SOME ASPECTS OF GLYCINE METABOLISM IN

ARTHROBACTER GLOBIFORMIS

by

Eric Stanley Bridgeland

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Department of Biochemistry,
University of Leicester.

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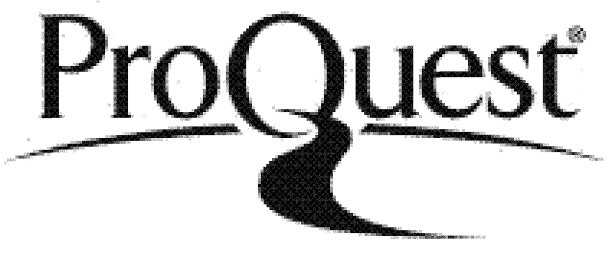

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Abbreviations and Symbols

A	Absorbance
AMP, ADP, ATP	5'-mono-, 5'-di- & 5'-triphosphates of adenosine
C_2-compound etc.	A compound containing two carbon atoms.
CoA (CoASH), acetyl CoA	Coenzyme A & its acetyl derivative
DEAE-cellulose	Diethylaminoethylcellulose
DNPhydrazine	2,4-dinitrophenylhydrazine
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetra-acetate
GSH	Glutathione, reduced
GDP, GTP	5'-di- & 5'-triphosphates of guanosine
H_4PteGlu, methylene-H_4PteGlu & methenyl-H_4PteGlu	Tetrahydropteroylglutamate & its 5,10-methylene- & 5,10-methenyl-derivatives.
IDP	Inosine 5'-diphosphate
K_m	The concentration of substrate when either v, or v_s = Vmax/2 (Michaelis constant)
NAD, NADH	Nicotinamide-adenine dinucleotide, oxidized and reduced
NADP, NADPH	Nicotinamide-adenine dinucleotide phosphate, oxidized and reduced
Pi	Orthophosphate
PEP	Phosphoenolpyruvate
PHMB	p-hydroxymercuribenzoate

Rf	The migration rate of a substance during chromatography relative to that of the solvent front
R_{SA}	The migration rate of a substance during gel filtration relative to that of bovine serum albumin
RNA	Ribonucleic acid
$S_{20,w}$	Sedimentation coefficient (constant) corrected to 20° in water
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle
tris	2-Amino-2-hydroxymethyl propane-1,3-diol
v,Vmax.	v is the velocity of an enzymic reaction at a given substrate concentration and Vmax., its velocity at a saturating concentration of substrate
v_{15},v_s	These two symbols refer specifically to L-serine dehydratase. v_{15} is the amount of pyruvate or ammonia formed during a 15 min. assay and v_s, the steady state velocity of pyruvate (or ammonia) formation (see p.116)
V_e	Elution volume (see p.186)

Other abbreviations and symbols employed are in accord with the recommendations of the Editorial Board of the Biochemical Journal, as set out in volume 102, page 1 (1967).

SECTION I

THE PATHWAY OF GLYCINE UTILIZATION IN ARTHROBACTER GLOBIFORMIS

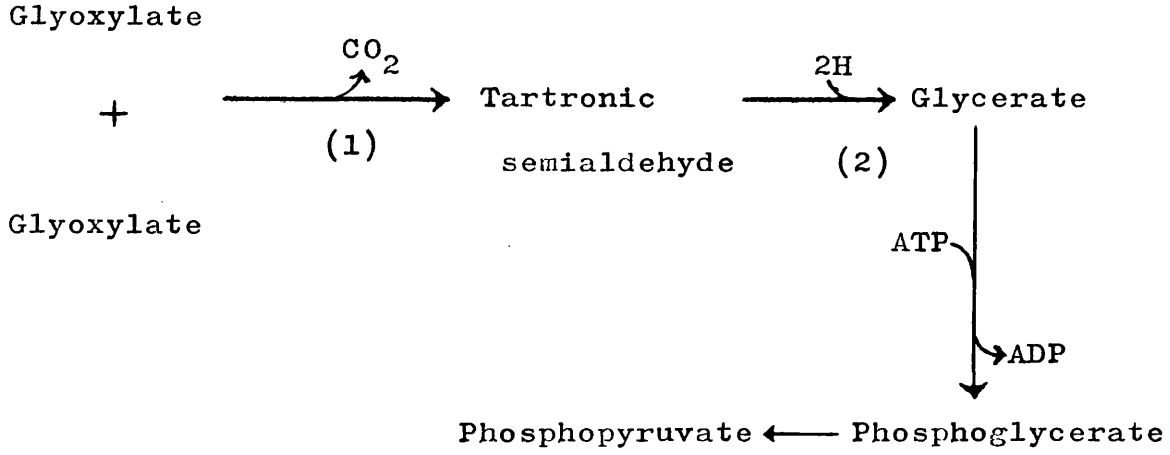
CHAPTER I

INTRODUCTION

A number of micro-organisms possess the remarkable ability to derive all their carbon and energy from a simple, organic compound. Compounds which can be oxidized to acetate will usually, in aerobic organisms, be further catabolized by way of the tricarboxylic acid (TCA) cycle. In organisms growing on substrates of this type, the TCA cycle serves two essential functions. The first of these is to provide the organism with energy, in the form of ATP. This is achieved as follows. Acetate formed from the growth substrate is converted by one turn of the cycle to carbon dioxide, protons and electrons. The electrons are transferred via a sequence of carrier substances to molecular oxygen, and the energy released in the process is harnessed to the synthesis of ATP. The second function of the

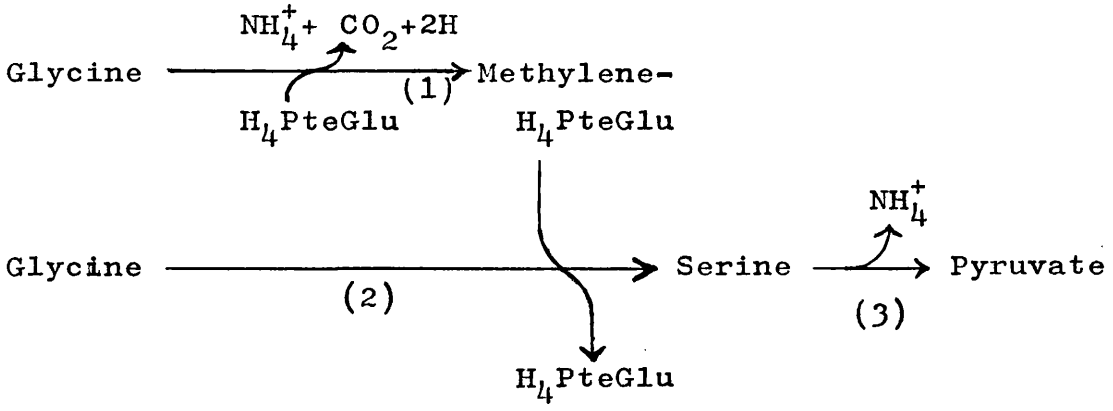
Fig.1. The glycerate, serine and β-hydroxy-aspartate pathways.

The glycerate pathway



(1) Glyoxylate carboligase (2) Tartronate semialdehyde reductase

The serine pathway

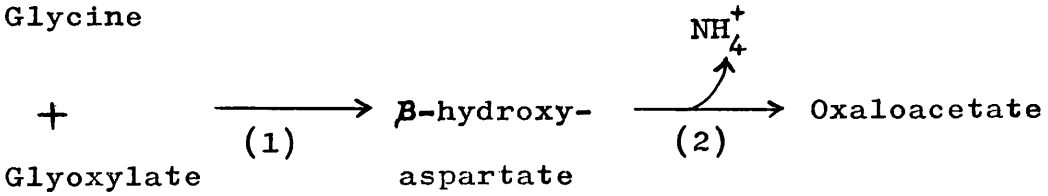


(1) Glycine cleavage system, (2)Serine hyrdroxymethyl-transferase, (3) L-serine dehydratase

contd.....

Fig.1. contd..

<u>The β-hydroxyaspartate pathway</u>



(1) β-hydroxyaspartate aldolase

(2) β-hydroxyaspartate dehydratase

cycle is to provide a source of chemical intermediates for the synthesis of cell constituents. As intermediates of the cycle are consumed by biosynthetic pathways, they have to be replenished at an equivalent rate from the growth substrate, otherwise the energy-yielding function of the cycle will not be maintained. The sequences of reactions which serve to convert the growth substrate, or a product of its catabolism, to an intermediate of the cycle are termed anaplerotic sequences (Kornberg, 1966).

Compounds which are too highly oxidized to be oxidized directly to acetate present special problems for organisms utilizing them as growth substrates (Kornberg and Elsden, 1961). The present discussion will be limited to the problems associated with growth on glyoxylate and glycine, which are both C_2-compounds more highly oxidized than acetate. Bacteria have developed two novel routes for the catabolism of glyoxylate and precursors thereof. Pseudomonads and *Escherichia coli* use the dicarboxylic acid cycle (Kornberg and Sadler, 1961) whereas *Micrococcus denitrificans* uses the β-hydroxyaspartate pathway (Kornberg and Morris, 1965). In the dicarboxylic acid cycle ~~(fig.1)~~, glyoxylate condenses with acetyl CoA to form L-malate. The latter compound is oxidized via oxaloacetate, phosphoenolpyruvate (PEP) and pyruvate, with the regeneration of acetyl CoA. Energy is derived from a substrate level phosphorylation in going from PEP to pyruvate, and from oxidative phosphorylation coupled to the transfer of the electrons released in the oxidative steps. In the β-hydroxyaspartate pathway (fig.1), glyoxylate condenses with glycine, formed by transamination

of glyoxylate, to form *erythro*-β-hydroxyaspartate. This compound is deaminated by a dehydratase to form oxaloacetate, which can be converted to acetyl CoA for catabolism by way of the TCA cycle. Since oxaloacetate is itself an intermediate of the cycle, no anaplerotic route is necessary. On the other hand, if the dicarboxylic acid cycle is to provide a source of biosynthetic precursors, it must be served by an anaplerotic sequence. The glycerate pathway (Kornberg and Gotto, 1959) fulfils this function by converting glyoxylate to PEP (fig.1).

Growth on glycine

Glycine can be converted to glyoxylate by oxidative deamination, and further metabolized by way of the routes just described. For example, Callely and Dagley (1959) found high levels of two enzymes of the glycerate pathway, i.e. glyoxylate carboligase and tartronate semialdehyde reductase (EC1.1.1.60), in cell-free extracts from a glycine-grown pseudomonad. Malate synthase (EC 4.1.3.2), an enzyme of the dicarboxylic acid cycle, was also present in these extracts (Dagley, Trudgill and Callely, 1961).

Although the β-hydroxyaspartate pathway might in theory provide a route for the utilization of glycine, it has not been found to perform this function in nature.

An alternative route for glycine utilization, which does not involve glyoxylate as an intermediate, has been shown to operate in the obligate anaerobe *Peptococcus glycinophilus* (Sagers and Gunsalus, 1961). Glycine is split to form a C_1-unit with the loss of ammonia and the carboxyl carbon as carbon dioxide. The C_1-unit is transferred to the coenzyme tetrahydropteroylglutamate (H_4Pte Glu), to form

methylene-H_4Pte Glu, and thence to another molecule of glycine to form L-serine (fig.1). The serine is then deaminated to pyruvate by L-serine dehydratase (EC 4.2.1.13). The initial cleavage of glycine is catalysed by an enzyme complex which has been fractionated into four distinct proteins (Sagers and Klein, 1965). Sagers and Gunsalus suggested that the pyruvate formed by this route might supply the energy necessary for growth by undergoing a phosphoclastic cleavage to yield acetate, hydrogen and carbon dioxide. These were the ultimate products of the glycine fermentation. The routes whereby *P.glycinophilus* converts glycine to biosynthetic precursors are not known. The serine pathway, just described, is also employed in the terminal stages of purine fermentation in *Clostridium acidi-urici* (Sagers and Beck, 1956; Radin and Barker, 1953), and in the formation of carbohydrates from glycollate by photosynthesizing plant material (Rabson, Tolbert and Kearney, 1962; Kearney and Tolbert, 1962).

Another route, which could in theory be employed for the complete catabolism of glycine, is the succinate-glycine cycle proposed by Nemeth, Russell and Shemin (1957). These workers have obtained evidence that this cycle contributes to the catabolism of glycine in avian red cells. However, there is no evidence to suggest that the cycle serves this function in bacteria (Kornberg, 1966). An analogous, acetate-glycine cycle has been proposed by Elliott (1959), who observed that washed suspensions of *Staphylococcus aureus* formed aminoacetone when shaken in air with glycine plus glucose, or with threonine alone. The cycle involves an initial condensation between glycine and acetyl CoA to

form α-amino-acetoacetic acid, a compound which is known to decarboxylate spontaneously to aminoacetone. Acetyl CoA is regenerated from the aminoacetone in a sequence of reactions involving methylglyoxal, lactate and pyruvate as intermediates. Kornberg (1966) has stressed the fact that this cycle is purely speculative, and that there is no convincing evidence to suggest that it plays an important role in the utilization of glycine as an energy source.

The metabolism of *Arthrobacter globiformis*

A. globiformis is a coryneform bacterium which is widely distributed in soils. It is obligately aerobic and metabolically versatile, being able to utilize any one of a wide range of compounds as carbon and energy source for growth. Among these compounds are sugars, sugar alcohols, dicarboxylic acids, and a number of amino acids including glycine, L-serine and L-threonine (Morris, 1960).

Kornberg and Morris (Morris, 1963) showed that cells of this bacterium, when growing in a medium containing glycine as growth substrate, rapidly incorporated ^{14}C from [$2^{14}C$] glycine into serine, alanine and glutamate. Cell-free extracts from glycine-grown cells formed pyruvate, alanine and carbon dioxide from glycine, when provided with NAD, NADP, pyridoxal phosphate and H_4Pte Glu. Kornberg and Morris concluded from these studies that *A. globiformis* probably utilizes glycine by way of the serine pathway, but they did not publish their results in detail.

The present studies were embarked upon, first to confirm and extend the results of Kornberg and Morris, and secondly to investigate the pathways by which *A. globiformis* synthesizes precursors of cell materials from glycine.

CHAPTER 2.

MATERIALS AND GENERAL METHODS.

Source of the organism and its maintenance.

A culture of *Arthrobacter globiformis* (N.C.I.B. Strain 8602) was obtained from Dr.J.G.Morris and maintained by subculturing at monthly intervals on slopes of glycine salts agar. These cultures were incubated at 30° for 36-72 hr., and stored at 4°.

Growth media

Glycine salts agar was prepared from the following stock solutions:

Solution B3 (g./l.): K_2HPO_4, 19.2 and KH_2PO_4, 27.2; (final pH 6.4).

Solution B4 (g./l.): NH_4Cl, 15; $MgSO_4 . 7H_2O$, 1.0; $CaCl_2 . 6H_2O$, 0.4; 5ml.of 10^{-2} M-$FeCl_3$ and 10 ml. of trace salts solution.

Trace salts solution (g./l.): H_3BO_3, 2.8; $MnSO_4.4H_2O$, 2.1; $CuSO_4.5H_2O$, 0.2; $Na_2MoO_4.2H_2O$, 0.75; $CoSO_4$, 0.24; $ZnSO_4.7H_2O$, 0.25; and EDTA (Na salt), 1.0.

These solutions were stored over toluene/CCl_4 at 4°. To prepare 100 ml. of glycine salts agar, Solution B3, 40 ml., was mixed with Solution B4, 20 ml., Ionagar No.2 (Oxoid), 1.5g, and 40 ml. of distilled water. This and other growth media were sterilized by autoclaving for 10 min. at 15lb/in^2.

Liquid growth media (final pH 7.0) were prepared from sterile basal salts medium containing (per 1.):

1M-potassium phosphate buffer, pH 7.0	125 ml.
5%(w/v) $MgSO_4.7H_2O$	4.0 ml.
10^{-2}M - $FeCl_3$	1.0 ml.

by adding the following sterile solutions (per 1.):

100 µg/ml. d-biotin (sterilized by filtration)	1.0 ml.
4% (w/v) $CaCl_2.6H_2O$	2.0 ml.
15% (w/v) NH_4Cl	20 ml.

Carbon sources were sterilized separately by autoclaving and added to give the following final concentrations: D-glucose, 42mM; sodium succinate, 50 mM; glycerol, 50 mM; D L-sodium lactate, 50 mM; L-serine, 50 mM; and glycine, 100 mM. NH_4Cl was omitted when the carbon source was L-serine or glycine. Large batches

of glycine salts medium (15 l. in a 20 l. carboy) were prepared with freshly distilled water and used at once without sterilizing.

Growth conditions

Cultures of *A. globiformis* were grown aerobically at 30°. Small batches of liquid medium (0.2 and 0.5 l. in 0.5 and 2 l. Erlenmeyer flasks, respectively) were inoculated from a freshly grown slope culture and grown in a "Gyrotary" shaking incubator (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Large batches of medium (15 l.) were inoculated with 1 l. of a logarithmic phase culture (0.4 - 0.8 mg. dry wt./ml) and aerated with sterile air supplied from a sparger. Cells were harvested in the log.phase of growth by centrifugation at 0-5°. Large batches of culture were centrifuged continuously in the Sharples centrifuge.

Estimation of bacterial cells

A curve relating the absorbance at 680nm. of suspensions of *A. globiformis* to their dry weight content was linear up to an absorbance of 1 and had a slope of 2.05 per mg. dry wt./ml.

Preparation of cell-free extracts

Harvested cells were washed by suspending them in 10mM-tris-HCl buffer, pH 7.5, and centrifuging the suspension. The washed cells were resuspended in the same buffer, usually at a concentration of 40 mg. dry wt/ml. The following methods were employed for preparing cell-free extracts. Method 1 was used routinely.

1. Ultrasonic treatment. The cell suspension was cooled to 0^{o} and exposed to vibrations from an M.S.E. 60W ultrasonic oscillator (Measuring and Scientific Equipment Ltd., London) operating at 1.2-1.5 amps. An exposure time of 1 min./ml. of suspension was employed. Care was taken to maintain the temperature of the suspension at $0-5^{o}$ during the treatment.

2. French press. Cell suspensions at 0^{o} were passed two or three times through a chilled French pressure cell (American Instrument Co., Silver Spring, Md., U.S.A.) at a pressure of 12,000lb/in^{2}.

3. Lysozyme treatment. The procedure is based on that of Sistrom (1958). A freshly prepared suspension of early log phase cells (5-20 mg. dry wt./ml.) was incubated with lysozyme (0.1 mg./ml. of suspension) at 30^{o} for 20 min. Sodium EDTA and tris-HCl buffer, pH 8.0, were then added to give respective final concentrations of 2.3 and 40mM, and incubation continued for a further 10 min.

Extracts prepared by each of these methods were centrifuged at 25,000g. for 5-10 min. at $0-4^{o}$ to sediment cell debris.

Chemical estimations

Protein. The protein content of cell-free extracts was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). Cell suspensions were heated with an equal volume of 2M-NaOH at 100^{o} for 10 min. The protein extracted by this treatment was estimated by the method above. Bovine serum albumin was used as the standard in both cases.

Ammonia was estimated with glutamate dehydrogenase according to the procedure of Holzer, Cennamo and Boll (1964).

