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COMPARATIVE STUDIES ON SEVERAL CATALYTIC PROPERTIES OF BIOSYNTHETIC L-THREONINE DEHYDRATASE (Deaminating) IN SEVEN SPECIES OF UNICELLULAR MARINE PLANKTONIC ALGAE

by

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ABSTRACT

Several aspects of L-threonine dehydratase from seven species of unicellular marine planktonic algae were investigated; (1) the disulfide group requirement for activity of the enzymes from two cryptomonads, (2) monovalent inorganic cation requirement for enzyme activity, (3) substrate specificity and substrate analog inhibitions, (4) allosteric activation and inhibition and diverse effects from other amino acids, (5) pH optima of the algal enzymes with particular emphasis on the elucidation of the unique pH-activity response of the enzyme from Hemiselmis virescens.

The threonine dehydratases from Chroomonas salina and Hemiselmis virescens require disulfide groups for enzyme activity as exemplified by the specific inhibition exerted by all thiol reagents tested, which inhibition could be partially reversed or prevented by the appropriate treatments. Sulfhydryl group requirement for enzyme activity was confirmed and it was demonstrated that these groups are essential for feedback inhibition from L-isoleucine.

All algal enzymes appear to require monovalent alkali-metal cations for full expression of activity, more specifically K^+ and NH_4^+. Anacystis marina was exceptional in showing maximal stimulation from Li^+. Organic cations were without effect whereas some inhibition from certain divalent cations (Zn^{2+}, Cu^{2+}) and anions (NO_3^-, I^-, ClO_3^-) were observed, whilst HPO_4^{2-} and SO_4^{2-} were stimulatory.

Aside from L-threonine, the algal enzymes extended substrate activity to L-serine and L-allothreonine. In addition to its known threonine dehydratase, Chroomonas salina appeared to produce a serine dehydratase which accounted for the relatively high substrate activity observed toward L-serine with this species. Inhibition from substrate analogs was limited to L-homoserine and L-serine despite the substrate activity of the latter. The mechanism for the peculiar

mode of inhibition evinced by L-homoserine remains unknown whereas that of L-serine appears to result from inactivation of the enzyme.

With the exception of Cyclotella nana and to a lesser extent Hemiselmis virescens, all the algal enzymes were subject to feedback inhibition from L-isoleucine, which inhibition was pH dependent, subject to reversal by L-valine, and could be duplicated by the analog L-O-methyl threonine. Several other amino acids (L-leucine, L-norvaline, L-valine) were able to inhibit most enzymes when present at high concentration. It was proposed that the mode of inhibition by these latter amino acids may occur via interaction at the site specific for allosteric inhibition. L-Valine at low concentration effected pronounced activation of the enzymes and was thusly assigned the role of allosteric activator, acting at a site distinct from that of L-isoleucine or L-threonine.

Hemiselmis virescens was distinctly unique in that, unlike the other algal enzymes, it displayed two pH-activity optima. The investigation of this phenomenon was pursued in two ways (i) examination of enzyme response to various potential effectors (nucleotides, L-methionine, L-aspartate, L-cystathionine) at a pH intermediate between the two optima, (ii) examination of enzyme response to known effectors (L-valine, L-isoleucine) at the two pH optima. It was concluded from these studies that Hemiselmis virescens may produce a culture-dependent mixture of two threonine dehydratases, one of which is generally similar to the other algal enzymes, the other of which is insensitive to the usual allosteric regulation yet is not a standard biodegradative isozyme.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

INTRODUCTION TO THE PRESENT INVESTIGATION

It was the purpose of this investigation to examine several interesting features of algal biosynthetic threonine dehydratase, some of which were indicated, but not adequately investigated in the earlier study from our laboratories (23). This earlier work involved the preliminary characterization of the enzyme from seven species of unicellular marine planktonic algae (belonging to five taxonomic divisions). These species were reported to exhibit considerable differences in specific activity as well as significant effects from the nutritional conditions of algal culture. The enzymatic production of α-ketobutyrate from L-threonine was confirmed 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 L-threonine concentration. With the exception of Cyclotella nana, all the algal enzymes were strongly inhibited by L-isoleucine; L-valine, EDTA, AMP, ADP, and cyclic 3',5'-AMP were reported to have no significant effect. Several carbonyl reagents 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 and these inhibitions were partially reversed by dithiothreitol. The enzymes of Chroomonas salina and Hemiselmis virescens were also markedly inhibited by dithiothreitol. These results characterized the algal threonine dehydratases generally as isoleucine-regulated, pyridoxal phosphate requiring, allosteric enzymes similar to the corresponding TDH (BS) 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.

When this present investigation was initiated, its scope was intended to include the study of the following features of the algal enzymes 1) the particular disulfide and sulfhydryl group requirements of the enzymes from two cryptophytes, 2) the effects of cations and anions on the activity of all the algal enzymes previously examined, 3) substrate specificity and substrate-analog inhibitions, 4) specificity of feedback regulation by L-isoleucine and structurally related amino acids, 5) the causes of the previously reported peculiar isoleucine-induced feedback inhibition of the enzyme from H. virescens. From this list, the study of the first two features yielded such fruitful results that they called for priority of publication. These have now been prepared as papers and submitted for publication, and are appended to this dissertation as Supplements I and II. Their results will be summarized in the next two paragraphs.

Disulfide and sulfhydryl group requirements of enzyme activity in two cryptophytes (see Supplement I). A systematic investigation of the cryptophyte's "disulfide requirement" revealed that both C. salina and H. virescens were sensitive to inhibition from all thiols tested (dithiothreitol, cysteine, etc.) but showed no effect from ascorbic acid or reduced NAD. By contrast, the enzyme activities from the five non-cryptophycean algae were generally not affected by any of these reagents. The thiol-reagent inhibition of the cryptomonad enzymes (i) achieved saturation with 60-70 % reduction in activity, (ii) was considerably reduced by pretreatment of the enzymes with L-threonine and L-isoleucine, and (iii) was partially reversed by subsequent treatment with arsenite and exposure to air. It was deduced that such inhibitions were caused by thiol-specific reduction of enzyme-protein disulfide groups essential for the full expression of activity

and that these groups were susceptible to ready reductive cleavage and oxidative restoration. The additional activity-requirement of the cryptomonad enzymes for sulfhydryl groups was confirmed a) by the study of their sensitivity to inhibition from mercurials and disulfide-sulfhydryl exchanging reagents, and b) by the partial reversal of these inhibitions from subsequent treatment with dithiothreitol. Both cryptophyte enzymes were desensitized to feedback inhibition from L-isoleucine by prior exposure to subinhibitory concentrations of $HgCl_2$ or dithiodipyridine.

Stimulation of TDH activity by monovalent inorganic cations and diverse effects from other ions (see Supplement II). Threonine dehydratases from the five classes of algae (seven species) were all activated to varying degrees by monovalent inorganic cations. The activation was generally the strongest (3-5 fold) with K^+ and NH_4^+, whilst Li^+, Rb^+, and Cs^+ showed intermediate orders varying with algal species. Anacystis marina was exceptional in showing strongest stimulation (5-fold) from Li^+ and more pronounced activation from Na^+ than Cs^+, whilst Tetraselmis maculata showed another type of response with the least effect from Li^+ and markedly greater activation from Rb^+ than NH_4^+. Activation was hyperbolic in response to ion concentration and specific for monovalent inorganic cations with indications of a coenzyme type of role. Organic cations were inert and the divalent cations Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+} were either inhibitory or without effect. Among the anions tested, chloride, bromide, fluoride, bicarbonate showed no effect; iodide, nitrate, chlorate were inhibitory, whilst phosphate and sulfate were slightly stimulatory. It was concluded that the algal threonine dehydratases may have an absolute K^+ or NH_4^+ requirement for in vivo expression of activity.

REVIEW OF LITERATURE

Differentiation of biosynthetic and biodegradative threonine dehydratases

L-Threonine dehydratase (L-threonine hydro-lyase, deaminating; EC 4.2.1.16) catalyses the conversion of L-threonine to α-ketobutyric acid and ammonia. The product, α-ketobutyrate may be further metabolized in two ways 1) sequential enzymic alteration ultimately leading to the biosynthesis of L-isoleucine (see Appendix A for details), 2) oxidative catabolism to yield energy. Threonine dehydratases (TDH) involved in the former process are appropriately termed "biosynthetic" (BS) and for the latter process, "biodegradative" (BD). Identification of the isozymes is based primarily on certain regulatory characteristics; TDH (BS) being subject to feedback inhibition by L-isoleucine and TDH (BD) being isoleucine insensitive but activated by AMP (72) or ADP (78). With two exceptions (46), the pH optima for the TDH (BS) have been reported within the range 8.0-9.5 whereas the TDH (BD) range widely in their optima from pH 6.2-10.5. Molecular weights for these enzymes have been reported as low as 147,000 (72) although they have generally been established in the vicinity of 200,000 (7,21, 28,35,38,56,73) with a dimeric or tetrameric subunit composition (35,38,56). The requirement of pyridoxal phosphate as a bound cofactor and a general sulfhydryl group requirement has been conclusively established. Although the simultaneous occurrence of both isozymes in one organism has been reported (46, 48,74,77), the usual circumstance is the presence of only one type. The ensuing descriptions therefore, pertain only to biosynthetic threonine dehydratases from various sources which have hitherto been studied and represents a condensation of the major physico-chemical and regulatory characteristics of this enzyme.

Bacterial TDH (BS)

Since the initial work on threonine dehydratase of Escherichia coli (76,77), numerous reports have appeared concerning this enzyme from a variety of bacterial sources (3,7,10,12-16,18,19,24,25,33-37,39,45,54-59,66,67,73).

Sensitive feedback inhibition from isoleucine was observed in all but a few instances (28,45) wherein high concentration of isoleucine was required to effect inhibition and then only with low levels of substrate concentration. On the other hand, mutant as opposed to the wild type TDH have frequently shown resistance to isoleucine (4,21,52). Feedback inhibition has shown pH dependency (3,12,19,22,26,54-56,73) with optimum effects in the vicinity of pH 8 and virtual abolition of response within pH 9.5-11.

Valine has been implicated as an allosteric effector since low concentration has been observed to activate the deaminating reaction (3,7,12,19,29,34) in the presence of sub-saturation levels of substrate. At high concentration of valine, however, inhibition of the enzyme reaction has been observed (3,18,19,39,54-56, 73), which inhibition has shown an analogous pH dependency to that of isoleucine (3,54). Valine has also displayed the ability to partially reverse the isoleucine-induced inhibition of TDH (7,12,19,26,39,67,73). Other amino acids reported to inhibit the enzyme at high concentration are leucine (3,15,56,73), norvaline (3, 15,73), norleucine (3,73) and α-aminobutyrate (3). The ability of these latter amino acids to either overcome isoleucine inhibition or activate at low substrate concentration has been occasionally described (15,29,56).

Substrate saturation kinetics have usually exhibited sigmoidal rather than hyperbolic profiles although the latter situation has also been reported (3,24, 28,38,58,59,67,72). In all instances, the presence of isoleucine either increased or conferred sinuosity upon the substrate saturation curves whereas valine

either increased or conferred hyperbolicity upon these curves (12,19,34,69). The opposing nature of the alterations to substrate saturation kinetics by isoleucine and valine has prompted the suggestion that the initial profile with threonine alone may reflect the relative amounts of contaminating isoleucine:valine in the enzyme preparation (37) or may be the net result of an inadequately stabilized enzyme (31,32).

Aside from L-threonine, L-serine is the only other amino acid able to serve as substrate, albeit with substantially lower reactivity relative to threonine (10,15,28,33,55,56,73). Conversely, the substrate analogs allo-threonine (15,29,59) and homoserine (15,29) appear to function as inhibitors of the enzyme activity toward threonine; such inhibition has also been reported from serine despite its capacity to function as a substrate (28,56,66).

Systematic investigations of any ionic requirements of the bacterial TDH have been infrequently performed, although high ionic strength is commonly employed to maintain enzyme stability. From reports which have studied this aspect, it appears that monovalent cations may assist in the full expression of enzyme activity (7,73).

Yeast TDH (BS)

Threonine dehydratase has been studied in yeast (5,6,9,22,41,42,49) and has exhibited gross similarity to the bacterial counterparts. Feedback inhibition from isoleucine has been reported in all cases and on one occassion, low concentration of isoleucine stimulated enzyme activity (22). Feedback inhibition was demonstrated to be pH dependent (9,22,41) and subject to reversal by valine (9,22), norleucine or α-aminobutyrate (9). At low concentration, valine serves to activate the enzyme (6,9,49) although inhibition from high concentration has not been reported. Other amino acids reported as inhibitory are allothreonine, homoserine,

and leucine (9,41). Substrate saturation kinetics were sigmoidal for all cases studied and attained a hyperbolic profile in the presence of valine (6,9,22,49) or the amino acids which reversed isoleucine inhibition (9). Monovalent cations appear to be required for the optimal expression of yeast TDH activity, particularly ammonium, which has been implicated as an "end-product activator" (9,42).

Plant and Fungal TDH (BS)

Threonine dehydratases of plants (26,46-48,53,60,69,70,74) and fungi (47) undergo feedback inhibition from isoleucine, which inhibition is pH dependent (26) and subject to reversal from valine (26,60,69,70). An interesting feature of these enzymes is that the role of allosteric activator has been assigned not only to valine (69,70), but also to norvaline, norleucine, aspartate (46,48,74) and in the case of the fungal TDH, only phenylalanine (47). Amino acids reported to inhibit the enzyme at high concentration are leucine (60), valine (26,69,70) and homoserine (47,74). Substrate saturation curves have been reported as hyperbolic (60,69,70) or sigmoidal (46-48,74); in the latter case, the presence of allosteric effector evinced a hyperbolic response to substrate concentration. Compounds other than L-threonine capable of functioning as substrates with lowered reactivity are L-serine (26,60,69) and for a rose tissue culture enzyme, L-allocystathionine (26). Monovalent cations (more specifically potassium and ammonium) appear to be required for the full expression of TDH activity (26,29).

MATERIALS AND METHODS

Algal cultures

Table 1 lists the algal strains employed in the investigations together with other relevant data. Details of the algae, their source and maintenance have been communicated (1) as have the techniques of mass culture, harvesting and freeze drying (23). Protein content of each algal culture was determined by micro-Kjeldahl N-determinations and were previously reported (23). Excepting the 2 cryptophytes, all the algae used in this study were grown photoautotrophically with added vitamins. C. salina was cultured under 3 sets of conditions, (i) photoautotrophic (vitamins, light), (ii) photoheterotrophic (glycerol, vitamins, light), (iii) chemoheterotrophic (glycerol, vitamins, darkness) (17). Being unable to use nitrate as N-source or to grow in darkness on organic substrates hitherto tested, H. virescens was cultured phototrophically with the same vitamins under 3 other conditions (i) with urea (2 mM) as N-source, (ii) with glycine (4mM) as N-source, (iii) with glycine (4mM) and glycerol (2,17). Where present, the glycerol concentration was 0.25 M. Unless otherwise stated, the enzyme tests concerning C. salina and H. virescens were normally made with the glycerol-light grown culture of the former and the glycine-light grown culture of the latter.

Algal extracts

Suspensions of algal powder in appropriate buffer were subjected to ultrasonic oscillation (5 min, 0-4 C) in a Raython 10 Kcycle magnetostrictive oscillator at a maximum output of 1.1 A.

Reaction mixtures

Unless otherwise indicated, all enzyme incubation mixtures contained unmodified whole algal sonicate (0.5 ml in 0.2 M potassium N-tris(hydroxymethyl) methylglycine (K-Tricine), pH 8.5), pyridoxal phosphate (0.1 μmole), and L-threonine (80 μmoles) in a final volume of 1 ml. For studies which involved the effects of pH, the following buffers were substituted for K-Tricine: tris(hydroxymethyl)methylamine (Tris-HCl), pH 7.0-8.7; 2-methyl-2-amino-propanol (Map-HCl), pH 9.0-10.0. When these latter buffers were employed, KCl (final concn 0.1 M) was included to satisfy the enzyme requirement for monovalent cation.

The mixtures were normally preincubated first with the test reagent (15 min, 22°C), then with pyridoxal phosphate (5 min, 37°C), and finally incubated with threonine at the latter temperature for periods varying with the algal species (usually as indicated in Table 1). Identical preincubations were effected on controls taken without the test reagent. All the tests included both unincubated and fully incubated controls. The enzyme reaction was terminated by the addition of aqueous trichloroacetic acid (0.5 ml, 50 % w/v) followed by centrifugation (3,000 g, 20 min).

Assay procedure

Keto acid produced was assayed according to the method of Friedemann and Haugen (30) with minor modifications. A 1 ml aliquot of the reaction mixture supernate was treated with 2,4,-dinitrophenylhydrazine (1 ml, 1 % in 2 N HCl) and allowed to react (5 min, 22°C) after which absolute ethanol (1 ml) and benzene (3 ml) were added and the mixture vortex-mixed at high speed (1 min) and centrifuged (2,000 g, 5 min). A 2 ml aliquot of the organic layer was then vortex-mixed for 1 min with sodium carbonate (3 ml, 10 % w/v) followed by

centrifugation (2,000 g, 5 min). A 2 ml aliquot of the aqueous layer was then combined with sodium hydroxide (2 ml, 1.5 N) and absorbance determined at 435 nm on a Beckman DU-2 spectrophotometer. From the corrected optical density, mμmoles keto acid produced was calculated by means of a conversion factor obtained from standard calibration curves of α-ketobutyrate and pyruvate (see Appendix C).

Chemicals

Chromatographically homogeneous L-threonine, other L-amino acids, and dithithreitol were obtained from Calbiochem (Los Angeles, Calif.); D-amino acids and L-O-methyl threonine from Sigma (St. Louis, Mo.); AMP, ADP, ATP and cyclic 3',5'-AMP from P-L Biochemicals (Milwaukee, Wis.). All other reagents used were of the highest purity grade commercially available.

Table 1. Culture and standard assay conditions of the algal species used.

Alga [φ]	Culture conditions		Assay conditions	
	Light*	Added organic nutrients**	Dry alga (mg)	Incubation (min @ 37°C)
CHLOROPHYTA				
Tetraselmis maculata (Te.ma.)	+	nil	2	40
BACILLARIOPHYTA				
Cyclotella nana (Cy.na.)[†]	+	nil	6	30
CRYPTOPHYTA				
Chroomonas salina (Ch.sa.(NA))	+	nil	4	20
(Ch.sa.)	+	glycerol	2	15
(Ch.sa.(Gly. D))	-	glycerol	2	10
Hemiselmis virescens (He.vi.)	+	glycine	2	15
(He.vi.(Gly.L))	+	glycine glycerol	4	15
(He.vi.(Urea)	+	urea	4	20
RHODOPHYTA				
Porphyridium cruentum (Po.cr.)	+	nil	2	30
CYANOPHYTA				
Agmenellum quadruplicatum (Ag.qu.)	+	nil	2	10
Anacystis marina (An.ma.)	+	nil	2	15

[φ] Abbreviations in parentheses are used to denote these species in figures and tables.

[†] Recently redesignated *Thalassiosira pseudonana* (see refs. 18 & 20 of Supplement I).

* Presence (+) or absence (-) of continuous illumination (*ca.* 16,500 lux).

** In addition to the vitamins normally added to culture medium (see methods for concentrations used).

PART I. Substrate Specificity Studies and Inhibition from Substrate Analogs. Evidence for Additional L-Serine Dehydratase Activity in the Cryptomonad C. salina

RESULTS

Substrate specificity

Several compounds of structural similarity to L-threonine were tested as to their substrate reactivity with the algal threonine dehydratases, the results of which are aummarized in Table 2. Of the amino acids tested, L-serine and L-allothreonine were deaminated by all species, the response of the former to graded concentration being sigmoidal with similar Km but lowered Vmax relative to L-threonine. D-Threonine, D-serine, L-homoserine and L-O-methyl threonine were not acted upon to any significant extent. The slight activity obtained with L-allothreonine (4-12 %) did not appear to be due to L-threonine contamination because the keto acid produced did not show the expected increase (as a constant % of the possible L-threonine impurity) on examining the saturation kinetics with L-allothreonine as substrate. Furthermore, the complete inhibition of this activity by L-isoleucine confirmed that the reaction was due to algal TDH acting inefficiently on L-allothreonine as substrate analog.

With the exception of C. salina, all species showed substantially less reactivity with L-serine as compared to L-threonine (17-26 % at equimolar concentration). C. salina showed double the activity from L-serine relative to L-threonine and appeared, therefore, to contain a serine dehydratase (SDH) in addition to its known TDH. Such simultaneous occurrence of both dehydratases has been previously reported for a pseudomonad (18). It was subsequently

observed that another nutritionaly different culture of this algal species (C. salina (NA)) behaved analogously to the standard culture in that all amino acids tested were ineffective as substrates excepting L-allothreonine and L-serine which showed activities of 7 % and 236 % respectively when compared to equimolar concentrations of L-threonine. Evidently, the ability of C. salina to deaminate L-serine is a species peculiarity and not a nutritionally-induced characteristic. To this end, a series of comparative studies involving several known properties of the cryptomonad and other algal TDH was initiated to compare the responses obtained in relation to the substrate used (ie. L-threonine or L-serine).

Optimum pH of the deaminase reaction in relation to substrate

The pH optima for the deamination of L-threonine by the algal TDH had been previously obtained (23) but were re-investigated under rigorously controlled conditions (see Part III). Examination of the pH optima when L-serine was used as substrate (Fig. 1B) yielded identical activity profiles when compared to those obtained using L-threonine as substrate (Fig. 1A), excepting the case of C. salina. With this species, the optimum with L-serine (pH 8) did not coincide with that using L-threonine (pH 8.5), which was indeed suggestive of an enzyme other than TDH assuming responsibility for L-serine deamination.

Sensitivity to L-isoleucine in relation to substrate

Feedback inhibition from L-isoleucine is a characteristic feature of all except one algal TDH (see Part II), a property alien to serine dehydratase (18). In the event that the C. salina enzyme preparation contained an active SDH in addition to the TDH, the overall dehydratase activity of this preparation on L-serine as substrate would be expected to be relatively insensitive to feedback

inhibition from L-isoleucine. The data presented in Table 3 clearly demonstrates that only C. salina (from both nutritionally different cultures) displayed any difference in response to L-isoleucine from L-serine versus L-threonine as substrate. It may be noted that the C. salina enzyme preparation did show some inhibition (12 %) when L-serine was used as substrate, which inhibition appears to be due to the L-isoleucine-sensitive activity of the TDH component known to be present. Based on this premise, the keto acid difference between the values obtained without and with L-isoleucine may be reasonably assumed as due to the action of the TDH component only. It was thus inferred that 12 % of the total keto acid produced from 50 mM L-serine as substrate was the result of TDH action, the remainder (88 %) being contributed from the activity of the SDH component. Stoichiometric computations, equating the keto acid contribution (12 %) from TDH action on L-serine to the known amount of keto acid produced from equivalent L-threonine by the same enzyme, give an estimate of 24 % conversion of L-serine relative to L-threonine, due to the TDH component only. Referring to Table 2, it is apparent that this estimated ability of the C. salina TDH to deaminate L-serine is in agreement with the values obtained for the other algal enzymes (17-26 %).

Substrate-related response of C. salina enzyme activity to various reagents and effectors

The C. salina TDH has shown certain characteristic properties, such as monovalent cation (see Supplement II) and disulfide group (see Supplement I) requirements and feedback regulatory effects from L-isoleucine and L-valine (see Part II). It was of interest to examine these and other effects on the the deamination obtained from L-serine by the C. salina enzyme preparation in order to further establish the presence of SDH activity in this system.

Table 4 shows the different responses obtained from such an examination comparing L-serine versus L-threonine as substrate. In the first place, K^+ was the more effective monovalent cation for L-serine deamination whilst NH_4^+ was most effective towards L-threonine as previously observed, secondly, the known inhibitory effects of L-isoleucine, L-valine and dithiothreitol with respect to L-threonine deamination were markedly decreased with L-serine as substrate. Moreover, the maximal inhibitions induced by these reagents with L-serine as substrate followed the same relative pattern (although substantially reduced) as was obtained when L-threonine functioned as the substrate. These observations confirm the occurrence of SDH in C. salina and also lend support to the previous inference that part of the total keto acid produced through the deamination of L-serine was the result of TDH action.

EDTA had been previously shown to be without effect on the algal TDHs (23) but was tested with L-serine as substrate in view of a previous report (8) of a ferrous ion requirement for a bacterial SDH, which requirement appeared to be exceptional in that no definite divalent cation involvement has been recorded for other serine dehydratases from bacterial, plant or animal sources (27,40, 65). The ineffectiveness of EDTA on the deamination of L-serine by the C. salina enzyme preparation suggests that the algal enzyme is no exception to the other SDHs and has no recognizable divalent cation requirement.

Inhibition of algal TDH activity by structural analogs of L-threonine

In view of previously reported inhibition (presumably via substrate antagonism) of other threonine dehydratases by L-serine, L-allothreonine or L-homoserine, these substrate analogs were tested for effects on the activity of the algal TDHs towards L-threonine. The results summarized in Table 5 show that L-serine and L-homoserine inhibit the enzyme reaction markedly whilst L-allothreonine has no

effect. It is interesting to note that whereas both L-allothreonine and L-serine could function as substrates to varying degrees, only the latter was inhibitory.

