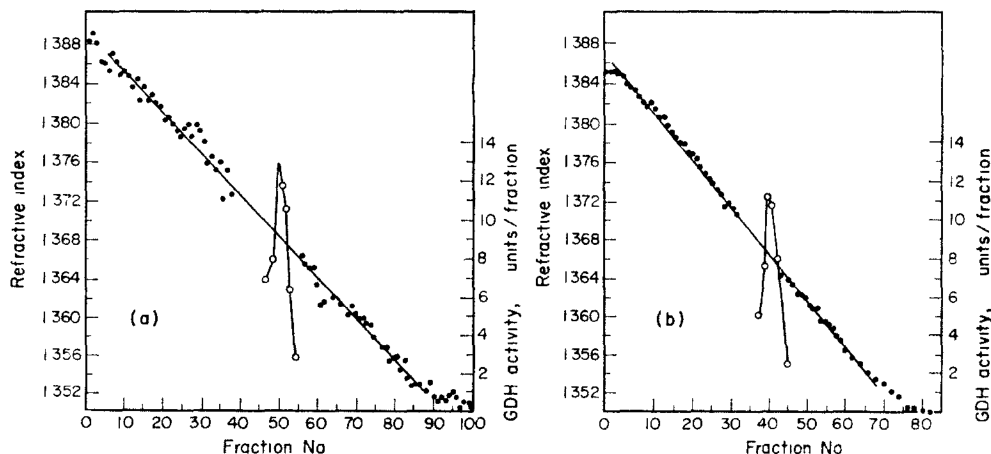

FIG 7 DISTRIBUTION OF NADH-DEPENDENT GDH ACTIVITIES ALONG A CAESIUM CHLORIDE GRADIENT (see Experimental)

The GDH was extracted from cultures pre-grown for 11 days, on glutamate (6 8 mM) medium and then transferred to either 50% deuterium oxide (99 8%, tritium-free)-ammonium medium (15 mM) (a), or to aqueous ammonium medium (b) for three days In each, 0 1 ml extract was added to the caesium chloride solutions and then centrifuged at 147 000 *g* for 68–72 hr at 4° Then single-drop fractions were collected for the determination of refractive indices and NADH-dependent GDH activities

TABLE 3 FURTHER BUOYANT DENSITIES OF *Lemna* GDH

[a]Buoyant density of GDH	[b]Buoyant density of marker	*a–b*	GDH density shift relative to marker	Fold increase in GDH activity over 3 days
Glucose-6-phosphate dehydrogenase as marker				
(i) Light 1 3159	1 3059	0 0100		
(ii) Heavy 1 3359	1 3222	0 0137	0 0037	4 5
1 3317	1 3069	0 0248	0 0148	
Alcohol dehydrogenase as marker		*b–a*		
(i) Light 1 3359	1 3651	0 0292		
(ii) Heavy 1 3638	1 4102	0 0464	0 0172	
1 3569	1 3850	0 0281	(−) 0 0011	6 3

As in Table 2 except that plants were transferred only from glutamate to ammonium medium Shifts in GDH density were then calculated relative to the density of markers, glucose-6-phosphate dehydrogenase or alcohol dehydrogenase included in the samples applied to the gradients

DISCUSSION

Our observations using *Lemna gibba* and a slightly different experimental procedure, have confirmed the observations of Joy[1] using *Lemna minor*, that when cultures are transferred from medium containing amino acids to one containing ammonium as sole nitrogen sources, an increase in GDH activity is observed

Joy[2] suggested that this increase was due to the fact that the enzyme extracted from amino acid-grown cultures was very sensitive to inhibition by EDTA, a constituent of his culture medium and enzyme extraction solutions This property was progressively lost upon culture on ammonium medium hence giving rise to the increase in GDH activity When EDTA was omitted from our growth medium, it never having been a constituent of our extraction solutions, the kinetics of increase in GDH activity upon transfer to ammonium were the same as in its presence We have, therefore, discounted changes in sensitivity of the enzyme to inhibition by EDTA as a mechanism for the observed increase

The observations of Puritch and Barker[3] that prolonged exposure of tomato leaves to ammonium sulphate causes deterioration of the ultra-structure of cells, especially notable in chloroplasts, suggested to us, together with the observed chlorosis in our cultures after 3 days growth on ammonium, that the observed rise in GDH activity might be due to an increase in the ease with which the enzyme could be extracted owing to deterioration in membrane structure To our knowledge, however, there are no existing reports upon the beneficial effects of salts upon the extraction of GDH

In so far as we have found that ammonium sulphate has no effect upon the amount of GDH activity extracted when included in the extraction buffer, it would appear that its behaviour is different from that of Triton X100, though the possibility exists that its effectiveness is only seen in long term Furthermore, we have never been able to obtain levels of GDH activity in extracts of cultures grown on L-glutamate-Triton-medium which are as high as those obtained on ammonium medium