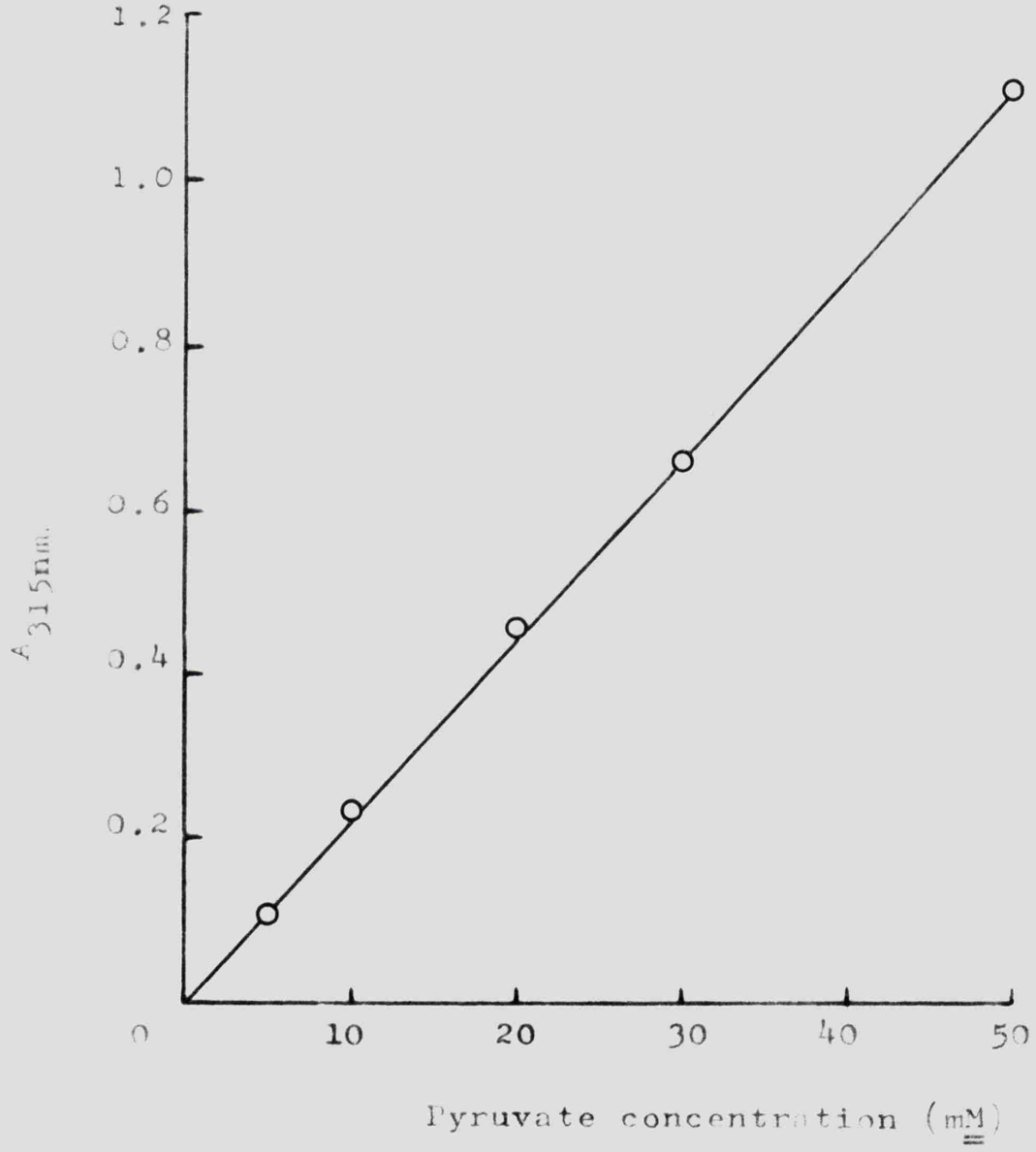
Serine was estimated by the chromotropic acid method (Frisell, Meech and Mackenzie, 1954).

Pyruvate was estimated by three separate methods:

1. As its 2,4-dinitrophenylhydrazone (DNP-hydrazone), by a method based on that of Friedemann and Haugen (1943). To a 1 ml. sample containing up to 0.2μmole of pyruvate was added 0.33 ml. of a solution of 0.1%(w/v) 2,4-dinitrophenylhydrazine in 2$\underline{\underline{M}}$-HCl (DNP-hydrazine reagent), followed, 5 min. later, by 2 ml. of 2$\underline{\underline{M}}$-NaOH. After a further 10 min. the solution was centrifuged, to remove any precipitate, and its absorbance at 445nm. determined against a ~~suitable~~ blank prepared by adding the enzyme after the DNPhydrazine reagent. A standard curve was prepared using a solution of sodium pyruvate which had been assayed by method 2 below. A plot of $A_{445nm.}$ against pyruvate concentration was linear and had a slope of 5.50 per μmole of pyruvate.

2. The lactate dehydrogenase method. A sample containing up to 0.1μmole of pyruvate was added to the following system and the change in absorbance at 340nm., due to oxidation of NADH, determined. The assay system (final volume, 3.0ml.) contained (μmoles): potassium phosphate buffer, pH 7.2, 150; NADH, 0.45; lactate dehydrogenase, 1.1 units. The molar absorbtivity of NADH at 340nm. was taken to be 6.22×10^3 $\underline{\underline{M}}^{-1}$ cm^{-1}.

Fig.2. Spectrophotometric estimation of pyruvate at 315nm. The pyruvate solutions contained 50m$\underline{M}$-tris-HCl buffer, pH8.2, and 10m$\underline{M}$-$MgCl_2$. From the slope of this plot a value of $22\underline{M}^{-1}$ $cm.^{-1}$ is obtained for the molar absorbtivity of pyruvate at 315nm.


1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
$A_{315nm.}$
Pyruvate concentration (mM)

3. The $A_{315nm.}$ method. Pyruvate exhibits a small absorption peak at 315nm. in the presence of 50m$\underline{M}$-tris-HCl buffer, pH 8.2, and 10m$\underline{M}$-$MgCl_2$. A curve relating $A_{315nm.}$ to pyruvate concentration was linear for concentrations up to 50m$\underline{M}$(fig.2). The molar absorbtivity of pyruvate at 315nm. under these conditions is 22 $\underline{M}^{-1}$ cm^{-1}. The usefulness of this method is limited because the absorbance of pyruvate solutions decreases slowly with time, and the absorbtivity of pyruvate at 315nm. is influenced by pH. Nevertheless, the method was reliably employed for following the appearance of pyruvate for periods of up to 15 min. in assays of $\underline{L}$-serine dehydratase.

Spectrophotometric methods

The cuvettes employed were made of silica and had a light path of 1cm. Continuous assays were carried out in an Optika CF4 recording Spectrophotometer, at room temperature (20-23°), or in a Unicam SP800 recording spectrophotometer fitted with a constant temperature cell housing (Unicam Instruments Ltd., York Street, Cambridge), at a temperature of 30°.

Enzyme assays

Lactate dehydrogenase The rate of NADH oxidation caused by the addition of the enzyme preparation to the following system was followed spectrophotometrically at 340nm. The assay system (final volume, 1.0ml) contained (μmoles): potassium phosphate buffer pH 7.5, 50; sodium pyruvate, 1; and NADH 0.1.

Malate dehydrogenase. The procedure and assay system were the same as those employed for lactate dehydrogenase except that oxaloacetate was substituted for pyruvate. When assaying the enzyme in the presence of lactate dehydrogenase and pyruvate (e.g. in fractions from sucrose gradients), the pyruvate was converted to lactate, before adding oxaloacetate to the system, by the addition of 1.5 units of lactate dehydrogenase and an excess of NADH.

Definition of an enzyme unit

The definition of the Enzyme Commission of the I.U.B. is adhered to: "One unit of any enzyme is defined as that amount which will catalyse the transformation of 1 micromole of the substrate per minute under defined conditions".

Preparation of oxo-acid DNPhydrazones.

Small amounts of oxo-acid DNPhydrazones, suitable for use as chromatographic markers, were prepared as follows. A mililitre of the oxo-acid solution (5μmoles) was incubated with 0.33 ml. of 0.1%(w/v) DNPhydrazine reagent at 30° for 10 min. The oxo-acid DNPhydrazone formed in this way was extracted into 1 ml. of ethyl acetate and thence into 4ml. of 10% (w/v) Na_2CO_3 solution. (Any unreacted DNPhydrazine remains in the ethyl acetate). The alkaline DNPhydrazone solution was acidified and the DNPhydrazone extracted into a small volume of ethyl acetate. The latter solution was applied to chromatograms.

Absorption spectra of oxo-acid DNPhydrazones were determined using a solution prepared by mixing equal volumes of 1.5M-NaOH and of the DNPhydrazone dissolved in 10% (w/v) Na_2CO_3 solution.

Crystalline pyruvate DNPhydrazone was prepared for melting point determinations by scaling up the method described above. The pyruvate DNPhydrazone was precipitated from the Na_2CO_3 solution by acidification, collected by filtration and crystallized from ethanol. The crystals were pale yellow, square-shaped plates.

Preparation and assay of acetyl CoA

CoA was acetylated with acetic anhydride as described by Stadtman (1957). The acetyl CoA content of this preparation was estimated by the method of Srere, Brazil and Gonen (1963).

Chemicals

Proteins, including enzymes: Alcohol dehydrogenase (EC 1.1.1.1) (from yeast, 180 units/mg), citrate synthase (EC 4.1.3.7) (from pig heart, 70 units/mg), cytochrome c (from horse heart), glutamate dehydrogenase (EC 1.4.1.2) (from beef liver, ammonia-free, 3 units/mg), aspartate transaminase (glutamate-oxaloacetate transaminase (EC 2.6.1.1.) (from pig heart, 180 units/mg), lactate dehydrogenase (EC 1.1.1.27) (from rabbit skeletal muscle, 360 units/mg) and malate dehydrogenase (EC 1.1.1.37) (from pig heart, 720 units/mg) were purchased from C.F. Boehringer & Soehne, G.m.b.H. Mannheim, Germany; lysozyme (from egg white, 40,000 units/mg) and crystalline serum albumin (bovine)

from British Drug Houses (B.D.H.)Ltd., Poole, Dorset; and avidin (2.5 units/mg) from the Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

Coenzymes: ATP(Na_2 - Salt), ADP (Na_3 - Salt), GTP (Li_3 - Salt) and GDP (Li_2 - Salt) were purchased from C.F.Boehringer; IDP (Na - Salt), d-biotin, pyridoxal phosphate and tetrahydropteroylglutamate (H_4PteGlu) from Sigma Chemical Co., St.Louis, Mo., U.S.A.; NAD, NADH, NADP & NADPH from C.F.Boehringer & Sigma.

Other chemicals: Sephadex and blue dextran 2000 were purchased from Pharmacia, Uppsala, Sweden; phosphoenolpyruvate (K-salt) sodium pyruvate, α-oxoglutaric acid and oxaloacetic acid from C.F.Boehringer; sodium glyoxylate and p-hydroxymercuribenzoate (Na-Salt) from Sigma; lithium hydroxypyruvate was a gift from Dr.J.M.Ashworth of this department; L-serine and glycine from B.D.H.Ltd; 5,5'-dithiobis (2-nitrobenzoic acid) was a gift from Professor P.A.Srere, University of California, Livermore, U.S.A., 2,5-diphenyloxazole and dimethyl-2,2-p-phenylenebis (5-phenyloxazole) from Nuclear Enterprises (GB)Ltd., Sighthill, Edinburgh 11, Scotland. Most other chemicals were of Analar grade.

Radioactive chemicals: These were purchased from the Radiochemical Centre, Amersham, Bucks. Radioactive impurities were removed from [2^{14}C] glycine and L-[3^{14}C] serine by chromatography on Dowex-50 (hydrogen form) cation exchange resin. The amino acid was absorbed on to a small column of the resin from neutral

aqueous solution. The column was washed with water, then with 0.2$\underline{\underline{M}}$-HCl, and finally with 0.4$\underline{\underline{M}}$-HCl to elute the amino acid.

CHAPTER 3.

IDENTIFICATION OF INTERMEDIATES ON THE MAIN PATHWAY OF GLYCINE UTILIZATION

INTRODUCTION

The experiments of Kornberg and Morris (Morris, 1963), in which isotopically labelled glycine was used to trace early intermediates in the pathway of glycine utilization in *A. globiformis*, have been repeated; the results are described in this chapter.

METHODS

Conditions for the incorporation of [2^{14}C] glycine by whole cells

Log. phase cells growing in glycine salts medium were harvested at room temperature and suspended to 7.7mg. dry weight/ml. in basal salts medium (pH 7.0), containing d-biotin and $CaCl_2$ but no carbon

source. Glycine (14.4μmoles) was added to the cell suspension (11.6ml) which, throughout the course of the experiment, was kept at 10° and aerated continuously by agitation. A preliminary experiment had shown that most of the glycine was taken up from the medium in a period of 9 min. under these conditions. After this period, a sample (1 ml.) was removed from the cell suspension and transferred to a tube containing ethanol (3ml.) at a temperature of 70°. A solution of [2-^{14}C] glycine (6μmoles; 8.1μc/μmole) was added to the bulk of the cell suspension at zero time and further samples withdrawn from it and added to hot ethanol at the times indicated in table 1. [2-^{14}C] glycine (0.6μmole) was subsequently added to the ethanolic extract of the first sample. All ethanolic extracts were centrifuged, the supernatant solutions kept, and the pellets extracted a second time with hot 20% (v/v) ethanol (3ml). The combined extracts from each sample were evaporated to dryness under reduced pressure at 35°, and the residue dissolved in distilled water (0.5ml).

Separation of radioactive products.

Oxo-acids. An aliquot (0.35ml) from the concentrated extract of each sample was added to a solution of 0.1% (w/v) DNPhydrazine (0.2ml). Oxo-acid DNPhydrazones were isolated and applied to chromatograms together with the DNPhydrazones of authentic oxaloacetate, α-oxoglutarate, pyruvate and glyoxylate. Chromatography was carried out in an ascending fashion using the solvent of El Hawary and Thompson (1953).

Amino acids and other products. Amino acids were separated by high voltage electrophoresis in an acetic-formic acid buffer, of pH 1.85, as described by Atfield and Morris (1961). An aliquot (0.02ml) of the concentrated extract from each sample was applied to an electrophoretogram (4cm.from the anode end) together with 5µg. of each of the following amino acids: glycine, L-α-alanine, L-serine, L-aspartate and L-glutamate. Electrophoresis at pH 1.85 produced a good separation of glycine (which contained the bulk of the ^{14}C in early samples) from other added amino acids, as well as separating these from one another.

In order to detect radioactive products which were anionic at pH 1.85, another aliquot from each extract was applied to a second electrophoretogram at a distance of 24cm. from the anode end. Pyruvate and succinate (1µmole of each)were applied as markers, in addition to the mixture of amino acids, and electrophoresis was carried out as before.

The extract remaining from each sample was chromatographed two-dimensionally on paper as described by Kornberg (1958) to separate TCA cycle intermediates and other products.

Identification of radioactive products.

Electrophoretograms and chromatograms were autoradiographed as described by Kornberg (1958), and then treated as described below to visualize marker substances. The identity of a radioactive product was considered established when the position and shape of the image

it produced on film corresponded exactly to those of the spot obtained on paper for the marker substance. The identity and purity of electrophoretically homogeneous, radioactive products was checked by eluting them from the electrophoretograms and subjecting them to two-dimensional chromatography. Marker substances were visualized as follows: amino acids, citrate and succinate as described by Kornberg (1958), pyruvate by spraying with 0.1%(w/v) DNPhydrazine solution, followed by 2% (w/v) NaOH in ethanol, and oxo-acid DNPhydrazones by spraying with the latter reagent, or by viewing under ultra-violet light.

Radioassay of products

Radioactive products were cut out from chromatograms and electrophoretograms, placed into glass counting vials together with 20ml. of scintillating fluid (5g.2,5-diphenyloxazole and 0.3g. dimethyl-2,2-p-phenylenebis (5-phenyloxazole) per 1. of toluene), and their radioactivity determined with a Tricarb, series 3000, liquid scintillation spectrometer (Packard Instrument Company, Inc. La Grange, Illinois, U.S.A.). The degree of quenching caused by oxo-acid DNPhydrazones was estimated as follows. Aliquots of a solution of [2^{14}C] glycine were applied to chromatography paper impregnated with the DNPhydrazone and to plain paper, and the radioactivities of the two pieces of paper compared.

Fig.3. Distribution of isotope among cellular pools of serine (●), alanine (■), aspartate (o), glutamate (▵), and citrate (□) during the incorporation of [2-^{14}C] glycine by glycine-grown A.globiformis. [2-^{14}C] glycine was added at zero time to the cell suspension immersed in a water bath at 10°. Samples were withdrawn from the suspension and added to hot ethanol at the times indicated. Radioactive products were separated by high voltage electrophoresis. Further details are given in the text.

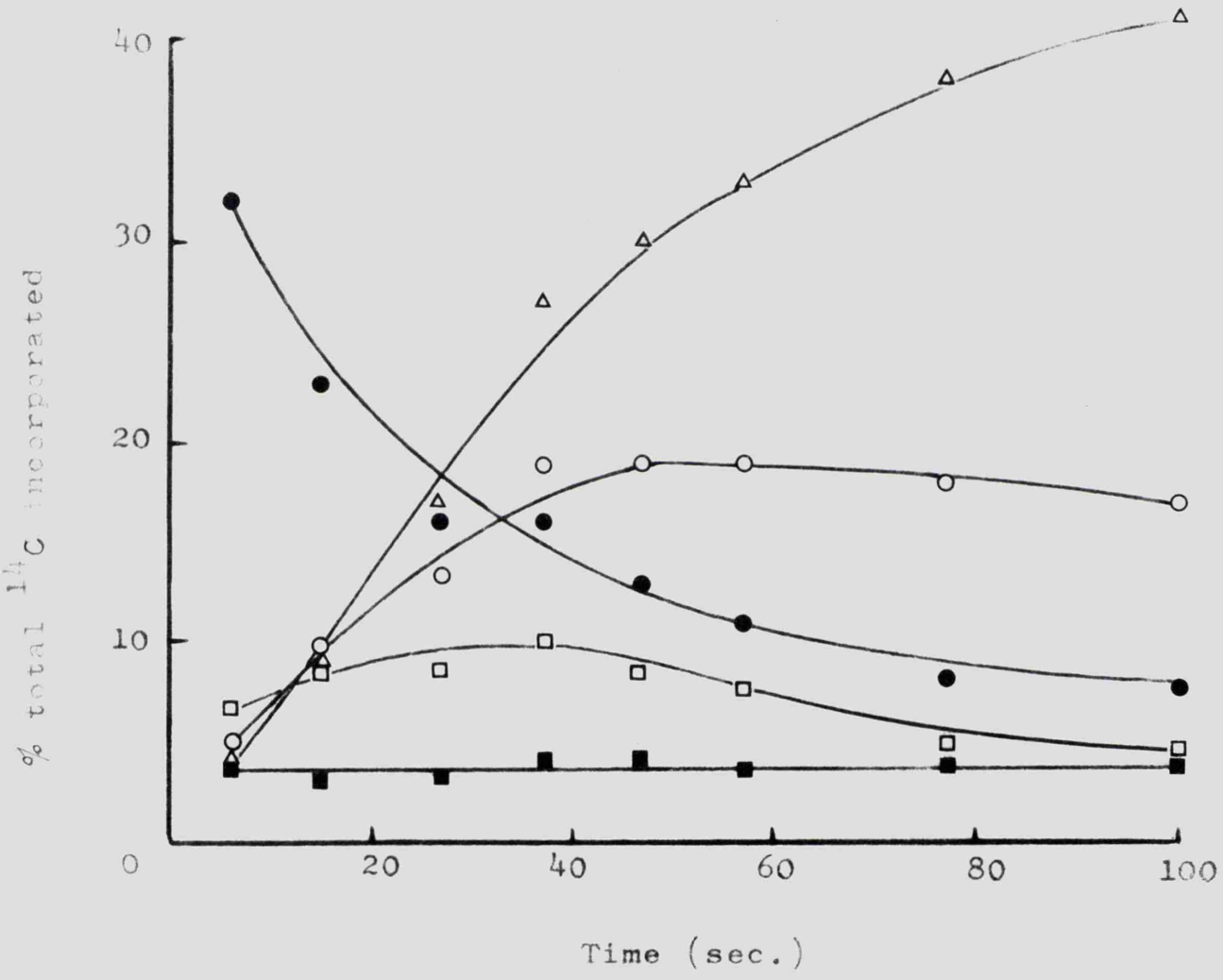

40
30
20
10
0
20
40
60
80
100
% total ^{14}C incorporated
Time (sec.)

