Phytochemistry, 1972, Vol 11, pp 277 to 287 Pergamon Press. Printed in England.

COMPARATIVE CHARACTERIZATION OF L-THREONINE DEHYDRATASE IN SEVEN SPECIES OF UNICELLULAR MARINE ALGAE*

I. D DESAI and D. LAUB

Division of Human Nutrition, School of Home Economics, University of British Columbia, Vancouver

and

N J ANTIA

Fisheries Research Board of Canada, Vancouver Laboratory, Vancouver, B C, Canada

(*Received* 20 *March* 1971)

Abstract—Crude extracts of the following marine algae, cultured axenically under controlled conditions, were examined for L-threonine dehydratase activity two cyanophytes (*Agmenellum quadruplicatum*, *Anacystis marina*), one rhodophyte (*Porphyridium cruentum*), two cryptophytes (*Chroomonas salina*, *Hemiselmis virescens*), one chlorophyte (*Tetraselmis maculata*), and one diatom (*Cyclotella nana*) The specific activities (4–85 mμmoles/min/mg protein) showed considerable species differences as well as significant effects from the nutritional conditions of algal culture (autotrophic vs heterotrophic) The enzymatic production of α-ketobutyrate from L-threonine was confirmed (with 2 species) by the isolation and chromatographic identification of its 2,4-dinitrophenylhydrazone The algal enzymes showed pH optima in the range of 8 5–9 5 and 'sigmoid' or 'paraboloid' kinetic response to threonine concentration With the exception of *C nana*, all the algal enzymes were strongly inhibited by L-isoleucine, L-Valine, EDTA, AMP, ADP, and cyclic 3′,5′-AMP had no significant effect Several carbonyl reagents (hydrazine, hydroxylamine, etc) strongly inhibited enzyme activity and this inhibition was reversed to varying degrees by pyridoxal 5′-phosphate Excepting iodoacetamide, all the reagents known to modify protein sulfhydryl groups inhibited the activity strongly and these inhibitions were partially reversed by dithiothreitol The enzymes of *C salina* and *H virescens* were also strongly inhibited by dithiothreitol These results characterized the algal enzymes generally as isoleucine-regulated, pyridoxal-phosphate requiring, allosteric threonine dehydratases similar to the corresponding 'biosynthetic' enzymes previously reported for bacteria, fungi, and higher plants In addition, the algal enzymes appeared to require sulfhydryl groups for the expression of activity The *C nana* enzyme appeared to be insensitive to isoleucine regulation, and the cryptophyte enzymes appeared to require disulfide groups for activity

INTRODUCTION

OUR INTEREST in the nutritive value of unicellular algae to man and members of the marine food chain has prompted us to examine their production of the essential amino acids, L-threonine and L-isoleucine, under controlled culture conditions Previous studies on bacteria[1,2] and yeasts[3] have shown that the production of these amino acids is closely controlled by the microbial cell's enzymatic machinery involving at least two feedback systems, one regulating the synthesis of appropriate enzymes and another their activity. A key enzyme in this control machinery linking threonine to isoleucine is L-threonine dehydratase (L-threonine hydro-lyase, deaminating; E C. 4 2 1 16), the first enzyme in the biosynthetic pathway to isoleucine in all forms of life hitherto studied. This enzyme has been

* Part I in the series "Threonine Deminase in Algae".

[1] J -P CHANGEUX, *Sci Am* **212** (4), 36 (1965)
[2] P DATTA, *Science* **165**, 556 (1969)
[3] H BUSSEY and H. E UMBARGER, *J. Bacteriol.* **98**, 623 (1969).

more or less extensively studied in certain bacteria,[4–11] yeasts,[12,13] fungi,[14] and higher plants,[15–18] but it does not appear to have been investigated in algae. We have surveyed 14 species of axenically cultured marine planktonic algae for the occurrence of this enzyme and have found widely varying levels of activity Seven species showing considerable activity were chosen from this survey and whole extracts were tested for properties of the enzyme which would enable comparison with previously reported threonine dehydratases

RESULTS

Culture conditions All the algae (Table 1) were mass cultured photoautotrophically (some were vitamin-auxotrophs), under continuous illumination at 20° in buffered enriched seawater (of salinity 2 6%) Nitrate was replaced by glycine or urea in the case of *Hemiselmis virescens*, which is unable to utilize nitrate as *N*-source for growth [19] The silicate enrichment was doubled for the culture of the diatom *Cyclotella nana* In addition, *H. virescens*, *Chroomonas salina*, and *Porphyridium cruentum* were similarly cultured phototrophically in the presence of glycerol[20] and *C salina* was also cultured heterotrophically on glycerol in darkness [21] All cultures were harvested at late log phase of growth and the freeze-dried, pulverized cells were stored dry under vacuum at −25° Such storage did not appear to affect the enzyme activity for several months.