To elucidate the nature of these inhibitions, the following enzymatic kinetics were examined (i) the effects of gradient concentrations of L-serine and L-homoserine at saturating concentrations of substrate, (ii) the response of graded substrate concentration to the presence of the analogs at concentration levels which were 50 % inhibitory at substrate saturation. The typical kinetic profiles obtained from this 2-fold examination are depicted in Figs. 2A and 2B, which profiles were closely comparable for all the algal species examined. These results showed a distinct difference in the nature of the inhibitory effect obtained from the two substrate analogs. In response to increasing concentration of the inhibitors, the enzyme activity drops rapidly with L-serine to attain a "saturating" degree of inhibition whereas with L-homoserine, there appears to be a threshold concentration prior to which no inhibition was evident and beyond which an apparently linear decrease in activity was observed (see Fig. 2A). Although the net inhibition evinced by 50 mM L-serine and L-homoserine approached comparable magnitude for each algal enzyme, the latter amino acid required approximately 3-fold greater concentration to effect 50 % inhibition of the enzyme.

Substrate saturation kinetics for A. marina have been shown to be sigmoidal in the absence of allosteric effector (see Part II). This pattern was found to be altered differently by the inhibitions obtained from L-serine and L-homoserine. As expected from their concentrations used, both compounds produced 50 % reduction of Vmax at substrate saturation levels (see Fig. 2B). At low substrate levels however, L-serine produced an even more pronounced inhibition whilst L-homoserine tended to weaken its inhibitory grasp with declining substrate concentration.

These effects on the substrate saturation kinetic profiles may be interpreted as caused by an overall decrease in Vmax with both substrate analogs but different effects on Km, which appears to be increased by L-serine but hardly altered by L-homoserine.

When the deamination of L-threonine was measured as a function of the incubation period and compared with those obtained from simultaneous addition of either L-serine or L-homoserine, a difference in the mode of inhibition from the substrate analogs (as exemplified by Fig. 3A) was again observed. Apart from the overall 50 % inhibition obtained, the rectilinear profile of the L-threonine deamination reaction was not altered by the presence of L-homoserine, whereas it was radically changed to a hyperbolic profile from the presence of L-serine. This latter profile reflected a similar course of the deamination of L-serine taken as a control without the L-threonine. In the case of C. salina (Fig. 3B), the deamination course was linear for L-threonine, L-homoserine + L-threonine and L-serine but the slope was considerably greater for L-serine relative to L-threonine, providing additional evidence for the simultaneous of an active serine dehydratase.

DISCUSSION

Biosynthetic threonine dehydratases from a variety of surces have been generally reported to extend substrate specificity to L-serine (10,15,26,28,33,55,56, 60,69,73) with a reactivity considerably less than that for L-threonine. A few reports have appeared describing TDHs which may deaminate other amino acids, notably allocystathionine for an enzyme from rose tissue culture (26) and allo-threonine in the case of a biodegradative TDH from sheep liver (65). The algal enzymes, being able to act upon both L-serine and L-allothreonine (although the

latter was affected to a very small extent), may be likened to the animal, rather than bacterial or plant enzymes in this respect. Further substantiation of this resemblance arises from the observation that the algal enzymes, like that from sheep liver, were not subject to the competitive inhibition from allothreonine previously reported for yeast (9) and bacterial (15,29,59) biosynthetic TDHs. The plant enzymes do not appear to have been sufficiently investigated in this respect.

Among the algae studied in the present investigation, the cryptomonad C. salina was unique in showing evidence of an enzyme (other than TDH) capable of readily deaminating L-serine. That the enzyme in question was an SDH was substantiated by studies wherein certain known properties previously characterized for the C. salina TDH were compared to those manifested when L-serine replaced L-threonine as substrate; (i) shift in pH optimum from 8.5 (for L-threonine) to 8.0 (for L-serine), (ii) abolition of the characteristic effects from dithiothreitol, L-isoleucine and L-valine (known for TDH activity) on substitution of L-serine for L-threonine, (iii) reversal of the order of activation by two monovalent cations on exchanging the two substrates. In addition, apparent linearity of L-serine deamination and the failure of L-homoserine to function as substrate negate the possibility of an active homoserine dehydratase which could conceivably deaminate L-serine (43). These findings are in agreement with previously reported properties of certain SDHs occurring either singly or in conjunction with a TDH (18,43,65).

Previous investigators have reported inhibitory effects from L-serine (28, 56,65,66) and L-homoserine (29,47,65,74) in spite of the former amino acid's ability to undergo deamination by TDH. Irreversible inactivation of the enzyme has been proposed to account for this paradoxical L-serine inhibition and this explanation may apply to the algal enzymes as well in view of the conversion of

the L-threonine deamination velocity from a rectilinear to a hyperbolic profile on simultaneous incubation with L-serine, which response is strikingly similar to that reported for the sheep liver TDH (65). This phenomenon of inactivation has been described for several bacterial biosynthetic TDHs (28,56,66) and animal biodegradative TDHs (44,65). Little is known regarding the mechanism of inactivation involved although it has been hypothesized that L-serine, by binding to the enzyme in a "tighter" manner than L-threonine, affects the binding constant of the cofactor (pyridoxal phosphate), ultimately leading to alteration in the balance of active holoenzyme and inactive apoenzyme (44).

The question of L-homoserine inhibition however, poses a completely different situation from that of L-serine since the inhibition became evident only at high substrate levels. This indicates that the L-homoserine inhibition may not be competitive with L-threonine for direct interaction at the substrate binding sites as has been suggested (29) since, if this were the case, inhibition would undoubtedly be greater, not nonexistent at low levels of substrate. Because of the immediate involvement of homoserine in the biosynthesis of threonine (see Appendix B), it is tempting to speculate that homoserine may be interrelated with threonine dehydratase in some presently unknown precurssor-product mechanism.

Table 2. Algal deaminase activity observed from structural analogs of L-threonine tested as substrate*

Substrate (50 mM)	Enzyme activity (% of control)						
	Ch.sa.	He.vi.	Ag.qu.	An.ma.	Po.cr.	Te.ma.	Cy.na.
L-threonine	100	100	100	100	100	100	100
L-allothreonine	6	8	9	12	9	4	6
L-O-methyl threonine	<2	<2	<2	<2	<2	<2	<2
D-threonine	<2	<2	<2	<2	<2	<2	<2
L-serine	197	24	19	17	18	20	26
D-serine	<2	<2	<2	<2	<2	<2	<2
L-homoserine	<2	<2	<2	<2	<2	<2	<2

*When tested, the structural analogs were added to the reaction mixture in place of the L-threonine normally used in the standard enzyme incubation procedure.

Table 3. Sensitivity of algal deaminases to L-isoleucine inhibition in relation to substrate.*

Alga	L-Ileu. (5 mM)	Enzyme activity (% of control) Substrate (50 mM) L-Serine	L-Threonine
Ch.sa.	-	100	100
	+	88	4
Ch.sa.(NA)	-	100	100
	+	84	6
He.vi.	-	100	100
	+	48	46
Ag.qu.	-	100	100
	+	8	10
An.ma.	-	100	100
	+	6	8
Po.cr.	-	100	100
	+	5	4
Te.ma.	-	100	100
	+	11	10

* Method involved pretreatment with L-isoleucine (15 min, 22°C) where indicated, prior to the regular assay procedure commencing with pyridoxal phosphate addition. 50 mM L-serine or L-threonine replaced the substrate normally used.

Table 4. Substrate-related response of C. salina deaminase activity to various reagents and effectors.*

Compound tested	Concn (mM)	Enzyme activity (% of control) Substrate (50 mM) L-Serine	L-Threonine
nil	-	100	100
L-isoleucine	0.01	99	88
	0.10	92	36
	1.0	88	5
	10	86	4
L-valine	0.10	101	112
	1.0	100	100
	10	96	48
	100	89	11
dithiothreitol	1.0	93	26
	10	92	25
EDTA	100	96	102
KCl†	100	428	310
NH_4Cl†	100	345	400

* Method involved pretreatment with the test compound (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition. 50 mM L-serine or L-threonine replaced the substrate normally used.

† Relative to controls of Tris-HCl buffer without added cation.

Table 5. Effects of substrate analogs on algal TDH activity towards L-threonine.*

Substrate analog (50 mM)	Enzyme activity (% of control)						
	Ch.sa.	He.vi.	Ag.qu.	An.ma.	Po.cr.	Te.ma.	Cy.na.
L-serine	-	40	13	16	22	18	27
L-allothreonine	99	101	103	104	103	98	99
L-homoserine	12	45	19	30	32	25	39

* Method involved pretreatment with the analog (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

Fig. 1. pH-activity profile of the deaminase reaction in relation to substrate; (A) L-threonine and (B) L-serine. Algal extracts were prepared at the appropriate pH (see methods) prior to the regular assay procedure commencing with the addition of pyridoxal phosphate. 50 mM L-serine or L-threonine was substituted for the substrate normally used.

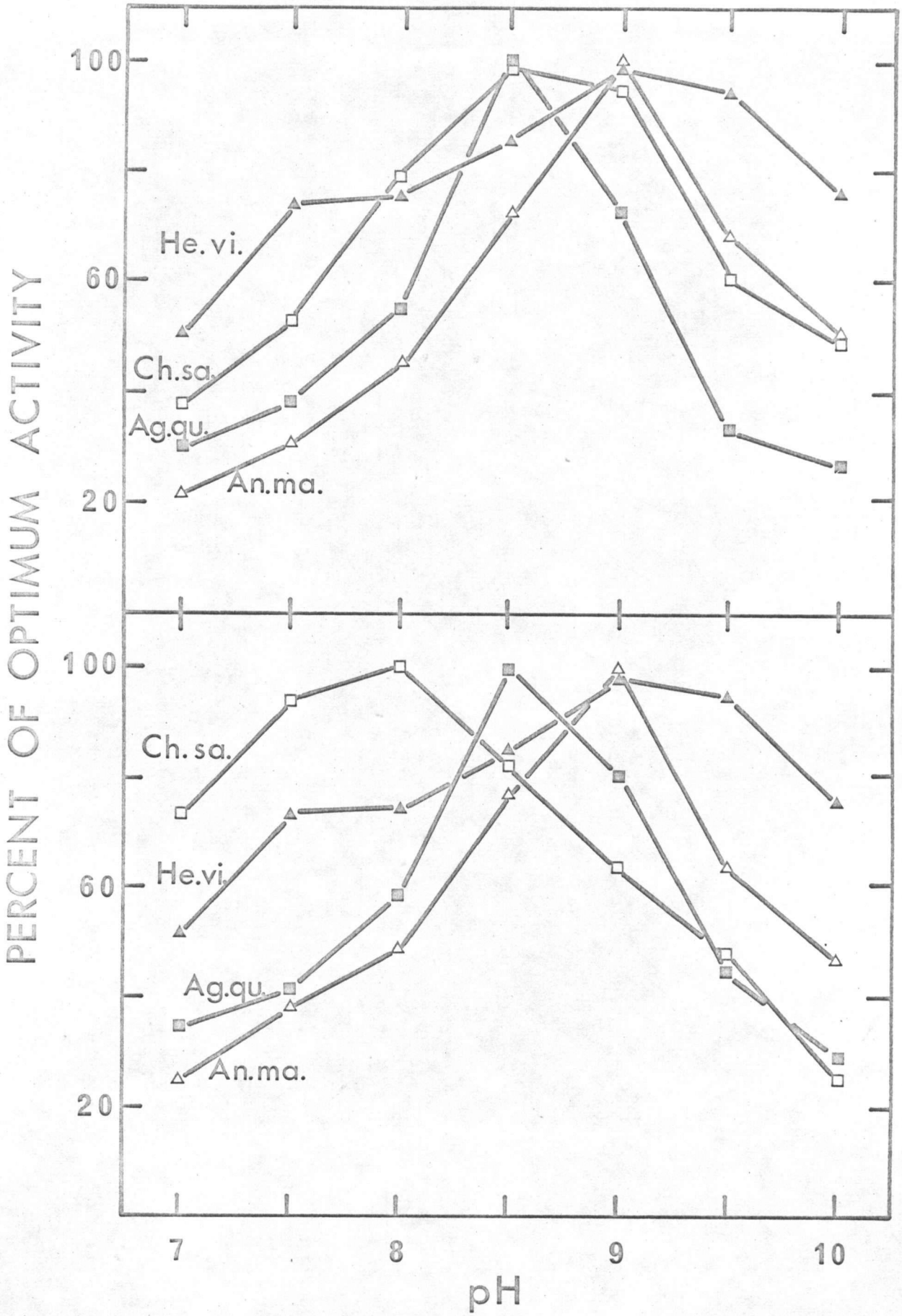
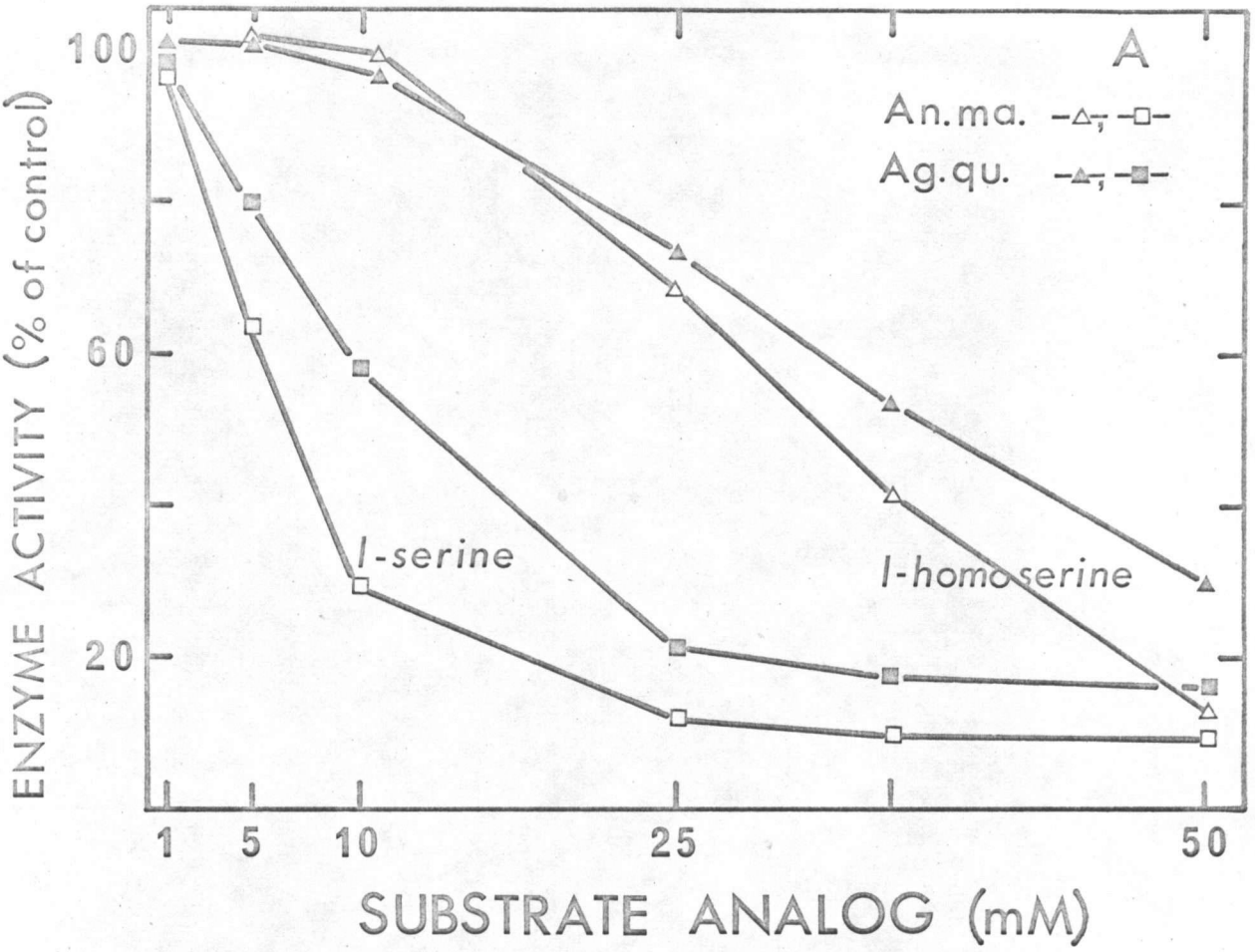

PERCENT OF OPTIMUM ACTIVITY
100
60
20
He. vi.
Ch.sa.
Ag.qu.
An.ma.
100
60
20
Ch. sa.
He.vi.
Ag.qu.
An.ma.
7
8
9
10
pH

Fig. 2. A. Effects of L-serine and L-homoserine concentration on threonine dehydratase activity of A. marina and A. quadruplicatum. Method involved pretreatment with the analog (15 min, 22° C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

Fig. 2. B. Effects of L-homoserine and L-serine on the substrate saturation kinetics of the A. marina enzyme. Method involved pretreatment with the required analog (15 min, 22° C) prior to the regular assay procedure commencing with pyridoxal phosphate addition. Appropriate concentration of L-threonine was substituted for that normally used.


A
An.ma.
Ag.qu.
ENZYME ACTIVITY (% of control)
100
60
20
l-serine
l-homoserine
1
5
10
25
50
SUBSTRATE ANALOG (mM)

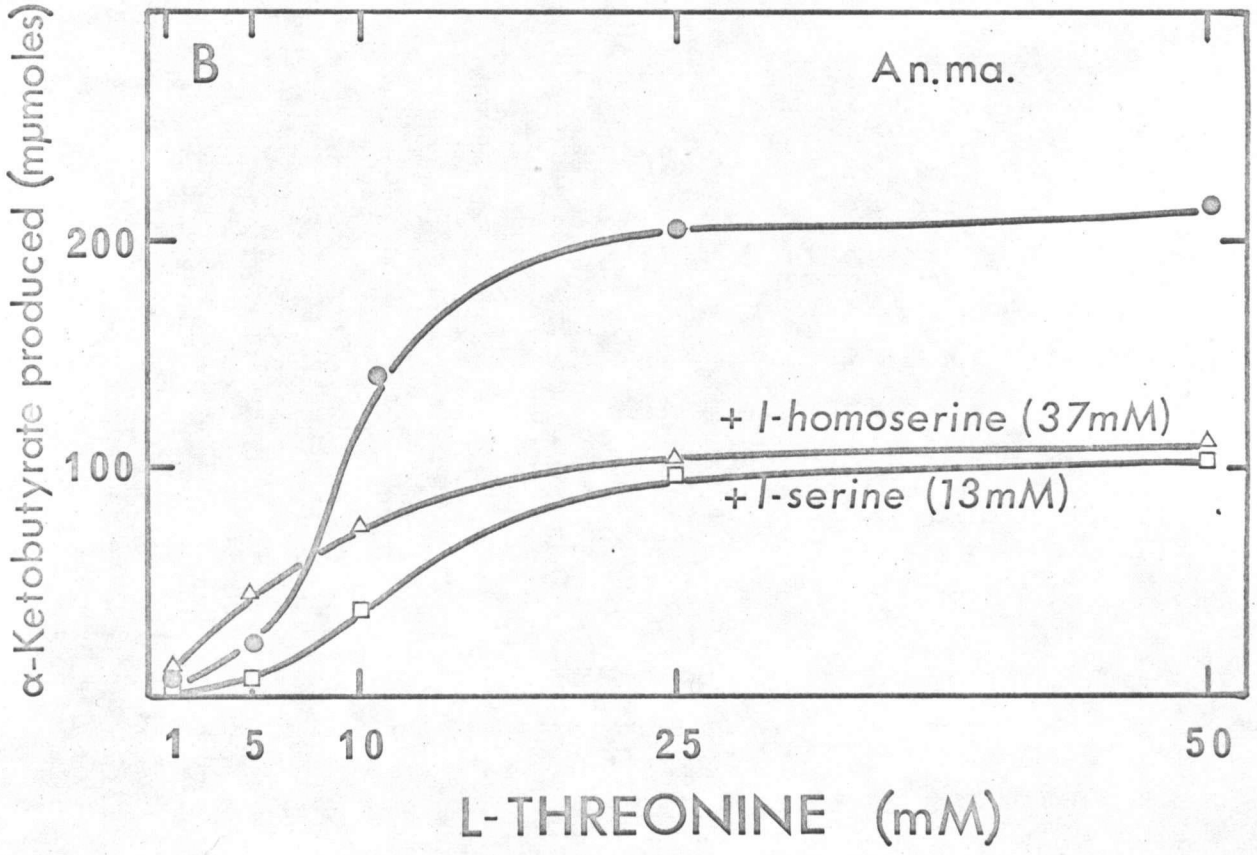
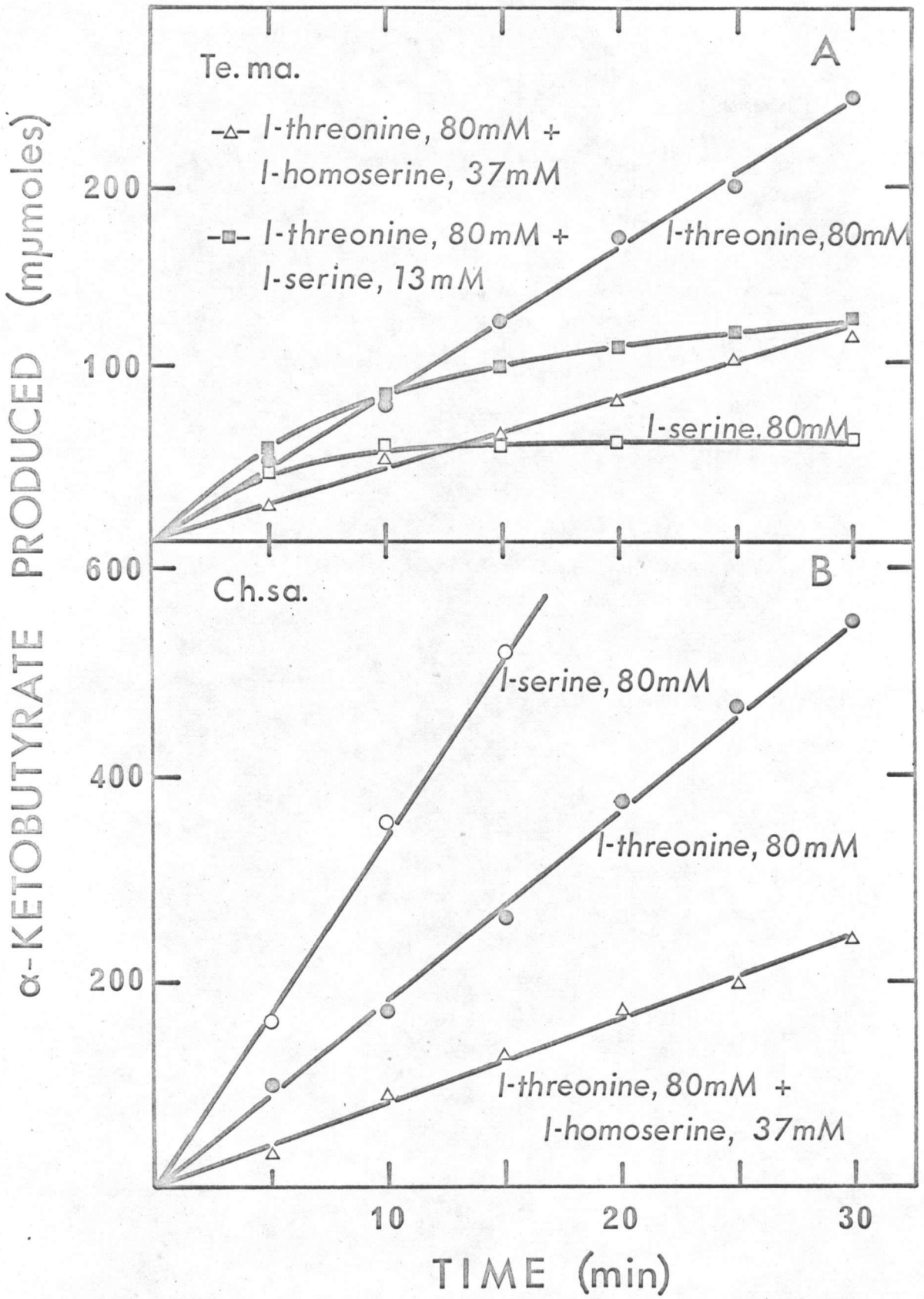

B
An.ma.
α-Ketobutyrate produced (mμmoles)
200
100
+ l-homoserine (37mM)
+ l-serine (13mM)
1
5
10
25
50
L-THREONINE (mM)

Fig. 3. Effect of L-homoserine and L-serine on the rate of L-threonine deamination by the enzymes from (A) T. maculata and (B) C. salina. Substrates were added alone or simultaneously with the analog as indicated.


Te. ma.
A
-△- l-threonine, 80mM + l-homoserine, 37mM
-■- l-threonine, 80mM + l-serine, 13mM
l-threonine, 80mM
l-serine, 80mM
200
100
600
Ch.sa.
B
l-serine, 80mM
400
l-threonine, 80mM
200
l-threonine, 80mM + l-homoserine, 37mM
10
20
30
α-KETOBUTYRATE PRODUCED (mμmoles)
TIME (min)

PART II. Feedback Inhibition, Allosteric Activation and Diverse Effects from Other Amino Acids

L-isoleucine is well known as the natural feedback inhibitor of the deaminating reaction catalysed by L-threonine dehydratase (16). Likewise, L-valine has been implicated as an allosteric activator of the same reaction (61). In view of the conflicting information reported in the literature concerning the effects of amino acids either analogous to, or structurally unrelated to L-isoleucine and/or L-valine, a systematic investigation was devised to elucidate the behaviour of the algal TDH in response to some of these amino acids.