Table 1. Distribution of isotope among cellular oxo-acids during incorporation of (2-^{14}C) glycine by Arthrobacter globiformis

Details of the experimental procedure are given in the legend to fig. 3 and in the text.

Time (sec.)	% total ^{14}C incorporated			
	Pyruvate	Glyoxylate+	Oxaloacetate*	∝-Oxoglutarate
6	2.8	(0.63)	(0.13)	0.28
15	1.7	(0.75)	(0.14)	0.34
27	1.3	(0.51)	(0.11)	0.42
37	1.5	(0.20)	(0.04)	0.33
47	1.8	(0.34)	(0.05)	0.45
57	1.7	(0.24)	(0.05)	0.45
77	1.5	(0.32)	(0.05)	0.35
100	1.3	(0.16)	(0.04)	0.33

+Glyoxylate was incompletely separated from pyruvate and so the results given for glyoxylate are maximal estimates.

*Oxaloacetate may have been partially decomposed by the extraction procedure, in which case these results will be minimal estimates.

Calculation of total, incorporated ^{14}C in each sample.

The total, non-glycine ^{14}C on each electrophoretogram was determined and corrected for that present on the electrophoretogram of the control sample, which had been extracted with ethanol before adding [$2^{14}C$] glycine to it. This gave the total amount of incorporated ^{14}C in the aliquot of the sample applied to the electrophoretogram.

RESULTS

Glycine-grown cells of *A. globiformis* rapidly incorporated isotope from [$2^{14}C$] glycine into serine, alanine, citrate, aspartate, glutamate (fig.3) and pyruvate (table 1)*. After 6 sec., serine contained 32% of the incorporated ^{14}C, four times

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* The results described here were obtained in an experiment where the cells were allowed to incorporate isotope at a temperature of 10°. Very similar results were obtained when a temperature of 30° was employed.

more than was present at this instant in any other component of the ethanol-soluble fraction. By 27sec., the proportion of incorporated ^{14}C in serine had decreased by half. During the same period the proportions of incorporated ^{14}C in citrate, glutamate and aspartate increased steadily. The levels of isotope in citrate and aspartate became maximal around 37 sec. and then declined slowly, whereas the level in glutamate continued to rise, though at a somewhat reduced rate, until the end of the experiment. Alanine contained a fairly constant proportion of the incorporated ^{14}C throughout the course of the experiment.

Of the oxo-acids (table 1), pyruvate was consistently found to contain substantially the highest proportion of incorporated ^{14}C in the earliest sample taken. By 15 sec., the proportion of incorporated ^{14}C in pyruvate had decreased significantly; thereafter it remained approximately constant. Glyoxylate initially contained a very small proportion of the incorporated ^{14}C, and this proportion decreased slowly with time.

Identified products together accounted for 55% of the incorporated ^{14}C in the 6 sec. sample and for proportions increasing up to 73-78% in later samples. Five unidentified spots were located on the autoradiographs of the electrophoretograms of all samples. The total ^{14}C content of these five "spots" accounted for 28% of the incorporated ^{14}C in the 6 sec. sample, and for decreasing proportions in later samples. The ^{14}C content of individual "spots" never exceeded 9% of the incorporated ^{14}C in any one sample.

The areas where glycerate and malate were usually found on two-dimensional chromatograms were not radioactive in the chromatograms of the earliest samples.

DISCUSSION

The following features of the time course for the incorporation of isotope from [2^{14}C] glycine into serine suggest that serine is the first stable product formed from glycine. In the first place, serine initially contained four times more ^{14}C than any other single product. Secondly, the proportion of incorporated ^{14}C in serine decreased throughout the course of the experiment, whereas, over the first 37 sec., the proportions of ^{14}C in most other products increased or remained constant. The rapid attainment of steady state levels of ^{14}C in pyruvate and alanine suggests that they are also early products of glycine metabolism. Since serine and pyruvate are intermediates of the serine pathway and alanine is derived directly from pyruvate, it is concluded that this pathway probably plays a major role in the utilization of glycine in *A. globiformis*. These findings confirm those of Kornberg and Morris.

The finding that, in early samples, glyoxylate, glycerate and malate did not contain substantial amounts of ^{14}C suggests that the glycerate and β-hydroxyaspartate pathways and the dicarboxylic acid cycle play little or no part in the utilization of glycine in *A. globiformis*.

The time courses for the incorporation of ^{14}C into citrate, glutamate and aspartate are consistent with those expected for the

operation of the TCA cycle.

SUMMARY

1. Glycine-grown cells of *A. globiformis* rapidly incorporated isotope from [2^{14}C] glycine into serine, pyruvate and alanine. This result is taken to indicate that the serine pathway plays a major role in the utilization of glycine by this bacterium.

2. Intermediates of the glycerate and β-hydroxyaspartate pathways and of the dicarboxylic acid cycle did not incorporate substantial amounts of ^{14}C in these experiments.

3. The time courses for the incorporation of ^{14}C into citrate, glutamate and aspartate were consistent with those expected for the operation of the TCA cycle.

CHAPTER 4.

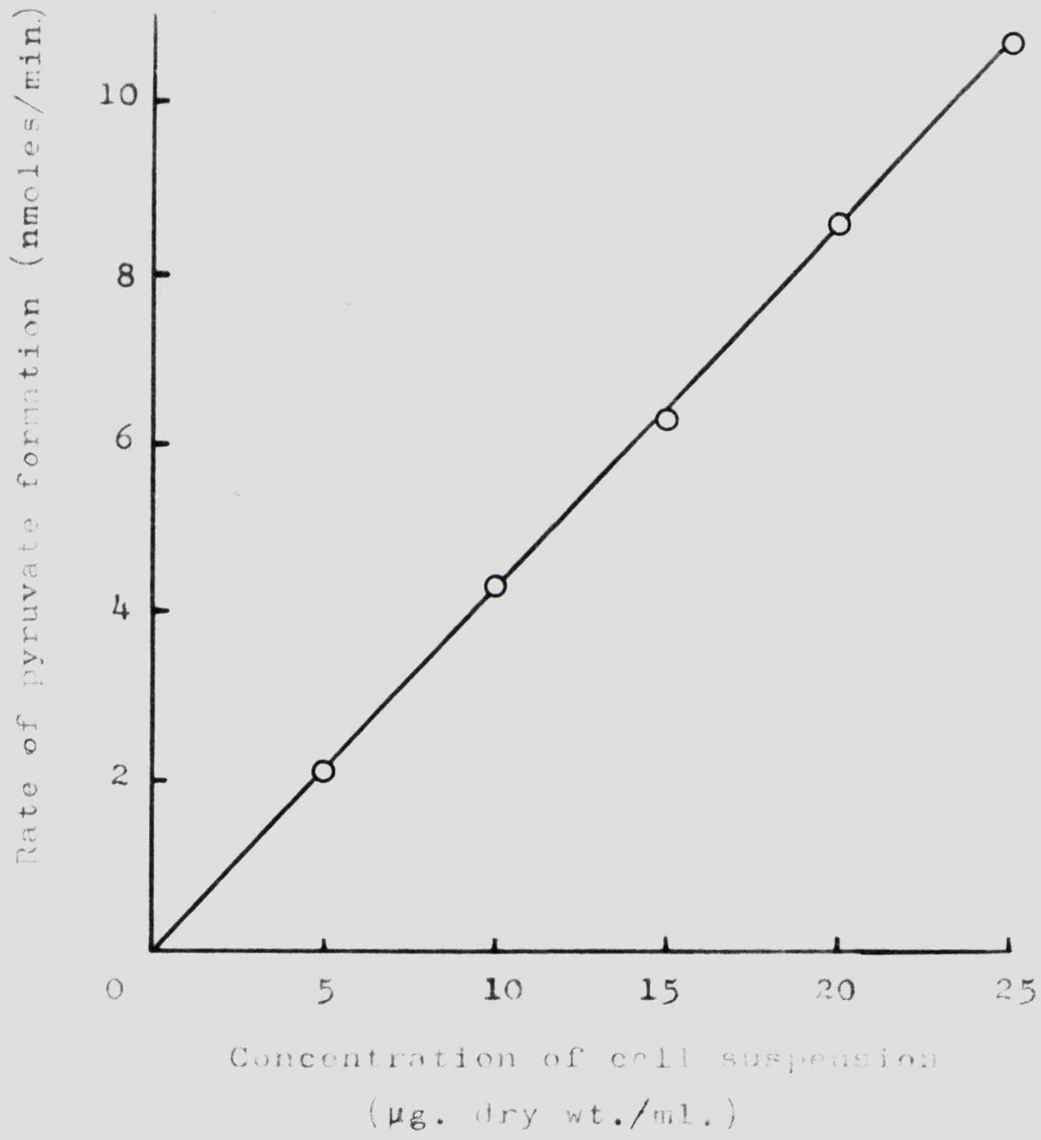
REACTIONS LEADING TO THE FORMATION OF SERINE AND PYRUVATE

INTRODUCTION

Further evidence that glycine is utilized by way of the serine pathway in *A. globiformis* is provided by the studies reported in this chapter.

The author is indebted to Dr.K.M.Jones and Messrs. D.F.Pindar and C.B.E.Lewis for providing the results reported in all sections of this chapter, except those concerning L-serine dehydratase.

Fig.4. The rate of formation of pyruvate from L-serine by toluene-treated, glycine-grown cells as a function of the concentration of the cell suspension. Assay systems were incubated for 15min. at 30°.


Rate of pyruvate formation (nmoles/min.)
10
8
6
4
2
0
5
10
15
20
25
Concentration of cell suspension
(μg. dry wt./ml.)

METHODS

Treatment of cells with toluene

Toluene-treated cells were employed in assays of L-serine dehydratase activity. Suspensions of washed cells (up to 5ml.), containing up to 1mg. dry wt./ml., were shaken vigorously with toluene (0.05ml.), incubated at 30° for 15min. and then stored at 0° until assayed.

Enzyme assays

L-serine dehydratase assays were performed on toluene-treated cells because of the difficulties encountered in assaying the extracted enzyme. The amount of pyruvate formed from L-serine during a 15min. incubation period at 30° was estimated by reaction with DNPhydrazine (Chapter 2 p.29).

Assay systems contained in 1.0ml. (μmoles): tris-HCl buffer, pH 8.2, 50; L-serine (adjusted to pH 8.2 with KOH), 50; and a suspension of toluene-treated cells. Assays were terminated by the addition of 0.33ml. of 0.1% (w/v) DNP-hydrazine reagent.

The time course for the formation of pyruvate was linear (fig.18), and the amount of pyruvate formed in 15 min. directly proportional to the quantity of cells present in the assay system (fig.4).

Malate synthase

acetyl-S-CoA + glyoxylate → L-malate + CoASH. This enzyme was assayed by estimating polarographically the rate of formation of CoA which accompanies the synthesis of L-malate from acetyl CoA and

Table 2. Oxidative ability of glycine-grown A.globiformis (K.M. Jones, unpublished results)

Manometer flasks contained 2 ml. of a 5mg. dry weight/ml. suspension of washed, glycine-grown cells in 0.1 M-potassium phosphate buffer, pH 7.1 (main compartment), 0.8 ml. of a 25mM-solution of the substrate (side arm), 0.2 ml. of 2M-KOH (centre well) and an atmosphere of air. The substrate was tipped in from the side arm and oxygen uptake followed with time. The incubation temperature was 30°.

Substrate	Rate of Oxygen uptake (μl O_2/min./mg. dry wt.)
Glycine	1.5-2.1
L-serine	1.0-1.5
Pyruvate	1.1
Glucose	0.58
Citrate	1.2
Succinate	1.2
Fumarate	0.96
L-malate	1.0
Acetate	0.33
Glyoxylate	0.08
Formate	0.26

glyoxylate (Weitzmann, 1966).

Tartronate semialdehyde reductase

$^{-}O_2C.CH(OH).CHO + NADPH\ (NADH) + H^{+} \rightarrow {}^{-}O_2C.CH(OH).CH_2OH + NADP^{+}(NAD^{+})$

This enzyme was assayed by estimating spectrophotometrically the rate of oxidation of NADPH which accompanies the reduction of tartronate semialdehyde to D-glycerate (Gotto and Kornberg, 1961).

Glyoxylate carboligase

$2\,{}^{-}O_2C.CHO \rightarrow {}^{-}O_2C.CH(OH).CHO + Co_2$

This enzyme was assayed by estimating manometrically the rate of evolution of CO_2 from glyoxylate under anaerobic conditions (Kornberg and Gotto, 1961).

β-hydroxyaspartate aldolase and dehydratase

Aldolase: erythro-β-hydroxyaspartate $\rightleftharpoons$ glyoxylate + glycine

Dehydratase: erythro-β-hydroxyaspartate $\rightarrow$ oxaloacetate + NH_4^{+}

The sum of the activities of these two enzymes was determined by estimating the total amount of oxo-acid formed from erythro-β-hydroxyaspartate, by reaction with DNPhydrazine (Gibbs and Morris, 1965).

RESULTS

Oxidation studies with whole cells

Washed, glycine-grown cells oxidized L-serine, pyruvate and intermediates of the TCA cycle, i.e. citrate, succinate, fumarate and L-malate, at rates comparable with that observed for glycine (table 2). In the case of the TCA cycle intermediates, there was

a brief lag period before a steady rate of oxidation was attained. Glyoxylate, acetate and formate were oxidized more slowly than glycine and the other compounds.

An examination of extracts for enzymes of the serine pathway

Glycine cleavage system. This multi-enzyme system catalyses the reaction: $^{+}NH_3.CH_2.CO_2^{-} + H_4Pte\ Glu \rightarrow (CH_2OH).\ H_4Pte\ Glu + CO_2 + NH_4^{+} + 2H$

Pindar and Jones (personal communication) have demonstrated that extracts of glycine-grown cells form methylene-H_4Pte Glu from H_4Pte Glu and glycine. The rate of this reaction was determined by adding an excess of NADP to the system and estimating the methenyl-H_4Pte Glu formed by its absorbance at 355nm. in acid solution. This assay is based on the knowledge that cell-free extracts contain methylene-H_4Pte Glu: NADP oxidoreductase (EC 1.5.1.5) with an activity greater than that of the glycine cleavage system. Using this assay method, the glycine cleavage activity of extracts from glycine-grown cells was estimated to be 5-13 nmoles/min/mg.protein at 30°. Extracts from glucose-grown cells had a considerably lower activity, i.e. 0.1-0.7 nmoles/min/mg. protein.

When cells were transferred from a glucose to a glycine salts medium, they ceased growing for several hours. During this period, the level of glycine cleavage activity in extracts from these cells rose rapidly. At the time when the cells began growing again, their activity was approximately one half that of cells which had been growing on glycine for a number of generations.

Serine hydroxymethyltransferase (EC2.1.2.1). This enzyme catalyses the reaction:

$(CH_2OH).H_4PteGlu + {}^{+}NH_3.CH_2.CO_2^{-} \rightleftharpoons HO.CH_2({}^{+}NH_3).CH.CO_2^{-} + H_4PteGlu.$

When an extract from glycine-grown cells was incubated with L-serine, H_4Pte Glu, NADP and pyridoxal phosphate, the formation of methenyl-H_4Pte Glu could be detected by its absorbance at 355nm.in acid solution (Lewis and Jones, personal communication). Methylene-H_4PteGlu, the immediate product of L-serine cleavage, is oxidized to methenyl-H_4Pte Glu by the methylene-H_4Pte Glu:NADP oxidoreductase activity of the extract. By estimating the methenyl-H_4Pte Glu formed in this way, the serine hydroxymethyltransferase activity of extracts from glycine-grown cells was estimated to be 18nmoles/min/mg.protein at 30°. A similar result was obtained for the activity of extracts from glucose-grown cells.

The serine hydroxymethyl-transferase activity of extracts has also been detected by its ability to cause a glycine and H_4Pte Glu dependent disappearance of formaldehyde (Lewis and Jones, personal communication). The latter substance reacts non-enzymically with H_4Pte Glu to form methylene-H_4Pte Glu which is a substrate in the subsequent enzymic reaction.

Table 3. Amounts of pyruvate and ammonia formed from L-serine by a cell-free extract

Assay systems (2.0ml.) contained 50mM-tris-HCl buffer, pH8.2, 50mM-L-serine, 10mM-$MgCl_2$ and the volumes indicated of a cell-free extract. Assays were started by the addition of the extract and terminated, after 15 min. at 30°, by the addition of 0.33ml.25% (w/v) perchloric acid. Precipitated protein was centrifuged down, the supernatant solutions neutralized with 0.35ml. of 2M-KOH, and crystals of $KClO_4$ centrifuged down. Aliquots from the neutral, supernatant solutions were assayed for pyruvate (0.2ml.) by reaction with DNPhydrazine, and for NH_3 (0.1ml.) as described in chapter 2.

Volume of extract added (ml.)	Ammonia (μmoles/ml.)	Pyruvate (μmoles/ml.)
0.5*	0	0
0.1	0.02	0.10
0.2	0.27	0.33
0.3	0.56	0.61
0.4	0.99	0.94
0.5	1.32	1.22

*In this assay the extract was added to the system after the perchloric acid.

Table 4. Simultaneous estimations of L-serine and pyruvate during the deamination of L-serine by a cell-free extract.

The assay system (25ml.) contained 2.5mM-L-serine, 50mM-potassium phosphate buffer, pH7.5, 10mM-$MgCl_2$ and a cell-free extract. The reaction was started by the addition of L-serine. Aliquots (2ml.) were taken and added, at the times indicated, to 20% (w/v) TCA (2ml.) Precipitated protein was removed by centrifugation and aliquots of the supernatant solutions assayed for pyruvate (0.2ml.) by reaction with DNPhydrazine, and for L-serine (2ml.) as described in Chapter 2.

Time (min.)	Pyruvate (μmoles/ml.)	L-serine (μmoles/ml.)	Pyruvate+L-serine (μmoles/ml.)
0	0.03	2.54	2.57
1.0	0.09	2.52	2.61
3.0	0.29	2.26	2.55
6.0	0.54	1.99	2.53
9.0	0.75	1.80	2.55
14.5	1.00	1.53	2.53
29	1.39	1.09	2.48
44	1.63	0.85	2.48
60	1.76	0.79	2.55

L-serine dehydratase. When L-serine was incubated with an extract from glycine-grown cells, the formation of an oxo-acid was detected by its reaction with DNPhydrazine to form a DNPhydrazone. The latter substance was crystallized and found to be indistinguishable in its following characteristics from the DNPhydrazone of authentic pyruvate:

1. Its absorption spectrum in alkaline solution had a peak at 447nm. with a shoulder at 515nm. (The absorption spectra of glyoxylate and hydroxypyruvate DNPhydrazones have peaks, without shoulders, at 445nm. and 452nm, respectively).
2. Its R_f value, following chromatography in the solvent of El Hawary and Thompson (1953), was 0.55 (slower moving spot). (An R_f value of 0.44 was obtained for glyoxylate DNPhydrazone (slower moving spot)).
3. It melted with decomposition at 215-217°. Mixtures of the DNPhydrazones of the oxo-acid and of authentic pyruvate (melting point, 216-218°) melted sharply at 215-216°.

Pyruvate and ammonia were formed in equimolar amounts when an extract from glycine-grown cells was incubated with L-serine (table 3). L-serine was also estimated under these conditions and the amounts consumed were found to be equimolar with the amounts of pyruvate formed (table 4).

It is therefore concluded that extracts from glycine-grown cells promote the following reaction:

$$\text{L-serine} \rightarrow \text{pyruvate} + NH_4^+$$

This is the reaction catalysed by the enzyme L-serine dehydratase.