Algal extracts. In view of the previously reported instability of the enzyme in bacterial[5,9,22] and yeast extracts,[23] freshly prepared algal extracts were used for all activity tests. Brief ultrasonication of algal cells in the test buffer was the preferred method of 'extraction' because of limited amount of algal material available, and whole sonicates were used for all tests since preliminary examination showed that the enzyme in some species was not readily solubilized leaving considerable activity in the pellet after high-speed centrifugation

Linearity of activity. All algal extracts from 2 mg cells showed linear production of α-ketobutyrate (in standard assay mixture) with incubation periods up to at least 20 min, and in the case of *P cruentum* and *Tetraselmis maculata* this linearity was found to extend to 45 min. The activity from 2 mg cells of *C nana* was too low for reliable enzyme assay and

[4] W. M HARDING, J A TUBBS and D MCDANIEL, *Can J Biochem.* **48,** 812 (1970)
[5] C LEITZMANN and R W BERNLOHR, *Biochim Biophys Acta* **151,** 449 (1968)
[6] C NING and H GEST, *Proc Natl Acad Sci. U.S* **56,** 1823 (1966)
[7] M REH and H. G SCHLEGEL, *Arch Mikrobiol* **67,** 110 (1969)
[8] P DATTA, *J Biol Chem.* **224,** 858 (1969)
[9] J LEIBOVICI and C ANAGNOSTOPOULOS, *Bull Soc Chim Biol* **51,** 691 (1969)
[10] L P LOSEVA, V I LYUBIMOV, Z S KAGAN and V L KRETOVICH, *Dokl Akad Nauk SSSR* **181,** 997 (1968)
[11] G W HATFIELD and R. O BURNS, *Science* **167,** 75 (1970)
[12] C CENNAMO, M BOLL and H HOLZER, *Biochem Zeit* **340,** 125 (1964)
[13] A BRUNNER and H DE ROBICHON-SZULMAJSTER, *FEBS Letters* **5,** 141 (1969).
[14] Z S KAGAN, E M SINELNIKOVA and W L KRETOVICH, *Biokhimiya* **34,** 1279 (1969).
[15] W S TOMOVA, Z S KAGAN and W L KRETOVICH, *Biokhimiya* **33,** 244 (1968)
[16] Z S KAGAN, E M SINELNIKOVA and W L KRETOVICH, *Enzymologia,* **36,** 335 (1969), *Dokl. Akad. Nauk SSSR* **185,** 1372 (1969).
[17] R K SHARMA and R MAZUMDER, *J Biol Chem* **245,** 3008 (1970)
[18] D K DOUGALL, *Phytochem* **9,** 959 (1970)
[19] N J ANTIA and V CHORNEY, *J Protozool* **15,** 198 (1968)
[20] J Y. CHENG and N J ANTIA, *J Fish Res Bd Can* **27,** 335 (1970)
[21] N J ANTIA, J. Y CHENG and F J R TAYLOR, *Proc Intern Seaweed Symp.* **6,** 17 (1969).
[22] W M HARDING, *Arch Biochem Biophys* **129,** 57 (1969)
[23] H HOLZER, M BOLL and C CENNAMO, *Angew Chem Intern Edit* **3,** 101 (1964).

TABLE 1. PROTEIN CONTENT, STANDARD ASSAY CONDITIONS, pH OPTIMA AND SPECIFIC THREONINE DEHYDRATASE ACTIVITIES OF ALGAL CULTURES

Alga	Culture conditions Light*	Added organic compounds†	Protein (wt % dry alga)	Assay conditions‡ Incub period (min)	pH optimum (±0 2)	Specific activity (units)§
Chlorophyta (green algae).						
Tetraselmis maculata	+	nil	42 7	40	8 7	4 3 ●
Bacillariophyta (diatoms)						
Cyclotella nana	+	nil	33 5	30	8 8	4 5●
Cryptophyta						
Chroomonas salina	+	nil	50 3	15		25 5
	+	glycerol (0 25 M)	31 9	15	8 8	44 2●
	−	glycerol (0 25 M)	50 3	10		84·7
Hemiselmis virescens	+	glycine (4 mM)	54 0	15	8 9	16 0●
	+	glycine (4 mM) + glycerol (0 25 M)	34 6	20		13 0
	+	urea (2 mM)	54 0	20		8 1
Rhodophyta (red algae).						
Porphyridium cruentum	+	nil	37 8	30	9 5	12 2●
	+	glycerol (0 5 M)	28 1	30		16 9
Cyanophyta (blue-green algae)						
Agmenellum quadruplicatum	+	nil	59·5	10	8 5	24 3●
Anacystis marina	+	nil	56 2	15	8 9	16·4●

* Presence (+) or absence (−) of continuous illumination (*ca* 16,500 lux) from cool-white fluorescent lamps; when absent, complete darkness was used

† In addition to the vitamins normally added to the culture medium (see Experimental).

‡ Each assay was carried out with a whole sonicate of algal material of 2 mg except for *C. nana*, 6 mg, in 0 5–0 7 ml buffer (0 2 M). The sonicate was preincubated at 37° for 5 min with pyridoxal phosphate (0·1 μmole) and incubated, at the same temperature, with L-threonine (80 μmoles) for the periods shown, in a final volume of 1 ml

§ mμmoles keto acid produced per min per mg protein in incubation mixture at pH 8 5 under assay conditions used. Type chosen for enzyme characterization shown by ●.

did not show linearity beyond 30 min incubation; it was not improved by incorporation of EDTA (1 mM) or dithiothreitol (10 mM) in the extraction buffer. This suggested instability of the diatom enzyme resulting from low concentration reminiscent of instability previously reported for bacterial[22] and yeast[23] threonine dehydratases; it was further evidenced by the finding of a paraboloid increase of activity with increase in algal material (in the same volume of extract) from 2 to 20 mg The instability was minimized by preparing extracts from 6 mg cells which showed sufficiently high activity for reliable enzyme assay with 30 min incubation.