RESULTS

Response to L-isoleucine at saturating substrate concentration

With the exception of C. nana and H. virescens, all species showed sensitive inhibition from L-isoleucine (Fig. 4). A. marina and A. quadruplicatum showed relatively higher threshold concentrations but the inhibitions were extremely rapid once the threshold was surpassed so that by 5 mM L-isoleucine, the degree of inhibition was comparable to that shown by the remaining species. T. maculata was the most sensitive to L-isoleucine. The response of the algal enzymes was found to be extremely rapid in that maximal inhibition could be effected in less than 60 sec indicating that the enzyme is non-hysteretic, similar to the TDH of Escherichia coli (34), but in contrast to that of Bacillus subtilis (36). The C. nana enzyme was insensitive to L-isoleucine and the suggested probability of it being a biodegradative type of TDH seemed unlikely in view of its lack of response to the nucleotides previously tested (23). The H. virescens enzyme was only 50 % inhibited by L-isoleucine and was unaffected by nucleotides like

the C. nana enzyme, but was unique of all the algal enzymes in that it displayed 2 pH optima (see Part III for details).

pH dependency of L-isoleucine inhibition

Since the feedback inhibition from L-isoleucine has been shown to be pH dependent in the case of several previously studied threonine dehydratases (3, 9,26), it was considered important to examine this property for the algal enzymes, particularly with a view to understand the exceptional cases of C. nana and H. virescens. The algal enzymes were tested at 2 L-isoleucine concentrations (giving maximal and 50 % inhibitions previously observed at pH 8.5) by introducing the inhibitor into the reaction mixture at the pH in question (Fig. 5). The results showed that optimal L-isoleucine effect occurs within the range of pH 7.0-8.5, beyond which a gradual decline occurs, eventually culminating in the complete lack of inhibition toward pH 10.0. Furthermore, within this effective pH range, a "finer" profile of pH-dependent inhibition maximum was discerned in the case of H. virescens known to give only 50 % inhibition with saturating L-isoleucine concentration. This "fineness" of inhibition pH optimum may apply to all the algal enzymes at submaximal inhibitor concentration since the C. salina enzyme showed a similar profile when tested at L-isoleucine concentration giving 50 % inhibition. However, the C. nana enzyme gave no evidence of any physiologically significant increase in sensitivity to L-isoleucine over the entire pH range tested, confirming previous suggestions (23) of a "desensitized" type of TDH in this alga. It is interesting to note that all species still manifest considerable activity toward L-threonine at pH 9.5-10.0 (see Part III) although sensitivity toward the feedback inhibitor is considerably diminished. This type of behavior has led to previous inferences on the existence of separate binding sites for the substrate and feedback

inhibitor in the case of other threonine dehydratases (20).

Response to L-valine at saturating substrate concentration

When tested in the presence of saturating L-threonine, low concentration of L-valine was slightly stimulatory (5-30 %) with four algal enzymes and at high concentration, inhibitory with all algae excepting C. nana (Fig. 6). The inhibition profiles obtained from L-valine concentration were remarkably similar to those evoked by L-isoleucine (cf. Fig. 4) after allowing for the sensitivity difference (100-fold) observed between the two amino acids. C. nana and H. virescens behaved similarly toward inhibition from L-valine as they did toward L-isoleucine in that the former enzyme remained uninhibited and the latter was suppressed by only about 40 % at the highest concentration tested.

As was the case with L-isoleucine, the inhibition exerted by L-valine was pH dependent and for concentrations selected to invoke 50 % inhibition, the pH-response profiles were identical. Conversely, the stimulation obtained from low concentration of L-valine appeared to be unaffected by pH (cf. Fig. 5).

Reversal of L-isoleucine inhibition by L-valine

The tests (made in this study) of the effects of L-valine on the algal enzymes, already inhibited by L-isoleucine, indicated that the former amino acid is able to partially reverse the inhibitory effect of the latter, and that these reversals were prescribed by certain conditions: a) the L-valine concentration must be sufficiently low to be non-inhibitory, b) the L-isoleucine concentration must be below that causing maximal inhibition. Figure 7 portrays the degree of reversal effected by a concentration gradient of L-valine from the algal enzymes half-maximally inhibited by L-isoleucine. No reversals were observed from similar tests of the effects of L-valine concentration on the maximally-

inhibited algal enzymes (L-isoleucine "saturated"), nor was the residual activity of the latter suppressed by the highest levels (50 mM) of L-valine tested, suggesting that after L-isoleucine "saturation" the algal enzymes were indifferent to L-valine.

Alteration of substrate saturation kinetics by L-isoleucine and L-valine

In order to acquire a fuller understanding of the principle by which L-valine and L-isoleucine exerted their effects, the substrate saturation kinetics of the algal enzyme was studied in the presence of these amino acids. This aspect was investigated for three algal species chosen as representing three "shades" of isoleucine-valine interrelated effects hitherto observed: (i) A. marina (maximal L-isoleucine inhibition, no L-valine stimulation), (ii) T. maculata (maximal L-isoleucine inhibition, significant L-valine stimulation), (iii) C. nana (little L-isoleucine inhibition, greatest L-valine stimulation). The results depicted in Fig. 8 appear to elucidate the mode of stimulation from L-valine. The saturation kinetics were sigmoidal with L-threonine alone and hyperbolic in the presence of L-valine. C. nana was found to be extremely sensitive to L—valine, since its saturation profile, which was highly "abnormal" with L-threonine alone, acquired a "normal" hyperbolic shape in its presence. The kinetic response of A. marina to low (0.1 mM) concentration of L-valine provides an explanation for the lack of stimulation observed earlier when tested in the presence of high L-threonine concentration; the activating effect of this level of L-valine, being manifest primarily at subsaturating levels of substrate, serves to decrease the Km while leaving the Vmax essentially unchanged. That certain species did exhibit stimulation from L-valine at the higher substrate concentration also becomes understandable from a similar explanation indicating that the full Vmax potential had not been attained by this substrate concentration

without L-valine, as exemplified by the response of T. maculata depicted in Fig. 8.

Further insights into the inhibitions caused by L-isoleucine and L-valine (high concentration) were gained from these kinetic studies. The data (Fig. 8) show that L-isoleucine retains the sinuosity of the original substrate saturation curve, decreasing Vmax and elevating the Km, whereas inhibitory levels of L-valine eliminate the sinuosity and decrease both Vmax and Km. In fact, such L-valine levels still exert a considerable degree of stimulation at low substrate concentration.

Effects of other amino acids on the algal enzymes

Several amino acids, some of which are closely related structurally to either L-isoleucine or L-valine, were tested for any capacity to activate or inhibit the enzyme reaction. From the data in Table 6 it is evident that aside from L-valine and L-isoleucine, L-leucine, L-norvaline and L-α-amino-n-butyrate showed inhibition at high concentration (with decreasing effectiveness as listed) while L-O-methyl threonine was equal in its inhibition to L-isoleucine at a comparable concentration. D-Isoleucine, L-alanine and L-glycine exerted no effects whatsoever. L-Aspartate, which could only be tested at low concentration because of solubility problems, showed slight stimulation (ca. 10 %) towards some species, similar to that reported for the TDH of Bacillus licheniformis (55).

The pronounced inhibition produced by 5 mM L-O-methyl threonine prompted a closer inspection of the concentration-sensitive response provoked by this structural analog of L-isoleucine. The profiles of this response, depicted in in Fig. 9, showed the effects of L-O-methyl threonine to be virtually indistinguishable from those of L-isoleucine.

Since the algal TDH were inhibited to varying degrees by L-leucine, L-norvaline and L-α-aminobutyrate, it was decided to verify whether any of these amino acids

were capable of mimicking the previously observed stimulation effects from L-valine. Substrate saturation curves in the presence of these compounds at low concentration (0.1 mM) were determined with T. maculata, which had shown a sensitive response to L-valine. These tests showed no recognizable activation or kinetic alteration of the saturation profile which was observed to maintain a virtually identical sigmoidality in the absence or presence of either L-norvaline, L-leucine or L-α-aminobutyrate. It was thus inferred that the previously noted activation was highly specific for L-valine.

DISCUSSION

One of the prime criteria for the classification of a threonine dehydratase as biosynthetic is its sensitivity to feedback inhibition from isoleucine according to the model originally developed by Changeux (16). All of the algal enzymes conform to this criterion, with the exception of C. nana, and to a lesser extent, H. virescens. However, evidence for the absence of effect from nucleotides (23) and presence of allosteric response to L-valine negates the possibility of a biodegradative TDH in the former species and likens it to the desensitized biosynthetic TDH of Rhodospirillum rubrum (28). In the case of the latter algal species, the situation is unique in that L-isoleucine inhibits enzyme activity by approximately 50 %, and the algal preparation displays a double activity-pH optima (suggestive of 2 enzymes) yet is nevertheless insensitive to nucleotides (see Part III). Apart from these exceptions, the L-isoleucine inhibition showed (i) considerable inter-species variation with respect to threshold concentration, (ii) non-hysteretic response and, (iii) effects on substrate saturation kinetics tending to increase homotropic enzyme-substrate interactions (indicated by increased Km).

As is the case with the algal enzymes, previous investigations had revealed that inhibition by L-isoleucine is pH dependent (3,9,26). That the inhibition exerted by higher concentrations of L-valine follows an identical pH dependency as that of L-isoleucine suggests that its inhibitory effect may be due to an ability to mimic isoleucine and occurs at the site specific for allosteric inhibition (3). It appears from structural similarity considerations that the trends of inhibition obtained from L-leucine and L-norvaline may be due to similar mechanistic reasons and that this inhibition tends to decrease in proportion to structural similarity. The effect of L-α-aminobutyrate may not be the result of binding at the isoleucine site, but through a degree of competition with L-threonine for the substrate site, which mode of inhibition has been ascribed to this amino acid in certain biodegradative threonine dehydratases (63,64).

Few investigation have been performed with respect to the absolute stereospecific nature of isoleucine inhibition. Certain compounds such as thiaisoleucine (4), L-O-methyl threonine (75), L-O-ethyl threonine (71) and isoleucine hydroxamate (51) have been examined for their effect upon the growth of isoleucine-requiring auxotrophs and concomitant biosynthesis of TDH following the incorporation of these antimetabolites into the growth media. However, excepting the early work of Changeux designed to reveal the specificity of substrate and inhibitor sites (15), the ability of isoleucine analogs to function as feedback inhibitors of the enzyme *in vitro* has been virtually unexplored. A recent publication has reported that both L-O-methyl threonine and thiaisoleucine do in fact markedly inhibit TDH activity but at 10-fold greater concentrations than the isoleucine achieving the same degree of inhibition (75). The algal enzymes appear to be incapable of distinguishing between the natural feedback inhibitor (L-isoleucine) and L-O-methyl threonine as the development of inhibition from graded concen-

trations of both compounds were identical. It may be inferred, therefore, that the prime criteria determining the ability of a compound to mimic an allosteric role is the overall similarity in molecular configuration and volume to the natural agent.

From the evidence obtained with algal threonine dehydratases, it appears that the activating effect of L-valine is exerted *via* a site distinct from either the substrate or inhibitor sites resulting in the abolition of cooperative interaction between threonine molecules (19), rather than functioning as a substrate analog as has been suggested (29,34). An alternate explanation of valine activation equates the observance of sigmoidal substrate saturation curves without effector to the presence of contaminating isoleucine, the inhibition from which is reversed by the addition of valine; thus, the apparent activation (37). The validity of this explanation is questionable in view of sigmoidal substrate saturation kinetics subject to normalization by valine reported for highly purified enzymes, such as the TDH of yeast (49).

In contrast to the majority of threonine dehydratases wherein valine was found to activate, certain plant enzymes have had L-norvaline (46,48,74) and to a lesser extent L-norleucine, L-aspartate (46) assume the role of allosteric activator. A fungal enzyme was specifically activated by phenylalanine (47). For the algal enzymes, only L-valine is implicated as allosteric activator, which role was not imitated by any of the amino acids tested including L-norvaline. Even when a 50 % inhibitory (at saturating substrate concentration) concentration of L-valine was incorporated with the enzyme, considerable activation occurred at non-saturating substrate concentrations, an effect previously observed with the TDH of *E. coli* K-12 (29).

The fact that *C. nana*, the isoleucine-insensitive algal TDH, was greatly

activated by L-valine imparts clear evidence of a distinct site for valine. The observed lack of inhibition of this isoleucine-insensitive enzyme from high concentration of L-valine may be extrapolated to the other algal enzymes in support of the premise that the inhibitory effect observed in these isoleucine-sensitive enzymes is likely the result of interaction from excess L-valine at the isoleucine site (39). Furthermore, the partial ability of L-valine to reverse L-isoleucine inhibition lends weight to the mechanistic view of an additional distinct site for valine (11). An earlier suggested reversal mechanism that valine may simply act by displacement of isoleucine at the latter site (4) appears unlikely from the overall evidence obtained with the algal enzymes.

An alternate explanation of the unusual responses of the C. nana TDH cannot be ignored. The substrate saturation curve with L-threonine alone appears to be essentially "inhibited" in that appreciable activity was not evident prior to approximately 35 mM L-threonine, after which the increase in activity appeared to be linear. Since L-valine dramatically altered the substrate saturation profile (14-fold stimulation at 25 mM L-threonine) and showed no sign of inhibition at high concentration, the impression is obtained that the enzyme lacks an active isoleucine site. However, it is conceivable that this site may be present already "filled" by endogenous isoleucine since the substrate saturation profiled is not too unlike that of the TDH from Bacillus stearothermophilus in the presence of near-maximal inhibitory concentration of isoleucine (73); this possibility implies that the crude enzyme preparation of C. nana may already have bound endogenous isoleucine unlike the other algal enzymes. Thus, the extremely sensitive stimulation of the C. nana TDH from L-valine may be due to a net reversal of this "native" isoleucine inhibition.

On an overall basis, therefore, the algal threonine dehydratases conform to the K-system of enzymes as developed by Monod (61), characterized by; a) velocity plots exhibiting homotropic interactions for substrate (sigmoidal response to concentration), b) increased sinuosity of the initial velocity plots in the presence of allosteric inhibitor, and c) conversion of the initial velocity plots to a hyperbola in the presence of allosteric effector.

Table 6. Effects of various amino acids on algal threonine dehydratase activity.*

Amino acid tested	Concn	Enzyme activity (% of control)						
	(mM)	Ch.sa.	He.vi.	Ag.qu.	An.ma.	Po.cr.	Te.ma.	Cy.na.
L-isoleucine	5	4	46	10	8	4	11	94
L-O-methyl threonine	5	6	47	7	7	5	10	98
L-valine	50	27	63	89	35	32	18	134
L-leucine	50	40	69	72	57	36	49	102
L-norvaline	50	60	81	83	70	59	61	97
L-α-amino-n-butyrate	50	77	89	92	80	73	70	96
L-aspartate	5	106	110	104	109	111	107	100
L-alanine	50	99	103	102	104	100	99	97
L-glycine	50	100	104	100	101	98	103	106
D-isoleucine	50	101	102	97	100	100	102	103

* Method involved pretreatment with the amino acid (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

Fig. 4. Effect of L-isoleucine concentration on algal threonine dehydratase activity. Method involved pretreatment with L-isoleucine (15 min, 22° C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

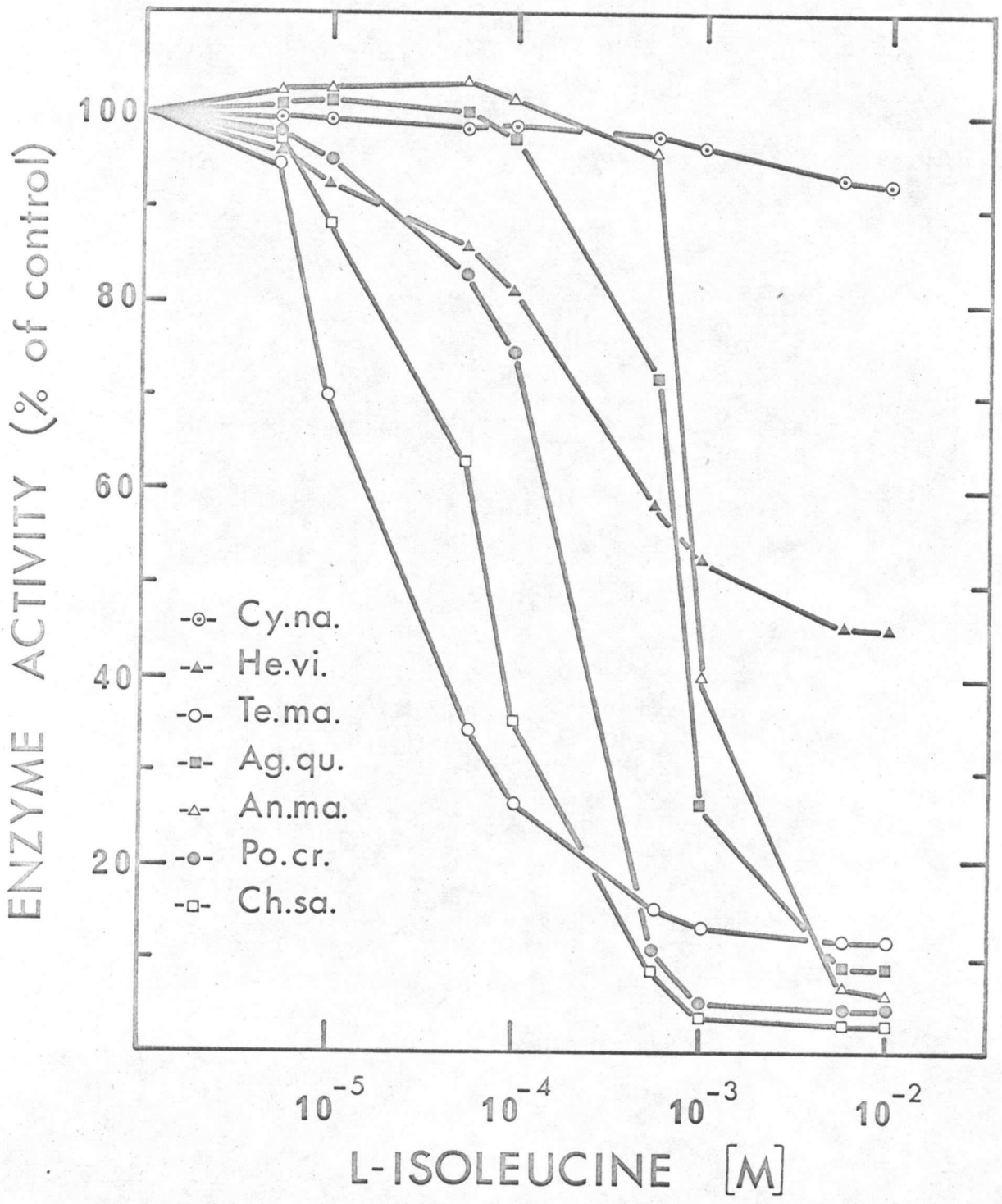

ENZYME ACTIVITY (% of control)
100
80
60
40
20
-⊙- Cy.na.
-△- He.vi.
-○- Te.ma.
-■- Ag.qu.
-△- An.ma.
-●- Po.cr.
-□- Ch.sa.
10^{-5}
10^{-4}
10^{-3}
10^{-2}
L-ISOLEUCINE [M]

Fig. 5. Influence of pH on the effects from L-isoleucine and L-valine on threonine dehydratase activity. Algal extracts at the appropriate pH (see methods) were pretreated with the required concentration of effector (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

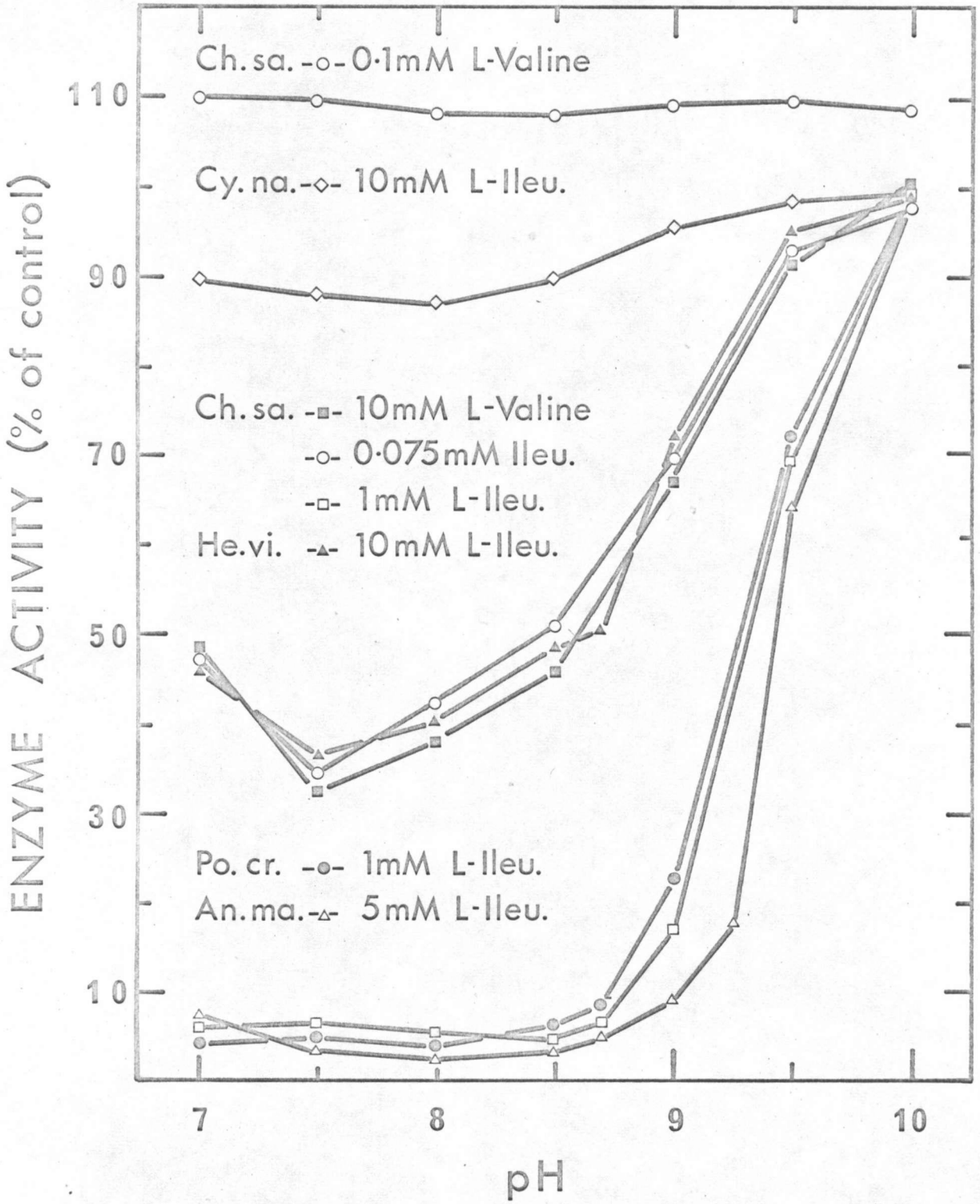

Ch. sa. -o- 0·1mM L-Valine
Cy. na. -◇- 10mM L-Ileu.
Ch. sa. -■- 10mM L-Valine
-o- 0·075mM Ileu.
-□- 1mM L-Ileu.
He. vi. -▲- 10mM L-Ileu.
Po. cr. -●- 1mM L-Ileu.
An. ma. -△- 5mM L-Ileu.
ENZYME ACTIVITY (% of control)
110
90
70
50
30
10
7
8
9
10
pH

Fig. 6. Effect of L-valine concentration on algal threonine dehydratase activity. Method involved pretreatment with L-valine (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