Fig.5. The level of L-serine dehydratase in cells of A.globiformis following their transfer from a glucose to a glycine salts medium. Log. phase cells growing in liquid medium, containing glucose as sole carbon source, were harvested at room temperature and suspended in 0.85% ($^{w}/v$) NaCl. This suspension was used to inoculate two flasks of media which had been prewarmed to 30° and which contained glycine (100mM) (●) or glucose (42mM) (o) as sole sources of carbon. These cultures were incubated aerobically at 30°. Samples were withdrawn from both cultures at intervals increasing from 15 to 60min., and their absorbance at 680nm. determined for assessment of growth. The samples were then treated with toluene and their L-serine dehydratase activity assayed. During the course of the experiment, the mean generation times of A.globiformis growing in the glucose and glycine salts media were estimated to be 2.4 and 2.6hr., respectively.

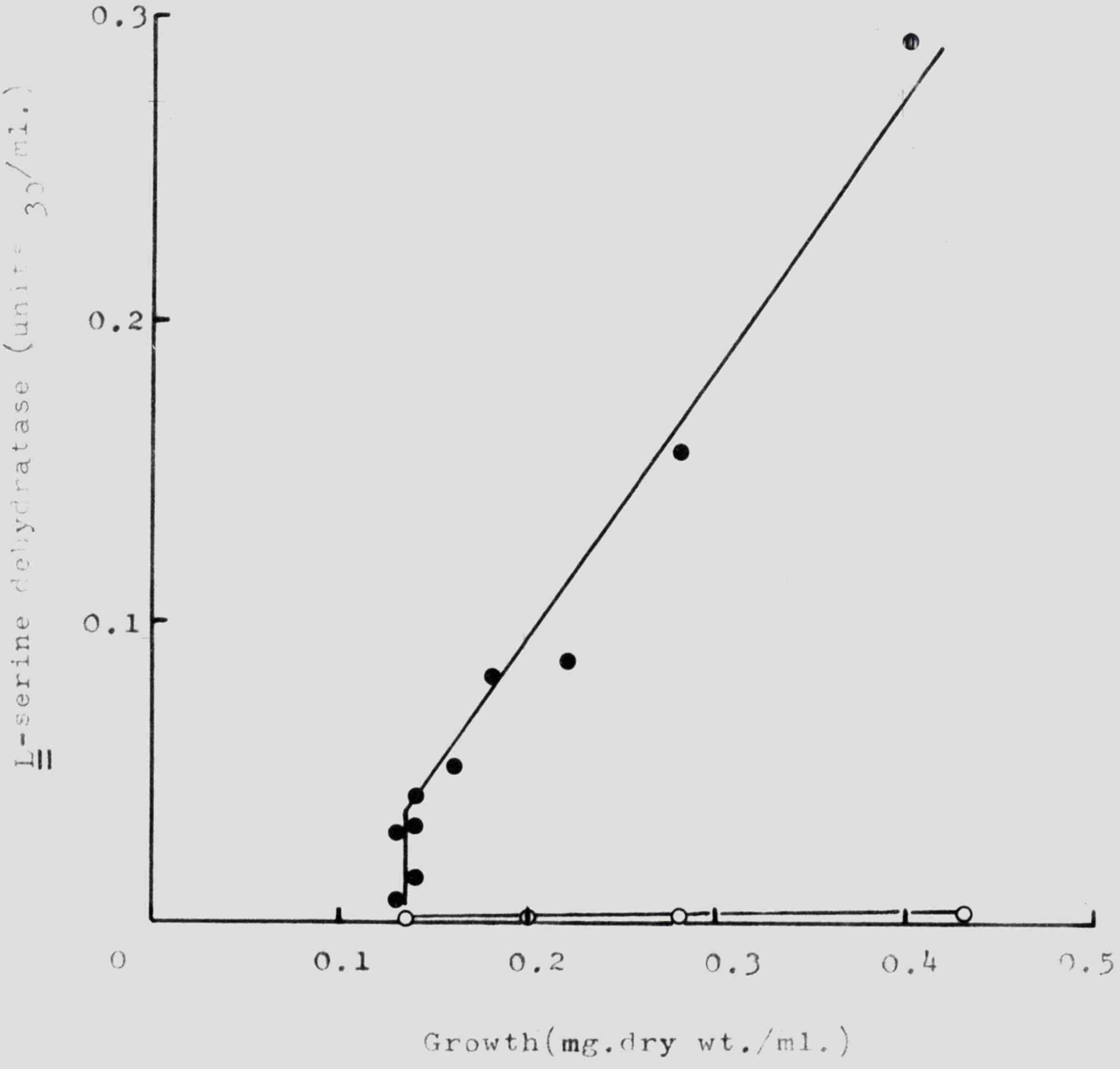

0.3
0.2
0.1
L-serine dehydratase (unit/ 30/ml.)
0
0.1
0.2
0.3
0.4
0.5
Growth(mg.dry wt./ml.)

Fig.6. The level of L-serine dehydratase in cells of *A. globiformis* following their transfer from a glycine to a glucose salts medium. Fresh media containing glycine (●) or glucose (○) as sole carbon sources for growth were inoculated with a suspension of log. phase, glycine-grown cells. Experimental details are given in the legend to fig.5.

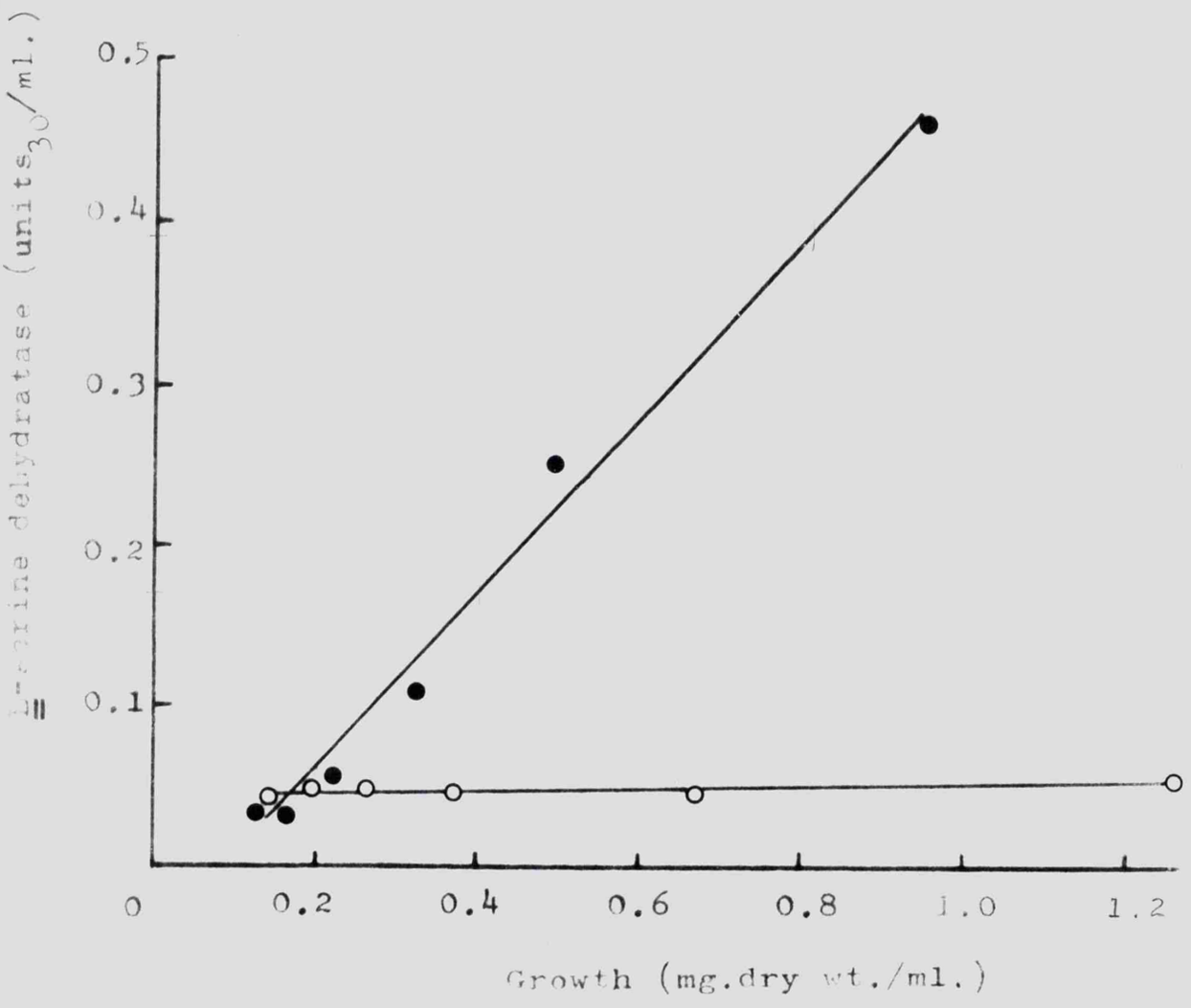

Threonine dehydratase (units$_{30}$/ml.)
0.5
0.4
0.3
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
1.2
Growth (mg.dry wt./ml.)

Table 5. Activities of malate synthase and enzymes of the glycerate and β-hydroxyaspartate pathways in extracts of glycine-grown A. globiformis (K.M.Jones, unpublished results)

Specific activities are given as μmoles of substrate transformed/min./mg. protein at 20 -30°. The sum total of the β-hydroxyaspartate aldolase and dehydratase activities of an extract was determined by estimating the total amount of oxo-acid (glyoxylate plus oxaloacetate) formed from erythro-DL-β-hydroxyaspartate. The figures in parentheses give the levels of the enzymes in bacteria where they are known to function for the utilization of the growth substrate. The figures for the first three enzymes in the table are for glycollate-grown Pseudomonas B_2aba (taken from Kornberg and Gotto, 1961), and those for the β-hydroxyaspartate enzymes are for glycollate-grown Micrococcus denitrificans (taken from Kornberg and Morris,1965)

Enzyme	Specific activity	
Glyoxylate carboligase	0.003	(0.68)
Tartronate semialdehyde reductase	0.01	(0.78)
Malate synthase	<0.01	(1.73)
β-hydroxyaspartate aldolase	0.0013	(3.1)
β-hydroxyaspartate dehydratase		(0.19)

Effect of growth conditions on the intracellular level of L-serine dehydratase.

The specific activities of L-serine dehydratase in glycine and L-serine-grown cells (toluene-treated) were 1-2 and 0.6 μmoles pyruvate/min/mg.cell protein at 30°, respectively. Considerably lower specific activities were obtained for this enzyme in glucose and succinate-grown cells, i.e. 0.015-0.029 and 0.067 μmoles pyruvate/min/mg. cell protein, respectively.

Cells transferred from a glucose to a glycine salts medium ceased growing for several hours following the transfer. During this period, the L-serine dehydratase activity per unit volume of culture rose rapidly (fig.5). By the time the cells began growing again, their intracellular level of L-serine dehydratase was at least one half that found in cells which had been growing on glycine for many generations. Cells transferred from a glycine to a glucose salts medium continued to grow without a pause. The L-serine dehydratase activity per unit volume of culture stopped rising from the time of transfer, and remained approximately constant for at least three generations (fig.6).

An examination of extracts for malate synthase and enzymes of the glycerate and β-hydroxyaspartate pathways.

The activities of malate synthase and enzymes of the glycerate and β-hydroxyaspartate pathways in extracts from glycine-grown cells were far lower (table 5) than the glycine oxidizing activity of the whole cells (see discussion).

Moreover, the activities of these enzymes are very low by comparison with their activities in bacteria grown under conditions where the respective enzymes are known to function for the utilization of the growth substrate (table 5).

DISCUSSION

Providing glycine-grown cells are able to take up rapidly intermediates on the pathway of glycine catabolism, they should oxidize these intermediates at least as rapidly as they oxidize glycine. Glycine-grown cells did, in fact, oxidize L-serine, pyruvate and TCA cycle intermediates at rates which were comparable with that for glycine. On the other hand, glyoxylate, which is an intermediate of the glycerate and β-hydroxylaspartate pathways and of the dicarboxylic acid cycle, was oxidized much more slowly than glycine.

Similarly, extracts from glycine-grown cells should possess sufficient activity of each of the enzymes employed by these cells for glycine catabolism to account for the overall glycine oxidizing activity of the whole cells. The specific activity of L-serine dehydratase in toluene-treated, glycine-grown cells, i.e. 1-2μmoles/min./mg. cell protein, is more than adequate to accommodate the glycine oxidizing activity of the whole cells, i.e. 0.2-0.4μmoles/min/mg. cell protein. Unfortunately, serine hydroxymethyltransferase was assayed in the direction of glycine formation, and so the specific activity obtained for it cannot be subjected to this kind

of scrutiny. The equilibrium for this reaction is known to favour L-serine formation (Jaenicke and Kutzbach, 1963), but a suitable value for the equilibrium constant has not been found in the literature. The estimated activity of the glycine cleavage system in extracts from glycine-grown cells is far below the glycine oxidizing activity of whole cells. However, this is a complex, multi-enzyme system and therefore it is quite likely that the conditions employed for its assay were far from optimal. Furthermore, the synthetic H_4PteGlu used in the assay (and in the assay of serine hydroxymethyltransferase) may have been a poor substitute for the natural coenzyme for two reasons. First, because the synthetic H_4PteGlu is a mixture of two stereoisomers, only one of which is a substrate for the enzyme; the other is possibly inhibitory. Secondly, because the natural coenzyme may be a poly-glutamyl rather than a monoglutamyl derivative of folic acid.

The sharp rise in the intracellular levels of L-serine dehydratase and the glycine cleavage system, which occurred during the growth lag following the transfer of cells from a glucose to a glycine salts medium, suggests that the intracellular levels of these enzymes have to be relatively high before glycine can be utilized as carbon source for growth. When cells were transferred from a glycine to a glucose salts medium, the need for a high intracellular level of L-serine dehydratase apparently disappeared. Thus, whereas the cells continued to grow at a virtually unaltered rate following the transfer, the total L-serine dehydratase activity of the culture ceased to rise.

Serine hydroxymethyltransferase was exceptional in that its activities in extracts from glycine and glucose-grown cells were very similar. Presumably, during growth on glucose, this enzyme plays a role in the synthesis of glycine and in the provision of C_1-units from L-serine for the synthesis of purines, thymine, methionine etc., as it is known to do in other organisms (Umbarger and Umbarger, 1962; Rabinowitz, 1960). It would be of interest to determine whether the synthesis of L-serine, during growth on glycine, and the cleavage of L-serine, during growth on substrates other than glycine, are brought about by one and the same enzyme, or whether each of these functions is served by a separate isoenzyme. In the event of there being isoenzymes, they would probably be subject to separate modes of control.

The finding that the level of L-serine dehydratase in L-serine-grown cells is comparable to that in glycine-grown cells suggests that this enzyme also plays a role in the utilization of L-serine as growth substrate.

The low activities found in extracts from glycine-grown cells of malate synthase and enzymes of the glycerate and β-hydroxyaspartate pathways confirms other evidence (Chapter 3 p46) which suggests that the corresponding pathways play little or no part in the utilization of glycine in *A. globiformis*.

SUMMARY

1. Glycine-grown cells oxidized glycine, L-serine, pyruvate, citrate, succinate, fumarate and L-malate at similar rates, whereas they oxidized glyoxylate, acetate and formate more slowly.

2. The activities of L-serine dehydratase and the glycine cleavage system were considerably higher in extracts from glycine-grown cells than in extracts from glucose-grown cells. Serine hydroxymethyltransferase was present in similar activities in extracts from both glycine and glucose-grown cells.

3. During the growth lag which accompanied the transfer of cells from a glucose to a glycine salts medium, the intracellular levels of L-serine dehydratase and the glycine cleavage system rose rapidly.

4. Extracts from glycine-grown cells contained low activities of malate synthase and enzymes of the glycerate and β-hydroxyaspartate pathways,

CHAPTER 5.

THE REPLENISHMENT OF TRICARBOXYLIC ACID CYCLE INTERMEDIATES AND THE SYNTHESIS OF PHOSPHOENOLPYRUVATE.

INTRODUCTION

During growth on glycine, *A. globiformis* might satisfy some of its energy requirements by oxidizing the hydrogen atoms, and possibly some of the methylene-H_4PteGlu, released from the cleavage of glycine. The methylene group of the methylene-H_4PteGlu could be oxidized via methenyl-H_4PteGlu and formate to carbon dioxide (Sagers and Gunsalus, 1961). However, the bacterium is likely to derive the bulk of its energy from the catabolism of the pyruvate formed from glycine. The following results suggest that the pyruvate is completely oxidized by enzymes of the pyruvate dehydrogenase complex and of the TCA cycle. These results were obtained from studies of

glycine-grown cells and extracts from these cells.

1. The metabolism of pyruvate by cell suspensions is completely inhibited by 5mM-arsenite, a potent inhibitor of pyruvate dehydrogenase.

2. Following the addition of [2-^{14}C] glycine to a cell suspension, ^{14}C was rapidly incorporated into the cellular pools of citrate, glutamate and aspartate (Chapter 3 p. 44).

3. Citrate, succinate, fumarate and L-malate were oxidized by cell suspensions at rates comparable with that observed for glycine (Chapter 4 p. 53)

4. Cell-free extracts incorporated ^{14}C from [^{14}C]-bicarbonate into citrate and α-oxoglutarate when provided with pyruvate and various cofactors (this chapter).

5. Citrate synthase and L-malate oxidase activities have been detected in extracts.

Morris (1960) had previously observed that glucose-grown cells of *A. globiformis* oxidize intermediates of the TCA cycle, and that fluoroacetate inhibits the oxidation of glucose by these cells [Fluoroacetate gives rise to fluorocitrate, an inhibitor of aconitate hydratase (EC 4.2.1.3)].

Replenishment of tricarboxylic acid cycle intermediates

The reactions which serve to replenish intermediates of the TCA cycle, during growth on precursors of pyruvate, have been reviewed by Kornberg (1966). Pyruvate may be carboxylated

directly by the biotin enzyme pyruvate carboxylase (EC 6.4.1.1) to yield oxaloacetate:

pyruvate + ATP + CO_2 → oxaloacetate + ADP + P_i

This anaplerotic route has been found to operate in avian and mammalian tissues; the enzyme occurs in a number of microorganisms. Pyruvate carboxylases from most sources show a partial or absolute requirement for acetyl CoA.

An alternative route for effecting anaplerosis from pyruvate and its precursors has recently been found to operate in E.coli (Cooper and Kornberg, 1965). This route involves a novel enzyme, PEP synthase, which converts pyruvate directly to PEP by the following reaction:

ATP + pyruvate ⇌ PEP + AMP + P_i

This reaction overcomes the energy barrier associated with a reversal of the pyruvate kinase (EC 2.7.1.40) reaction by making use of the additional energy released when a second phosphate group is split from ATP. The PEP formed in this way is carboxylated to form oxaloacetate in an essentially irreversible reaction catalysed by the enzyme PEP carboxylase (EC 4.1.1.31):

PEP + Co_2 → oxaloacetate + P_i

This enzyme also occurs in Salmonella typhimurium, a few autotrophic bacteria and in plants. The enzymes from S. typhimurium and E.coli require acetyl CoA for maximal activity (Cánovas and Kornberg, 1965).

Three other enzymes are known which could in theory effect the net synthesis of TCA cycle intermediates by carboxylation of pyruvate or PEP. However, there is now fairly strong evidence that two of these enzymes, namely the "malate enzyme" (EC 1.1.1.40) (Kornberg, 1965a) and PEP carboxykinase (EC 4.1.1.32) (Utter, 1963), function in the reverse direction to supply C_3-compounds by decarboxylation of C_4-acids of the TCA cycle under conditions where the latter intermediates are readily available. The third enzyme, PEP carboxytransphosphorylase (EC 4.1.1.38) has only so far been found in Propionibacterium spp. (Siu, Wood, and Stjernholm, 1961).