Specific activity. Table 1 summarizes the specific activities observed as well as the standardization of the extracts and incubation conditions used in all activity tests made in this investigation. The specific activities of the photoautotrophically cultured algae showed considerable species variation (4–85 units). Culture of the algae with glycerol caused considerable increase in the specific activities of the *C. salina* and *P. cruentum* enzymes, but appeared to diminish that of *H. virescens*. The illumination of culture during growth depressed the enzyme level of glycerol-grown *C. salina*, while glycine appeared to favour enzyme synthesis relative to urea as *N*-source for phototrophic growth of *H. virescens*.

Although desirable, it was beyond the scope of this investigation to characterize the enzyme from all the multiple (nutritionally-different) cultures listed in Table 1, and only one type of culture of each algal species was used for further studies of enzyme properties. This type of culture, indicated in Table 1, will be considered to represent its species in future references to the enzyme from that species

Identification of enzyme reaction product The product of reaction from extracts of *C. salina* and *A quadruplicatum* was isolated as the 2,4-dinitrophenylhydrazone from the penultimate stage (see Experimental) of the regular assay procedure based on the method of Friedemann[24] for the determination of keto acid At this stage, one-tenth of the reaction product in aq Na_2CO_3 was treated with aq NaOH for the usual colorimetric determination and the remainder was extracted with benzene after acidification. TLC of this extract by the method of Stan and Schormuller[25] showed a major yellow product with R_f value and visible absorption spectrum identical with those obtained from similarly-treated α-keto-butyrate standards and significantly different from those of pyruvate standards, together with a minor yellow product with properties similar to those of acetaldehyde dinitrophenyl-hydrazone. These products were not detected in extracts from 'zero incubation-time' controls made with algal material and threonine The identity of the major product with α-ketobutyrate dinitrophenylhydrazone was confirmed by co-chromatography after elution. The minor product (presumably propionaldehyde dinitrophenylhydrazone) appeared to arise from decarboxylation of the major product during the acidification step of the isola-tion procedure, since it was obtained from α-ketobutyrate standards, was reproduced by the major product after elution with aq $NaHCO_3$, acidification, benzene extraction, and rechromatography, and was not detected from acetaldehyde standards submitted to the complete keto acid isolation procedure. The evidence obtained indicated that α-ketobutyrate was the only enzyme reaction product measured by the assay method used, and that acetaldehyde, possibly arising from threonine aldolase activity,[26–28] was excluded by the method. This does not preclude the possibility of interference from threonine aldolase acting simultaneously with the dehydratase on the substrate during the incubation, and other methods will be required to determine the occurrence of the aldolase in the algal extracts. A similar interference from the possible occurrence of threonine dehydrogenase[29,30] was not expected however, since the pyridine nucleotide required by this enzyme was not included in the incubation mixture

pH optimum. All the algal enzymes showed pH optima (Table 1) in the range of 8·5–9·0 on testing with 0·1 M potassium phosphate buffer in the pH range 6·0–8 0 and with potas-sium Tricine [*N*-tris (hydroxymethyl)methylglycine] buffer in the range 7·0–10·5. These buffers showed comparable activities at the same pH, but Tris-HCl (pH 8–9) and $NaHCO_3$-Na_2CO_3 (pH 9 5–10 5) buffers were inhibitory The pH-activity profiles showed sharp optimum peaks for the enzymes of *A marina*, *A. quadruplicatum*, and *H. virescens* but broad flattened optima for the other algae, particularly in the case of *P. cruentum* enzyme which displayed near maximal activity between pH 8 5 and 10·5. In general, the algal en-

[24] T. E FRIEDEMANN, in *Methods in Enzymology* (edited by S P COLOWICK and N O. KAPLAN), Vol 3, p 414, Academic Press, New York (1957)
[25] H -J STAN and J SCHORMULLER, *J Chromatog* **43**, 103 (1969).
[26] J G MORRIS, *Biochem J.* **115**, 603 (1969)
[27] R H DAINTY, *Biochem J* **117**, 585 (1970)
[28] H YAMADA, H. KUMAGAI, T NAGATE and H YOSHIDA, *Biochem Biophys. Res. Commun* **39**, 53 (1970)
[29] D MCGILVRAY and J G MORRIS, *Biochem J* **112**, 657 (1969)
[30] A J WILLETTS and J M TURNER, *Biochem J* **117**, 27 P (1970)

zymes showed steep decline in activity towards pH 7·0 dwindling to insignificance at pH 6·0, in contrast to considerable activity still manifest at the basic end (pH 10·5) of the pH spectrum.

Substrate concentration. When tested at (or near) the optimal pH and in the presence of an adequate concentration of pyridoxal phosphate, 4 algal species showed typical sigmoid-shaped enzyme-threonine saturation curves and 3 species showed paraboloid-shaped curves (Fig. 1) The highest substrate concentration (80 mM) tested was that concentration normally used for enzyme characterization in this investigation; it was not inhibitory to any algal enzyme and appeared to reach substrate saturation in the case of 4 species (Fig. 1).