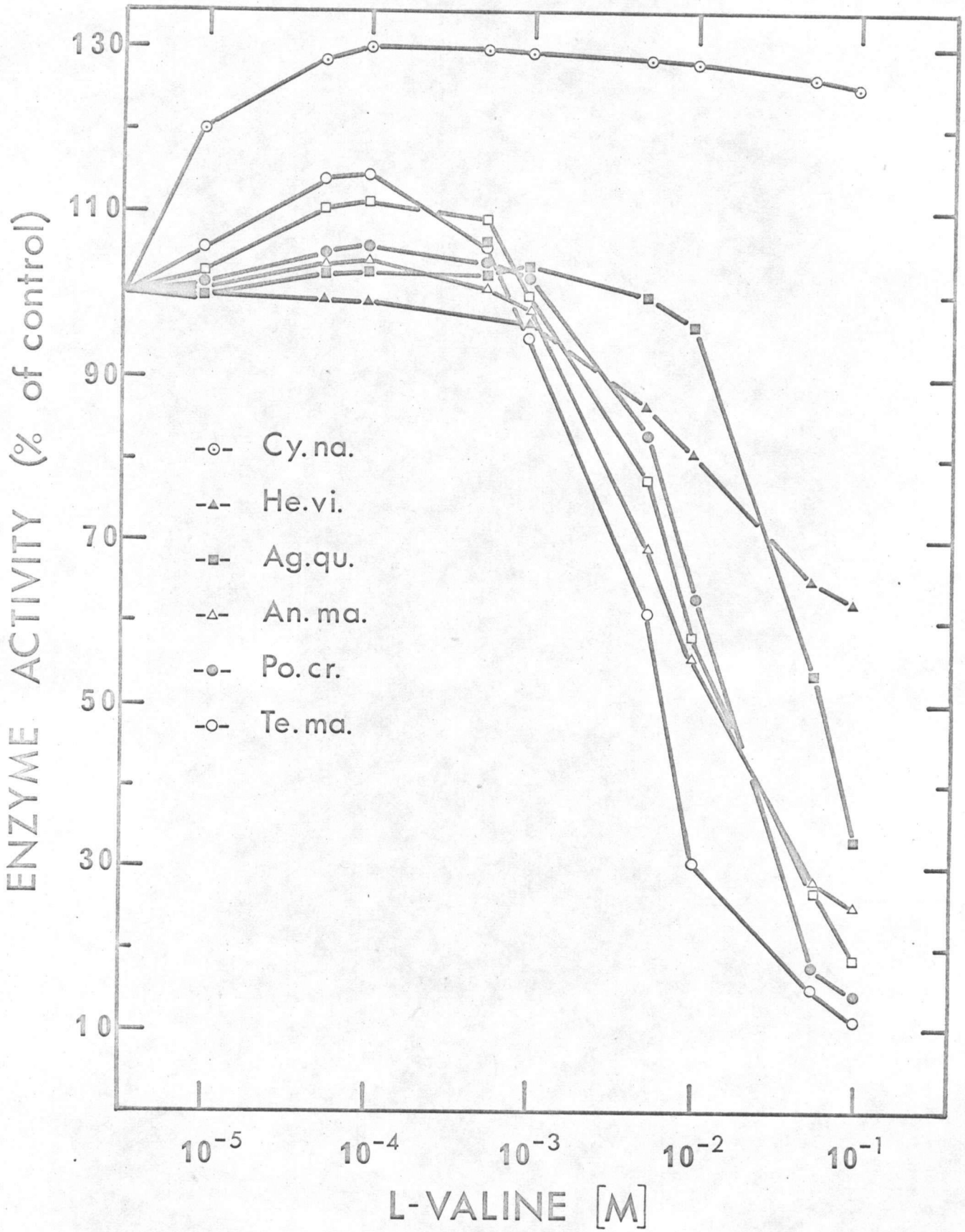

ENZYME ACTIVITY (% of control)
130
110
90
70
50
30
10
-⊙- Cy. na.
-▲- He. vi.
-■- Ag. qu.
-△- An. ma.
-●- Po. cr.
-○- Te. ma.
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
L-VALINE [M]

Fig. 7. Reversal of L-isoleucine inhibition of the algal enzymes by graded concentrations of L-valine. Method involved pretreatment with half-maximal inhibitory L-isoleucine concentration (1 min, 22°C) followed by preincubation with L-valine (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

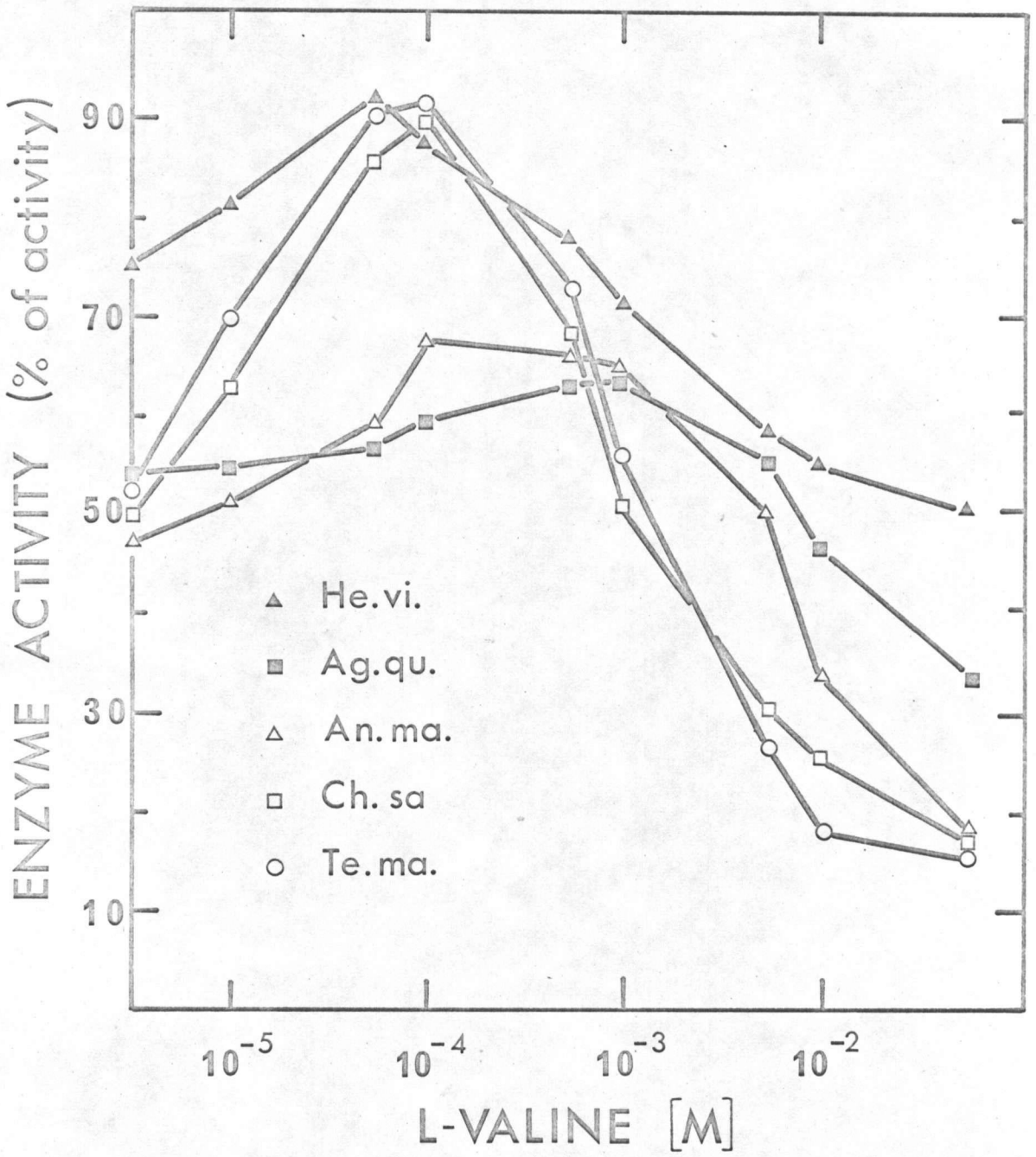

ENZYME ACTIVITY (% of activity)
90
70
50
30
10
He.vi.
Ag.qu.
An.ma.
Ch. sa
Te.ma.
10^{-5}
10^{-4}
10^{-3}
10^{-2}
L-VALINE [M]

Fig. 8. Effects of L-valine and L-isoleucine on the substrate saturation kinetics of the enzymes from T. maculata, C. nana and A. marina. Where required, the algal extracts were pretreated with the indicated concentrations of effector (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition. Appropriate L-threonine was substituted for the concentration normally used.

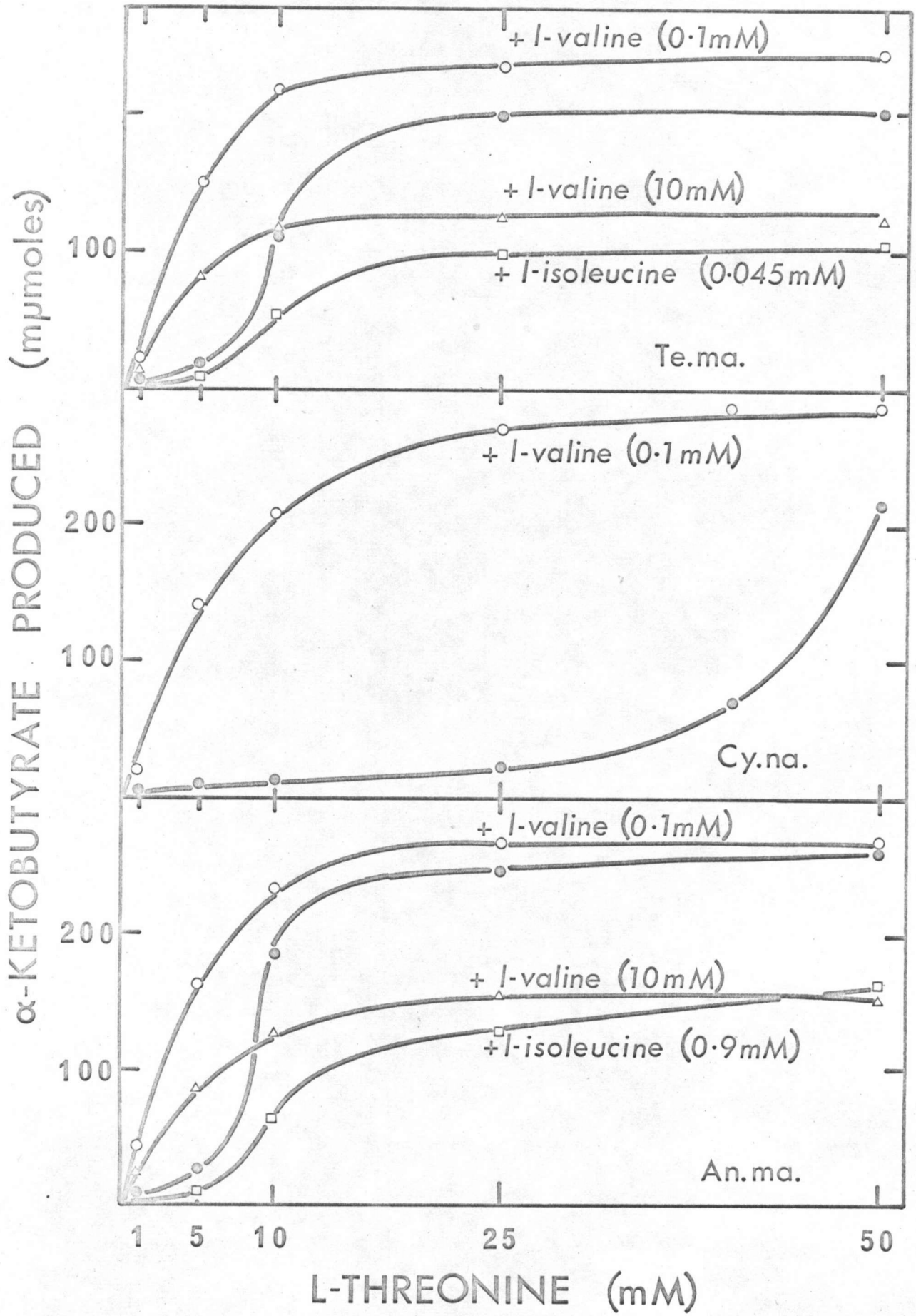
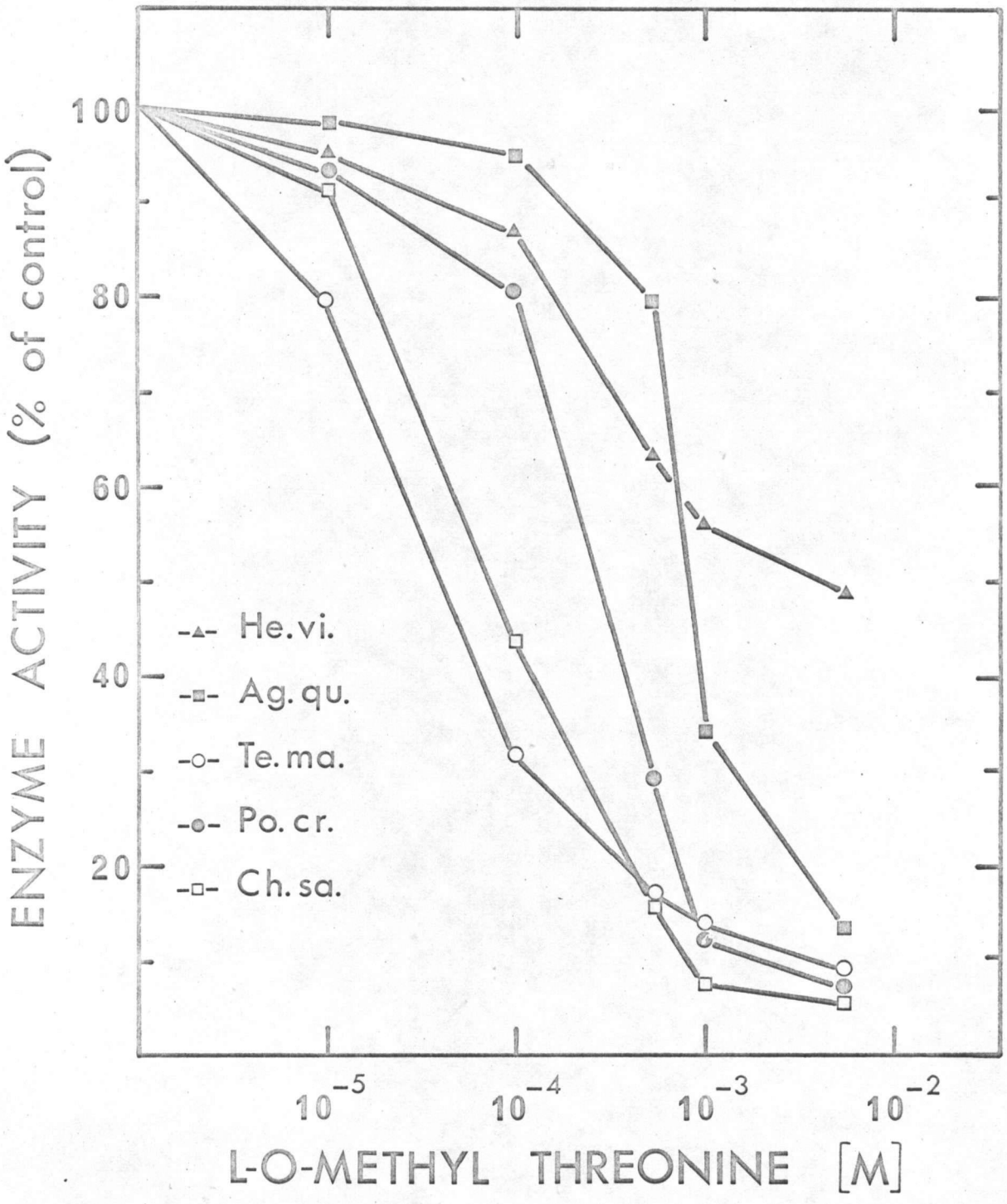

+ l-valine (0·1mM)
+ l-valine (10mM)
+ l-isoleucine (0·045mM)
Te.ma.
+ l-valine (0·1mM)
Cy.na.
+ l-valine (0·1mM)
+ l-valine (10mM)
+l-isoleucine (0·9mM)
An.ma.
100
200
100
200
100
α-KETOBUTYRATE PRODUCED (mμmoles)
1
5
10
25
50
L-THREONINE (mM)

Fig. 9. Effect of L-O-methyl threonine concentration on algal threonine dehydratase activity. Method involved pretreatment with L-O-methyl threonine (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.


ENZYME ACTIVITY (% of control)
100
80
60
40
20
He.vi.
Ag.qu.
Te.ma.
Po.cr.
Ch.sa.
10^{-5}
10^{-4}
10^{-3}
10^{-2}
L-O-METHYL THREONINE [M]

PART III. Re-investigation of pH Optima with Strict Buffer-Ion Control. Evidence for 2 pH Optima with Differential Sensitivity to Allosteric Effectors in the Cryptomonad Hemiselmis virescens.

RESULTS

pH optima of the deaminase reaction

With the establishment of the algal TDHs requirement for monovalent inorganic cations (see Supplement II), it became necessary to re-evaluate the previously obtained pH profiles as they were performed without adequate control of cation concentration (23). From this point of view, it was considered desireable to use buffers (such as Tris-HCl and Map-HCl) which contained no alkali-metal cations and to supplement these with a constant level of KCl (100 mM), thereby eliminating the activity deviations previously obtained (23) from unpredictable cation variations such as present in K-phosphate, K-Tricine, K-bicarbonate etc. (in spite of their molarity equivalence). Preliminary tests, in addition to those reported in Supplement II, have shown that the activating effect from monovalent cations is unaffected by pH in the range 7-10. Figure 10 shows the pH-activity profiles for six algal enzymes, all of which exhibited a single optimum in the pH range 8.5-9.0. However, the examination of H. virescens showed a "shoulder" in the vicinity of pH 8 in addition to the expected optimum at pH 9 (Fig. 11). To verify this observation, two other nutritionally different cultures of this species were similarly examined. It was found that both the urea-grown and glycerol-grown cultures showed two distinct pH optima, one peak being in the vicinity of pH 8 (corresponding to the "shoulder" observed in the standard culture), the other in the vicinity of pH 9. Identical pH-activity studies with the enzymes from nutritionally different cultures of C. salina and P. cruentum

showed no difference as compared to the profiles observed for the standard cultures of these species.

Response of H. virescens enzyme to potential effectors at pH intermediate between the two optima (8.5)

In view of the ability of the H. virescens enzyme (from three nutritionally different cultures) to undergo only 50 % inhibition from L-isoleucine, the observation of two pH optima was highly suggestive of the simultaneous presence of a biodegradative TDH. However, when several nucleotides were tested in the presence or absence of dithithreitol* at pH 8.5, no activation was observed with either high or low concentration of substrate (Table 7). The same lack of effect was noted when the nucleotides were tested at pH 8 and 9, all of which cast doubt on the possibility of a biodegradative TDH in the enzyme preparation. Because of the relationships of aspartate, methionine and cystathionine to the metabolism of threonine (see Appendix B), these amino acids were tested with the H. virescens enzyme in order to assess the possibility of (i) unknown precurssor-product interactions with this TDH and, (ii) contaminating enzymes capable of eliciting a substrate response with L-threonine (eg. cystathionine synthase (62), capable of deaminating threonine). The results of these examinations (Table 7) showed no untoward effects from L-aspartate, L-methionine or L-cystathionine when tested at low and high concentration at two substrate concentrations. Finally, the previously performed studies of substrate specificity (Part I), clearly indicated that, unlike the other cryptophyte C. salina, the simultaneous presence of an SDH or even homoserine dehydratase potentially capable of deaminating L-threonine

*Several investigators have reported the necessity of sulfhydryl-protective reagents for full expression of nucleotide activation (46).

is improbable.

Differential effects from allosteric compounds at the two pH optima

The prior observations that the inhibition obtained from L-isoleucine, L-valine, L-leucine, and L-norvaline were invariably about half of those generally observed with the other algae (excepting C. nana), prompted the investigation of L-isoleucine inhibition at the two pH optima. However, since it was already demonstrated that feedback inhibition is pH dependent and approaching elimination by pH 10 (Part II), little could be gained from simply incorporating L-isoleucine at each pH and comparing the results. For this reason, the algal extract was first treated with maximally inhibiting levels of L-isoleucine in buffer of low strength (0.01 M) at pH 7.5 (the optimal inhibitory pH) and after allowing an interaction interval, the mixture was readjusted to the required pH with buffer of high strength (0.1 M). From the results (Fig. 12), it is apparent that because the inhibition by L-isoleucine was effected at pH 7.5, the enzymes from C. salina and P. cruentum remained fully inhibited irregardless of the pH at which the subsequent incubation with L-threonine was performed. On the other hand, the enzymes from the three nutritionally different cultures of H. virescens showed progressively less inhibition from L-isoleucine as the more alkaline optimum was approached. An interesting culture-dependent correlationship appears between the activity profiles without L-isoleucine at the two pH optima and the inhibition profiles obtained with L-isoleucine in the vicinity of the pH 9 optimum. In the absence of L-isoleucine, each of the two cultures (Gly. L and Urea) showed comparable activity maxima at the two pH optima whereas the standard culture was considerably more active at the pH 9 optimum with a consequential "shoulder" (rather than a peak) at pH 8. In the presence of L-isoleucine, the two former

cultures evinced more pronounced inhibition in the vicinity of the pH 9 optimum relative to the latter culture. Such a correlationship appears to be more than a coincidence and suggests that (i) all three cultures may possess at least two enzymes, one sensitive to L-isoleucine with a pH optimum near 8 and another insensitive to L-isoleucine with a pH optimum near 9, (ii) these enzymes may occur in different proportions in the three cultures, (iii) the isoleucine-insensitive enzyme (pH 9 optimum) may be present in the greatest proportion in the standard culture.

Since the activating effect of L-valine was shown to be independent of pH in the range 7-10 for the algal enzymes (Part II), examination of any differential effects to this amino acid at the two pH optima could be pursued without pH modification as was required for the above studies with L-isoleucine. In consequence, substrate saturation kinetics with L-threonine alone, and in combination with either L-valine, L-homoserine or L-serine were examined at pH 8 and 9, the results of which investigations are portrayed in Fig. 13 for the enzyme from the standard culture of H. virescens. The data revealed the following features (i) the response to inhibition from L-serine and L-homoserine were similar at each pH and analogous to that depicted for A. marina (cf. Fig. 2B, Part I) and, (ii) while substantial activation (through curvature alteration) by L-valine is evident at pH 8, no such effect was observed at pH 9.

DISCUSSION

With few exceptions (18,46), the pH optima for biosynthetic threonine dehydratases have been reported within the range of 8-9.5. In the case of the algal enzymes, a single optimum pH for enzyme activity was within the narrow range of 8.5-9.0 excepting H. virescens. The latter species showed an unusual response

to pH with two optima, one in the vicinity of pH 8, the other around pH 9. The sharpness of the pH optima appeared to depend on the conditions of algal culture. However, different culture conditions did not affect the pH optima of C. salina and P. cruentum enzymes, suggesting that the overall pH response of H. virescens may be independent of culture conditions. Since the degree of this "two peak" pH response does show some dependence on culture conditions, it appears likely that this alga is producing a mixture of at least two TDHs. This inference is also supported by the culture-related degrees of inhibition obtained from L-isoleucine. Assuming such a mixture, the two enzymes present may not differ basically in their physiological function since no evidence was obtained of the "standard" type of biodegradative TDH.

This two-enzyme hypothesis was extensively tested within the framework of the known characteristic behaviour of the other algal enzymes, as well as in previously unexamined peripheral areas, with the expectation that possible unique differences in behaviour may be delineated at the two pH optima. In designing these tests, it was rationalized that any real difference between the H. virescens and other algal TDHs or any real effects from potential substrates and unknown effectors would be manifested to a recognizable degree at a pH (8.5) between the two optima. Once such differences or effects were recognized, it was imperative to submit them to a finer examination at the actual optima. The results of such tests and finer examination have already been stated earlier (Parts I & II, where such tests were concerned with routine examination of all algal species) and immediately prior to this section (Part III, Results, where the tests were more specifically concerned with H. virescens). In reviewing these results within the broad perspective of the above-proposed two-enzyme hypothesis for H. virescens, the overall picture obtained appears to further support this postulation

without any serious contraindication. The overall evidence so obtained from H. virescens is summarized below:

1) In contrast to the other algal enzymes, L-isoleucine, after ensuring its binding to the enzyme, gave 50 % inhibition at pH 8.5 and this inhibition was considerably enhanced at pH 7.5-8 but was markedly reduced at pH 9-9.5, indicating clearly differentiable responses to this effector at the two optima.
2) Again in contrast to the other algal enzymes, high concentrations of L-valine, L-leucine and L-norvaline gave only half the degree of inhibition generally obtained from these enzymes at a pH intermediate between the two optima, leading to the inference that these amino acids were mimicking the pH-dependent L-isoleucine effects noted above.
3) Low concentration of L-valine converted the substrate saturation kinetic profile from sigmoidal to hyperbolic at pH 8, but had no effect on the saturation profile at pH 9, which again indicated a differential response from another effector at the two pH optima.
4) The substrate specificity study at pH 8.5 and the substrate analog inhibitions observed at the two pH optima showed no recognizable difference from the other algal species, confirming that the two enzymes of H. virescens were TDHs similar in this respect to one another as well as to the other algal enzymes.
5) The H. virescens enzyme showed no effects from certain nucleotides, nor from a number of amino acids (L-methionine, L-aspartate, L-cystathionine) that might be expected to bear an energy control or precurssor-product relationship to L-threonine in the presently known maps of metabolic pathways centering around this amino acid. The absence of effect from L-cystathionine also eliminated the possibility of nonspecific deaminations of L-threonine or specific formation of α-ketobutyric acid reported for certain enzymes

specifically acting on the former amino acid as substrate (eg. cystathionine synthase (62), cystathionine-cysteine-lyase (50)).