The synthesis of phosphoenolpyruvate

During growth on glycine A. globiformis will need a supply of PEP, in excess of that normally required for the synthesis of aromatic amino acids etc., for the synthesis of hexoses, pentoses and glycerol. The pyruvate formed from glycine via the serine pathway could give rise to PEP in either of two ways. The direct route would involve the enzyme PEP synthase. The indirect route would involve oxaloacetate as an intermediate and the two enzymes pyruvate carboxylase and PEP carboxykinase. The latter enzyme catalyses the reaction:

oxaloacetate + NuTP $\rightleftharpoons$ PEP + NuDP + CO_2

where Nu denotes nucleoside. The PEP carboxykinases from animal sources and from some micro-organisms require guanosine or inosine nucleotides, whereas those from plant sources and other micro-organisms require adenosine nucleotides.

METHODS

Estimation of ^{14}C

The ^{14}C content of "infinitely thin" samples on planchettes was determined with a thin, mica, end-window, Geiger-Muller tube (General Electric Company of England) connected to a decade scaler (ECKO Electronics, Ltd., Southend-on-Sea, Essex). Measured counts were corrected for background counts (100-140 counts/100 sec). A coincidence correction was also applied where this was significant. Using this method 2.51 x 10^5 counts were recorded per 100 sec. per μC of ^{14}C. This represents a counting efficiency of 6.8%.

Preparation of [^{14}C] bicarbonate solutions and estimation of their ^{14}C content

Solutions of 0.15M-$KH^{14}CO_3$ (2.0ml. volume; 0.4μc/μmole) were freshly prepared for each set of assays by adding to a tube with a tightly fitting ground glass stopper: 0.6ml. of $Na_2{}^{14}CO_3$ solution (38.4μc/μmole), 0.04ml. of 0.5M-tris-HCl buffer, pH 8.5, 1.36ml. of distilled water and 30mg.of solid $KH\ CO_3$. In calculating the specific activities of these solutions, the $KH\ CO_3$ added was assumed to be the sole source of the bicarbonate ion.

An aliquot (0.10ml) from a freshly prepared 1/100 dilution (in 10mM-K_2HPO_4) of the $KH^{14}CO_3$ solution was added to a planchette containing a circle of lens tissue soaked in 0.06ml. of 50mM-$Ba(OH)_2$ and a drop of a dilute solution of bovine serum albumen (the protein sticks the lens tissue to the planchette). The planchette was dried under an infra red lamp and its ^{14}C content estimated as described earlier.

The estimate obtained by this method for an aliquot of $Na_2\ ^{14}CO_3$ solution, taken from a freshly opened vial, was in good agreement with the ^{14}C content quoted by the Radiochemical centre.

Enzyme Assays

Pyruvate carboxylase was assayed by two methods, both of which were based on those described by Utter and Keech (1963).

1. Estimation of the pyruvate dependent rate of incorporation of ^{14}C from [^{14}C] bicarbonate into acid-stable material. Assay systems contained in 1.0ml. (μmoles): tris-HCl buffer, pH 8.0, 100; sodium pyruvate, 10; $MgCl_2$, 10; ATP, 10; acetyl CoA, 0.4; citrate synthase, 1.4 units; $KH^{14}CO_3$ (0.4μc/μmole), 15; and the enzyme preparation (up to 1mg. of protein from a cell-free extract). A control from which pyruvate had been omitted was included with every set of assays. The assay systems were brought to 30° before adding the enzyme preparation to initiate the reaction. Aliquots (0.1ml) were withdrawn from the systems at 2min. intervals and added to planchettes, each of which contained a circle of lens tissue soaked in one drop of 60% (w/v) TCA. The planchettes were dried under an infra red lamp and their ^{14}C content estimated as described earlier.

Under the conditions of this assay, ^{14}C was incorporated into acid-stable material at a constant rate for periods of at least 8-10min. This rate was directly proportional to the amount of extract added to the assay system for amounts containing up to 1mg. of protein.

2. Partially purified preparations of the enzyme were assayed spectrophotometrically, by coupling oxaloacetate formation to NADH oxidation with malate dehydrogenase. Assay systems contained in 1.0ml. (μmoles): tris-HCl buffer, pH 7.5, 100; sodium pyruvate, 10; $KHCO_3$, 20; $MgCl_2$, 5; ATP, 5; acetyl CoA, 0.4; NADH, 0.1; malate dehydrogenase (freed from $(NH_4)_2SO_4$ by dialysis), 3.6 units; and the enzyme preparation (0.05-0.5mg. protein). NADH oxidation was followed continuously at 340nm.

PEP carboxykinase was assayed by estimating the PEP dependent rate of incorporation of ^{14}C from [^{14}C] bicarbonate into acid-stable material. Assay systems contained in 1.0ml. (μmoles): tris-maleate-NaOH buffer (containing equimolar proportions of tris and maleic acid) pH 7.5, 100; PEP (K salt), 5; GDP, 5; $MnCl_2$, 5; L-potassium glutamate, 10; aspartate transaminase, 3.6 units; $KH^{14}CO_3$ (approximately 0.4μc/μmole), 15; and the enzyme preparation (up to 1 mg. of protein from a cell-free extract). A control from which PEP had been omitted was included with every set of assays. The assay procedure was the same as that described for pyruvate carboxylase except that aliquots were removed from the systems at 3-4min. intervals.

Under the conditions of this assay ^{14}C was incorporated into acid-stable material at a constant rate for a period of at least 15min. This rate was directly proportional to the amount of extract added to the assay system for amounts containing up to 1mg. of protein.

Malate enzyme was assayed spectrophotometrically, by estimating the rate of NADP reduction in the presence of L-malate. Assay systems contained in 1.0ml. (μmoles): tris-HCl buffer, pH 8.0, 20; L-potassium malate, 5; NADP, 0.1; $MgCl_2$, 5; and the enzyme preparation. The L-malate was added last to initiate the reaction and NADPH formation followed continuously at 340nm. Cell-free extracts were found to contain very little NADPH oxidase activity.

CoA acetylating activity was assayed by estimating the acetate and ATP dependent rate of disappearance of CoA. Free CoA was estimated colorimetrically by reaction with 5,5'-dithiobis (2-nitrobenzoic acid)(DTNB) (Srere, Brazil and Gonen, 1963). Assay systems contained in 1.5ml.(μmoles): tris-HCl buffer, pH 8.0, 150; potassium acetate, 15; $MgCl_2$, 15; ATP, 15; CoA, 0.8; and the enzyme preparation (0.5 - 2.5 mg. of protein from a cell-free extract). A control from which ATP had been omitted was included with every assay. The assay systems were brought to 30° before adding the ATP to initiate the reaction. Aliquots (0.2ml) were removed from the systems at 2-3min. intervals and diluted into 2.8ml. of distilled water containing 0.3μmole of DTNB. These solutions were left to stand for 10 min. at room temperature before measuring their absorbance at 412nm. In calculating the CoA content of the aliquots, a value of $1.36 \times 10^4 M^{-1} cm.^{-1}$ was taken for the molar absorbtivity at 412nm. of 5-thiol-2-nitrobenzoic acid.

Citrate synthase was assayed by estimating the rate of appearance of CoA which accompanies the condensation of acetyl CoA with oxaloacetate. Free CoA was estimated colorimetrically by reaction with DTNB.

PEP synthase was assayed by estimating the ATP dependent rate of disappearance of pyruvate. The method was devised by Dr. R.A.Cooper (personal communication). Assay systems contained in 0.5ml. (μmoles): tris-HCl buffer, pH 8.0, 50; sodium pyruvate, 0.75; ATP, 5; $MgCl_2$, 5; and the enzyme preparation (approximately 1mg. of protein from a cell-free extract). A control from which ATP had been omitted was included with every assay. The assay systems were brought to 30° before adding the ATP to start the reaction. Aliquots (0.1ml) were removed from the systems at 5min. intervals and added to 0.9ml. distilled water plus 0.33ml. 0.1%(w/v) DNPhydrazine reagent. Pyruvate DNPhydrazone was estimated spectrophotometrically as described in Chapter 2 (p. 29).

Separation of pyruvate carboxylase from citrate synthase

1. Fractionation with ammonium sulphate. This part of the procedure employs a temperature of 0° throughout. It should be modified in view of the finding that pyruvate carboxylase from *A. globiformis* is cold labile.

To 30ml. of an ultrasonic extract from glycine-grown cells (containing 10-15mg. protein/ml) was added, with constant stirring, 20ml. of a solution of saturated $(NH_4)_2SO_4$ which had been adjusted to pH 7.2 with ammonia solution and which contained 1mM-EDTA.

When the addition of this solution was complete, the resulting suspension, which was 40% saturated with respect to $(NH_4)_2SO_4$, was stirred for a further 15min, centrifuged at 25,000g. for 10min. and the precipitate discarded. To the supernatant solution was added a further 10ml. of the neutral, saturated $(NH_4)_2SO_4$ solution (50% saturation); the resulting suspension was stirred and centrifuged as before. The precipitate was dissolved in 2ml. of 50mM-potassium phosphate buffer, pH 7.0, and its temperature allowed to rise to that of the room (20-22°). This fraction contained most of the pyruvate carboxylase activity and very little citrate synthase activity. The bulk of the citrate synthase remained in the final supernatant solution.

2. Chromatography on G-200 sephadex. The following procedure was carried out at room temperature. A column of G-200 sephadex (2.1cm. diameter x 55cm) was prepared as described by Andrews (1965) and equilibrated with 50mM-potassium phosphate buffer, pH7.0. The enzyme preparation from the previous stage was mixed with cytochrome c and blue dextran, which both served as markers, and applied to the column. The phosphate buffer used above was run through the column at a flow rate of approximately 30ml/hr. and the effluent collected in 3ml. fractions. Pyruvate carboxylase eluted in a single peak, largely in two 3ml. fractions, with an "elution volume" (for definition see Chapter 11, p.186) of 81ml. Citrate synthase had an elution volume of 95ml. The preparation of pyruvate carboxylase obtained in this way did not contain detectable citrate synthase activity, but

Table 6. Requirements of pyruvate carboxylase in cell-free extracts.

The complete assay system (1.0ml.) contained tris-HCl buffer, pH7.8, $MgCl_2$, sodium pyruvate, sodium acetate, ATP, CoA, $KH^{14}CO_3$ and an extract of glycine-grown cells (1-2mg. protein). The extract employed in systems 7 & 8 had been passed through a column of G-25 sephadex. The ultrafiltrate added to system 8 had been freshly prepared from a concentrated cell-free extract. Assay systems were incubated for 20 min. at 30°. Further details are given in the text.

Number	Contents of assay system	^{14}C incorporated (counts/100sec.)
1	Complete	1911
2	Pyruvate omitted	8
3	ATP omitted	0
4	$MgCl_2$ omitted	10
5	CoA omitted	21
6	Acetate omitted	247
7	Complete	1406
8	Complete:ultrafiltrate of extract added	1702

Table 7. Inhibition of pyruvate carboxylase by avidin

Extracts from glycine-grown cells (4-8mg. protein) were preincubated (in 10mM-tris-HCl buffer, pH7.8; final volume, 0.5ml.) for 5 min. at 30° together with either varying amounts of avidin (2.5 units/mg.), or mixtures of avidin and varying amounts of d-biotin. The mixtures of avidin and biotin had been preincubated for 5 min. before adding the extract to them. An aliquot (0.3ml.) from each preincubation mixture was assayed for pyruvate carboxylase activity. Assay systems 1-5A were incubated for 20 min. and systems 1-5B for 45 min, all at 30°.

Number	Extract preincubated with: Avidin(μg.)	Biotin(nmoles)	^{14}C incorporated (counts/100sec.)
1A	-	-	3216
2A	14	-	3226
3A	28	-	1997
4A	71	-	73
5A	142	-	0
1B	-	-	500
2B	142	-	0
3B	142	10	529
4B	142	50	638
5B	142	100	627

did contain low activities of acetyl CoA hydrolase and NADH oxidase.

RESULTS

Pyruvate carboxylase

Studies with cell-free extracts. The following results indicate the presence of pyruvate carboxylase activity in extracts from glycine-grown cells of *A. globiformis*. Similar results were obtained with extracts from glucose-grown cells.

Cell-free extracts caused a pyruvate dependent incorporation of ^{14}C from [^{14}C] bicarbonate into acid-stable material when provided with ATP, Mg^{2+} and CoA (table 6). The addition of acetate to this system stimulated, by approximately eight-fold, the amount of ^{14}C incorporated during a 20min. incubation period. Acetate and CoA could be replaced with acetyl CoA without significantly altering the amount of ^{14}C incorporated, and without affecting the requirement shown by the system for ATP.

Preincubating an extract with avidin completely inhibited its ability to cause a pyruvate dependent incorporation of ^{14}C from [^{14}C]-bicarbonate into acid stable material (table 7). On the other hand, the avidin caused no inhibition if it was preincubated with d-biotin before adding it to the extract.

The acid-stable radioactive products from these assay systems were separated by two-dimensional, paper chromatography and identified as described in Chapter 3 (p. 39). Oxo-acids were converted to their

DNPhydrazones and analysed in this form by paper chromatography. Citrate was found to contain the greatest proportion of the incorporated ^{14}C, α-oxoglutarate, a relatively small proportion, and oxaloacetate, none at all. Approximately half of the incorporated ^{14}C could be diverted from citrate into malate by adding to the assay system malate dehydrogenase and an NADH generating system comprising ethanol, NAD and alcohol dehydrogenase.

The formation of both [^{14}C] malate and [^{14}C] citrate in the experiment above suggests that [^{14}C] oxaloacetate is the immediate product of the reaction incorporating ^{14}C from [^{14}C] bicarbonate. Presumably, the [^{14}C] oxaloacetate is condensed with the acetyl CoA added to the system to form [^{14}C] citrate by the citrate synthase activity of the extract. This explanation alone might be sufficient to account for the absolute requirement shown by the system for acetyl CoA. Alternatively, there may be an additional requirement for catalytic amounts of acetyl CoA to activate the pyruvate carboxylase. In order to investigate these two possibilities, cell-free extracts were fractionated to obtain a pyruvate carboxylase preparation free from citrate synthase activity.

Studies with the partially purified enzyme. Partially purified pyruvate carboxylase incorporated ^{14}C from [^{14}C] bicarbonate into acid-stable material when provided with pyruvate, ATP, Mg^{2+}, acetyl CoA, aspartate transaminase and L-glutamate. The addition to the system of an ultrafiltrate from a cell-free extract did not increase the amount of ^{14}C incorporated. Acetyl CoA could not be replaced by CoA

Table 8. Requirements of pyruvate carboxylase after separation from citrate synthase.

The complete assay system (1.0ml.) contained tris-HCl buffer, pH8.0, $MgCl_2$, sodium pyruvate, ATP, acetyl CoA, L-potassium glutamate, aspartate aminotransferase (AAT), $KH^{14}CO_3$ and a partially purified pyruvate carboxylase preparation (0.06mg protein) which was free from citrate synthase activity. After 33 min. incubation at 30° aliquots were taken from the assay systems and their content of acid-stable ^{14}C estimated.

Number	Ommissions	Additions (μmoles)	^{14}C incorporated (counts/100 sec.)
1	None	None	2908
2	Pyruvate	None	17
3	ATP	None	33
4	Acetyl CoA	None	0
5	Acetyl CoA	CoA(0.5)	2
6	$MgCl_2$	None	0
7	$MgCl_2$	$MnCl_2$(5)	519
8	AAT & L-glutamate	None	106
9	None	Ultrafiltrate of cell-free extract	2334

Systems 1 and 8 were incubated for a further 82 min.

contd.....

Table 8. contd.

Acid-stable ^{14}C was again estimated in an aliquot from system 1. To system 8 was then added 3ml. 0.1% ($^{w}/v$) DNPhydrazine in 2M-HCl and the DNPhydrazones formed were extracted into ethyl acetate. An aliquot of this solution, equivalent in amount to the material taken for the estimation of acid-stable ^{14}C, was plated on a planchette and its radioactivity determined.

Number	^{14}C incorporated (counts/100sec.):	
	into acid-stable material	into DNPhydrazones
1 above	14094	-
8 above	-	9039

in this system, and Mn^{2+} was less effective than Mg^{2+} (table 8). The amount of ^{14}C incorporated during a 33 min. incubation period was considerably reduced by omitting the aspartate transaminase and L-glutamate from the system. However, when the incorporation of ^{14}C in this incomplete system was terminated by the addition of DNPhydrazine reagent, rather than TCA, a relatively large amount of ^{14}C was found in the ethyl acetate-soluble, oxo-acid DNPhydrazone fraction. In fact, the ^{14}C content of this fraction was comparable with that of the acid-stable material from a similar, but complete, assay system (table 8). Almost all the ^{14}C in the oxo-acid DNPhydrazone fraction was found to chromatograph with the DNPhydrazone of authentic oxaloacetate. On the other hand, the acid-stable ^{14}C from the complete assay system, containing aspartate transaminase and L-glutamate, chromatographed with authentic aspartate. These results confirm that [^{14}C] oxaloacetate is the immediate product of the pyruvate dependent, ^{14}C incorporation reaction.

The nature of the acetyl CoA requirement. Partially purified pyruvate carboxylase could be assayed spectrophotometrically, by coupling oxaloacetate formation to NADH oxidation with malate dehydrogenase. In this system, NADH oxidation was shown to be dependent upon the presence of pyruvate, ATP and acetyl CoA. The requirement for acetyl CoA was shown to be for catalytic amounts by the following experiment. In an assay system containing 0.10μmole of acetyl CoA, a change in absorbance at 340nm. corresponding to the oxidation of 0.21μmole of NADH was recorded.

Table 9. Effect of temperature, acetyl CoA and sucrose on the stability of pyruvate carboxylase

Solutions containing a cell-free extract (final concentration 1.6-3.2mg. protein/ml.), 50mM-potassium phosphate buffer, pH7.0, and, where indicated, acetyl CoA (0.5mM) or sucrose (1M) were stored for 18hr. at the temperatures indicated. Aliquots (0.2ml.) of these solutions were assayed for pyruvate carboxylase activity before and after the period of storage. Assay systems were incubated at 30° for 20 min.

Storage temperature (°C)	Additions to storage system	^{14}C incorporated (counts/100sec.): initially	after 18hr.	% initial activity remaining after 18hr.
20-23	None	1152	667	58
0	None	1152	282	24
~ -20	None	1152	824	71
20-23	Acetyl CoA	974	584	60
0	Acetyl CoA	974	710	73
20-23	Sucrose	708	474	67
0	Sucrose	708	527	74

The quantity of acetyl CoA remaining in the assay system at this point was determined by estimating the CoA released, by reaction with DTNB, following the addition to the system of a little cell-free extract containing acetyl CoA hydrolase activity. This assay detected 0.07 µmole of the 0.10µmole of acetyl CoA originally added to the assay system. The remaining 0.03µmole could be accounted for as having been hydrolysed by the acetyl CoA hydrolase activity of the pyruvate carboxylase preparation.

The catalytic nature of the acetyl CoA requirement was confirmed by a second experiment in which 1.8µmoles of ^{14}C from [^{14}C] bicarbonate was incorporated into acid-stable material in an assay system containing only 0.4µmole of acetyl CoA.

Molecular weight of the enzyme. An estimate for the molecular weight of pyruvate carboxylase was obtained from its behaviour during gel filtration on a column of G-200 sephadex. The ratio of the elution volume (Ve) to that of blue dextran (Vo), which is completely excluded from the gel particles, was (Ve/Vo) 1.40-1.45. From this value, a value of 4-5 x 10^5 was obtained for the molecular weight of the enzyme, by interpolation on the plot of Andrews (1965). This plot relates log [molecular weight] to Ve/Vo for columns of G-200 sephadex.