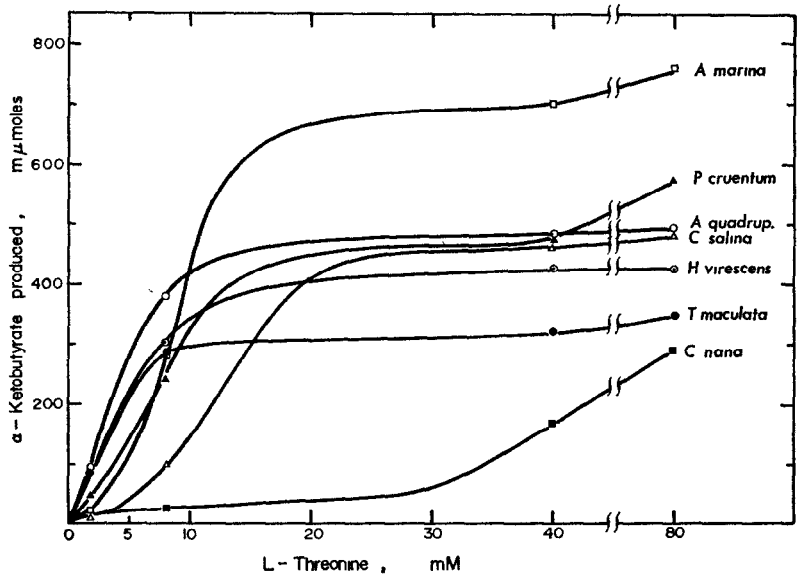

FIG. 1. EFFECT OF SUBSTRATE CONCENTRATION ON THREONINE DEHYDRATASE ACTIVITY OF ALGAL EXTRACTS.
The assay conditions indicated in Table 1 were used with K-Tricine buffer (pH 8 5) and substrate concentrations as shown

The saturation curves of *A. marina*, *P. cruentum*, and *C. nana* showed pronounced intermediary plateau regions similar to those previously reported for certain enzymes with multiple substrate binding sites [31]

Pyridoxal phosphate requirement. This requirement for algal enzyme activity was tested by studying the effects of known antagonists[32,33] and carbonyl reagents in the absence of pyridoxal phosphate normally added to the assay incubation mixture (Table 2). The algal enzymes generally showed little effect from added pyridoxal phosphate, excepting the 40–60% stimulation obtained in the cases of *C salina* and *C. nana* (Table 2). However, all the carbonyl reagents tested showed marked inhibition of activity, the degree of inhibition

[31] J. TEIPEL and D E KOSHLAND, JR., *Biochem.* **8**, 4656 (1969).
[32] A E. BRAUNSTEIN, in *The Enzymes* (edited by P D. BOYER, H. LARDY and K. MYRBÄCK) 2nd Edition Vol 2, p 113, Academic Press, New York (1960).
[33] F.-J LEINWEBER, *Mol Pharmacol* **4**, 337 (1968)

Table 2 Effect of pyridoxal phosphate and carbonyl reagents on algal threonine dehydratase activity

Reagent (1 mM)	Pyridoxal phosphate (mM)	Enzyme activity (% of control)*						
		C nana	*T maculata*	*C salina*	*H virescens*	*A quadrup*	*A marina*	*P cruentum*
Nil	0 1	164 8	108 6	138 7	103 7	114 4	107 0	107 6
Hydroxylamine	—	0	0	0 5	0	0	0	1 2
	2 0	—	20 2	69 7	82 0	7 4	—	86 5
Methoxylamine	—	0	0	30 8	28 1	7 0	4 4	33 1
	2 0	—	—	85 3	—	—	—	—
NSD-1055	—	0	0	0	0	0	1 1	7 6
	2 0	—	21 9	45 4	44 9	41 8	—	83 1
Hydrazine	—	26 0	31 0	39 4	34 8	0	1 3	16 1
	2 0	—	98 7	124 2	106 0	56 0	—	115 0
Semicarbazide	—	35 2	44 4	98 5	89 0	0	62 4	94 9
	2 0	—	—	100 0	—	—	—	—
Isoniazid	—	42 9	72 4	85 7	86 7	22 5	75 9	81 2

* Assay conditions indicated in Table 1 with the extracts prepared in 0 2 M K-Tricine buffer (pH 8 5) and pyridoxal phosphate omitted from the incubation mixture except where shown Test samples were preincubated at room temperature for 15 min with each reagent shown and for another 15 min with the reversal agent Controls were similarly treated without the reagents