It is concluded from these considerations that H. virescens is exceptional among the algal TDHs in producing a K-type (according to Monod (61)) biosynthetic TDH as well as an undefined type TDH insensitive to all know effectors tested (resembling certain bacterial mutant variants (4,21,52)) and whose physiological function remains unknown. In fact, the case of H. virescens is strongly reminiscent of a mutant of Rhodopseudomonas spheroides (19,20) reported to simultaneously contain two TDHs, both insensitive to nucleotides and showing similar substrate specificity, but differing in their sensitivity to effectors so that the one (PRTDI) enzyme is completely inhibited by L-isoleucine and activated by L-valine while the other (PRTDII) is 1000-fold less sensitive to L-isoleucine and not subject to activation by L-valine. It is pointed out that this conclusion is largely based on very indirect inferences drawn from the data obtained, and will require large-scale attempts at purification and separation of the enzymes in question. Among enzymes other than threonine dehydratases that have shown two pH optima in crude extracts, differential responses to inhibitors and activators have provided similar indirect evidence to indicate the occurrence of two enzymes in such extracts (eg. acid phosphatase in seminal fluid of rabbits (79)). In the case of aspartate transcarbamoylase (80) , the two pH optima also showed differential response to the principal allosteric effector involved, but these authors appear to have ignored the possibility of occurrence of more than one enzyme without adequate justification, presumably because the second and more pronounced pH optimum was observed at the high and relatively non-physiological pH of 10.5. Nevertheless, in the absence of the final definitive proof, the question of two threonine dehydratases, each with a single pH optimum, or one enzyme with two pH optima is still open to conjecture.

Table 7. Effects of certain nucleotides and amino acids on enzyme activity of H. virescens at pH 8.5.*

Compound tested	Concn (mM)	DTT† (0.01 mM)	L-Threonine (mM)	Enzyme activity (% of control)
nil	-	-	50	100
	-	-	5	100
	-	+	50	80
	-	+	5	74
AMP	5	+	50	80
	5	+	5	81
ADP	5	+	50	83
	5	+	5	72
ATP	5	+	50	78
	5	+	5	83
cyclic-AMP	5	+	50	81
	5	+	5	74
L-methionine	50	-	50	99
	50	-	5	95
	5	-	50	102
	5	-	5	91
L-cystathionine	50	-	50	104
	50	-	5	96
	5	-	50	99
	5	-	5	103
L-aspartate	5	-	50	110
	5	-	5	114

* Method involved pretreatment with DTT (15 min, 22°C) followed by preincubation with the test compound (15 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition. Appropriate L-threonine concentration was substituted for that normally used.

† Dithiothreitol

Fig. 10. pH-activity profiles for six algal threonine dehydratases. Algal extracts were prepared at the required pH (see methods) prior to the standard assay procedure commencing with the addition of pyridoxal phosphate.

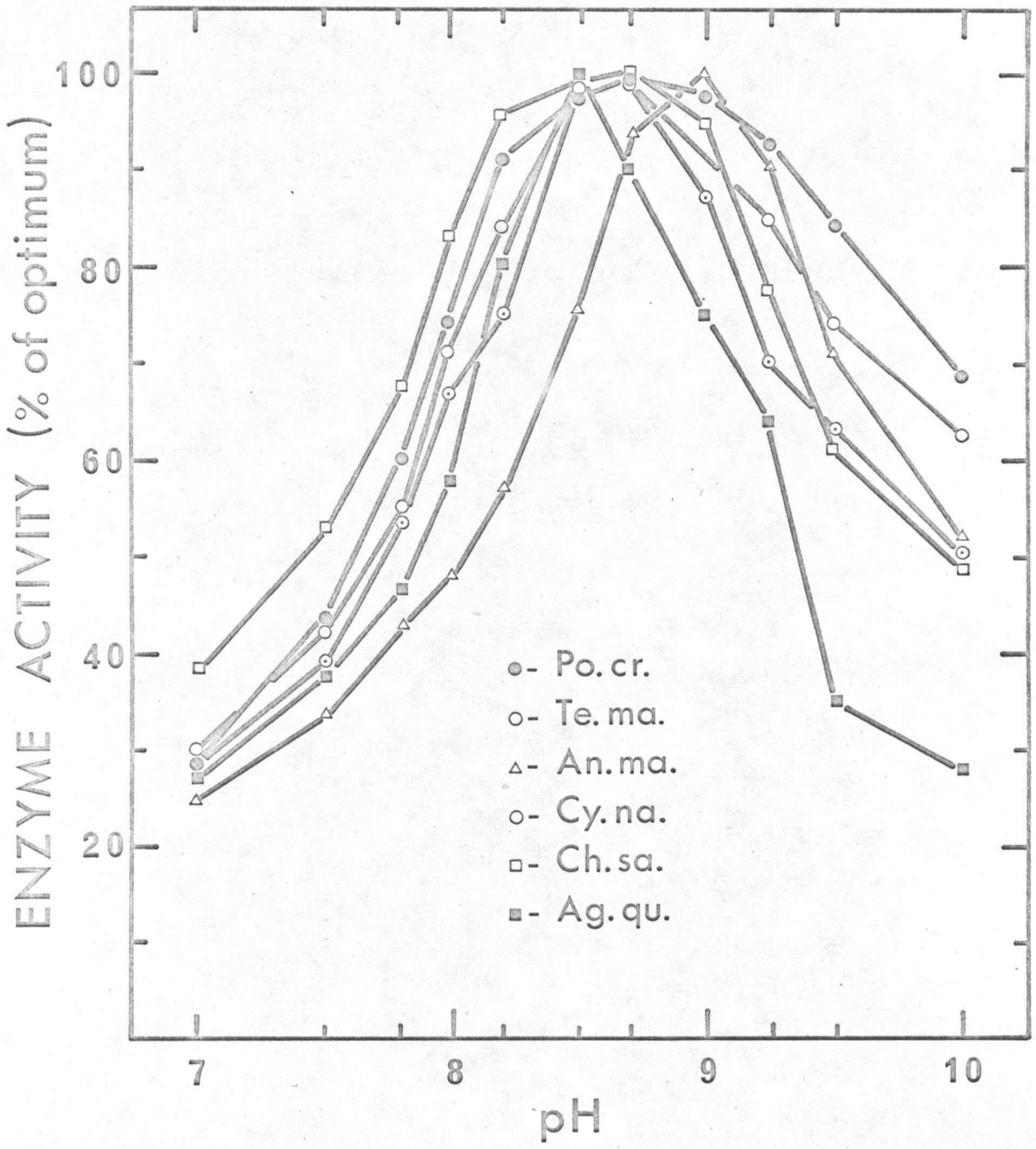

ENZYME ACTIVITY (% of optimum)
100
80
60
40
20
7
8
9
10
pH
- Po. cr.
- Te. ma.
- An. ma.
- Cy. na.
- Ch. sa.
- Ag. qu.

Fig. 11. pH-activity profiles for the enzymes from three nutritionally different cultures of H. virescens. Algal extracts were prepared at the required pH (see methods) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

Fig. 12. pH-activity profiles in the presence of L-isoleucine for the enzymes from three nutritionally different cultures of H. virescens. C. salina, P. cruentum, shown for comparison. The algal extracts were prepared in 0.01 M Tris-HCl buffer (pH 7.5) and preincubated with 10 mM L-isoleucine (15 min, 22°C). The pH was then adjusted as required by the addition of appropriate buffer (see methods) prior to the regular assay procedure commencing with the addition of pyridoxal phosphate.

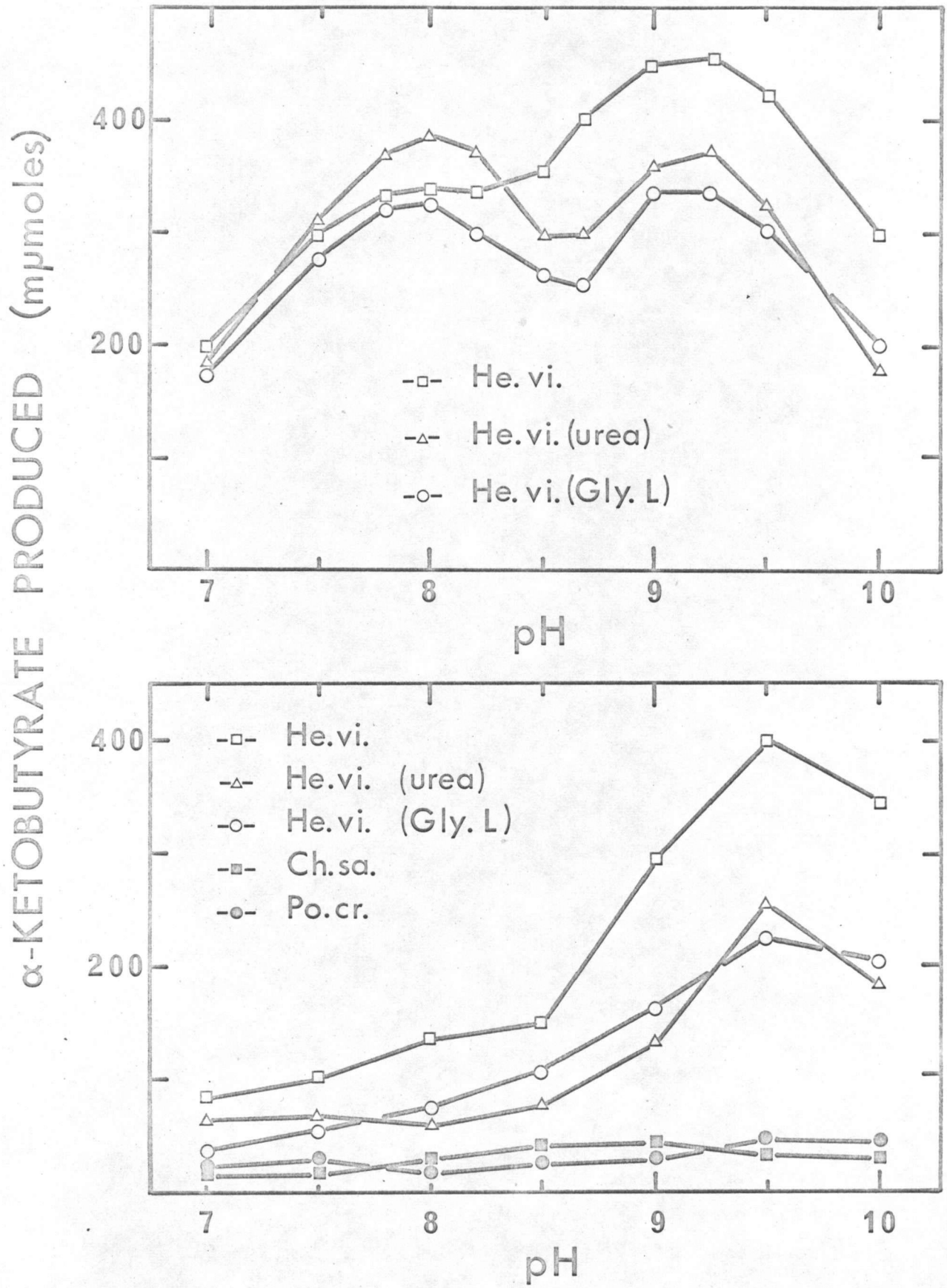
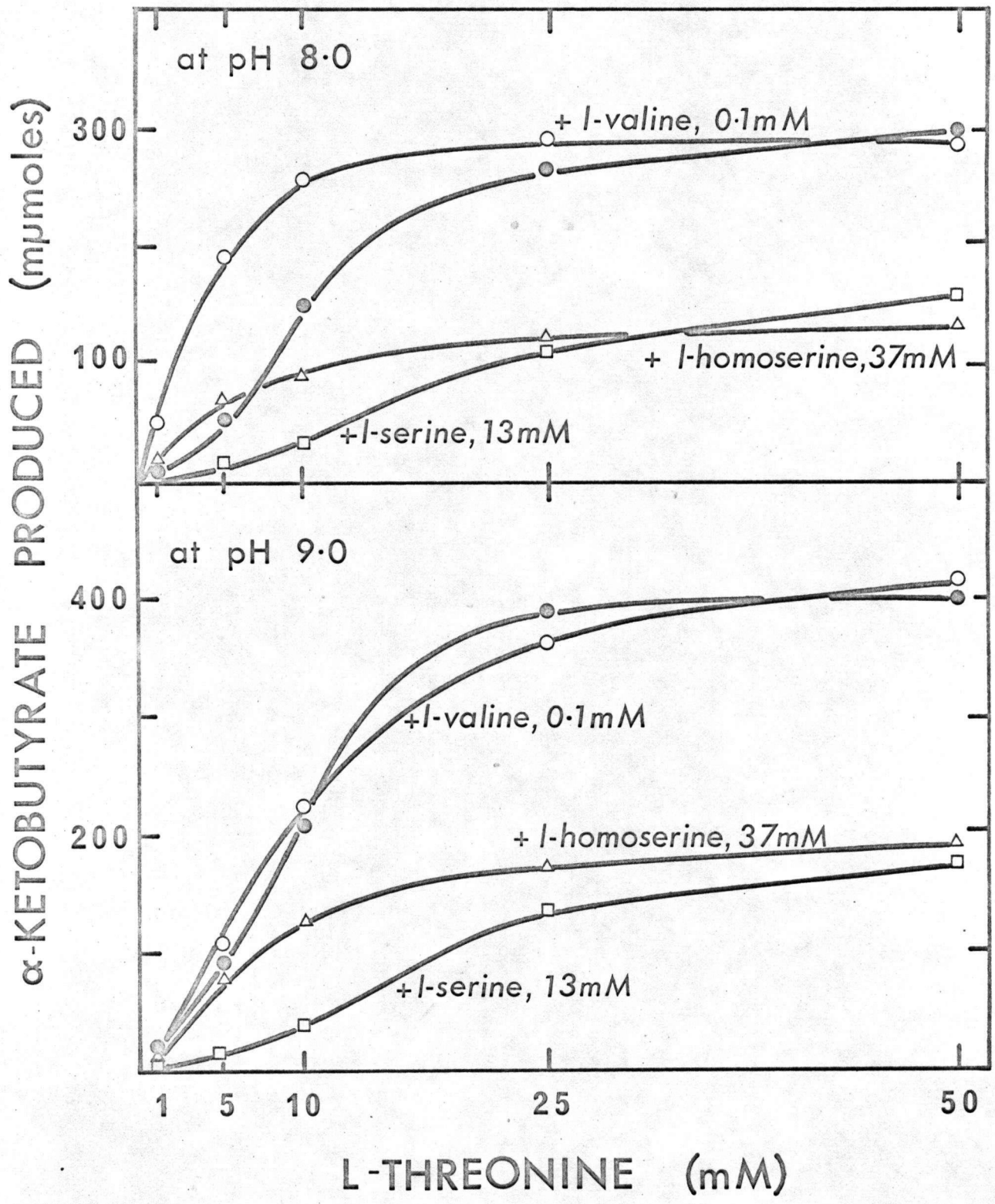

α-KETOBUTYRATE PRODUCED (mμmoles)
400
200
-□- He. vi.
-△- He.vi. (urea)
-○- He. vi. (Gly. L)
7
8
9
10
pH
400
200
-□- He.vi.
-△- He.vi. (urea)
-○- He.vi. (Gly. L)
-■- Ch.sa.
-●- Po.cr.
7
8
9
10
pH

Fig. 13. Effects of L-homoserine, L-serine and L-valine on the substrate saturation kinetics of the H. virescens enzyme at pH 8 and pH 9. Algal extracts were prepared at the desired pH (see methods) and pretreated with the indicated concentration of effector (15 min, 22° C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.


at pH 8·0
+ l-valine, 0·1mM
+ l-homoserine, 37mM
+l-serine, 13mM
at pH 9·0
+l-valine, 0·1mM
+ l-homoserine, 37mM
+l-serine, 13mM
300
100
400
200
1
5
10
25
50
α-KETOBUTYRATE PRODUCED (mμmoles)
L-THREONINE (mM)

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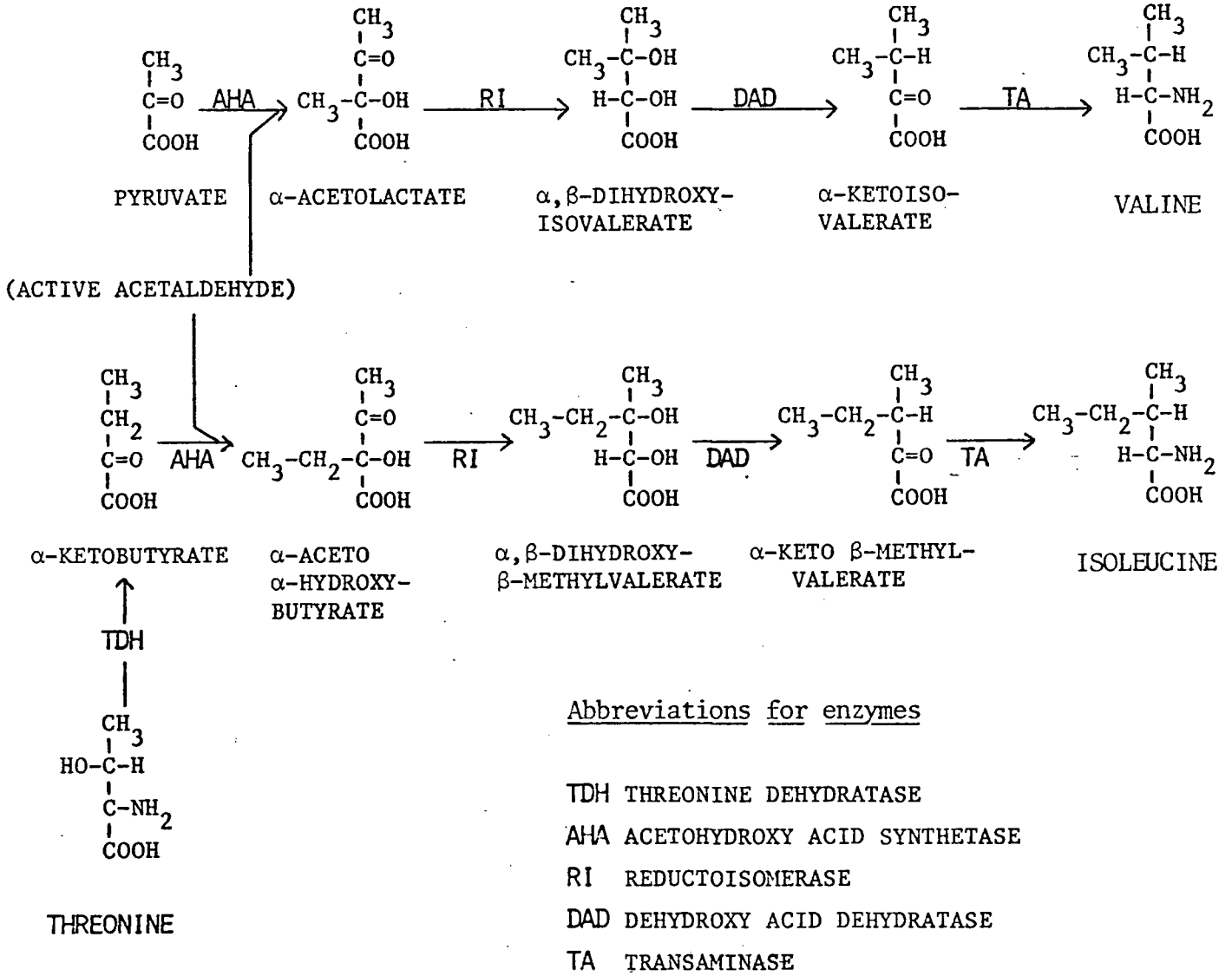
APPENDIX A.

The Common Locus of Threonine Dehydratase in Branched-chain Amino Acid Biosynthesis



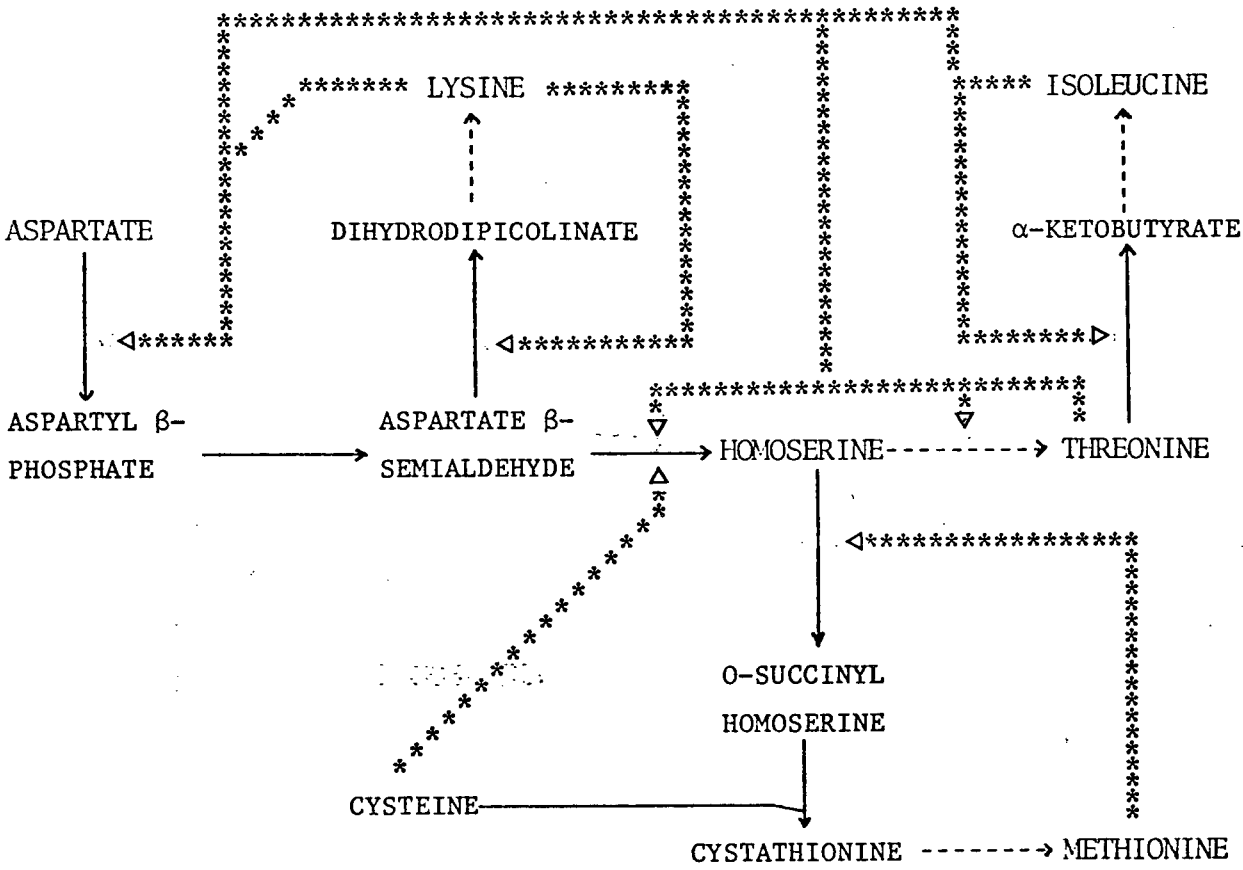
Abbreviations for enzymes

TDH THREONINE DEHYDRATASE
AHA ACETOHYDROXY ACID SYNTHETASE
RI REDUCTOISOMERASE
DAD DEHYDROXY ACID DEHYDRATASE
TA TRANSAMINASE

Adapted from: ALLAUDEEN, H.S. & RAMAKRISHNAN, T. 1968. Arch. Biochem. Biophys. 125: 199-209.

APPENDIX B.

Schematic Representation of Threonine Biosynthesis and the Major Peripheral Regulatory Circuits



(——) single enzymatic step

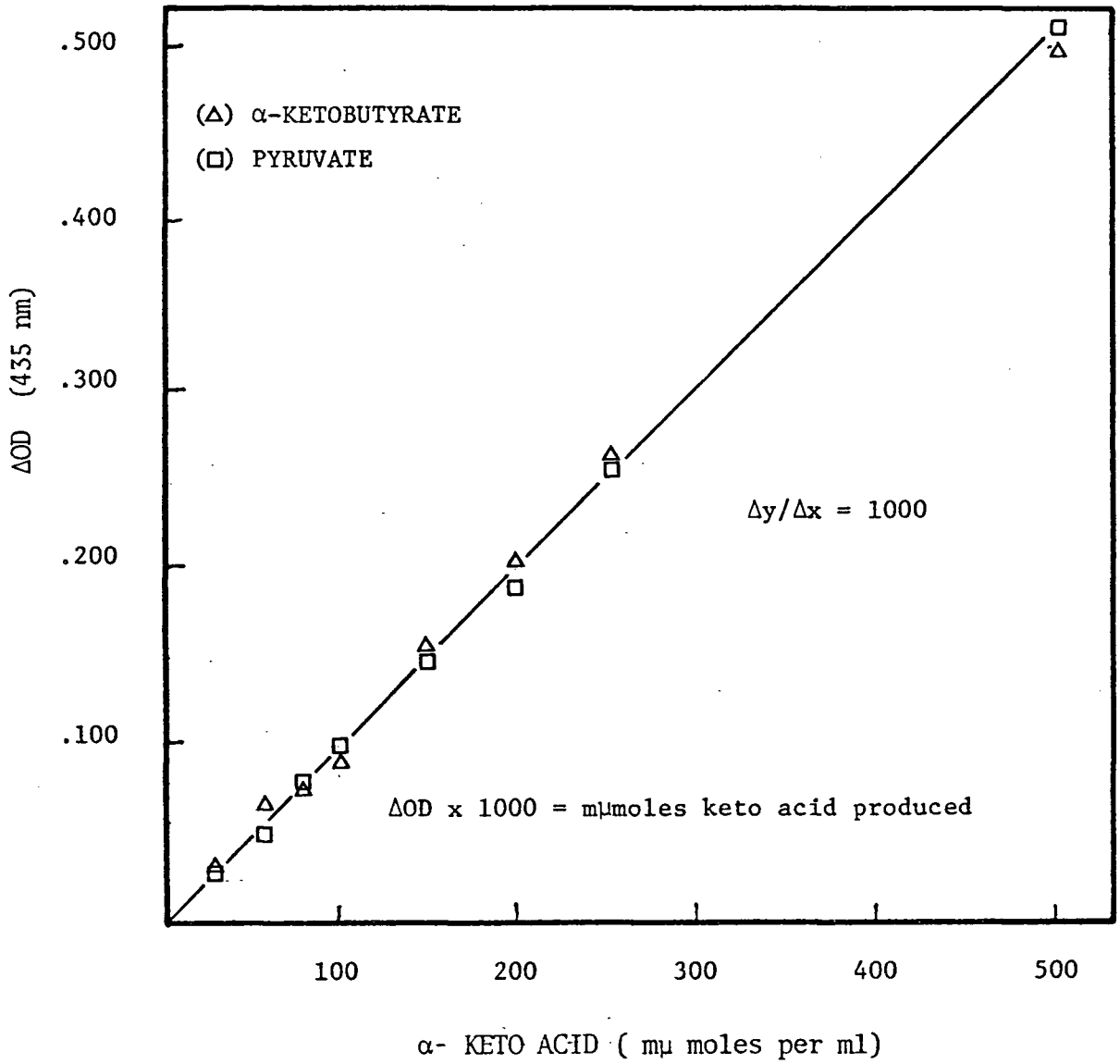
(----) more than one enzymatic step

(****) major regulatory sequences

Adapted from: DATTA,P. 1969. Science, 165: 556-62.