Evidence that Triton X100 produces an apparent increase in GDH activity when included in glutamate medium by facilitating enzyme extraction is as follows GDH activity present

[3] PURITCH, G S and BARKER, A V (1967) *Plant Physiol* **42**, 1229

in extracts increases with increasing concentrations of Triton X100 in extraction buffer, 0 5% Triton in the latter is almost equivalent in its effect to 0 1 % included in glutamate medium Since Triton X100 has no *in vitro* effects upon GDH activity, its sole action would appear to be its influence upon the liberation of enzyme from the tissue It is, we feel, reasonable to suppose that increasing time of culture of *Lemna* upon glutamate-Triton X100 medium will cause increasingly greater amounts of enzyme to be liberated owing to accumulation of the detergent by the plant Lower amounts of detergent will be required therefore, to liberate equivalent amounts of enzyme compared with when Triton X100 is included in the extraction buffer Our results support this hypothesis although other explanations are possible

Even though used at concentrations which strongly inhibit growth, D-*threo*-chloramphenical, lincomycin and erythromycin did not prevent the rise in GDH activity when cultures were transferred to ammonium medium This indicates that *de-novo* synthesis of GDH does not take place, if at all, on 70*S* ribosomes The slight stimulatory effect of D-*threo*-chloramphenicol and lincomycin may indicate that there are present in *Lemna*, proteins synthesized on 70*S* ribosomes, which normally inhibit the activity or synthesis of GDH and which are themselves being inhibited by these compounds Although both azetidine-2-carboxylic acid and *p*-fluoro-DL-phenylalanine inhibit the activity rise, their effects are difficult to interpret, owing to their inhibition of respiration and possibly other metabolic processes [4] [5] In the absence of any known side-effects, puromycin is thought to be a fairly specific inhibitor of protein synthesis This compound inhibited the ammonium-stimulated increase of GDH activity by 17% after 2 days and 49% after 3 days It has been suggested[6] that owing to its size and low lipid solubility puromycin penetrates slowly into plant cells and this may account for the delayed action of the compound observed here If it is accepted that this inhibitory effect of puromycin is mediated through an action on protein synthesis, then we may conclude, in the absence of any effects of inhibitors of protein synthesis on 70*S* ribosomes, that the observed rise in GDH activity is due to enzyme synthesis on 80*S* ribosomes

The average shift in the buoyant density of GDH, determined from three separate experiments, when plants were transferred from aqueous glutamate medium to deuterium oxide-ammonium medium was found to be 0 017 when density shifts were calculated directly by subtraction of the relevant 'light' GDH density values from the corresponding 'heavy' values The maximum shift to be expected when all carbon-bound hydrogen exchanges with deuterium in a protein of mean density 1 33 is about 0 06 [7] For several reasons we could not expect to record such shifts under the experimental conditions employed here The fact that much smaller shifts were recorded (0 004, the average of two experiments) when plants were transferred from aqueous glutamate to deuterium oxide glutamate medium suggests that only a small part of the density shift observed upon transfer to deuterium-ammonium medium is due to exchange or turnover Providing that the rate of hydrogen–deuterium exchange between GDH and deuterium oxide is the same in deuterium oxide–ammonium medium as that in deuterium oxide–glutamate medium, we are led to interpret these observed shifts in density in terms of incorporation of deuterated amino acids during *de novo* synthesis of enzyme in these experiments Why this conclusion cannot be wholly

[4] WALTON, D C (1966) *Plant Physiol* **41**, 1549
[5] MILLER, J and ROSS, C (1966) *Plant Physiol* **41**, 1185
[6] NOODEN, L D and THIMANN, K V (1966) *Plant Physiol* **41**, 157
[7] HU, A S L, BOCK, R M and HALVORSON, H O (1962) *Anal Biochem* **4**, 489

substantiated when density shifts are calculated with reference to the densities of commercial enzymes used as markers is not fully understood, but there are two possible explanations Impurities in the GDH extract (e g carbohydrates) may be complexing with the markers on the gradient resulting in the production of species of variable density Additionally, these impurities may also be deuterated and able to undergo deuterium–hydrogen exchange with the markers producing varying degrees of density labelling These ideas are supported by the fact that, in each case, the density of markers is greater when they are present with deuterated GDH extracts However, since the experiments without enzyme markers all indicated significant density shifts, we conclude that *de novo* synthesis of GDH is occurring upon 80*S* ribosomes

Similar effects[8] of ammonium have been described for rice roots In this case, the rise was claimed to be not only due to synthesis of constitutive enzyme, but also to the appearance of a new form of the enzyme, detected by electrophoresis and present in the soluble fraction of the root extracts The results of our electrophoretic studies on *Lemna gibba* GDH also suggest the appearance of a new species of the enzyme present in extracts of ammonium-grown cultures However, this observation could also be explained on the basis that the ammonium treatment changes the shape and/or charge of a proportion of preexisting GDH molecules Further investigation on this point is required