varying with the reagent as well as with the algal species. The possibility of interference from the binding of enzyme-produced keto acid by the carbonyl reagents, resulting in false inhibition effects, was eliminated by showing that no significant effect on the keto acid measured was observed when α-ketobutyrate was incubated with the reagents under standard conditions without threonine and pyridoxal phosphate The enzymes of *A. quadruplicatum* and *C. nana* appeared to be the most vulnerable to all the carbonyl reagents. The hydroxylamine-derived reagents were consistently more inhibitory than the hydrazine derivatives. Apart from hydroxylamine, NSD-1055 (4-bromo-3-hydroxybenzyloxyamine, H_3PO_4) proved to be the most potent general inhibitor, causing virtually complete abolition of the activity of all algal enzymes This reagent has been considered to be a relatively specific inhibitor of pyridoxal–phosphate activated enzymes[33,34] (although not previously applied to threonine dehydratase) and its behaviour in the present study may represent a particularly successful demonstration of the pyridoxal phosphate requirement of algal threonine dehydratase. However, in view of recently reported cases of enzymes with keto-acid prosthetic groups[35] or with carbonyl character at active sites[36] which were also inhibited by carbonyl reagents but did not require pyridoxal phosphate, more rigorous proof of this requirement of algal threonine dehydratases was required This was obtained by demonstration of the reversal of the carbonyl-reagent induced inhibitions by subsequent incubation with pyridoxal phosphate. The results in Table 2 show that such reversals (partial to total) were obtained in all cases tested. In this connection, it is pointed out that the magnitudes of the inhibitions and reversals reported in Table 2 may be considerably

[34] A E Pegg and H G Williams-Ashman, *J Biol Chem* **244**, 682 (1969)
[35] D J George and A T Phillips, *J Biol Chem* **245**, 528 (1970)
[36] I L Givot and R H Abeles, *J Biol Chem* **245**, 3271 (1970)

suboptimal for some species, since they were obtained with only one set of test conditions which could hardly be expected to be optimal for all species.

Sulfhydryl group requirements. Proof of this requirement for algal enzyme activity was obtained by testing the effects of known —SH group protectants and modifying agents. The results, summarized in Table 3, show that the protective reagent dithiothreitol[37] had little effect on the activity of 4 algal enzymes, but significantly stimulated that of *T. maculata* while strongly inhibiting those of *C. salina* and *H. virescens* (both cryptophytes). The —SH group modifying agents were chosen to represent 3 types of chemical interaction: (a) —SH

TABLE 3 EFFECT OF SULFHYDRYL REAGENTS ON ALGAL THREONINE DEHYDRATASE ACTIVITY, AND REVERSAL OF INHIBITIONS BY DITHIOTHREITOL

Reagent	conc (mM)	Dithiothreitol (mM)	Enzyme activity (% of control)*						
			C. nana	*T. maculata*	*C. salina*	*H. virescens*	*A. quadrup*	*A. marina*	*P. cruentum*
Dithiothreitol	1	—	100 0	121 5	35 6	30 7	98 4	92 4	100 0
	10	—	113 4	120 0	10 7	22 1	99 3	91 9	110 1
N-Ethylmaleimide	1	—	8 5	10 3	0 6	14 8	0	0	0
	1	10	—	43 5	—	—	3 6	5 1	0
Iodoacetamide	1	—	83 1	109 0	84 0	100 0	26 9	59 9	110 8
	1	10	—	—	—	—	81 0	89 0	—
p-Chloromercuriphenyl sulfonate	1	—	9 8	0	51 2	4 9	2 2	18 0	10 1
	1	10	—	19 9	—	—	51 5	28 3	62 0
2,2′-Dithiodipyridine	1	—	5 2	2 5	3 6	0 8	3 1	0 3	4 7
	1	10	—	22 9	—	—	22 9	7 4	31·4

* Assay conditions indicated in Table 1 with extracts prepared in 0 2 M K-Tricine buffer (pH 8 5). Test samples were preincubated at room temperature for 15 min with each reagent shown and for another 15 min with the reversal agent Controls were similarly treated without the reagents

capture by the Hg of *p*-chloromercuriphenyl sulfonate, (b) —SH alkylation by *N*-ethylmaleimide or iodoacetamide, (c) —SH exchange with the disulfide grouping of dithiodipyridine.[38] Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] was found unsuitable for the sulfhydryl–disulfide exchange test because it interfered by showing strong absorption at the wavelength used for the colorimetric determination of keto acid. All the modifying agents caused significant reduction in activity, the degree of inhibition varying with the reagent and algal species tested. Iodoacetamide was generally the least effective and dithiodipyridine the most potent inhibitor. Among the algal enzymes, those of the blue–green species appeared to be the most consistently sensitive. That these reagents inhibited enzyme activity by —SH modification was further evidenced by the partial reversals obtained on subsequent treatment with dithiothreitol (Table 3). In no case was complete reversal

[37] W W CLELAND, *Biochem* **3**, 480 (1964)
[38] D R GRASSETTI and J F MURRAY, JR., *Arch Biochem Biophys* **119**, 41 (1967).

obtained and in some cases the reversal was unconvincingly low, suggesting the probability of nonspecific effects of these reagents in addition to —SH modification. Such reversals were not attempted with the cryptophyte enzymes because of their inhibition by dithiothreitol itself; this latter effect as well as their sensitivity to the —SH modifying agents (excepting iodoacetamide) suggests that the cryptophyte enzymes may require both sulfhydryl and disulfide groupings for the manifestation of activity, since dithiothreitol is known to cause reductive cleavage of disulfides [39]

Allosteric and other effects. Excepting *C nana*, all the algal enzymes showed sigmoid-type concentration-dependent inhibition by L-isoleucine (Fig. 2) The threshold concentration of isoleucine required to produce appreciable inhibition varied widely with algal species. The *C nana* enzyme was insensitive to isoleucine concentration and the low degree of inhibition obtained casts doubt on its specific nature The blue–green algal enzymes showed the most sensitive inhibition progressing from 0 to 80% with critical ranges of isoleucine concentrations, although relatively high threshold concentrations were required Apart from *C nana*, the enzyme of *H virescens* was exceptional in persisting with 45% residua