APPENDIX C.

Standard Calibration Curve for α-Ketobutyrate and Pyruvate.



SUPPLEMENT I

L-Threonine Deaminase in Marine Planktonic Algae. Disulfide and sulfhydryl group requirements of enzyme activity in two cryptophytes

Submitted and accepted for publication

in

The Journal of Phycology

SUMMARY

The 'biosynthetic' L-threonine (deaminating) dehydratase of 2 cryptophytes (Chroomonas salina and Hemiselmis virescens) showed sensitive inhibition from all thiols tested (dithiothreitol, cysteine, etc.) but no effect from ascorbic acid or reduced NAD. By contrast, the enzyme activities from 5 non-cryptophyceaen unicellular algae (2 cyanophytes, 1 rhodophyte, 1 diatom, 1 chlorophyte) were generally not affected by any of these reagents. The thiol-reagent inhibition of the cryptophyte enzymes (i) achieved saturation with 60-70 % reduction in activity, (ii) was considerably reduced by pretreatment of the enzymes with L-threonine and L-isoleucine, and (iii) was partially reversed by subsequent treatment with arsenite and exposure to air. It was deduced that such inhibitions were caused by thiol-specific reduction of enzyme-protein disulfide groups essential for the full expression of activity and that these groups were susceptible to ready reductive cleavage and oxidative restoration. This disulfide requirement, unique to the cryptophytes, may be the first recorded case of such a property of threonine dehydratase from all forms of life hitherto studied. The additional activity-requirement of the cryptophyte enzymes for sulfhydryl groups (which requirement was common to all the algal enzymes) was confirmed a) by the study of their sensitivity to inhibition from mercurials and disulfide-sulfhydryl exchanging reagents, and b) by the partial reversal of these inhibitions from subsequent treatment with dithiothreitol. Both cryptophyte enzymes were desensitized to feedback inhibition from L-isoleucine by prior exposure to subinhibitory concentrations of $HgCl_2$ or dithiodipyridine.

INTRODUCTION

The occurrence and catalytic properties of the 'biosynthetic' type of L-threonine dehydratase (L-threonine hydro-lyase, deaminating; EC 4.2.1.16) in 7 marine unicellular algae from 5 taxonomic divisions were reported in the previous communication of this series (13). In general, the algal enzymes showed broad resemblance in most properties (pH optima, substrate saturation kinetics, pyridoxal-phosphate and sulfhydryl-group requirements, isoleucine-sensitive allosterism) to the corresponding enzymes from bacteria, fungi, and higher plants. In particular, the alkali-metal cation (more specifically K^+ or NH_4^+) requirement of these algal enzymes indicated a closer property resemblance to the threonine dehydratases of yeast and higher plants (1). However, the algal enzymes themselves showed certain individual differences, one of which appeared to be taxonomically related. Whereas the enzyme activities from a chlorophyte, a diatom, a rhodophyte, and 2 cyanophytes were slightly stimulated or not affected by dithiothreitol [DTT], those from 2 cryptophytes were markedly inhibited by this thiol reagent (13). DTT, known to protect protein sulfhydryl groups (10), was expected to favour, or at least maintain, the activity of all the algal enzymes, since they had uniformly indicated sulfhydryl group requirement for the expression of activity (13). However, since DTT is also known to cause reductive cleavage of disulfides (31), this unexpected behaviour of the cryptophyte enzymes suggested that they may require both sulfhydryl [-SH] and disulfide [-S-S-] groupings for the manifestation of activity. This inference has now been confirmed by further tests with a number of thiol and related reagents, the results

of which studies are reported below.

MATERIALS AND METHODS

Algal species

CHLOROPHYTA: Tetraselmis maculata.

BACILLARIOPHYTA: Cyclotella nana (clone 3H of Guillard and Ryther (18) recently redesignated as Thalassiosira pseudonana (20)).

CRYPTOPHYTA: Chroomonas salina; Hemiselmis virescens.

RHODOPHYTA: Porphyridium cruentum.

CYANOPHYTA: Agmenellum quadruplicatum; Anacystis marina.

Details of the algal strains used, their source and maintenance have been reported (2). The algae were mass-cultured, harvested, and freeze-dried as previously described (13). Excepting the 2 cryptophytes, all the algae used in this study were grown photoautotrophically with added vitamins. C. salina was cultured under 3 sets of conditions, (i) photoautotrophic (vitamins, light), (ii) photoheterotrophic (glycerol, vitamins, light), (iii) chemoheterotrophic (glycerol, vitamins, darkness) (9). Being unable to use nitrate as N-source or to grow in darkness on organic substrates hitherto tested, H. virescens was cultured phototrophically with the same vitamins under 3 other conditions (all presumably heterotrophic, but this has not yet been established), (i) with urea (2 mM) as N-source, (ii) with glycine (4 mM) as N-source, (iii) with glycine (4 mM) and glycerol (3,9). Where present, the glycerol concentration was 0.25 M. Unless otherwise stated, the enzyme tests reported below for C. salina and H. virescens were normally made with the glycerol-light grown culture of the former and the glycine-light grown culture of the latter.

Enzyme assay

Suspensions of algal powder in appropriate buffer were submitted to ultrasonic oscillation and whole sonicates were assayed for enzyme activity as previously described (13), the assay method being based on that of Friedemann (15) for the colorimetric determination of keto acid produced.

Unless otherwise stated, all enzyme incubation mixtures contained algal sonicate (0.5 ml in 0.2 M potassium N-tris(hydroxymethyl)methyl glycine buffer, pH 8.5), pyridoxal phosphate (0.1 µmole), and L-threonine (80 µmoles) in a final volume of 1 ml. The mixtures were normally preincubated first with the test reagent for 15 min at 22°C, then with pyridoxal phosphate for 5 min at 37°C, and finally incubated with threonine at the latter temperature for periods varying with the algal species (usually as indicated by Desai et al. (13)). Identical preincubations were effected on controls taken without the test reagent. All the tests included both unincubated and fully incubated controls. All reagents used were of the highest purity grade commercially available.

RESULTS

Effects of thiol and reducing reagents

The response of the 2 cryptophyte enzymes to an extensive range of concentrations (0.01-10.0 mM) of the following reagents was systematically investigated: DTT, dithioerythritol [DTE], 2,3-dimercaptopropan-1-ol [BAL], dihydrolipoic acid [$Lip(SH)_2$], reduced L-glutathione [GSH], L-cysteine [Cys], L-ascorbic acid [$Asc(OH)_2$], reduced nicotinamide-adenine dinucleotide [$NADH_2$]. The results, depicted in Figs.

1 and 2, show that both cryptophyte enzymes are sensitively inhibited by all the thiol reagents but are not affected by ascorbic acid or $NADH_2$. By contrast, the enzyme activities of the non-cryptophyceaen algae were either slightly stimulated (10-20 % from DTT and DTE in the case of T. maculata) or not at all affected by the highest concentration (10 mM) of both the thiol and non-thiol reducing agents (Table 1). The concentrations of the thiol reagents required to produce 50 % inhibition (Figs. 1 and 2) revealed the following order of effectiveness of the reagents towards both cryptophyte enzymes: DTT = DTE = BAL > $Lip(SH)_2$ > GSH > Cys. This order of diminishing reactivity of the thiol reagents recalled a similar order of diminishing electronegativity of their redox potentials (10) and indicated that the thiol reagents were acting on the cryptophyte enzymes by a process of reduction. In view of the total absence of effect from $NADH_2$ known to possess comparable redox potential, it appeared that the reductive action of the thiol reagents was more specifically linked to their -SH groups interchanging with sensitive disulfide groups of the cryptophyte enzymes.

The inference that -SH sensitive disulfide groups were required for the activity of the cryptophyte enzymes was verified by tests designed to reverse the thiol reagent inhibition by a subsequent oxidative treatment effecting restoration of the original disulfide groups. Minimal concentrations of DTT were used to produce 30-50 % inhibitions, the excess thiol reagent was neutralized by specific complexing with sodium arsenite (31), and the partially inactivated enzyme was reoxidized by prolonged incubation in presence of air. This mild reversal treatment was considered necessary for valid interpretation of the tests, since the algal enzymes were known to possess

-SH groups also required for activity (13), which should be left undamaged under the conditions effecting the overall transformation R_1-S-S-R_2 → R_1-SH + R_2-SH → R_1-S-S-R_2. Air oxidation has been successfully used for reformation of the disulfide bonds of papaya lysozyme previously reduced by 2-mercaptoethanol (4). The test results, summarized in Table 2, indicate that (i) 40-70 % reversal of inhibition was obtained with both cryptophyte enzymes under the different conditions examined, (ii) the reversal was favored by higher temperature and longer incubation in air. It was established from appropriate controls a) that sodium arsenite alone had no effect on the enzyme activity but was required in adequate concentration to ensure the reversal, and b) that active aeration (surface agitation of the enzyme mixture with an air stream) made no difference to the reversal. No attempt was made to obtain complete reversal of the thiol reagent inhibitions, since the more drastic reoxidation conditions indicated by these tests were themselves expected to have deleterious effects on the enzymes.

Certain observations were made on the thiol-reagent induced inhibitions and their relationship to the cryptophyte enzyme substrate and allosteric effector, which might shed light on the nature of involvement of the essential disulfide groups in the mechanism of enzyme action. In the first place, all the tested thiol reagents tended to show saturation of inhibition with 60-70 % reduction in activity; the residual activity with 10 mM concentration of the various reagents ranged 25-29 % for C. salina and 29-37 % for H. virescens (Figs. 1 and 2). This residual activity from DTT inhibition was still sensitive to feedback inhibition from isoleucine in the case of C. salina but was completely desensitized in the case of H. virescens (Table 3). It

appears likely that this difference between the 2 cryptophyte enzymes may be related to their original marked difference in sensitivity to the allosteric effector. In the second place, it was observed that the inhibition from DTT was markedly prevented by prior treatment of both cryptophyte enzymes with threonine and isoleucine (Table 4), implying that protection of the sensitive disulfide groups from cleavage was obtained. Such protection was afforded best by the combination of both substrate and allosteric effector and significantly less by either alone. Although known to be essential for activity of the enzymes, added pyridoxal phosphate showed no protective effect (Table 4), which result may be expected from our previous inference of bound pyridoxal phosphate in the algal enzymes (13). An overall view of these observations suggested that the sensitive disulfide groups are not directly involved in the mechanism of enzyme action but appear to be in close proximity to the sites binding the substrate and allosteric effector, with the implication that such location enables them to favour enzyme action by facilitating enzyme-substrate binding.

The possibility was considered that this unique disulfide-activity requirement observed from the cryptophyceaen algae may have been due to the special nutritional (photoheterotrophic) status of their cultures hitherto studied, in contrast to the photoautotrophic cultures of the other algae examined. This implication was disproven by comparative tests of the effects of 1-10 mM DTT on 3 nutritionally different cultures of each cryptophyte. The results (Table 5) showed that the enzymes from the different cultures of the same species suffered the same degree of inhibition and retained the same order of residual activity on saturation of such inhibition.

Effects of reagents binding or modifying -SH groups

The previous indications of -SH group requirement of the cryptophyte enzymes could not be satisfactorily verified at that time because of unforeseen difficulties created by the unexpected finding of thiol-reagent inhibition of these enzymes (13). However, the interesting observation was then made that the organomercurial p-chloromercuriphenyl sulfonate [PCMPS] showed a marked difference between the 2 cryptophyte enzymes (maximal inhibition: 49 % for C. salina, 95 % for H. virescens), unlike the other -SH modifying agents. This difference was now confirmed by tests of the 2 enzymes with a range of concentrations of PCMPS, and comparable studies were made with another mercurial, $HgCl_2$, and the disulfide reagents, 2,2'-dithiodipyridine [DTDP] and 6,6'-dithiodinicotinic acid [DTDNA], both of which are known to modify protein -SH groups by exchange with their disulfide groups (16, 17). The results (Figs. 3 and 4) showed that, excepting the behaviour of PCMPS towards C. salina, all the reagents effected extremely sensitive inhibition of both cryptophyte enzymes, attaining 90-98 % inactivation at 0.1-0.5 mM reagent levels. $HgCl_2$ was consistently the most potent towards both enzymes, while the other reagents matched more evenly in their degree of action. Their overall 50 %-inhibition effectiveness (inversely related to molar concentration) averaged 1 to 2 orders of magnitude greater than that of the thiol reagents (cf. Figs. 1, 2,3,4). PCMPS produced marked difference in response of the 2 enzymes, achieving saturation with 50 % inhibition in the case of C. salina but effecting close to 100 % inhibition of H. virescens. Since $HgCl_2$ was uniformly totally inhibitory towards both enzymes, this difference cannot be attributed to the mercurial nature of PCMPS but may indicate

the presence of 2 types of essential -SH groups in the enzyme protein of C. salina, one type accessible to all the reagents (and presumably the only type in H. virescens) and another type inaccessible to PCMPS but approachable by the other reagents. Such differences in reactivity of an organomercurial towards different types of -SH groups on the same enzyme protein have been previously noted (19). The specific involvement of enzyme -SH groups in these inhibitions was further verified by tests designed to reverse the observed inactivations by subsequent treatment with DTT. The enzymes were first inactivated 40-60 % by PCMPS or DTDP and then exposed to minimal excess of the reversal reagent, the concentration of which was circumscribed by its own inhibitory action on the activities. The results (Table 6) showed that 50-60 % reversals were obtained from the test conditions approaching optimum tolerable concentration of the reversal agent.

The treatment with subinhibitory concentrations of $HgCl_2$ or p-chloromercuribenzoate has been previously used to obtain desensitization (to feedback inhibition from isoleucine) of several bacterial and plant threonine dehydratases (8,11,24,25,29,30). Such desensitizations have been generally attributed to the binding by the mercurial of -SH groups that are part of the isoleucine binding site on the enzyme. However, the yeast enzymes failed to show such desensitization despite their inactivation by mercurials (7,12). It was therefore of interest to conduct similar tests on the cryptophyte enzymes partially inactivated from $HgCl_2$, PCMPS, or DTDP by subsequent exposure to isoleucine. The results (Table 7) showed that the expected desensitization was obtained in all cases, excepting that of C. salina treated with PCMPS.

This exception may be consequential to the unique behaviour of PCMPS towards the C. salina enzyme noted above, suggesting that the very same -SH groups inaccessible to this reagent may govern the isoleucine binding site. Otherwise, the cryptophyte enzymes resemble their bacterial and plant counterparts, but differ from yeast, in their capacity for desensitization towards isoleucine inhibition.

DISCUSSION

These studies have established beyond doubt that the cryptophyte enzymes require both disulfide and sulfhydryl groups for the full expression of catalytic activity. The disulfide requirement is unique to the cryptophytes in that it is not shown by the other algal enzymes nor has it been previously recorded for any threonine dehydratase from all forms of life hitherto studied. The nature of this requirement deserves a few comments. Our observations indicated that the thiol-inhibited enzymes retain 25-35 % of their maximal activity after complete cleavage of the sensitive disulfide groups, suggesting that the latter are not absolutely essential to the basic mechanism of enzyme action but that they promote the reaction rate presumably by facilitating the enzyme binding to the substrate. Such an influence may be exercised by assistance in maintaining the enzyme molecule in its most favorable conformation state for activity, and, in this respect, the role of these disulfide groups may be similar to that of allosteric effectors activating enzymes by inducing conformational alterations (26,27). Viewed in this light, it is tempting to speculate that these disulfide groups, with their high potential for reversible reduction, may serve to regulate in vivo activity of the

cryptophyte enzymes which regulation may be intertwined with the metabolic control of oxidation-reduction states of physiological thiols such as glutathione or even of pyridine nucleotides; in the latter case the regulatory interaction could be indirectly transmitted through the mediation of transhydrogenase-type enzymes such as the $NADH_2$-dependent disulfide reductase of Bacillus cereus (5). In this connection it must be pointed out that the extraordinary thiol-sensitivity of these disulfide groups of the cryptophyte enzymes distinguishes them from those normally involved in bridging polypeptide chains of the enzyme molecule shown to be present in some purified bacterial threonine dehydratases (21,22) for which no disulfide group requirement was reported. The redox sensitivity of the former disulfide groups is reminiscent of similar sensitivity of the unusual type of disulfide bond "looping" 2 half-cysteine residues in the same polypeptide chain of insulin (6,14,23,28). The close proximity of such sensitive disulfide groups to the cryptophyte enzyme sites binding threonine and isoleucine is suggested by our observations of the protection from thiol-induced inhibition afforded by both the substrate and allosteric effector.

The sulfhydryl group requirement of the cryptophyte enzymes is common not only to all the algal species examined but appears to be a standard requirement of all threonine dehydratases hitherto tested (for citation of the literature, see ref. 13). The generality of this requirement and our observations, that reagents trapping or modifying such groups usually cause extremely sensitive and total inactivation, suggest that these particular -SH groups must be directly involved in the basic mechanism of enzyme action. The "desensitiza-

tions" to feedback inhibition from isoleucine obtained in this study certainly indicate their involvement at the enzyme site binding the allosteric effector.

ACKNOWLEDGMENT

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.

TABLE 1. Effects of thiol and reducing reagents on L-threonine dehydratase activity of 7 algal species.

Alga	Enzyme activity (% of control)*				
	DTE	GSH	Cys	$Asc(OH)_2$	$NADH_2$
C. salina	28	27	28	101	98
H. virescens	30	33	37	97	104
C. nana	105	104	105	99	101
T. maculata	112	107	108	99	96
P. cruentum	102	100	101	99	102
A. quadruplicatum	98	103	102	99	98
A. marina	102	99	100	100	98

*Activity obtained from treatment with 10 mM concentration of the reagents shown, under standard assay conditions.

TABLE 2. Reversal of DTT-induced cryptophyte enzyme inhibition by treatment with sodium arsenite and prolonged air exposure.*

DTT concn (mM)	$NaAsO_2$ concn (mM)	Preincubation with $NaAsO_2$		Enzyme activity			
				C. salina		H. virescens	
		Temp. (°C)	Period (min)	% inhib.	% reversal	% inhib.	% reversal
0.44	-	37	30	50	-	49	-
0.44	1.0	37	30	23	54	14	72
0.44	2.5	37	30	23	54	19	60
0.44	5.0	37	30	21	58	18	60
0.44	-	22	30	49	-	48	-
0.44	5.0	22	30	28	44	26	46
1.0	-	22	30	73	-	67	-
1.0	5.0	22	30	36	51	39	42
0.44	-	22	60	49	-	50	-
0.44	5.0	22	60	15	70	12	71
1.0	-	22	60	74	-	67	-
1.0	5.0	22	60	27	64	25	63

*Method involved treatment with DTT (15 min, 22°C) followed by preincubation with $NaAsO_2$ at the indicated temperature and period, prior to the regular assay procedure commencing with pyridoxal phosphate addition.

TABLE 3. Effects of isoleucine on residual activity of the cryptophyte enzymes obtained after maximal inhibition from DTT.*

Reagent concn (mM)		Enzyme activity (% of control)	
DTT	Isoleucine	C. salina	H. virescens
1.0	-	23	31
-	1.0	4	48
1.0	1.0	1	30

*Method involved treatment with DTT (15 min, 22°C) followed by preincubation with isoleucine (1 min, 22°C), prior to the regular assay procedure commencing with pyridoxal phosphate addition.

TABLE 4. Protective effects of threonine and isoleucine on the cryptophyte enzymes from DTT-induced inhibition.*

Concentration (mM)				Enzyme activity (% of control)	
Pyridoxal phosphate	Threonine	Isoleucine	DTT	C. salina	H. virescens
-	-	-	0.1	27	33
0.1	-	-	0.1	28	33
-	20	-	0.1	41	41
-	-	0.01	0.1	62	61
0.1	20	-	0.1	41	42
0.1	-	0.01	0.1	64	62
-	20	0.01	0.1	73	72
0.1	20	0.01	0.1	73	70

*Method involved pretreatment with pyridoxal phosphate, threonine, isoleucine, or combinations thereof (1 min, 22°C) followed by preincubation with DTT (15 min, 22°C). The regular assay procedure was modified to adjust for the amounts of pyridoxal phosphate and threonine used in the pretreatment, such that the final concentrations in the incubation mixture remained as usual.

TABLE 5. Effects of DTT on the activity of the cryptophyte enzymes from nutritionally different cultures.

Alga	Culture conditions		DTT concn	Enzyme activity
	Light*	Nutrients†	(mM)	(% of control)
H. virescens	+	glycine	1	35
			10	34
	+	glycine & glycerol	1	36
			10	35
	+	urea	1	38
			10	35
C. salina	+	nil	1	28
			10	26
	+	glycerol	1	26
			10	25
	-	glycerol	1	24
			10	25

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

†These nutrients correspond to the added organic compounds (see text) in addition to the vitamins normally included in all cultures.

TABLE 6. Reversal by DTT of cryptophyte enzyme activity inhibition from -SH binding agents.*

-SH binding agent		DTT concn	Reversal of inhibition (%)†	
	concn (mM)	(mM)	C. salina	H. virescens
DTDP	0.02	0.02	13	0
	0.02	0.05	67	33
	0.02	0.10	58	31
PCMPS	0.02	0.02	20	15
	0.02	0.05	62	53
	0.02	0.10	52	39
	0.05	0.05	12	-
	0.05	0.10	29	-

*Method involved pretreatment with -SH binding agents (15 min, 22°C) followed by preincubation with DTT (30 min, 22°C), prior to the regular assay procedure commencing with pyridoxal phosphate addition.

†Calculated by equating the inhibition from -SH binding agent alone to 0 % reversal, the inhibition from DTT alone to 100 % reversal, and scaling the net inhibition from the sequential action of both reagents between these extremes.

TABLE 7. Desensitization of the cryptophyte enzymes to isoleucine feedback inhibition by -SH binding agents.*

-SH binding agents		Isoleucine concn	Enzyme activity (% of control)†	
	concn (mM)	(mM)	C. salina	H. virescens
nil	-	1.0	2	48
PCMPS	0.005	1.0	6	51
	0.015	1.0	-	72
	0.050	1.0	9	98
$HgCl_2$	0.001	1.0	10	60
	0.005	1.0	67	84
	0.010	1.0	99	101
DTDP	0.005	1.0	27	52
	0.010	1.0	65	68
	0.020	1.0	87	89

*Method involved pretreatment with -SH binding agent (15 min, 22°C) followed by preincubation with isoleucine (1 min, 22°C) prior to the regular assay procedure commencing with pyridoxal phosphate addition.

†Note that 100 % control in these tests is represented by the activities obtained from all corresponding pretreatments effected without isoleucine.

Figure Legends

FIG. 1. Effects of thiol and reducing reagents on L-threonine dehydratase activity of Chroomonas salina under standard assay conditions. Reagent abbreviations are indicated in the text.

FIG. 2. Effects of thiol and reducing reagents on L-threonine dehydratase activity of Hemiselmis virescens under standard assay conditions. Reagent abbreviations are indicated in the text.

FIG. 3. Effects of -SH binding or modifying agents on L-threonine dehydratase activity of Chroomonas salina under standard assay conditions. Reagent abbreviations are indicated in the text.

FIG. 4. Effects of -SH binding or modifying agents on L-threonine dehydratase activity of Hemiselmis virescens under standard assay conditions. Reagent abbreviations are indicated in the text.