Cold inactivation of the enzyme. The pyruvate carboxylase activity of extracts was found to be more stable at room temperature than at 0^o. The enzyme was completely protected from cold inactivation by the presence of either 0.5m$\underline{M}$-acetyl CoA, or 1$\underline{M}$-sucrose (table 9).

Table 10. Levels of pyruvate carboxylase and PEP carboxykinase in A. globiformis grown on various carbon sources.

Cell-free extracts were prepared from log. phase cells (grown in liquid media containing the compound indicated as sole source of carbon) and assayed without delay. Pyruvate carboxylase and PEP carboxykinase were assayed by following the incorporation of ^{14}C from $KH^{14}CO_3$ into acid-stable materials, dependent upon the presence of pyruvate and PEP respectively. Initial rates of incorporation of ^{14}C were determined from plotes of ^{14}C incorporated against time. Specific activities are given as μmoles of ^{14}C incorporated/min./mg. protein at 30°.

Growth substrate	Specific activity	
	PEP carboxy-kinase	Pyruvate carboxylase
Glycine	0.016	0.030
DL-lactate	0.021	0.049
Succinate	0.011	0.063
Glycerol	0.0016	0.063
Glucose	0.00019	0.030

Table 11. Requirements of PEP carboxykinase in cell-free extracts.

The complete assay system (1.0ml.) contained tris-maleate-NaOH buffer, pH7.5, $MgCl_2$ (10µmoles), PEP, GDP, L-potassium glutamate, aspartate aminotransferase (AAT), $KH^{14}CO_3$ and an extract of glycine-grown cells (2-3 mg. protein).
Assay system were incubated for 30 min. at 30° before estimating the ^{14}C incorporated into acid-stable material. Systems 1 and 5 were incubated for a further 7 min. and 0.33 ml. of DNPhydrazine solution added to them. The DNPhydrazones were extracted into ethyl acetate and found to be free from detectable radioactivity. Further details are given in the text.

Number	Contents of assay system	^{14}C incorporated (counts/100 sec.)
1	Complete	4443
2	PEP omitted	0
3	GDP omitted	196
4	$MgCl_2$ omitted	219
5	L-glutamate & AAT omitted	2321
6	Complete; GSH (0.5µmoles) added	4573

Table 12. Effect of Mn^{2+}, Mg^{2+} and pH on the activity of PEP carboxykinase.

Assay systems (1.0ml.) contained 0.1 M-tris-maleate -NaOH buffer, at the pH's indicated, $MnCl_2$ or $MgCl_2$, at the concentrations indicated, an extract from glycine-grown cells (1-2mg. protein) and other components at the usual concentrations. Systems were incubated for 16 min. at 30°

Metal salt added	pH of buffer	^{14}C incorporated (counts/100sec.)
None	7.5	123
5mM-$MnCl_2$	"	1687
10mM-$MnCl_2$	"	1124
5mM-$MgCl_2$	"	552
10mM-$MgCl_2$	"	862
5mM-$MnCl_2$	6.5	2376
" "	7.0	2612
" "	8.0	593
" "	8.5	261

CoA did not afford this protection. The enzyme was most stable when frozen solid at approximately -20°.

Levels of the enzyme in cells grown on various carbon sources

Similar levels of pyruvate carboxylase activity were found in freshly prepared extracts from cells grown on either glycine, DL-lactate, succinate, glycerol or D-glucose as carbon source (table 10). In each of these cases, the specific activity of the enzyme fell in the range 0.030 - 0.063μmoles of ^{14}C incorporated/min/mg. protein at 30°.

PEP carboxykinase

The following results indicate the presence of PEP carboxykinase activity in extacts from glycine-grown cells of A.globiformis.

Cell-free extracts caused a PEP dependent incorporation of ^{14}C from [^{14}C] bicarbonate into acid-stable material when provided with guanosine diphosphate (GDP) and Mg^{2+} (table 11). Mn^{2+} was found to be more effective than Mg^{2+} in satisfying the requirement for a divalent cation (table 12). The addition of aspartate transaminase and L-glutamate to the system stimulated, by two-fold, the amount of ^{14}C incorporated during a 30 min. incubation period. Nevertheless, when these two components were omitted from the assay system, there was no incorporation of ^{14}C into the oxo-acid DNPhydrazone fraction. The amount of ^{14}C incorporated in the complete assay system over a period of 32 min. was not increased by adding to it an ultrafiltrate of a concentrated extract from glycine-grown cells.

Table 13. Nucleoside diphosphate specificity of PEP carboxykinase.

Assay systems (1.0ml.) contained nucleoside diphosphates, as and where indicated, each at a final concentration of 5mM, an extract from glycine-grown cells(0.9mg. protein) and other components at the usual concentrations. The initial rate of incorporation of ^{14}C (at 30°) was determined from a plot of ^{14}C incorporated against time of incubation.

Nucleoside diphosphate	rate of incorporation of ^{14}C (nc/min./ml.)
None	2.4
ADP	3.5
GDP	12.0-12.3
IDP	12.4-16.5
IDP+GDP	11.2-12.4

Chromatographic separation of the acid-stable products from a complete assay system revealed that most of the incorporated ^{14}C was present in citrate and aspartate. This result is consistent with [^{14}C] oxaloacetate being the immediate product of the PEP dependent, ^{14}C incorporation reaction.

Unfortunately, it was not possible to demonstrate that extracts would cause a GTP dependent decarboxylation of oxaloacetate, i.e. a reversal of the reaction studied above, because they decarboxylated oxaloacetate rapidly even in the absence of GTP.

Specificity of the enzyme towards nucleoside diphosphates. The requirement shown by the PEP carboxykinase activity of extracts for a nucleoside diphosphate was satisfied equally well by a 5mM concentration of either GDP or inosine diphosphate (IDP), but not nearly so well by the same concentration of ADP (table 13). The rate of incorporation of ^{14}C obtained with GDP and IDP present together was no greater than that obtained with either present alone.

Effect of pH. The PEP carboxykinase activity of extracts was maximal at pH 6.5-7.0 (table 12).

Levels of the enzyme in cells grown on various carbon sources. The specific activity of PEP carboxykinase in extracts from glycine-grown cells, i.e. 0.016μmoles ^{14}C incorporated/min/mg. protein at 30°, was comparable with the activities obtained for extracts from cells grown on DL-lactate and succinate as sole carbon sources.

Table 14. Assays of PEP carboxylase and PEP carboxytransphosphorylase activities in an extract from glycine-grown cells

The basal assay system (1.0ml.) contained (μmoles): tris-HCl buffer, pH7.5, 100; $MgCl_2$, 10; $KH^{14}CO_3$ (0.4μc/μmole), 7.5; an extract from glycine-grown cells (2-3mg. protein); L-potassium glutamate, 15; and aspartate aminotransferase, 3.8 units. Other su bstances were added to the assay systems as indicated; they were incubated for 32 min. at 30°. An assay of PEP carboxykinase activity is included for comparison. The assay system for PEP carboxylase is based on that of Suzuki and Werkman (1958), and the system for PEP carboxytransphosphorylase on that of Siu et al (1961).

No.	Enzyme assayed	Additions to basal assay system (μmoles)	^{14}C incorporated (counts/100sec.)
1	PEP carboxylase	PEP (5)	145
2	PEP carboxytransphosphorylase	PEP(5), P_i(10)	139
3	PEP carboxykinase	PEP(5), GDP(5)	2664

The activities of the enzyme in extracts from glycerol and glucose-grown cells were approximately one tenth and one hundredth, respectively, of the activity in glycine-grown cells (table 10).

An examination of extracts for the malate enzyme, PEP carboxylase, PEP carboxytransphosphorylase and PEP synthase.

An extract from glycine-grown cells was assayed for PEP carboxylase and PEP carboxytransphosphorylase activities by estimating its ability to incorporate ^{14}C from [^{14}C] bicarbonate into acid-stable material when provided with the appropriate substrates and cofactors (see table 14 for details). Only small amounts of ^{14}C were incorporated in these assays, suggesting that the extract did not contain significant activities of the two enzymes (table 14). However, the assay system employed for PEP carboxylase could be criticized because it did not contain acetyl CoA which is a necessary cofactor for some PEP carboxylases (Cánovas and Kornberg, 1965).

An extract from glycine-grown cells was observed to cause a slow, L-malate dependent reduction of NADP in an assay of malate enzyme activity. However, chromatographic analysis of the oxo-acids formed during the assay revealed a large proportion of oxaloacetate and only a small proportion of pyruvate.

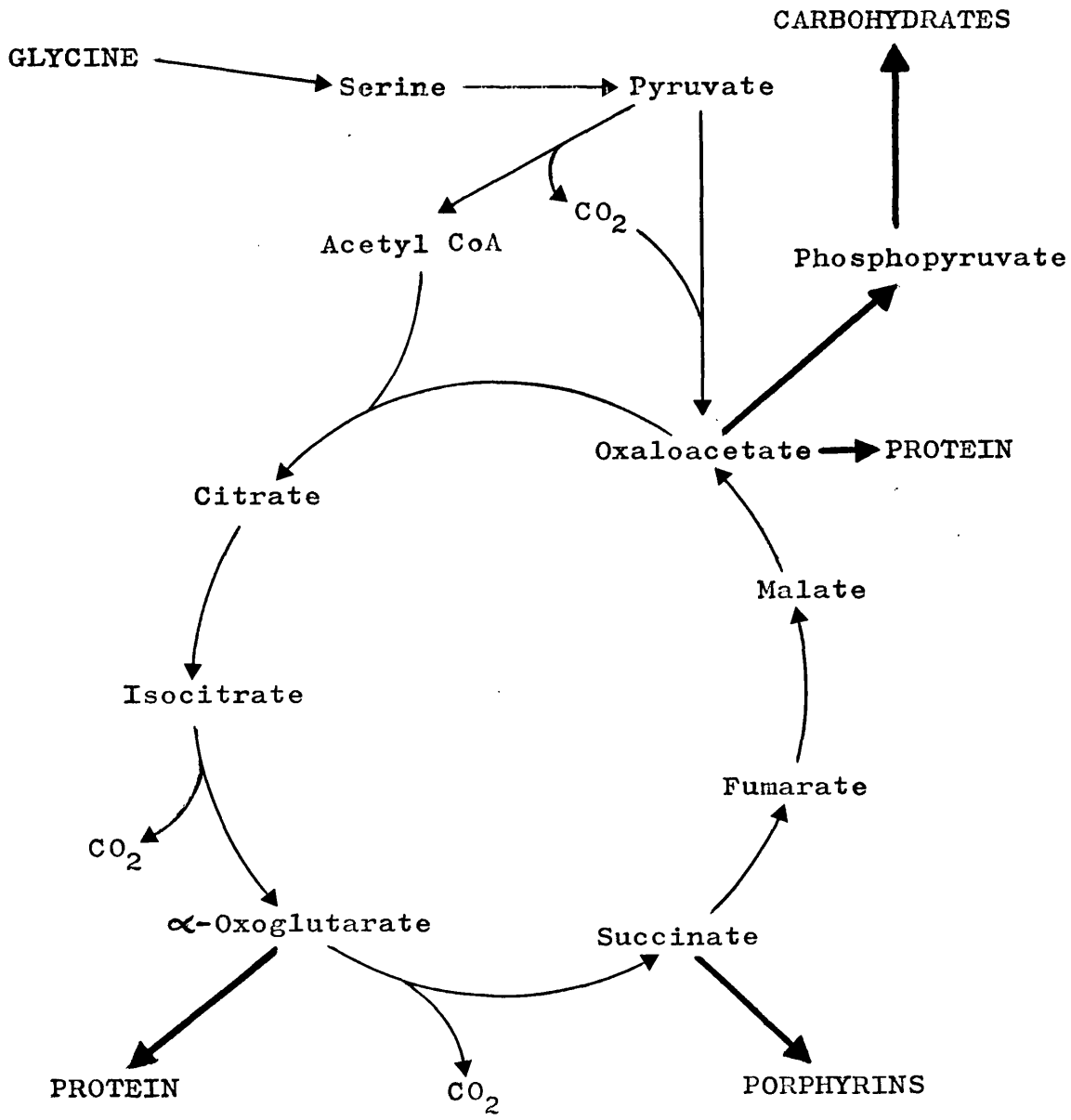


Fig.7. Suggested routes for the provision of energy and cell materials in A. globiformis utilizing glycine as carbon source for growth.

Extracts from glycine-grown cells did not possess detectable PEP synthase activity.

DISCUSSION

The replenishment of tricarboxylic acid cycle intermediates

The finding that A. globiformis contains pyruvate carboxylase suggests a route for the replenishment of TCA cycle intermediates during growth on glycine (fig. 7) and other precursors of pyruvate. Under each of these growth conditions, the intracellular level of pyruvate carboxylase is probably sufficient for it to serve the entire anaplerotic needs of the TCA cycle. Thus the activity of the enzyme in extracts from glycerol-grown cells, i.e. 0.063 units/mg. protein at 30^{o}, is comparable with the level of PEP carboxylase in extracts from E.coli grown on this substrate, i.e. 0.058 units/mg. protein at $20\text{-}22^{o}$ (Cánovas and Kornberg, 1966). PEP carboxylase has been shown to play an essential anaplerotic role in E.coli, during growth on glycerol and other PEP precursors, from studies of mutants deficient in this enzyme (Ashworth and Kornberg, 1966). The mean generation times of E.coli and A.globiformis growing in glycerol salts media are 1.5 (Kornberg, 1965b) and 2.6 hr., respectively.

The relatively high level of pyruvate carboxylase activity found in extracts from succinate-grown cells suggests that the enzyme may be formed constitutitvely, since it would appear to serve no useful function during growth on this and other TCA cycle intermediates. L-malate-grown Rhodotorula glutinis is

also reported to contain a relatively high level of the enzyme (Ruiz-Amil, de Torrontegui, Palacián, Catalina and Losada, 1965).

The conclusion that pyruvate carboxylase plays a major anaplerotic role during growth on pyruvate precursors is also supported by the tentative finding that extracts from glycine-grown cells do not contain significant activities of PEP carboxylase, PEP carboxytransphosphorylase and the malate enzyme. Each of these enzymes might in theory have contributed to anaplerosis.

The synthesis of PEP

During growth on glycine and other pyruvate precursors, *A. globiformis* will require an increased supply of PEP to serve as the precursor of carbohydrates. This requirement will presumably be satisfied by PEP carboxykinase, through decarboxylation of part of the oxaloacetate formed by pyruvate carboxylase. The relatively low levels of PEP carboxykinase found in extracts from cells grown on PEP precursors, e.g. glycerol and glucose, are consistent with this view.

The finding that pyruvate carboxylase from *A. globiformis* is activated by low concentrations of acetyl CoA (0.08mM was sufficient to produce maximal activation) suggests a mechanism for regulating the synthesis of oxaloacetate, and hence of PEP, along the lines of that suggested by Utter, Keech and Scrutton (1964) for the avian system. The formation of acetyl CoA from pyruvate and its precursors will stimulate pyruvate carboxylase to form oxaloacetate

more rapidly. This will increase the supplies of oxaloacetate available for condensing with acetyl CoA (thereby increasing its rate of oxidation) and for conversion to PEP by PEP carboxykinase.

Pyruvate carboxylase

In its absolute requirement for catalytic amounts of acetyl CoA, the pyruvate carboxylase from *A. globiformis* resembles the avian enzyme (Utter and Keech, 1963) more closely than enzymes from other micro-organisms. Thus the enzyme from baker's yeast (Ruiz-Amil, et al, 1965; Cooper and Benedict, 1966) is active in the absence of acetyl CoA, though stimulated several-fold by its presence or that of CoA. The activity of other microbial enzymes, e.g. those from *Aspergillus niger* (Bloom and Johnson, 1962) and *Pseudomonas citronellolis* (Seubert and Remberger, 1961), is completely unaffected by the presence of acetyl CoA.

The enzyme from *A. globiformis* also resembles the avian enzyme in being cold labile, and in being protected from cold inactivation by the presence of a low concentration of acetyl CoA, or a high concentration of sucrose (Scrutton and Utter, 1965). Recent studies (Valentine, Wrigley, Scrutton, Irias and Utter, 1966) have shown that inactivation of the avian enzyme at low temperatures is accompanied by its dissociation into four subunits. The fact that the enzyme from *A. globiformis* has a high molecular weight

($4\text{-}5 \times 10^5$) and is cold labile suggests that it also may possess a subunit structure which is modified by exposure to low temperatures.

PEP carboxykinase

In showing a greater activity with guanosine or inosine nucleotides than with adenosine nucleotides, the PEP carboxykinase from A. globiformis resembles the enzymes from a minority of the microorganisms which have been investigated (Quayle and Keech, 1959; Baugh, Claus and Werkman, 1960; Suzuki and Werkman, 1958), and also those from animal sources (Kurahashi, Pennington and Utter, 1957; Simpson and Awapara, 1964). The enzymes from the majority of microorganisms examined (Woronick and Johnson, 1960; Cannata and Stoppani, 1963; Baugh, Lanham and Surgalla, 1964; Theodore and Engelsberg, 1964) and those from plant sources (Mazelis and Vennesland, 1957) show a much greater activity with adenosine nucleotides than with guanosine or inosine nucleotides. It is interesting that several organisms which possess PEP carboxykinases with different nucleotide requirements each possess a succinyl CoA synthase (EC 6.2.1.4) with a nucleotide requirement which matches that of their PEP carboxykinase. For example, the succinyl CoA synthase (Gibson, Upper and Gunsalus, 1967) and PEP carboxykinase (L. Sage, personal communication) from E. coli are both dependent upon adenosine nucleotides for maximal activity. On the other hand, the two enzymes from mammalian liver mitochondria

are dependent upon guanosine or inosine nucleotides (Mazumder, Sanadi and Rodwell, 1960; Chang, and Lane, 1966). In the latter case, there is evidence to suggest that the reactions catalysed by the two enzymes are effectively coupled through their common nucleotide requirements (Mudge, Neuberg and Stanbury, 1954; Nordlie and Lardy, 1963). It would be of interest to determine whether or not these two enzymes have the same nucleotide requirements in *A. globiformis*.

SUMMARY

1. Evidence has been presented that glycine-grown cells of *A. globiformis* contain a functioning TCA cycle.

2. Extracts from *A. globiformis* contain a pyruvate carboxylase which shows an absolute requirement for catalytic amounts of acetyl CoA, and which is cold labile except in the presence of low concentrations of acetyl CoA or high concentrations of sucrose. Extracts from cells grown on a variety of substrates contain similar levels of this enzyme.

3. Cell-free extracts also contain a PEP carboxykinase which shows substantially more activity towards GDP and IDP than towards ADP. The levels of this enzyme in extracts from cells grown on glycine or other pyruvate precursors are approximately ten-fold and one hundred-fold higher than the levels in extracts from cells grown on glycerol and glucose, respectively.

4. Extracts from glycine-grown cells do not contain significant PEP carboxytransphosphorylase, malate enzyme and PEP synthase activities and probably do not contain significant PEP carboxylase activity.

CHAPTER 6.

GENERAL CONCLUSIONS

It has been established that *A. globiformis*, in utilizing glycine as carbon source for growth, converts it in the first place to pyruvate by way of the serine pathway. The only other organism which is known to employ this route for utilizing glycine as growth substrate is the obligate anaerobe *Peptococcus glycinophilus* (Sagers and Gunsalus, 1961). In the serine pathway part of the glycine is cleaved to form a C_1-unit which, in the form of the methylene group of methylene-H_4Pte Glu, condenses with more glycine to produce L-serine. The L-serine is converted to pyruvate by L-serine dehydratase. The dehydratase from *A. globiformis* possesses some interesting kinetic properties which are reported in section 2 of this thesis.

There is evidence to suggest that some of the pyruvate formed from glycine is oxidized to acetyl CoA, and that this is further oxidized by way of the TCA cycle. These reactions will presumably provide most of the energy needed for growth. The finding that *A. globiformis* has a pyruvate carboxylase means that pyruvate can also be converted to oxaloacetate for the replenishment of TCA cycle intermediates (fig.7). The absolute requirement shown by this enzyme for catalytic amounts of acetyl CoA will provide a control mechanism for regulating the supply of oxaloacetate to the level of acetyl CoA.