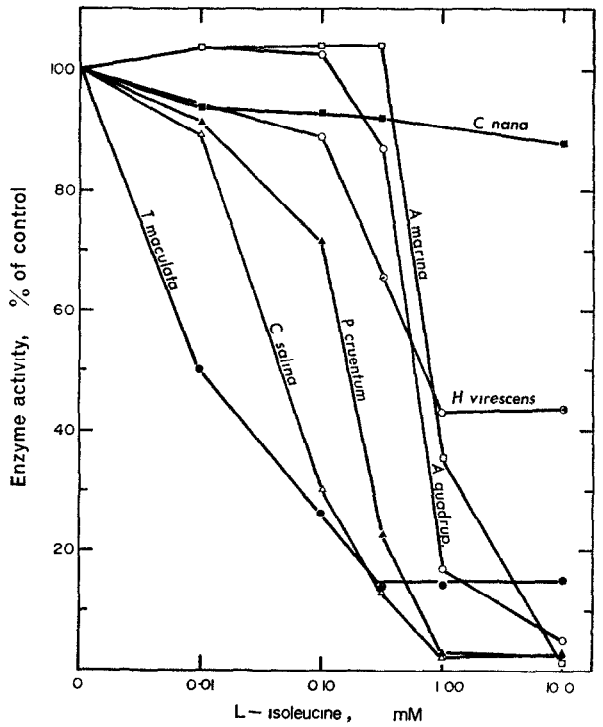

FIG 2 EFFECT OF L-ISOLEUCINE CONCENTRATION ON THREONINE DEHYDRATASE ACTIVITY OF ALGAL EXTRACTS

The assay conditions indicated in Table 1 were used with K-Tricine buffer (pH 8 5) and isoleucine additions ranging from zero (taken as control) to 10 mM as shown All test samples (including controls) were preincubated at room temperature for 15 min with isoleucine before the usual preincubation with pyridoxal phosphate (0 1 mM) and subsequent incubation with threonine (80 mM) at 37°

[39] W L ZAHLER and W W CLELAND, *J. Biol Chem.* **243**, 716 (1968)

activity after maximal inhibition, while the other algal enzymes were maximally inhibited to the extent of 85–95%. In general, the inhibition was similar to that previously recorded for 'biosynthetic' threonine dehydratases from bacteria,[5,8–10,40] yeast,[23] fungi,[14] and higher plants,[15,17,18] indicating similar allosteric properties for the algal enzymes with respect to regulatory feedback control from isoleucine.[1]

Among other effectors tested, L-valine (0·1–10 mM) had no significant effect on the activity of the algal enzymes. This was also the case with adenosine 5′-monophosphate, 5′-diphosphate, and cyclic 3′,5′-monophosphate, when tested at a concentration of 1m M. EDTA (1–10 mM) had no significant effect on the algal enzymes, excepting that of *C. nana* the activity of which was stimulated about 23%. The results with EDTA indicated that the algal enzymes, like previously reported threonine dehydratases, may have no divalent-metal ion requirement for activity, but that the *C. nana* enzyme may be sensitive to inhibition from heavy-metal ion contaminants in the assay mixture, which inhibition would be annulled by chelation of the contaminants by EDTA resulting in net stimulation of enzyme activity.

DISCUSSION

Two types of L-threonine dehydratases have been previously reported from bacteria[5–10,41,42] and higher plants,[15–18] usually occurring singly[5–10,17,18,42] and rarely simultaneously[15,16,41] in the same organism: (1) the 'biosynthetic' type subject to end product inhibition by L-isoleucine, (2) the 'biodegradative' type (catabolic enzymes) insensitive to isoleucine and activated by ADP[42] or AMP.[43] The latter type has also been found in certain animal tissues (sheep[44] and rat[45] liver) incapable of isoleucine biosynthesis, but the animal enzymes appear to be further variants, being insensitive to nucleotides and allosteric effectors tested[45] and extending substrate specificity to L-serine.[44,45] Both types of enzymes have shown indications of pyridoxal–phosphate and sulfhydryl-group requirements where tested. With two exceptions,[16] the pH optima of the 'biosynthetic' enzymes have been reported in the pH region 8·0–9 5, while those of the 'biodegradative' enzymes have ranged widely from pH 6·2 to 10 5.

The results of our studies have shown that, apart from the case of *C. nana*, the algal enzymes generally resemble bacterial, fungal, and higher-plant 'biosynthetic' threonine dehydratases in pH optima, substrate saturation, inhibition by isoleucine, pyridoxal–phosphate and sulfhydryl-group requirements. The *C. nana* enzyme is neither sensitive to isoleucine nor to AMP and ADP but shows the usual pH optimum profile, pyridoxal–phosphate and sulfhydryl-group requirement of other algal enzymes. Its substrate-saturation kinetics and weak reaction to isoleucine indicate relatively diminished affinity for substrate and allosteric effector and suggest that it may be an aberrant form of the 'biosynthetic' type reminiscent of desensitized[6,16] and mutant enzymes [1,2,7,8,40] The *H. virescens* enzyme is generally similar to the other algal enzymes but differs in resisting inhibition from isoleucine after 55% loss of activity; the possibility that this may be due to simultaneous occurrence of 'biosynthetic' and 'biodegradative' types appears unlikely from