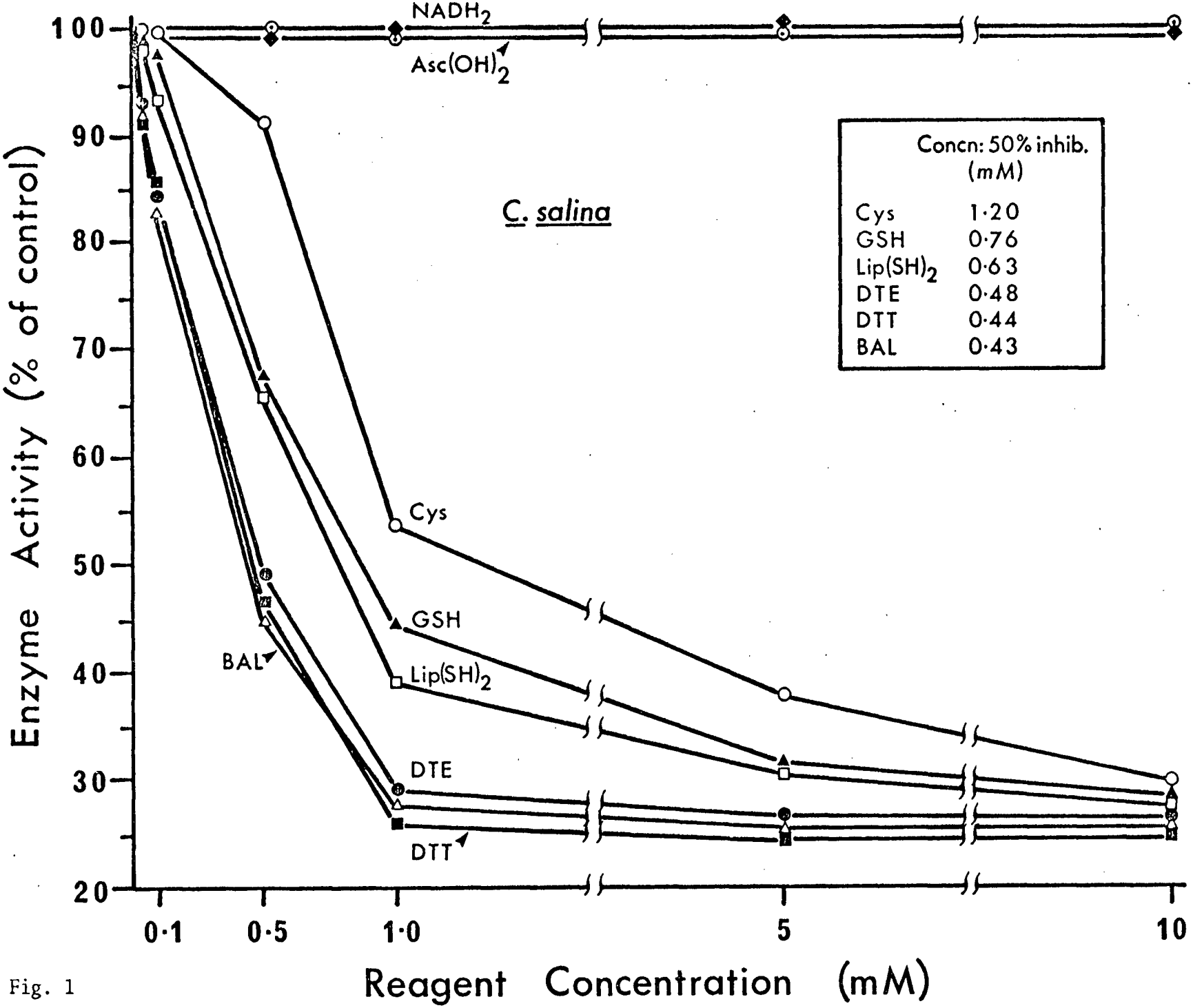

NADH2
Asc(OH)2
C. salina
Concn: 50% inhib. (mM)
Cys 1·20
GSH 0·76
Lip(SH)2 0·63
DTE 0·48
DTT 0·44
BAL 0·43
Cys
GSH
Lip(SH)2
BAL
DTE
DTT
Enzyme Activity (% of control)
100
90
80
70
60
50
40
30
20
0·1
0·5
1·0
5
10
Reagent Concentration (mM)

Fig. 1

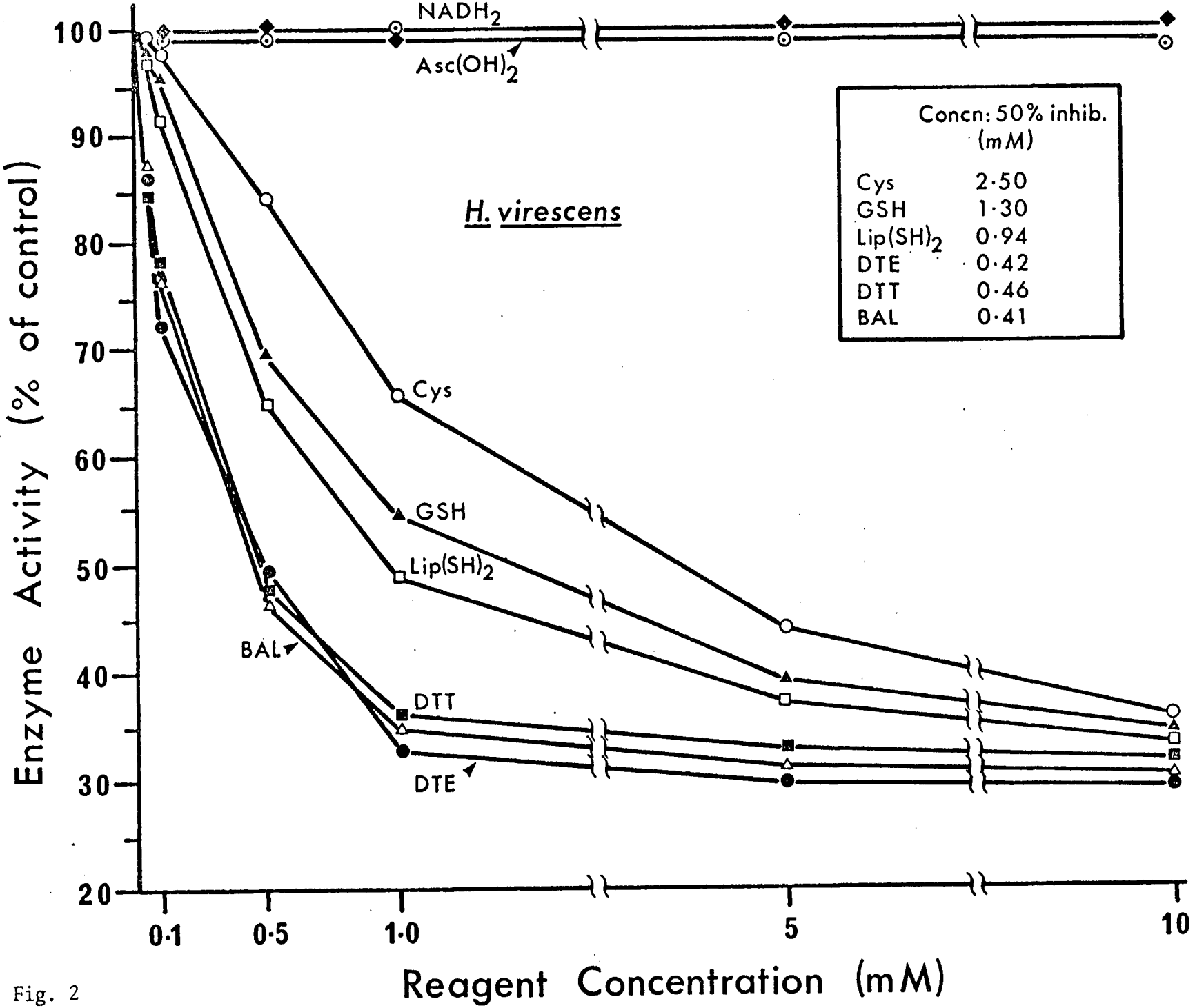

NADH2
Asc(OH)2
H. virescens
Concn: 50% inhib. (mM)
Cys 2·50
GSH 1·30
Lip(SH)2 0·94
DTE 0·42
DTT 0·46
BAL 0·41
Cys
GSH
Lip(SH)2
BAL
DTT
DTE
Enzyme Activity (% of control)
100
90
80
70
60
50
40
30
20
0·1
0·5
1·0
5
10
Reagent Concentration (mM)

Fig. 2

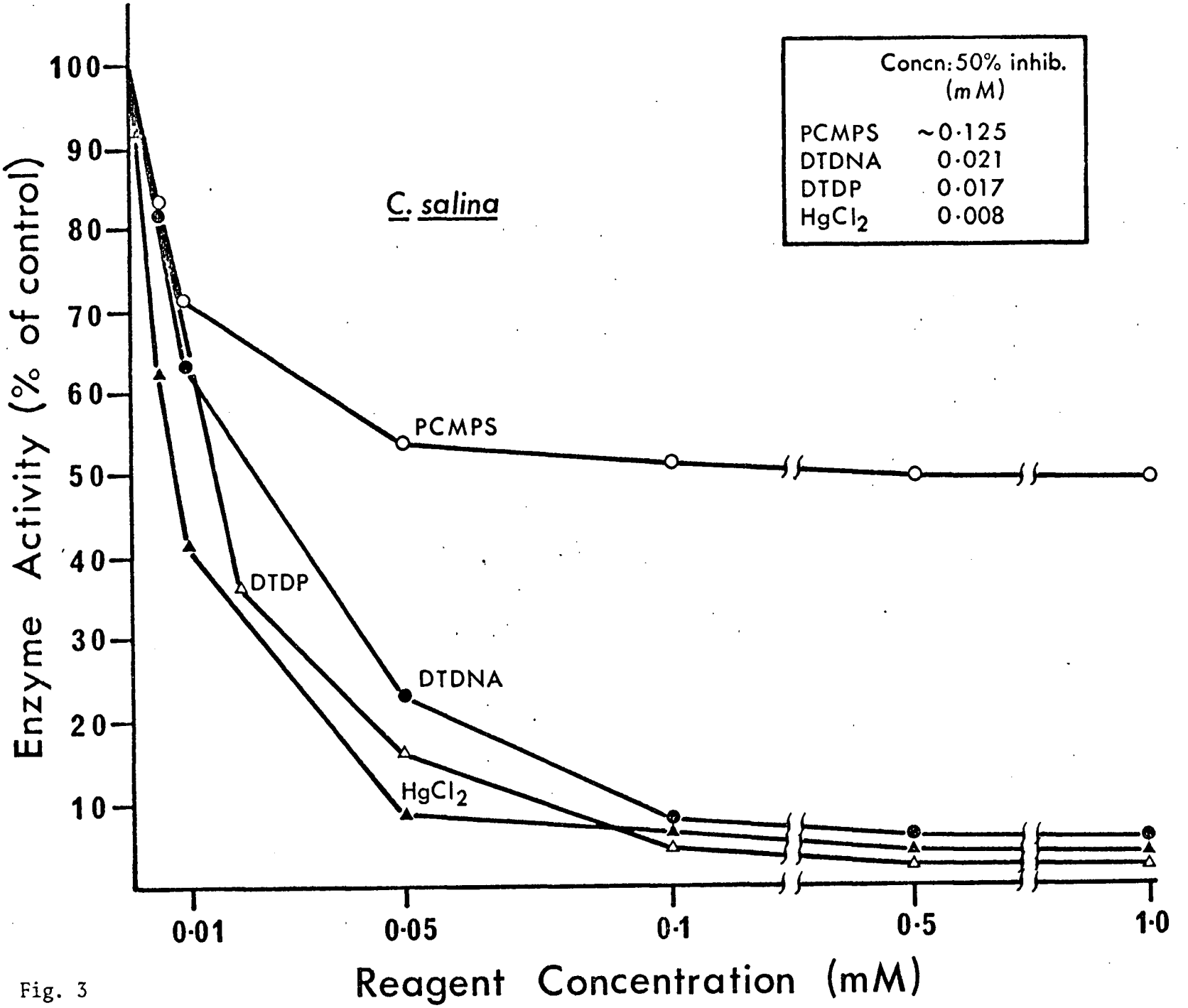

Concn: 50% inhib. (mM)
PCMPS ~0·125
DTDNA 0·021
DTDP 0·017
HgCl2 0·008
C. salina
PCMPS
DTDP
DTDNA
HgCl2
Enzyme Activity (% of control)
100
90
80
70
60
50
40
30
20
10
0·01
0·05
0·1
0·5
1·0
Reagent Concentration (mM)

Fig. 3

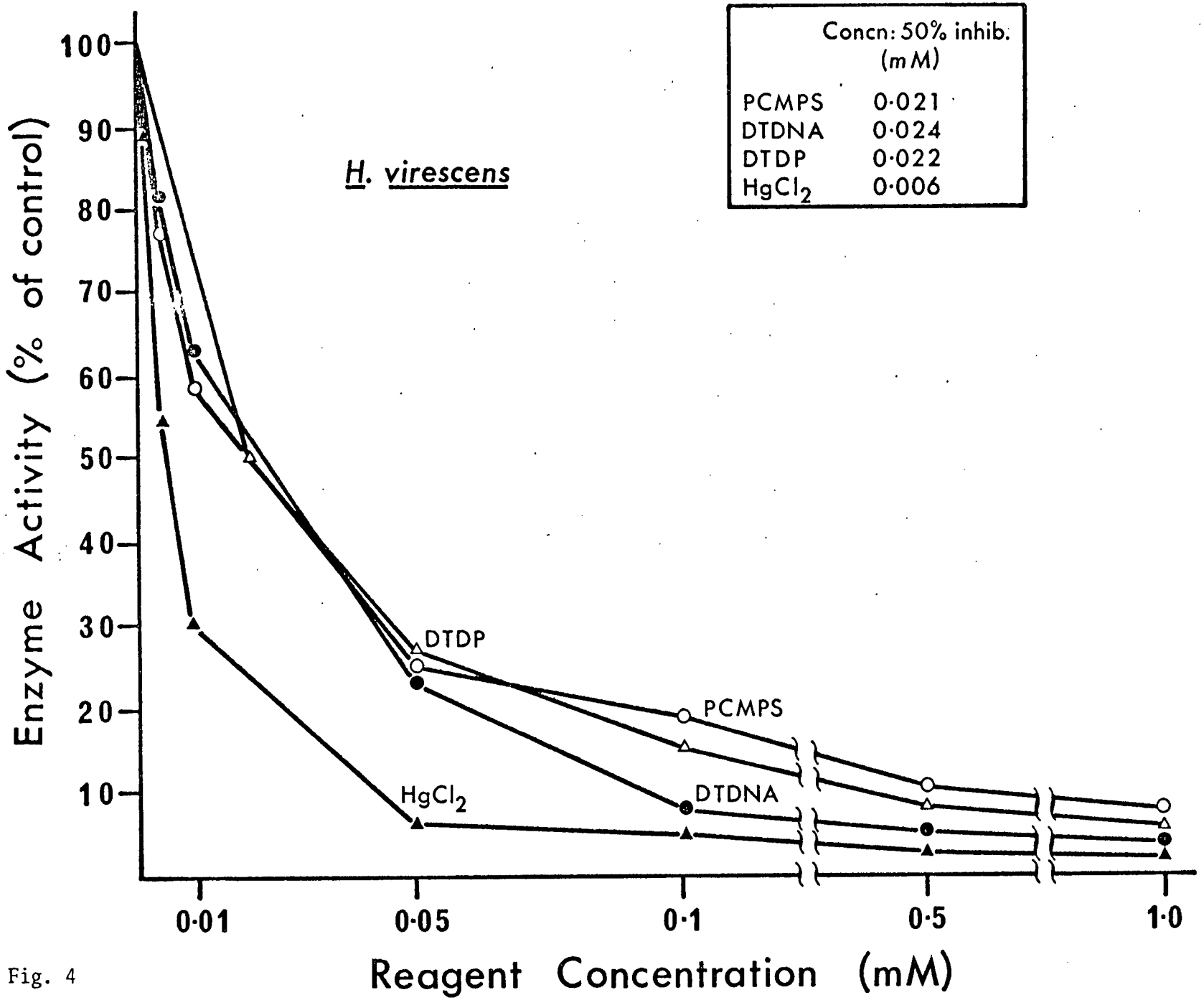

Concn: 50% inhib. (mM)
PCMPS 0·021
DTDNA 0·024
DTDP 0·022
HgCl2 0·006
H. virescens
Enzyme Activity (% of control)
100
90
80
70
60
50
40
30
20
10
DTDP
PCMPS
DTDNA
HgCl2
0·01
0·05
0·1
0·5
1·0
Reagent Concentration (mM)

Fig. 4

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SUPPLEMENT II

L-Threonine Deaminase in Marine Planktonic Algae. Stimulation of activity by monovalent inorganic cations and diverse effects from other ions

Submitted and accepted for publication

in

Archives fur Mikrobiologie

Summary 1. The 'biosynthetic' L-threonine (deaminating) dehydratase of 7 marine planktonic species from 5 classes of algae showed several degrees of activation from monovalent inorganic cations. The activation was generally the strongest (3 to 5-fold) with K^+ and NH_4^+, and the weakest (1 to 2-fold) with Na^+ and Tl^+, whilst Li^+,Rb^+, and Cs^+ showed intermediate orders varying with algal species. One blue-green alga was exceptional in showing strongest stimulation (5-fold) from Li^+ and more pronounced activation from Na^+ than Cs^+, whilst a green alga showed another type of response with the least effect from Li^+ and markedly greater activation from Rb^+ than NH_4^+.

2. The cation activation showed (i) 'hyperbolic' kinetic response to ion concentration, and (ii) high specificity for monovalent inorganic cations, with indications of a coenzyme type of role for the alkali-metal type of ions.

3. Organic cations were inert and the divalent cations Mg^{2+},Ca^{2+},Zn^{2+},Cu^{2+} were either inhibitory or without effect.

4. Among the anions tested, chloride, bromide, fluoride, bicarbonate showed no effect, iodide, nitrate, chlorate were inhibitory, whilst phosphate and sulfate were slightly stimulatory.

5. It was concluded that the algal enzymes may have an absolute K^+ or NH_4^+ requirement for *in vivo* expression of activity.

The identification and characterization of the 'biosynthetic' type of L-threonine dehydratase (L-threonine hydro-lyase, deaminating; EC 4.2. 1.16) in 7 marine planktonic species from 5 classes of algae was reported in the first paper of this series (Desai et al., 1972). Apart from certain individual differences (Antia et al., 1972), the algal enzymes showed broad resemblance in properties (pH optima, substrate saturation kinetics, pyridoxal-phosphate and sulfhydryl-group requirements, isoleucine-sensitive allosterism) to the corresponding enzymes from bacteria, fungi, and higher plants. During this study (Desai et al., 1972), the determination of the pH optima of the algal enzymes was beset by problems of inhibition of activity from Tris-HCl (pH 8-9) and sodium bicarbonate (pH 9.5-10.5) buffers; the inhibition was clearly evident from comparison with potassium phosphate and Tricine buffers, which in turn showed smaller but disturbingly significant deviations. The nature of the inhibitions suggested undefined effects from the ionic composition and concentration of the buffers used, which needed to be effectively controlled for reliable study of the algal enzymes. The systematic investigation of these effects, reported in this communication, has revealed

that (i) the algal enzymes require certain monovalent inorganic cations for the full expression of activity, (ii) the apparent inhibition obtained from Tris-HCl was due to the absence of these ions in the buffer used, (iii) the apparent inhibition observed in the sodium bicarbonate buffers was due to markedly lower activation of the enzymes by Na^+ relative to K^+, and (iv) the divergences observed between potassium phosphate and Tricine buffers of comparable molarity were due to differences of K^+ concentration in these buffers (apart from slight stimulation by phosphate anion), which differences are normally ignored but assume importance in the concentration-sensitive response of the algal enzymes.

Materials and Methods

Algal species

CHLOROPHYTA (green algae): *Tetraselmis maculata* (Te.ma.).

BACILLARIOPHYTA (diatoms): *Cyclotella nana* (Cy.na.).

CRYPTOPHYTA: *Chroomonas salina* (Ch.sa.); *Hemiselmis virescens* (He.vi.).

RHODOPHYTA (red algae): *Porphyridium cruentum* (Po.cr.).

CYANOPHYTA (blue-green algae): *Agmenellum quadruplicatum* (Ag.qu.); *Anacystis marina* (An.ma.).

Abbreviations shown after species names are used to denote these species in Figures and Tables. Details of the algal strains used, their source and maintenance have been reported (Antia and Cheng, 1970). The algae were mass-cultured, harvested, and freeze-dried as previously described (Desai et al., 1972). Excepting C. salina and H. virescens, all the cultures used in this study were grown photoautotrophically with added vitamins. C. salina and H. virescens were grown phototrophically with enrichments of glycerol and glycine, respectively, in addition to the vitamins.

Enzyme assay

Suspensions of algal powder in appropriate buffer were submitted to ultrasonic oscillation and whole sonicates were assayed for enzyme activity as previously described (Desai et al., 1972), the assay method being based on that of Friedemann (1957) for the colorimetric determination of keto acid produced.

All enzyme incubation mixtures contained algal sonicate (0.5 ml in buffer of strength double the final molarity required), pyridoxal phosphate (0.1 µmole), and L-threonine (80 µmoles) in a final volume of 1 ml. The mixtures were preincubated with pyridoxal phosphate for 5 min at 37°C, then incubated with threonine at

the same temperature for periods varying with the algal species (10-40 min as indicated by Desai et al., 1972). When tested, the salts of cations or anions were incorporated into the mixtures at the same time as the buffered algal sonicate. All such tests included unincubated and fully incubated controls made without the salts. Chromatographically homogeneous L-threonine was purchased from Calbiochem (Los Angeles, Calif.). The salts, buffer and other reagents used were of the highest purity grade commercially available.

Results

All the algal enzymes showed very low activity in 0.1 M Tris-HCl buffer, which was enhanced two to five-fold by the addition of several monovalent inorganic cations as their chloride salts (Fig. 1). At a concentration level of 0.1 M, NH_4^+ and K^+ were generally the most stimulatory causing 220-390% increase in activity, Li^+, Rb^+, Cs^+ showed intermediate degrees of activation with their order of effectiveness varying with the algal species, while Na^+ was usually the least effective with 3-115% increase in activity. The cyanophyte A. marina was exceptional in showing

the highest activity enhancement (384%) from Li^+ among the cations as well as in obtaining greater degree of activation from Na^+ (189%) than Cs^+ (166%). By contrast, the chlorophyte T. maculata showed the least effect from Li^+ (96%), the strongest stimulation from K^+ (390%), and considerably greater activation from Rb^+ (285%) than NH_4^+ (160%). The diatom C. nana showed the most specific requirement for K^+, with little or no effect from Rb^+, Li^+, Cs^+, Na^+, and the lowest activation from NH_4^+ observed among the algae.

Tl^+ could not be compared directly with the other cations as the chloride salt, because of its extremely low solubility. The more soluble $TlNO_3$ offered a means of comparison with KNO_3 and NH_4NO_3 at a concentration level of 0.05 M. The results (Table 1) showed no effect from Tl^+ on the diatom enzyme and relatively minor stimulation (26-61%) of the other algal enzymes. It was interesting to note that the strongest activation from Tl^+ was observed with the alga (A. marina) which had shown anomalous order of response to the other cations.

The possibility was considered that the lesser degree of activation obtained from some of the cations may be due to net inhibition from excess concentration

of these ions at the level used for comparison in Fig. 1. This was disproved by studying the effects of cation concentration gradations on 5 algal species (examples shown in Fig. 2). In all cases, enzyme activity showed hyperbolic increases with ionic concentration. Certain algal species-cation combinations tended to show ion saturation at the upper concentration levels tested but there was no evidence of inhibition. Estimates of the Km values for the cations showing the strongest effects (see Fig. 2) indicate high affinity of the algal enzymes for these ions.

An interesting outcome of the concentration gradient study was the explanation it offered for the minor but significant divergences obtained in enzyme activity values determined with 0.1 M K-phosphate, K-Tricine, K-Hepes, and Tris-HCl (containing 0.1 M KCl) buffers at comparable pH (Table 2). Although the molarity of the first 3 buffers is the same with respect to their buffering anions, they differ sufficiently in their K^+ concentration to produce the observed divergences in response of the more sensitive algal enzymes. Excepting the case of K-phosphate, these divergences were virtually eliminated whenever the KCl concentration, taken in

conjunction with Tris-HCl buffer, was adjusted to correspond closely with the K^+ concentration of the other buffers (Table 2). K-phosphate still showed a small deviation because of the stimulation obtained from the phosphate anion (see below). The corresponding comparison of Na-bicarbonate and K-bicarbonate buffers (Table 2) showed that the apparent inhibition of the algal enzymes previously obtained (Desai et al., 1972) from the former buffer was due to the lesser activation of the enzymes by Na^+ than K^+. When these buffers were compared with their Na^+ or K^+-controlled counterparts in Map-HCl buffer, excellent agreement was observed (Table 2).

The divalent cations Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} showed no activation of the algal enzymes when tested with Tris-HCl and K-Tricine buffers. With the former buffer, the activity observed was too low to assess other effects from these ions. But the latter buffer, due to its K^+ content, elevated activity levels sufficiently to indicate definite patterns of inhibition from the divalent cations (Table 3). Zn^{2+} and Cu^{2+} were strongly inhibitory (50-90%), while Ca^{2+} and Mg^{2+} showed little effect excepting the case of A. quadruplicatum. This cyanophyte was consistently

sensitive to all the 4 divalent cations, being inhibited about 30% by Ca^{2+} or Mg^{2+} and about 90% by Zn^{2+} or Cu^{2+}.