During growth on glycine and other pyruvate precursors, *A. globiformis* will need a supply of PEP, in excess of its normal requirements, for conversion to glycerol and carbohydrates. The relatively high levels of PEP carboxykinase found in cells grown under these conditions should meet this requirement by decarboxylating some of the oxaloacetate formed by pyruvate carboxylase.

SECTION 2.

L-SERINE DEHYDRATASE FROM ARTHROBACTER GLOBIFORMIS

CHAPTER 7

INTRODUCTION

Gale and Stephenson (1938) first recognized that the deamination of serine by *E. coli* differed from that of glycine, alanine and glutamate in that it would occur in the absence of oxygen. Chargaff and Sprinson (1943) identified pyruvic acid as the product of serine deamination and suggested that α-aminoacrylic acid might be an intermediate. The latter compound hydrolyses spontaneously to pyruvate and ammonia. Subsequently, Wood and Gunsalus (1949) partially purified an enzyme from *E.coli* which deaminated L-serine and L-threonine, forming pyruvate and α-oxobutyrate, respectively.

Enzymes which deaminate L-serine to pyruvate have been found widely distributed among micro-organisms and animal tissues. Of these enzymes, some, termed L-serine dehydratases [L-serine hydro-lyase (deaminating) (EC4.2.1.13)], will not deaminate any other amino acid. An enzyme from sheep liver (Sayre and Greenberg, 1956) and one from *Clostridium acidi-urici* (Benziman, Sagers and Gunsalus, 1960) are of this type. Other enzymes possessing L-serine dehydratase activity show greater activity towards L-threonine and are properly called L-threonine dehydratases (EC4.2.1.16). Examples of the latter type of enzyme are more numerous. These include a second enzyme from sheep liver (Sayre and Greenberg, 1956), enzymes from *Neurospora* (Yanofsky and Reissig, 1953) and yeast (Boll and Holzer, 1965), and two enzymes from *E.coli*, i.e. an inducible, catabolic L-threonine dehydratase and a biosynthetic one (Umbarger and Brown, 1957). L-serine dehydratase activity is also possessed by the B protein of tryptophan synthetase (EC4.2.1.20) from *E.coli* (Crawford and Ito, 1964), and by a crystalline preparation of tryptophan-inducible tryptophanase from the same bacterium (Newton, Morino and Snell, 1965). Some micro-organisms form enzymes for deaminating the D-isomers of serine and threonine (Yanofsky, 1952; Metzler and Snell, 1952b; Umbarger and Brown, 1956). These enzymes are quite distinct from those which deaminate the L-isomers, but nevertheless have many properties in common with them.

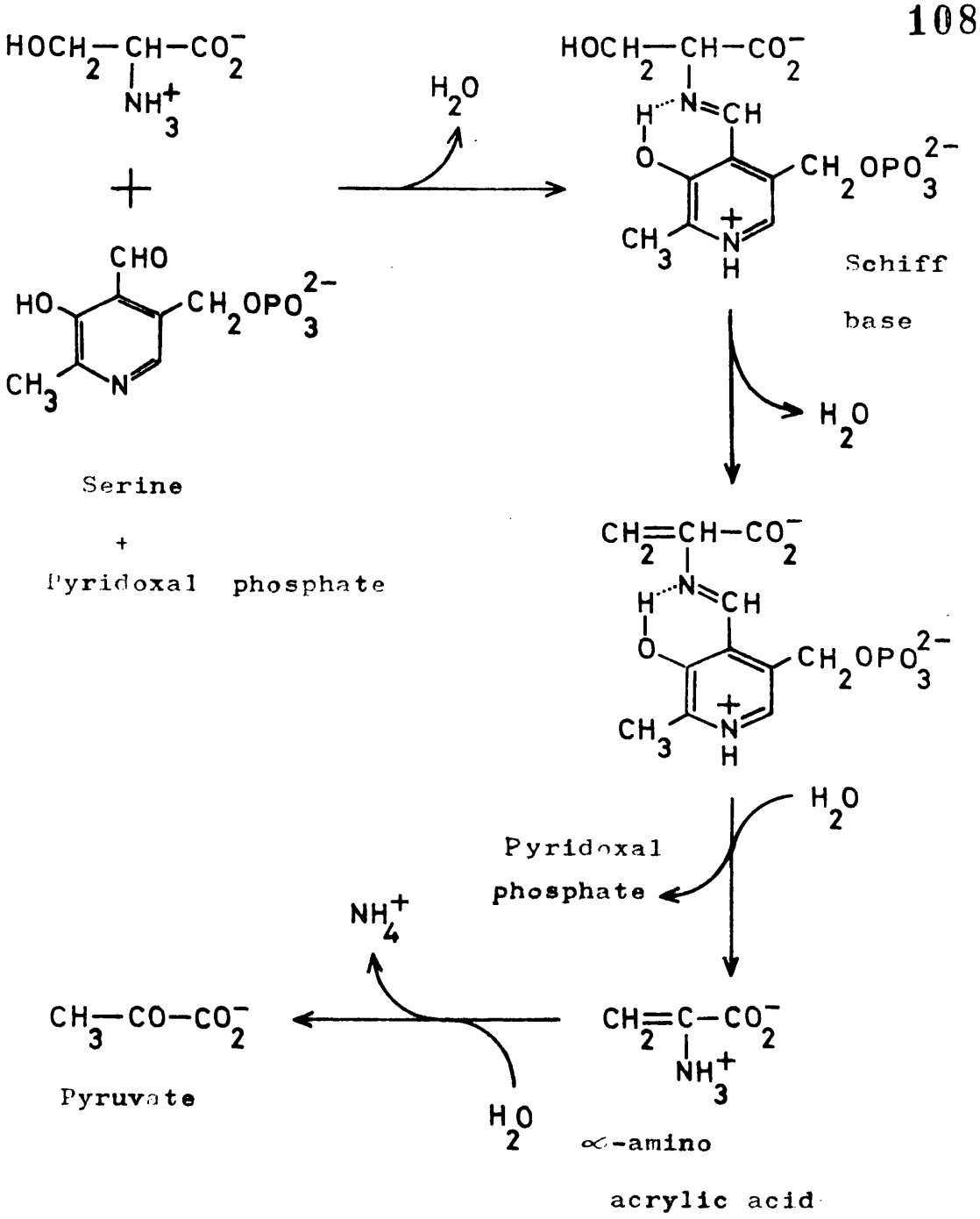


Fig.8. Mechanism for the dehydration of serine by pyridoxal phosphate enzymes, as proposed by Metzler and Snell (1952b).

The possibility that pyridoxal phosphate might be a coenzyme for serine deamination was first suggested by the results of nutritional studies (Fishman and Artom, 1944). Subsequently, a pyridoxal phosphate requirement was demonstrated for an apparently analogous deamination reaction catalysed by the enzyme tryptophanase (Wood, Gunsalus and Umbreit, 1947). Later, Yanofsky (1952) and Reissig (1952) both showed that pyridoxal phosphate stimulated the deamination of serine by cell-free extracts of Neurospora, while Metzler and Snell (1952b) obtained similar results with extracts from E.coli. At the same time, Metzler and Snell (1952a) showed that serine and other amino acids could be deaminated non-enzymically by pyridoxal phosphate in the presence of a divalent or trivalent cation. On the basis of their studies, Metzler and Snell (1952b) proposed a common mechanism for the enzymic and non-enzymic deamination of serine which is still favoured today. This mechanism, which is outlined in figure 8, involves as intermediates the condensation products (Schiff bases) formed between pyridoxal phosphate and serine and α-aminoacrylic acid, respectively, in addition to free α-aminoacrylic acid.

A pyridoxal phosphate requirement has been demonstrated for almost all enzymes which are known to deaminate serine and threonine. In some cases a partial requirement is readily demonstrated using a cell-free extract as the source of the enzyme, suggesting that the pyridoxal phosphate is only loosely bound to the enzyme (Yanofsky,1952;

Yanofsky and Reissig, 1953; Metzler and Snell, 1952b). In other cases the coenzyme appears to be more firmly bound, because it was found necessary to treat the enzyme with a pyridoxal phosphate-binding reagent before a requirement could be demonstrated (Umbarger and Brown, 1957; Nishimura and Greenberg, 1961; Dupourque, Newton and Snell, 1966). The L-serine dehydratase from Clostridium acidi-urici appears to be rather exceptional in this respect. First, it has not proved possible to resolve the enzyme and demonstrate a pyridoxal phosphate requirement for it. Secondly, the enzyme is quite insensitive to general inhibitors of pyridoxal phosphate enzymes, e.g. hydroxylamine, semicarbazide and cysteine (Benziman et al., 1960).

The majority of L-serine and L-threonine dehydratases do not require the presence of a cation for full activity. Nevertheless, the L-serine dehydratase from Clostridium acidi-urici has been activated twelve fold by Fe^{2+} and to a lesser extent by Mn^{2+} (Benziman et al., 1960). Also, the L-threonine dehydratases from yeast (Holzer et al., 1964) and from sheep liver (Nishimura and Greenberg, 1961) are activated by a number of monovalent cations, of which NH_4^+ and K^+ are the most effective.

Most enzymes with L-serine dehydratase activity form pyruvate (or ammonia) at a constant rate over a considerable period. However, it is of interest, in view of the results described in this thesis, that there are a few reports of enzymes which are exceptional in this respect. For example, an enzyme from rat liver is reported to exhibit a variable lag period in forming pyruvate from L-serine

(Fallon, Hackney and Byrne, 1966). On the other hand, the deamination of L-serine by L-threonine dehydratases from Sheep liver (Nishimura and Greenberg, 1961) and yeast (Boll and Holzer, 1965) ceases several minutes after L-serine is added to the enzyme. L-serine inactivates the latter two enzymes, apparently by reacting irreversibly with enzyme-bound pyridoxal phosphate.

The majority of L-serine and L-threonine dehydratases exhibit classical, Michaelis-Menten kinetics with respect to substrate concentration. That is to say, a plot of the velocity of the enzyme catalysed reaction against substrate concentration has the form of a rectangular hyperbola. On the other hand, biosynthetic L-threonine dehydratases from *E. coli* (Umbarger and Brown, 1957) and yeast (Boll and Holzer, 1965), both of which are sensitive to feedback inhibition by isoleucine, exhibit sigmoid saturation curves for both L-threonine and L-serine. A sigmoid saturation curve is taken to indicate the existence of co-operative interactions between several ligand-binding sites on a single protein molecule. The sites interact in a way such that, as they become successively occupied by ligand molecules, the affinity of vacant sites for the ligand is progressively increased. Thus the binding of a small amount of ligand to the protein facilitates the binding of more.

It is of interest, in view of the results to be discussed in this thesis, that the abnormal kinetic behaviour shown by L-threonine dehydratase from *E. coli*, towards both substrate and feed-back inhibitor, provoked one of the lines of investigation which

led to the concept of "allosteric interactions". The finding, that the inhibition of this enzyme by L-isoleucine is competitive with respect to L-threonine, came as a surprise in view of the dissimilarity between the structures of these two amino acids (Changeux, 1961). Further investigation revealed that after various treatments the enzyme lost its sensitivity to L-isoleucine, and that concomitantly the kinetics with respect to L-threonine concentration became normal. Similar results were observed at about the same time for aspartate transcarbamylase (EC 2.1.3.2) (Gerhart and Pardee, 1961) and phosphoribosyl-ATP pyrophosphorylase (Martin, 1963). These observations led both Monod and Jacob (1961) and Gerhart and Pardee (1962) to propose the existence of distinct but interacting sites for substrate and inhibitor. Monod, Changeux and Jacob (1963) emphasized the fact that the inhibitor and substrate are usually not "isosteric", but rather "allosteric", with respect to one another. For this reason inhibitors of this type, and activators also, became known as "allosteric effectors". The term allosteric is now used in a wider sense to describe the *indirect* interactions occurring between ligand molecules, whether of dissimilar or identical type, when bound at *distinct* sites on a protein molecule.

During studies on the pathway of glycine utilization in *A. globiformis*, difficulties were encountered in assaying the levels of L-serine dehydratase activity in cell-free extracts. The time course for the formation of pyruvate from L-serine was found to be non-linear. The rate of pyruvate formation increased continuously

at first and eventually, after a variable period, became constant. The duration of the initial "lag period" was found to be reduced by the presence of Mg^{2+}. It was also found that the substrate saturation curve, which was obtained by plotting the maximal linear rate of pyruvate formation against L-serine concentration, had a sigmoid form. These properties are in marked contrast to those exhibited by the same enzyme in toluene-treated cells. Under the latter conditions the enzyme formed pyruvate at a constant rate from the time of starting the assay, and exhibited classical, Michaelis-Menten kinetics with respect to L-serine concentration. Its activity was not stimulated by Mg^{2+}.

The first object of the studies reported in the following chapters was to determine the reason for the lag period in pyruvate formation. It was also of interest to know why the kinetic properties of the enzyme differ so radically when it is extracted from the cell.

In the course of these investigations it was found that L-serine causes a slow dimerization of the extracted enzyme. The rate of dimerization is increased by the presence of Mg^{2+}, and probably by other cations. A model which explains many of the properties of this enzyme has been devised on the basis of the model proposed by Monod, Wyman and Changeux (1965) for allosteric proteins.

CHAPTER 8

PROPERTIES OF L-SERINE DEHYDRATASE IN CELL-FREE PREPARATIONS

INTRODUCTION

The general enzymological properties of L-serine dehydratase from *A. globiformis* reported in this chapter were observed with cell-free extracts and partially purified preparations of the enzyme. Emphasis is placed on the properties which differ from those exhibited by this enzyme when it is studied in toluene-treated cells.

Glycine-grown cells were used as a source of the enzyme, except where stated to the contrary, because they contain a particularly high level of it.

METHODS

L-serine dehydratase assay methods.

Basal assay system. This contained in 1.0 ml.(μmoles): tris-HCl buffer, pH 8.2, 50; L-serine (adjusted to pH 8.2 with KOH) 50; $MgCl_2$, 10; and the enzyme preparation. The reaction was started by adding either L-serine or the enzyme preparation.

Discontinuous assay, estimating pyruvate as its 2,4-dinitrophenylhydrazone. The amount of pyruvate formed during a measured period of incubation at 30° (usually 15min) was estimated as its DNPhydrazone. The reaction was stopped by the addition of 0.33ml. of 0.1%(w/v) DNPhydrazine in 2M-HCl. Pyruvate DNPhydrazone was estimated as described in Chapter 2 (p. 29). This method was tested under the conditions of the L-serine dehydratase assay by adding known amounts of pyruvate to assay systems lacking either L-serine or the enzyme preparation and incubating for 15 min. before estimating pyruvate as its DNPhydrazone. The absorbance at 445nm. thus obtained was found to be directly proportional to the quantity of pyruvate added over the range 0.005-0.20μmole.

Continuous assays. Pyruvate formation was followed continuously by either of the following methods:

Lactate dehydrogenase method. Pyruvate formation was coupled to NADH oxidation with lactate dehydrogenase, and NADH disappearance followed spectrophotometrically at 340 nm. To the basal assay system (1.0ml) was added excess lactate dehydrogenase (the amount was determined empirically, but was usually 0.7 - 1.4 units), and 0.1 μmole NADH. This method is only suitable for L-serine dehydratase preparations which have

been freed from NADH oxidase activity.

$A_{315nm.}$ method. The molar absorbtivity of pyruvate at 315nm. is only 22 $\underline{M}^{-1}$ $cm.^{-1}$ (Chapter 2 p.32) and so very high rates of pyruvate formation can be estimated at this wavelength. The method has the advantage that it can be used with crude extracts.

Definitions of v_s and v_{15}

In assay systems containing the extracted enzyme, pyruvate formation eventually attains a constant, maximal rate. This rate will be called the "steady state velocity" and symbolized by v_s.

For some purposes the amount of pyruvate (or ammonia) formed during the first 15min. of an assay was estimated; this quantity has been symbolized v_{15}. It should be noted that v_{15} is a function of two independent variables: the rate of attainment of v_s and v_s itself. Thus the v_{15} estimation does not distinguish changes in one of these variables from changes in the other, nor yet from changes in them both. Neverless, v_{15} estimations have been found satisfactory for studying the effect of a variety of factors on one or other of these variables in cases where it is known, from parallel measurements, that the other variable remains constant.

Unit of L-serine dehydratase.

It should be noted that all estimates for the activity of the extracted enzyme are necessarily only approximate. This is because the specific activity of the enzyme, as determined from measurements of v_s, increases with its concentration, and only approaches a constant value

at extremely high concentrations (fig.11). Nevertheless, it was necessary to have some basis, however approximate, for comparing the activities of different enzyme preparations, and so the following unit has been defined.

A unit of extracted L-serine dehydratase is defined as that quantity of "fully activated" enzyme which forms 1μmole of pyruvate from L-serine per minute under the conditions of the assay. An enzyme preparation was considered to be "fully activated" at the point in an assay where the rate of pyruvate formation was constant and maximal, and when its specific activity was not increased by more than 10% by doubling its concentration. The latter condition was fulfilled when the rate of pyruvate formation in a 1 ml. assay system was in excess of 1μmole/min. at 23°.

Enzyme preparations were sometimes assayed at 30° and sometimes at room temperature (20-23°). The temperature employed in any particular instance is given as a subscript to the enzyme unit, e.g. a $unit_{30}$ of enzyme denotes a unit of enzyme at an assay temperature of 30°.

Separation of L-serine dehydratase from NADH oxidase.

The following procedure yields a preparation of L-serine dehydratase which is free from NADH oxidase activity, and so can be assayed by the lactate dehydrogenase method.

A column of G-100 sephadex (2.1 cm. diameter x 55 cm.) was equilibrated with 50mM-tris-HCl buffer, pH 7.5, containing 0.15M-KCl. Up to 5ml. of an extract from glycine-grown cells, which had been concentrated to 20-30mg. protein per ml. by ultrafiltration, was mixed with cytochrome c (2mg.) and blue dextran (2mg.) and applied to the column. The column was eluted with the buffer used above at a flow rate of approximately 30ml/hr. The column effluent was collected in 3ml. fractions. The single peak of L-serine dehydratase activity eluted between the peaks for blue dextran and cytochrome c with an elution volume relative to that for blue dextran of 1.5.

Purification of L-serine dehydratase

All operations in this procedure were carried out at 0-4°.

1. Extraction. Glycine-grown cells (72g. wet weight; approximately 23g. dry wt.) were suspended in 10mM-tris-HCl buffer, pH 8.0, containing 0.5mM-EDTA (500 ml.) and subjected to ultrasonic vibrations as described elsewhere (Chapter 2, p. 28). Cell debris was removed by centrifugation and the clear extract obtained was dialysed overnight in two batches, each against 2 l. of the tris-EDTA buffer.

2. Chromatography on DEAE-cellulose. A column of DEAE-cellulose (Whatman DE11)(4cm. diameter x 22.3cm.) was prepared and packed under slight pressure as described by Peterson and Sober (1962), and equilibrated with the tris-EDTA buffer used above. The dialysed extract (524ml. containing 3.3g. protein) was applied to the column, and the column washed with tris-EDTA buffer until the absorbance

Table 15. Purification of L-serine dehydratase from glycine-grown cells

The purification procedure is described in the text. Enzyme activities are based on v_s measurements at high enzyme concentrations, using the $A_{315nm.}$ method to follow pyruvate formation.