[40] I D Desai and W. J. Polglase, *Can J. Biochem* **45**, 11 (1967)
[41] H E Umbarger and B. Brown, *J Bacteriol* **73**, 105 (1957)
[42] H R Whiteley and M Tahara, *J Biol Chem* **241**, 4881 (1966)
[43] Y. Shizuta, A. Nakazawa, M Tokushige and O Hayaishi, *J Biol Chem.* **244**, 1883 (1969).
[44] J S. Nishimura and D M Greenberg, *J Biol. Chem* **236**, 2684 (1961).
[45] H. Nakagawa and H Kimura, *J Biochem* (*Tokyo*) **66**, 669 (1969).

the sharp pH optimum observed[15,16] and absence of effect from the nucleotides tested[41] but cannot be excluded without further examination

It is interesting to note the absence of any significant effect from L-valine on the algal enzymes. Previous work on 'biosynthetic' threonine dehydratase from bacteria[8,9,11] and yeast[12] has implicated valine as an allosteric effector causing stimulation at low and inhibition at high concentrations, but the validity of these effects is being questioned in relation to the instability of the enzyme under the test conditions used[4,13] The 'biosynthetic' enzyme of *Bacillus licheniformis*[5] offers the closest bacterial parallel to the algal enzymes in being unaffected by valine, as well as in several other properties tested. Unlike those of most bacteria,[7,9,46,47] yeast,[12,48] and higher plants,[17,18] this enzyme also failed to show reversal of isoleucine inhibition by valine,[5] indicating its total indifference to valine as an allosteric effector. Similar studies are required to detect any allosteric relationship of valine toward the algal enzymes.

With few exceptions, the earlier workers have not attempted to establish the sulfhydryl-group requirement of the threonine dehydratases studied and some have assumed its role in 'desensitization' to allosteric effectors obtained on treatment with mercurials ($HgCl_2$ or *p*-chloromercuribenzoate)[15,17,49] In the few cases tested, inhibitions were obtained from *N*-ethylmaleimide[12,42] and iodoacetamide[12] in addition to the mercurials, and the mercurial-induced inhibitions were prevented or reversed by mercaptoethanol,[5,46,50] 2,3-dithiol-propanol,[12] or dithiothreitol.[45] Our systematic approach in the present investigation has provided convincing evidence of a general sulfhydryl-group requirement of the algal enzymes and further studies are required to elucidate the nature of this requirement. In this connexion, it is interesting to note that the enzymes of two taxonomically-related algal species (belonging to Cryptophyta) have shown indications of an additional requirement for the disulfide grouping, not previously reported in the literature Such a requirement may imply particularly vulnerable cystine bridges between essential sub-units of the enzyme protein or the close proximity of such bridges to the active site of the cryptophyte enzymes.

EXPERIMENTAL

Algal cultures Details of the algal strains used, their source and maintenance have been reported[51] Each alga was mass-cultured in 5 l seawater (buffered with Tris (8 3 mM) at pH 7 9–8 1 and enriched with nitrate (2 5 mM), orthophosphate (0 25 mM), metasilicate (0 5 mM), vitamins (B_{12}, 2 7 nM, thiamine, 3 0 μM, biotin, 8 2 nM), and chelated trace-metal ions (EDTA, 21 8 μM, Fe^{3+}, 10 μM, Mn^{2+}, 5 μM, Zn^{2+}, 2 μM, MoO_4^{2-}, 1 μM, Cu^{2+}, 0 1 μM, Co^{2+}, 0 05 μM)) in a temperature-controlled (at 20°) chamber, under continuous illumination (see Table 1), with continuous magnetic stirring and periodic aeration (5% CO_2-enriched air), using previously described equipment and methods[52] The cultures were visually monitored and were harvested after 5–15 days of growth, depending on the algal growth rate All cultures were harvested by centrifugation at *ca* 13,000 *g* for 20 min and the pellets of algal material collected were freeze-dried in the presence of P_2O_5 The protein content of cultured material was estimated from duplicate micro-Kjeldahl N-determinations on 2-mg aliquots of dry algal powder

Algal extracts The dry algal powder (2–6 mg) was suspended in 0 5–0 7 ml buffer (0 2 M) and sonicated for 5 min at 0–4° in a Raytheon 10-kcycle magnetostrictive oscillator at a maximum current output of 1 1 A The whole sonicates were used directly for enzyme assays without further treatment

Threonine dehydratase assays The incubations, effected under standard conditions (Table 1) in 1 ml assay