Other reports on the stimulatory effect of ammonium upon the activity of GDH have been reported for various species [9] Such increases make available a mechanism whereby the plant can increase the rate of removal of ammonium and possibly avoid toxic effects Clonal, sterile and easily cultured material offer many attractions to those interested in enzyme induction in green plants and this is the reason why the plant *Lemna* is chosen for these experiments However, though our data strongly suggest *de novo* synthesis of the GDH, in this case, the fact that the substrate causes the side-effects noted may very well outweigh some of these advantages

EXPERIMENTAL

Plant material Lemna gibba was grown aseptically at 22–25° under 'Warm White' and 'Gro-Lux' fluorescent tubes on 30 ml of modified Hillman's medium[10] containing 1% w/v sucrose, pH 4 6, in 100 ml Erlenmeyer flasks The medium was sterilized by autoclaving at 15 psi for 15 min Medium containing inhibitors was sterilized using a 'Millipore' filter assembly Nitrate was replaced by chloride ions, nitrogen being supplied either as (a) L-glutamic acid, 6 8 mM, (b) $(NH_4)_2SO_4$, 15 mM with respect to ammonium, or (c) KNO_3, 15 mM with respect to nitrate

Enzyme extraction and assay The plants were rinsed with distilled water and then homogenized at 4° in a porcelain dish with a glass mortar in 2 5 vols of 0 2 M Tris-acetate buffer, pH 8 2 The resulting brei was strained through two layers of No 13 gauge muslin and the filtrate centrifuged for 12 5 min at 2000 *g* The resulting supernatant was centrifuged for 30 min at 45 000 *g* and the supernatant obtained was used for GDH assays as described by Lea and Thurman [11] The unit of GDH activity was defined as a 0 001 absorbance unit change per min at 340 nm Disc electrophoresis followed the method of Ornstein and Davies[12] and GDH activity was detected on the gels by the method of Thurman *et al* [13]

Respiratory measurements These were made in darkness at 27° in the presence of 1% sucrose, 20% NaOH in the centre well of the flask using a Gilson respirometer

Isopycnic density gradient centrifugation of GDH Gradients similar to those described by Longo[14] were used 2 42 ml of a solution of caesium chloride (0 76694 g/ml) in 0 2 M Tris-acetate, pH 8 2 was overlayered

[8] KANAMORI, T, KONISHI, S and TAKAHASHI, E (1972) *Physiol Plant* **26**, 1

[9] WEISSMAN, G S (1972) *Plant Physiol* **49**, 138

[10] HILLMAN, W S (1961) *Am J Botany* **48**, 413

[11] LEA, P J and THURMAN, D A (1972) *J Exp Botany* **23**, 440

[12] ORNSTEIN, L and DAVIES, B J (1961) *Disc Electrophoresis*, Preprint distributed by Kodak Ltd, Kirby, Liverpool

[13] THURMAN, D A, PALIN, C and LAYCOCK, M V (1965) *Nature* **207**, 193

[14] LONGO, C P (1968) *Plant Physiol* **43**, 660

by 2 48 ml of the same buffer in a 5 5 ml cellulose nitrate tube, 0 1 ml of enzyme extract was added and the tube centrifuged at 147 000 *g* for 68–72 hr at 4° In some experiments, commercial preparations of either glucose-6-phosphate dehydrogenase or alcohol dehydrogenase were included upon the gradients as density reference markers In each case *ca* 100 units of enzyme activity was applied to the gradients After centrifugation the gradient was fractionated into single drop fractions Some of these were used to locate GDH activity and marker enzyme where appropriate while the remainder were used for the determination of refractive index at 20° The densities of GDH and markers were calculated from these measurements using a conversion formula derived from a computer double regression analysis of data in *Handbook of Physics and Chemistry* [15] This indicated that, although a linear relationship, similar to that quoted by Ifft, Voet and Vinograd[16] for 25° can be derived at 20°, a more accurate quadratic solution is to be preferred The relationship employed was

$$^{20}\rho = 5\,6937 - 16\,922\,\mu + 10\,052\,(\mu^2), \text{ where } \mu = \text{refractive index}, \; ^{20}\rho = \text{density at } 20^\circ$$

Acknowledgement—One of us (D V S) was a recipient of a Science Research Council Postgraduate Research Studentship Award during the period of this work

[15] ANON (1967–8) *Handbook of Physics and Chemistry* (WEAST, R C , ed) 48th Edn, p D149, The Chemical Rubber Co , London
[16] IFFT, J B , VOET, D H and VINOGRAD, J (1960) *J Phys Chem* **65**, 1138