All the algal enzymes showed insignificantly low activities in Tris-HCl and Map-HCl buffers (irrespective of pH) in the absence of alkali-metal cations. It was necessary to establish whether this was due to enzyme inhibition or indifference from the organic cation strength of these buffers. The effects of a range of concentrations (10, 25, 50, 100 mM) of Tris-HCl (pH 8.5) and Map-HCl (pH 9.5), both with and without added KCl (5 mM), were tested on 2 algal species (C. salina and P. cruentum). The observed activities showed no significant effect from buffer concentration in all the cases tested. Similar tests were also made with 2 concentrations (10, 50 mM) of methylamine-HCl or trimethylamine-HCl incorporated into Tris-HCl (0.1M, pH 8.5;± 5 mM KCl) and again no effects were obtained from these additional organic cations. The evidence indicated an overall indifference of organic cations to the algal enzymes.

Tests of the effects of inorganic anions on the algal enzyme activity presented difficulties in maintaining simultaneous control over the pH and accompanying cation, since the usual buffers contained unequivalent concentrations of either or both ions at any chosen pH. Tris·H^+ was the obvious choice of enzymically inert but buffer-effective cation, but this required buffers of the chosen pH to be made up with acids of the anion tested (HBr, H_2SO_4, HNO_3), where strict control over the anion concentration was not possible. However, tests with such buffers showed poor enzyme activity of levels comparable to those obtained with Tris-HCl and indicated that any effects from these anions were too low for satisfactory evaluation in the absence of an activating monovalent cation. Eventually, the Tris-HCl buffer containing negligible chloride ion was chosen for the tests with K^+ salts of the anions at a conveniently controllable pH and the anion effects were evaluated against KCl controls. The bicarbonate effects were obtained from direct comparison of K-bicarbonate buffer with the corresponding KCl-enriched Map-HCl buffer. The results (Table 4) showed that fluoride, bromide, bicarbonate had no effect on the algal enzymes,

iodide was strongly (81-88%) inhibitory, nitrate and chlorate were moderately (18-45%) inhibitory, whilst phosphate and sulfate were slightly (8-25%) stimulatory. Whereas the chlorate inhibition was relatively uniform, the nitrate inhibition showed taxonomically-related differential response from the algae tested, being most pronounced with the 2 cyanophytes and relatively small with the 2 cryptophytes. Anion concentration increase in the range 0.05-0.20 M produced little change in the inhibitions or stimulations obtained, indicating that these concentrations were saturating in their effects. When nitrite was tested analogously to nitrate, it showed virtually total abolition of algal enzyme activity, but this was traced to its interference in the assay method when control determinations made with α-ketobuyric acid showed similar inhibition from nitrite of the colour normally developed.

Discussion

The results of this investigation lead to the inevitable conclusion that monovalent inorganic cations (more specifically alkali-metal type) are absolutely required for the expression of activity

by algal threonine deaminases. Organic cations ranging from Tris·H^+ to mono- and tri-methylated ammonium ions have shown no effects whatsoever and may be considered inert towards these enzymes. Tris has been generally reported to inhibit several enzyme systems (other than threonine deaminase) activated by monovalent cations (Betts and Evans, 1968); in one rare instance it proved stimulatory at low concentration while being inhibitory at high concentration (Bewley and Marcus, 1970). These effects of Tris have been attributed to its antagonistic competition with the required cation (generally K^+) or its non-competitive contribution to the total cationic strength. The entirely negative response of the algal enzymes to tests designed to elucidate such effects of Tris and Map buffers compels us to infer that a) these buffers are indifferent to the enzymes, and b) the insignificantly low, though measureable, activity obtained in these buffers is not due to inhibition from them but rather a consequence of contaminating alkali-metal type cations expected to be present in the algal preparations used. This interpretation is supported by the extreme sensitivity of response of the algal enzymes to minute amounts of added K^+ or NH_4^+ in the presence of large excess

of buffer cations. The low magnitude of the Michaelis constants obtained for the more potent alkali-metal type cations reflects their high specificity of enzyme "binding" comparable to that obtained from a natural substrate or cofactor, and further confirms our inference that the algal enzymes have an absolute requirement for these cations. Viewed in the physiological context, the specificity of this alkali-metal type cation requirement is further narrowed down to the ions K^+ and NH_4^+, either of which only may be expected to play a significant role in governing in vivo activity of the algal enzymes. Whether this role involves enzyme regulation by product activation in the case of NH_4^+ (as suggested for yeast threonine deaminase (Holzer et al., 1964)) or participation in enzyme-substrate complex formation in the case of K^+ (as suggested for whole categories of enzymes catalyzing elimination reactions (Suelter, 1970)) remains a question for conjecture.

The degree of activation obtained from the non-physiological cations Li^+, Rb^+, Cs^+, Tl^+ may be mechanistically significant to the comparative enzymology of several types of enzyme reactions promoted by monovalent cations (Suelter, 1970),

since some investigators have managed to correlate the degree of activation (or stabilization) of certain exemplary enzymes with the crystal radii of such ions (Bothwell and Datta, 1971). Prompted by the close similarity of ionic radii of Tl^+ and K^+, Kayne (1971) demonstrated that the former ion could indeed replace the latter in monovalent cation activation of pyruvate kinase and that the consequent kinetics from Tl^+ were closest to those from the cation Rb^+ with identical ionic radius. The evidence from the present study indicates that the algal enzymes show no such correlationship of cation activation to ionic radius; this is particularly seen in their generally poor response to Tl^+ and in the case of A. marina, where the order of activation was $Li^+ > K^+ > NH_4^+ > Rb^+ > Na^+ > Cs^+ > Tl^+$.

The probable monovalent cation requirement of previously studied 'biosynthetic' threonine deaminases from bacteria, yeast and plants may have escaped the notice of many earlier investigators (see Desai et al., 1972, for literature citations), who have generally used enzyme extracts prepared in phosphate buffers or $(NH_4)_2SO_4$ suspensions; however, the influence (related to ionic strength) of these cations in stabilizing or protecting the enzymes from inactivation

by dilution has been frequently noted (Changeux, 1964; Cennamo et al., 1964; Leitzmann and Bernlohr, 1968; Lessie and Whiteley, 1969). In the case of the yeast enzyme the stabilizing influence of the monovalent cations was found to be related to their order of specific enzyme activations subsequently observed (Holzer et al., 1964), whilst the enzyme of a pseudomonad (Lessie and Whiteley, 1969) was stabilized and activated from mere increase of total ionic strength, irrespective of monovalent (Na^+, K^+), divalent (Mg^{2+}, Ca^{2+}, Mn^{2+}), or organic ($Tris \cdot H^+$) cations present. A careful scrutiny of the scanty ion-activation observations reported in the literature indicates that none of the bacterial enzymes studied may have an essential requirement for monovalent cations. The threonine deaminase of Hydrogenomonas was unaffected by NH_4^+ (Reh and Schlegel, 1969), that of Bacillus stearothermophilus was inhibited (15%) by NH_4^+ but stimulated (18-75%) in increasing order by K^+, Na^+, Li^+ (Thomas and Kuramitsu, 1971), whereas the Salmonella typhimurium enzyme was only slightly stimulated (20%) by NH_4^+, K^+, Na^+, Li^+ (Burns and Zarlengo, 1968). On the other hand, the reported properties of the plant and animal enzymes suggest that they may have an

essential requirement, similar to the algal enzymes, for monovalent cations. The enzymes from a rose tissue culture (Dougall, 1970) and spinach leaf (Sharma and Mazumder, 1970) indicated such a requirement, being stimulated 5- to 10-fold by K^+, with lesser activation from NH_4^+, Rb^+, or Li^+, and least or no effect from Na^+. The enzyme of a yeast (Holzer et al., 1964) was activated 6-fold by NH_4^+, with other cation effects in the order $NH_4^+ > K^+ \geq Rb^+ > Li^+ \geq Cs^+ > Na^+$; the Michaelis constants reported for NH_4^+ and K^+ are of similar magnitude to those of the algal enzymes. Among animals, it is interesting to note that the 'biodegradative' threonine deaminase of sheep liver (Nishimura and Greenberg, 1961) was strongly activated by the cations in a manner ($K^+ > NH_4^+ \geq Rb^+ > LI^+ > Na^+$) similar to the higher-plant 'biosynthetic' enzymes and with Michaelis constants of magnitude comparable to those reported for the yeast enzyme. It thus appears that the algal enzymes resemble more closely those of the eucaryotic plants and animals in their monovalent cation activation/requirement properties than those of the procaryotic bacteria. Since the algae examined include both procaryotic (blue-green algae) and eucaryotic (other classes) organisms, it is tempting to speculate that

the monovalent cation 'promotion' of threonine deaminase was developed from a primitive non-specific ionic strength effect to a specific requirement during procaryotic evolution from bacterial ancestors to blue-green algae and that this 'selected' requirement was subsequently retained as a conservative characteristic during further evolution, despite subsequent modifications of the enzyme function ('biosynthetic' versus 'biodegradative') in conjunction with its allosteric properties.

That the algal deaminases do not have a divalent-metal cation requirement was indicated by our earlier observation (Desai et al., 1972) of no significant effect from ethylenediamine tetraacetate on their activity and is confirmed by the absence of any stimulatory effect from such cations tested in the present study. In this respect, the algal enzymes resemble all previously reported threonine deaminases. The rare case of activation of a pseudomonad enzyme (Lessie and Whiteley, 1969) by salts of Mg^{2+}, Ca^{2+}, Mn^{2+} is attributed to its stabilization from their non-specific enhancement of the total ionic strength. On the other hand, sensitive inhibition of these

enzymes from Hg^{2+} has been frequently reported (Leitzmann and Bernlohr, 1968; Sharma and Mazumder, 1970; Maeba and Sanwal, 1966; Nakazawa and Hayaishi, 1967), which inhibition has been generally attributed to mercaptide-producing modification of essential sulfhydryl groups; this mechanism of Hg^{2+}-induced inhibition has been verified with a bacterial enzyme (Datta, 1966) by regeneration of activity from subsequent treatment with 2-mercaptoethan-1-ol. It appears likely that the inhibitions of the algal enzymes from Cu^{2+} and Zn^{2+} may be due to similar interference with sensitive sulfhydryl groups known to be essential for activity (Desai et al., 1972). The general absence of effects from Ca^{2+} and Mg^{2+} on the algal enzymes finds a parallel in the similar behaviour of the yeast enzyme (Holzer et al., 1964). The extreme sensitivity of the enzyme of A. quadruplicatum to all the 4 divalent cations may be related to similar extreme inhibitory response consistently obtained from this enzyme on exposure to all the -SH group modifying agents and pyridoxal phosphate antagonists previously tested (Desai et al., 1972).

Apart from the enzyme of rose tissue culture (Dougall, 1970), the effects of anions have been

ignored in the literature reports on threonine deaminases. The former enzyme was found to be strongly inhibited by nitrate, nitrite, iodide, and slightly stimulated by sulfate and phosphate, with the halides showing a pattern of increasing inhibitory action paralleling their atomic weight. The algal enzymes were generally similarly affected by these anions, excepting the cases of nitrite and the halide pattern. Unlike the rose culture enzyme, the algae have shown no significant difference between fluoride, chloride, bromide, while being strongly inhibited by iodide. In the case of nitrite, the inhibition reported for rose culture appears to be an erroneous interpretation of interference from this anion in the enzyme assay method, which interference was unequivocally established during our tests on the algal enzymes. An overall view of the anion effects on the algal enzymes indicates a pattern of inhibition arising only from the ions (I^-, ClO_3^-, NO_3^-) known to possess a high reducing or oxidation potential and suggests that their inhibitory action may be due to reductive or oxidative processes rather than ion interaction. Viewed in the physiological context, these effects are expected to be manifested only from unnaturally

high concentrations of such anions, and there was no indication of a regulatory role of anions comparable to that recently reported on the activation by bicarbonate, phosphate, and sulfate of glucose dehydrogenase (Horne and Nordlie, 1971). It is concluded that anions may have no significant influence on *in vivo* activity of algal threonine deaminases, excepting in environments polluted with reducing or oxidizing reagents.

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TABLE 1. Stimulation of algal threonine deaminase from Tl^+ relative to K^+ and NH_4^+.

Cation (0.05 M nitrate salt)	Enzyme activity (% of control)[a]						
	Ch.sa.	He.vi.	Ag.qu.	An.ma.	Po.cr.	Te.ma.	Cy.na.
K^+	280	260	279	288	346	446	281
NH_4^+	380	377	378	269	290	228	179
Tl^+	126	127	147	161	139	128	100

[a]Standard enzyme incubations made with Tris-HCl buffer (0.1 M, pH 8.5). The activities obtained without added cation were taken as 100% control.

TABLE 2. Enzyme activity of C. salina obtained with different buffers of same molar strength[a] at comparable pH.

Buffer			Added	Enzyme activity	
Type	pH	Estimated K^+ (or Na^+) content (M)	KCl (M)	Keto acid measured (mμmoles)	% of corresponding control[b]
K-Tricine	8.0	0.07	-	232	102
K-Hepes	8.0	0.13	-	241	101
K-Phosphate	8.0	0.19	-	297	115
Tris-HCl	8.0	-	0.07	227	-
	8.0	-	0.13	238	-
	8.0	-	0.19	258	-
K-Bicarbonate	9.5	0.12	-	150	99
Na-Bicarbonate	9.5	0.13	-	68	102
Map-HCl	9.5	-	0.12	152	-
	9.5	-	0.13[c]	67	-

[a] 0.1 M.

[b] Above Tris-HCl or Map-HCl buffers with corresponding pH and added KCl.

[c] KCl replaced by NaCl.

TABLE 3. Effects of divalent cations on algal threonine deaminase activity.

Cation (0.01 M dichloride salt)	Enzyme activity (% of control)[a]						
	Ch.sa.	He.vi.	Ag.qu.	An.ma.	Po.cr.	Te.ma	Cy.na.
Mg^{2+}	105	92	72	98	102	93	95
Ca^{2+}	103	94	67	97	100	85	97
Zn^{2+}	43	46	13	43	40	52	31
Cu^{2+}	20	34	11	38	27	27	25

[a]Standard enzyme incubations made with K-Tricine buffer (0.1 M, pH 8.5). The activities obtained without added cation were taken as 100% control.

TABLE 4. Effects of inorganic anions on algal threonine deaminase activity.

Anion (K^+ salt)	Conc. (M)	pH[a]	Enzyme activity (% of control)[b]				
			Ch.sa.	He.vi.	Ag.qu.	An.ma.	Po.cr.
Br^-	0.10	8.7	101	100	98	97	-
F^-	0.10	"	101	98	101	96	-
I^-	0.05	"	15	15	17	22	-
	0.10	"	15	10	18	18	-
	0.20	"	12	17	14	19	-
NO_3^-	0.05	"	90	88	56	60	-
	0.10	"	87	87	55	61	-
	0.20	"	82	84	55	55	-
ClO_3^-	0.05	"	78	74	70	79	-
	0.10	"	76	74	72	78	-
SO_4^{2-}	0.05	"	111	108	116	113	-
	0.10	"	117	108	117	110	-
HPO_4^{2-}	0.05	"	119	121	127	122	-
	0.10	"	121	122	125	118	-
HCO_3^-	0.07	9.5[c]	99	-	95	-	97

[a]Buffered with Tris-HCl(0.1 M).

[b]The activities obtained from standard incubations were expressed as percent of controls containing

corresponding K^+ concentrations as KCl in place of the anion salt.

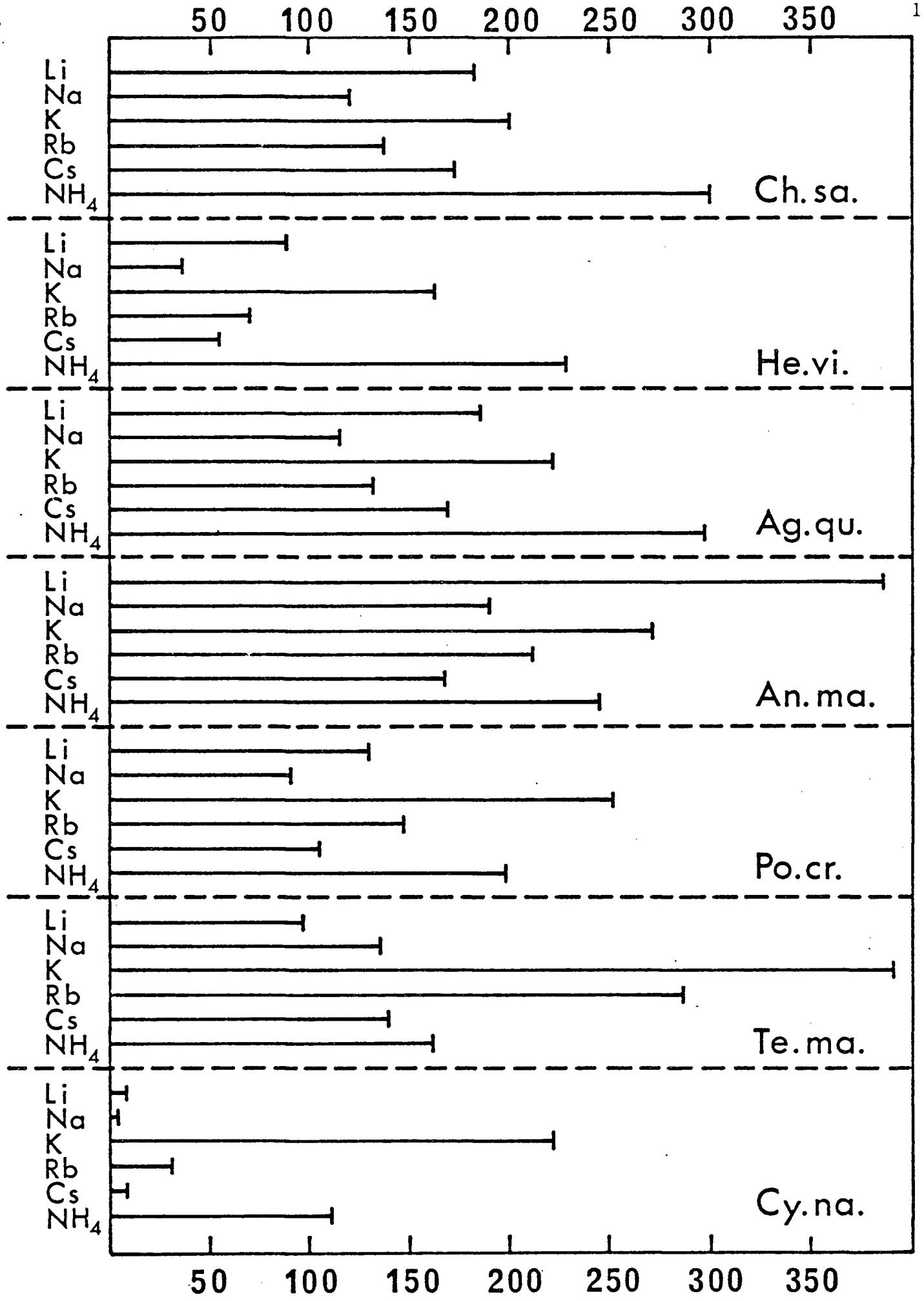
[c]The anion was part of 0.1 M K-bicarbonate buffer at this pH; in this case the control was made with Map-HCl buffer (0.1 M, pH 9.5) containing corresponding K^+ concentration (0.12 M) as KCl.

Figure Captions

Fig. 1. Stimulation of algal threonine deaminase activity by monovalent cations. The stimulation is shown as percent increase in activity from incorporation of 0.1 M chloride salts of the cations into standard incubation mixtures buffered with Tris-HCl (0.1 M, pH 8.5). The activity obtained from controls without the salts was equated to zero % stimulation.

Fig. 2. Effect of cation concentration on threonine deaminase activity of (A) Chroomonas salina, (B) Porphyridium cruentum, (C) Anacystis marina, (D) Tetraselmis maculata. Standard enzyme incubations made with Tris-HCl buffer (0.1 M, pH 8.5). Note that the activities are shown for the total incubation periods used (10-40 min varying with the algal species). The Km values were estimated from double reciprocal plots of reaction velocity (mμmoles keto acid produced per min per mg protein) versus cation concentration (moles per liter). In view

of the likelihood of contaminating cations in the enzyme extracts used, these Km values cannot be considered absolute but reflect the magnitude of cation concentration giving half-maximum velocity.


50
100
150
200
250
300
350
Li
Na
K
Rb
Cs
NH4
Ch.sa.
He.vi.
Ag.qu.
An.ma.
Po.cr.
Te.ma.
Cy.na.
Percent Stimulation

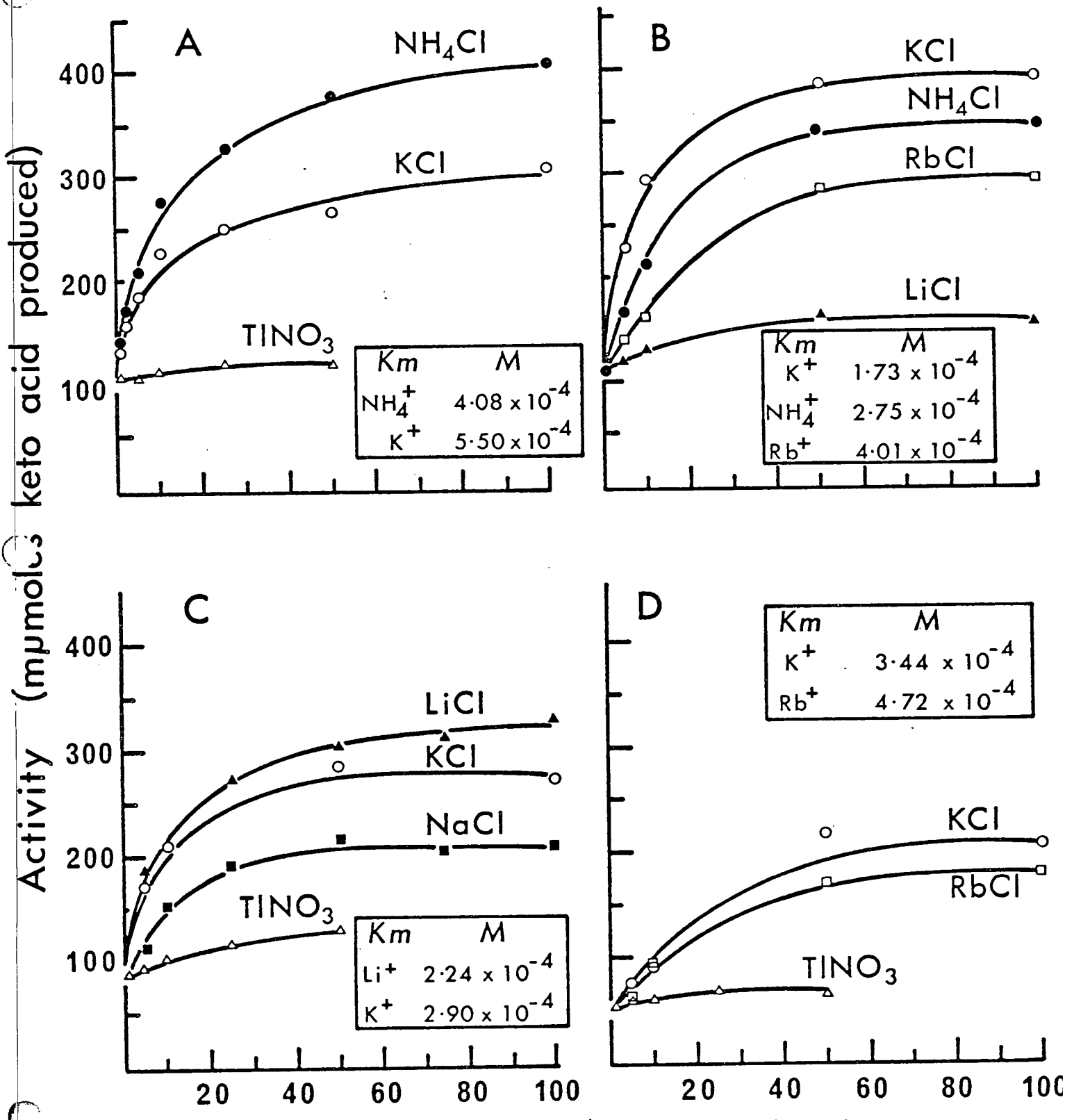

A
NH4Cl
KCl
TlNO3
Km M
NH4+ 4·08 x 10-4
K+ 5·50 x 10-4
B
KCl
NH4Cl
RbCl
LiCl
Km M
K+ 1·73 x 10-4
NH4+ 2·75 x 10-4
Rb+ 4·01 x 10-4
C
LiCl
KCl
NaCl
TlNO3
Km M
Li+ 2·24 x 10-4
K+ 2·90 x 10-4
D
Km M
K+ 3·44 x 10-4
Rb+ 4·72 x 10-4
KCl
RbCl
TlNO3
Activity (mμmols keto acid produced)
100
200
300
400
20
40
60
80
100
Salt Concentration (mM)

Fig. 2

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