[46] P DATTA, *J Biol Chem* **241**, 5836 (1966)
[47] G W HATFIELD and H E UMBARGER, *J. Biol Chem* **245**, 1742 (1970).
[48] H DE ROBICHON-SZULMAJSTER and P T MAGEE, *Europ J Biochem* **3**, 492 (1968)
[49] J-P CHANGEUX, *Cold Spring Harbor Symp Quant Biol* **26**, 313 (1961)
[50] A NAKAZAWA and O HAYAISHI, *J Biol Chem* **242**, 1146 (1967)
[51] N J ANTIA and J Y CHENG, *Phycologia* **9**, 179 (1970)
[52] N J ANTIA and J KALMAKOFF, *Fish Res Bd Can Manuscript Rept Ser (Oceanog Limnol)* No 203 (1965)

mixture, were terminated by adding 50% (w/v) aq. trichloroacetic acid (0 5 ml) and centrifuging at 2300 *g* for 20 min An aliquot (1 ml) of the supernate was treated with 1 ml of 1% 2,4-dinitrophenylhydrazine solution in 2N HCl for 5 min at room temp , then mixed with benzene (3 ml) and centrifuged at 1500 *g* for 3–4 min to obtain a clear extract. An aliquot (2 ml) of this extract was vortex-mixed for 1 min with 10% aq. Na_2CO_3 (3 ml) and the aqueous organic layers were separated by centrifugation at 1500 *g* for 3–4 min An aliquot (2 ml) of the aq Na_2CO_3 extract was mixed with 1 5 N NaOH (2 ml) and, 5 min later, the absorptivity was determined at 435 nm on a Beckman DU-2 spectrophotometer Calibration curves were constructed with standards of sodium α-ketobutyrate (Sigma) and pyruvate

Chromatographically homogeneous L-threonine, L-isoleucine and L-valine were purchased from Calbiochem (Los Angeles, Calif), AMP, ADP, and cyclic 3′,5′-AMP from P-L Biochemicals (Milwaukee, Wis). All other reagents were of the highest purity grade commerically available NSD-1055 was kindly donated by Smith and Nephew Research Ltd (Gilston Park, Harlow, Essex, U K)

Identification of enzyme reaction product Sonicated suspensions of 10 mg aliquots of *C. salina* and *A. quadruplicatum* cell powders were incubated as usual (Table 1) with pyridoxal phosphate and threonine for 30 min at pH 8 5 The following controls were simultaneously taken, all without incubation (a) 'blank' with pyridoxal phosphate only, (b) 'zero incubation-time algal controls' with algal powders and substrates as above, (c) separate standards (2 mM α-ketobutyrate, 2 mM pyruvate, 20 mM acetaldehyde) with pyridoxal phosphate only Incubations were terminated and the reaction mixtures were submitted to the regular assay procedure (see above) up to the aq Na_2CO_3 extraction step Two series of aliquots were taken from the Na_2CO_3 extracts and treated as follows (i) 0 2 ml aliquot was diluted to 2 ml with 10% aq. Na_2CO_3 and treated with 1·5 N NaOH (as per regular assay procedure) for spectrophotometric determination of keto acid, (ii) 2 ml aliquot was treated dropwise with conc HCl (*ca* 0 5 ml) to adjust the pH to 1–2 and then extracted with benzene (2 5 ml); the benzene extract was washed with H_2O, dried, redissolved in EtOAc (30 μl), and submitted to TLC on unactivated silica gel G plates (0 25 mm thick) developed with ethyl formate-light petroleum (60–80°)–HOAC (50 50·7 v/v) [25] The 'zero incubation-time algal controls' as well as the 'blank' and acetaldehyde standard controls showed no detectable colored spots on the chromatoplates. The controls of pyruvate and α-ketobutyrate standards showed each a 'major' lemon-yellow spot (pyruvate R_f 0 60, α-ketobutyrate 0 68) and a 'minor' brown–yellow spot (R_f 0 84 for both keto acids). The incubated algal samples gave 2 spots similar to those from the α-ketobutyrate standard and co-chromatographing with them On elution with 3% aq $NaHCO_3$, the 'major' spot products from algal samples, α-ketobutyrate, and pyruvate standards showed similar spectra with λ_{max} at 382, 382, and 378 nm respectively, and the 'minor' spot products also showed similar spectra with λ_{max} at 370–372 nm 2,4-Dinitrophenylhydrazine (DNPH) and its acetaldehyde derivative showed only single TLC spots corresponding to the above-obtained 'minor' spots in R_f values and absorption maxima When the above-eluted 'major' spot products in $NaHCO_3$ solution were acidified, extracted with benzene, and rechromatographed as before, they again produced 'major' and 'minor' spots similar to those originally obtained It was concluded that the 'major' spot products are the true dinitrophenylhydrazones of the keto acids The 'minor' spot products have resembled DNPH and acetaldehyde–DNPH so closely in chromatographic mobility and spectral properties that they appear to be decomposition or decarboxylation artefacts of the 'major' products, probably produced during the acidification step of the isolation procedure; the possibility is not precluded, however, that the 'minor' products may represent minor proportions of *cis-trans* isomers of the dinitrophenylhydrazones of the keto acids [53,54]

Acknowledgements—Thanks are due to Mr J. Y. Cheng and Miss Eileen Wiseman for mass-culturing the algae, to Miss Carol Gregorash for the preliminary experiments, and to Smith and Nephew Research Ltd. for the gift of NSD-1055 One of us (I D D) acknowledges financial support from the Fisheries Research Board of Canada and the Research Committee of the University of British Columbia.

[53] F A Isherwood and D H Cruickshank, *Nature, Lond.* **173**, 121 (1954).
[54] H. Katsuki, T. Yoshida, C Tanegashima and S Tanaka, *Anal Biochem* **24**, 112 (1968)

Key Word Index—Algae, L-threonine dehydratase, biochemical systematics, chemotaxonomy