Fraction	Volume (ml.)	Total protein content (mg.)	Total enzyme content ($units_{30}$)	Specific activity ($units_{30}$/mg. protein)	Recovery (%)
Crude sonic extract	524	3270	3500	1.07	100
Pooled fractions from DEAE-cellulose column	234	283	2740	9.7	78
*Fraction above	234	283	1420	5.0	41
*Material precipitated by $(NH_4)_2SO_4$ (35-55% saturation)	14.7	79	1050	13.3	30

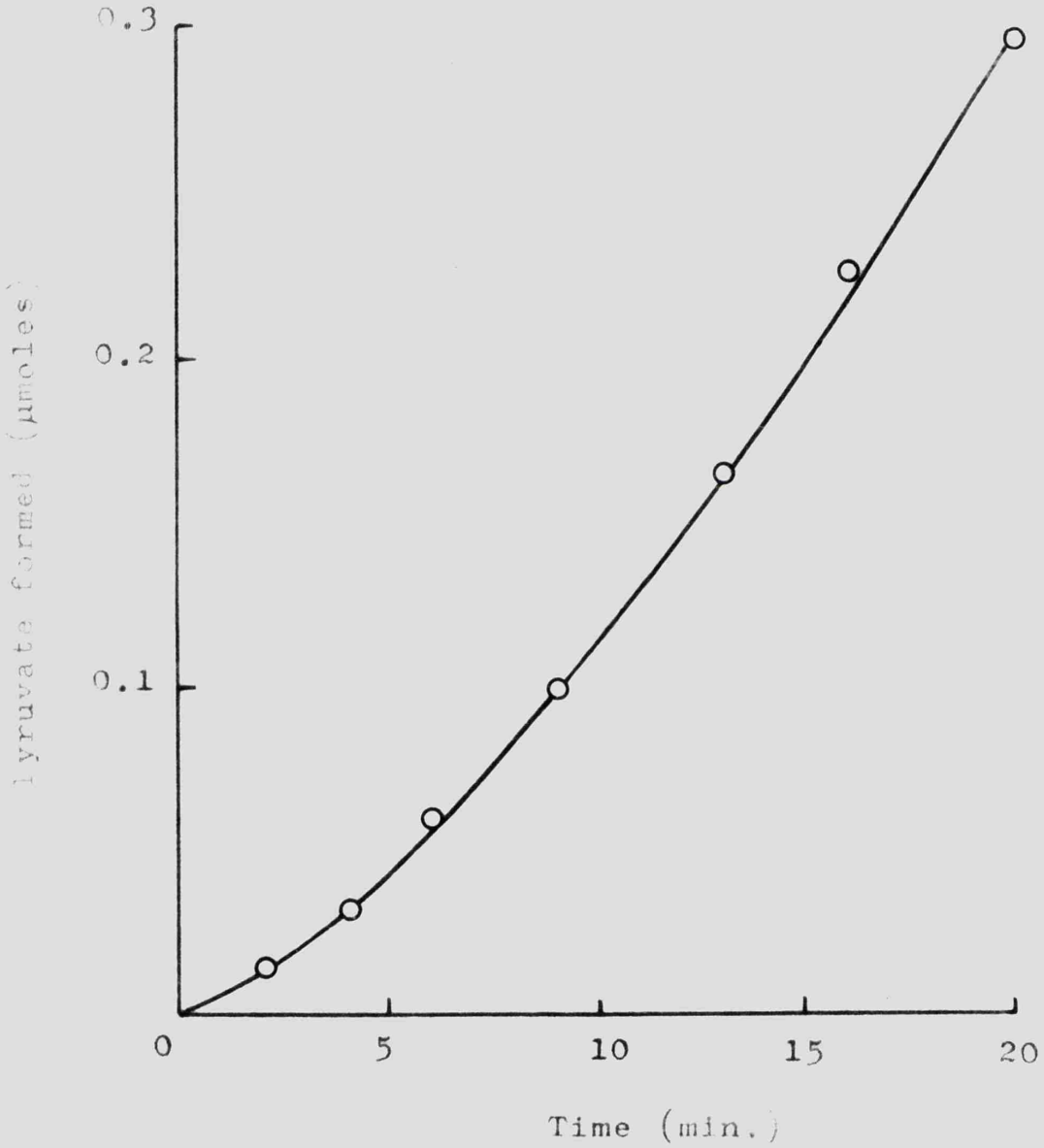
*The enzyme assays on which these results are based were performed 18hr. after those on which the other results are based.

of the effluent at 280nm. fell to a constant value of approximately 0.1. The washings did not contain detectable L-serine dehydratase activity. The column was then washed with tris-EDTA buffer containing 0.25M-KCl, until the absorbance of the effluent at 280nm. fell to a constant value as before. This eluted about 1.5% of the L-serine dehydratase activity originally applied to the column. Finally the column was washed with tris-EDTA buffer containing 0.43M-KCl to elute the enzyme. The effluent was collected in 15ml. fractions and those containing the bulk of the enzymic activity (fractions 15-28) were pooled (total volume 234ml).

3. Fractionation with ammonium sulphate. To the preparation from the previous stage, which had been made up to 250ml. with distilled water, was added, slowly, with constant stirring, 52.3g. of $(NH_4)_2SO_4$ (35% saturation). The resulting suspension was stirred for a further 15 min. and then centrifuged at 16000g. for 15 min. To the supernatant solution was added a further 35.5g. of $(NH_4)_2SO_4$ (55% saturation). The resulting suspension was stirred for 15 min. and centrifuged as before. The precipitate was dissolved in 12ml. of 0.1M-tris-HCl buffer, pH7.5, and dialysed overnight against 1 l. of 10mM-tris-HCl buffer pH 7.5. This preparation could be concentrated by ultrafiltration.

The results of this purification are presented in table 15. The low overall yield of the enzyme appears to be the consequence of leaving the preparation from stage 2 of the procedure overnight.

Fig.9. Time course for the formation of pyruvate from L-serine by a cell-free extract. The reaction was started by the addition of the extract. Aliquots (1.0ml.) were removed from the assay system (10ml.) at the times indicated and their pyruvate content estimated. The incubation temperature was 30°.


0.3
0.2
0.1
Pyruvate formed (μmoles)
0
5
10
15
20
Time (min.)

Thus it was noticed that the pooled fractions from the DEAE-cellulose column lost 50% of their activity in 18hr. at 0^{o}. It might be possible to improve the purification by defining more precisely the limits for precipitating the enzyme with $(NH_4)_2SO_4$.

In purifying the enzyme further, it might be possible to take advantage of the L-serine-induced change in its molecular weight, since other proteins are unlikely to be similarly affected. The partially purified enzyme preparation would be fractionated according to molecular weight, once in the presence of a saturating concentration of L-serine, and a second time in its absence. Either gel filtration (Chapter 11) or high-speed, zonal centrifugation could be employed to this end.

RESULTS

Kinetics of pyruvate formation

Figure 9 shows a typical time course for the formation of pyruvate from L-serine in an assay system containing a relatively low concentration of enzyme. The initial non-linear part of the time course, over which the rate of pyruvate formation increases continuously, is termed the "lag period". The gradient of the subsequent linear portion of the time course is the "steady state velocity"(v_s). A non-linear time course of this type is unusual for enzyme catalysed reactions. It was observed whether the enzyme was partially pure or not, and whether pyruvate appearance was followed discontinuously, by reaction with DNPhydrazine reagent, or continuously, by either the lactate dehydrogenase or $A_{315nm.}$ methods.

Fig.10. Effect of L-serine dehydratase concentration on the time course for pyruvate formation. Assays were started by adding to the assay system (1.0ml.), in the amounts indicated (µg. protein), a partially purified preparation of the enzyme (approximately 13units $_{30}$/mg. protein). The systems were incubated at 30° and pyruvate formation followed continuously by the lactate dehydrogenase method.

N.B. Zero on the abcissa only approximates to within ±0.5min. of the times of starting the assays.

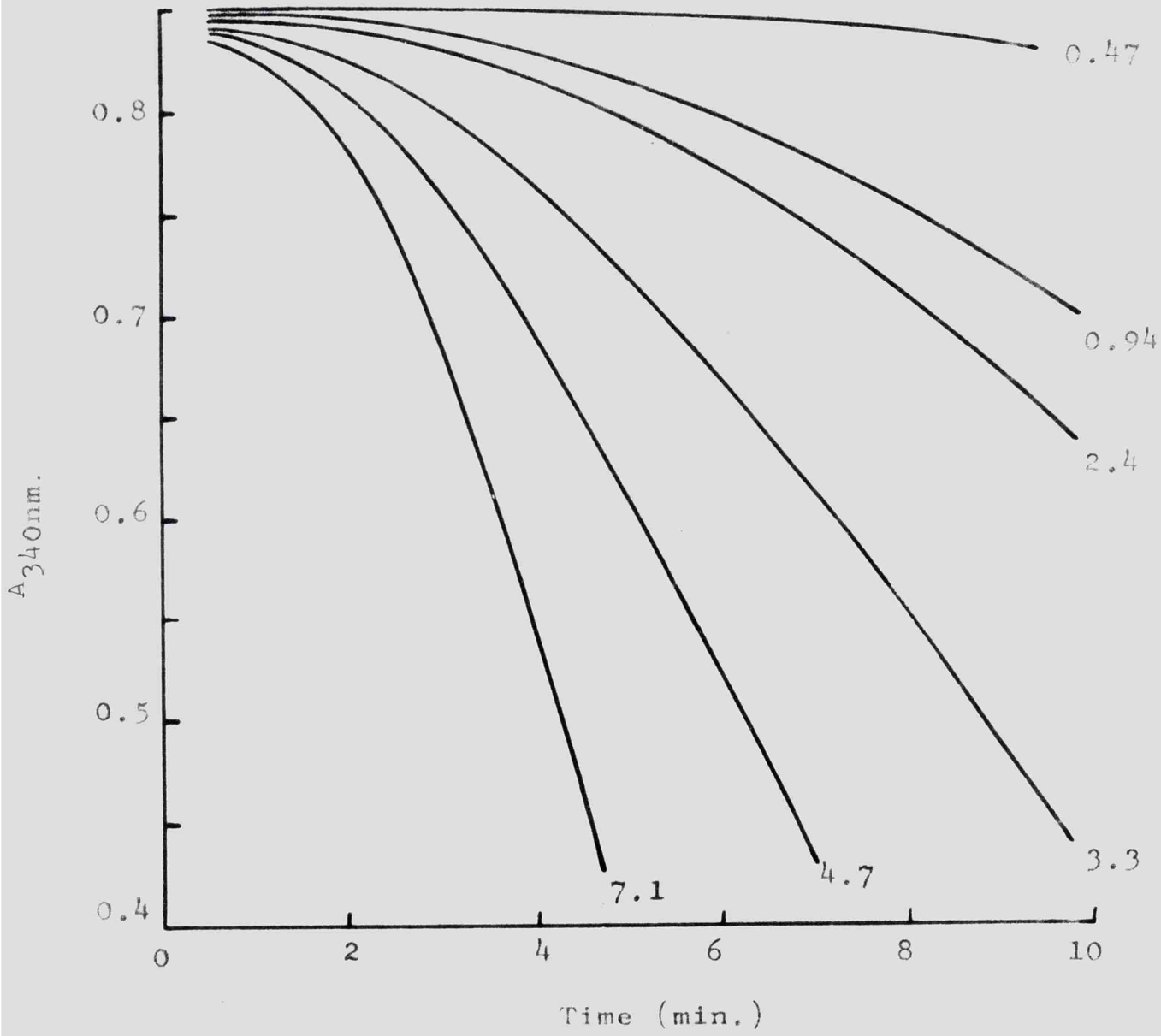
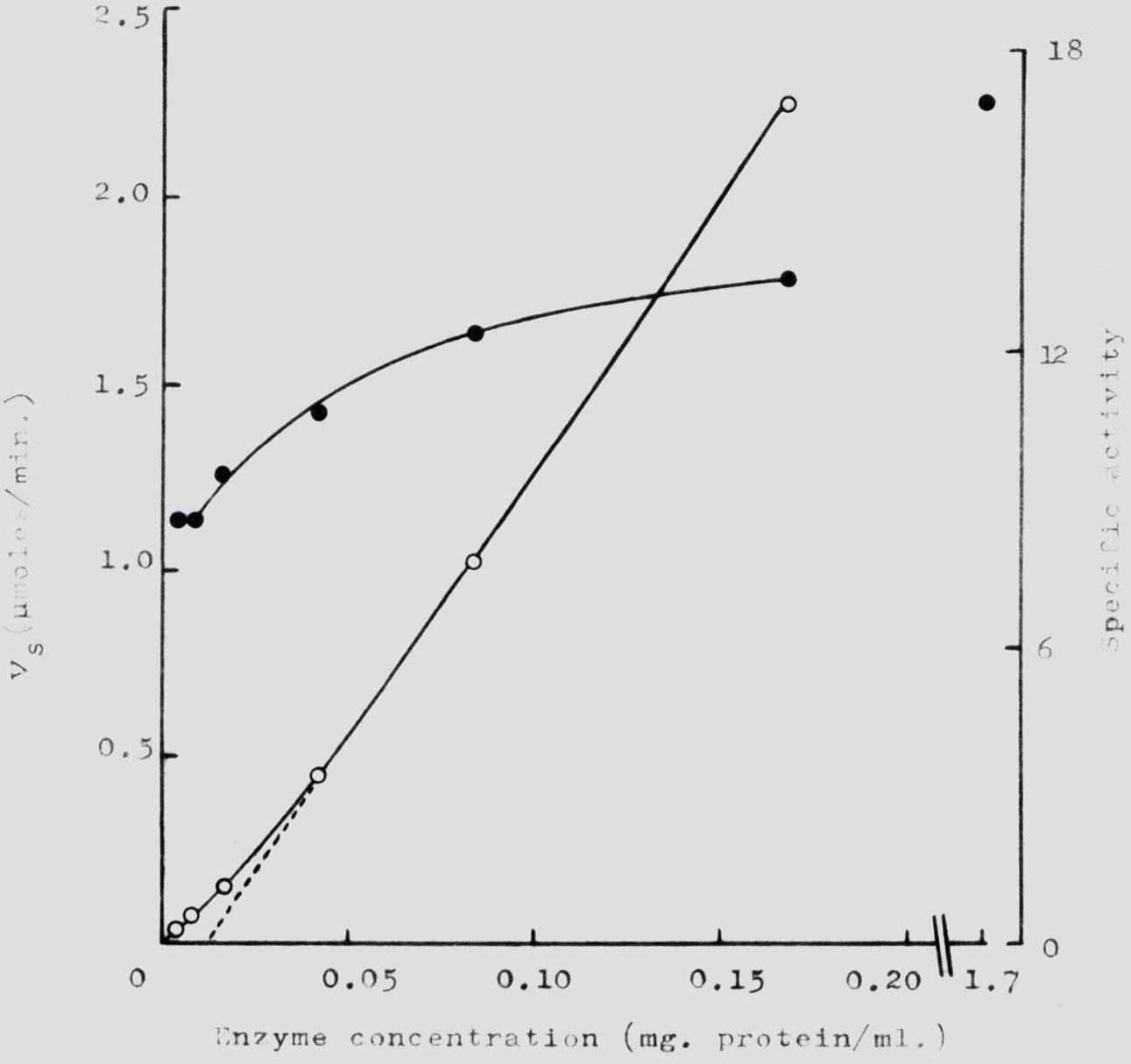

0.47
0.94
2.4
3.3
4.7
7.1
0.8
0.7
0.6
0.5
0.4
$A_{340nm.}$
0
2
4
6
8
10
Time (min.)

Fig.11. Effect of L-serine dehydratase concentration on v_s (o), and on the specific activity of the enzyme (●). Pyruvate formation was followed (at 23°) by the $A_{315nm.}$ method in assay systems containing a partially purified preparation of the enzyme, at the concentrations indicated, until a maximal, linear rate (v_s) was obtained. Specific activites (μmoles/min/mg. protein) were calculated from the values obtained for v_s.

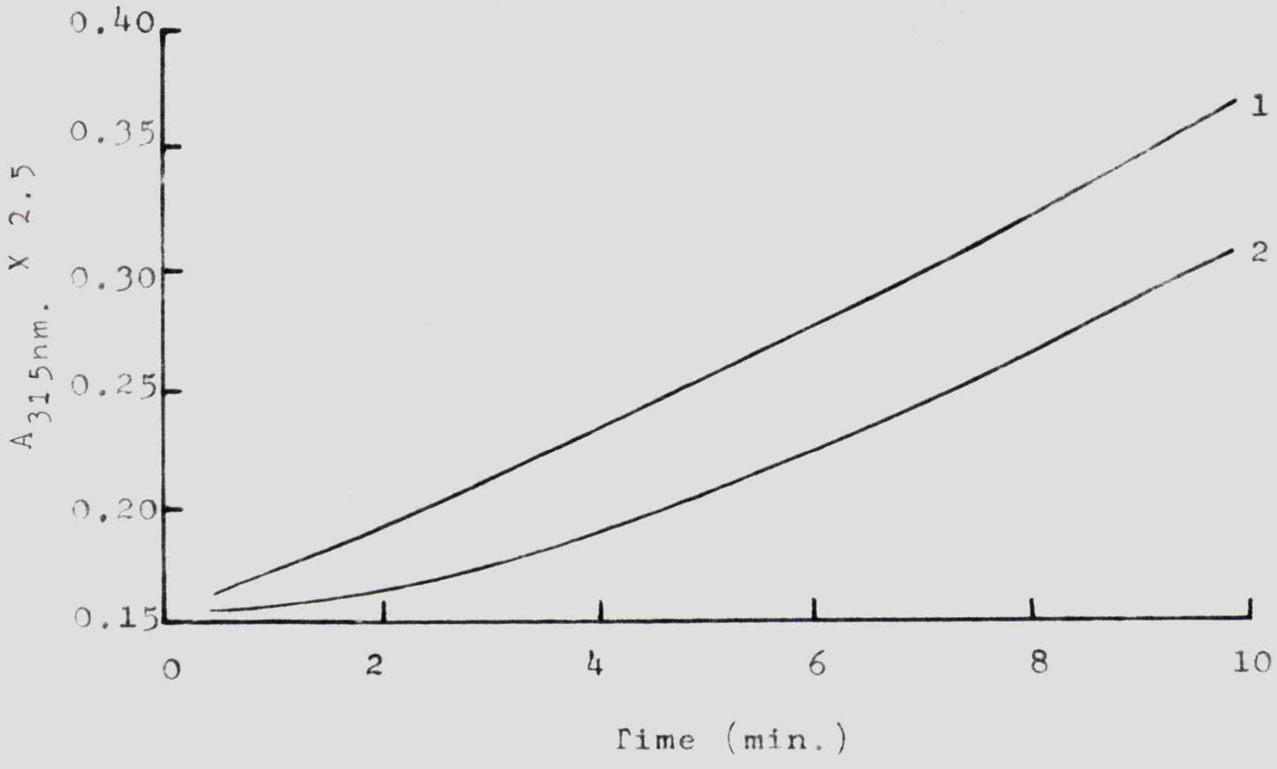

2.5
2.0
1.5
1.0
0.5
V_S (μmoles/min.)
18
12
6
0
Specific activity
0
0.05
0.10
0.15
0.20
1.7
Enzyme concentration (mg. protein/ml.)

Effect of enzyme concentration. The family of curves presented in figure 10 shows the effect of enzyme concentration on the time course for pyruvate formation. A mathematical analysis of the relationship between the form of these curves and enzyme concentration is delayed until chapter 12. At this point, it may be noted that the lag period becomes less pronounced as the enzyme concentration is increased, until it becomes quite undetectable at very high concentrations, e.g. at 14 $units_{23}$ of enzyme per ml.

When the v_s values attained at each of a range of enzyme concentrations are plotted against enzyme concentration, a curve is obtained which approaches linearity at high concentrations (fig.11). Consequently the slope of this curve, that is to say the specific activity of the enzyme, increases with increasing enzyme concentration to approach a limiting value at high concentrations (fig.11).

It was found that two assay systems containing identical amounts of enzyme protein showed different initial rates of pyruvate formation if the enzyme was added to one system as a concentrated preparation and to the other as a dilution of that preparation. For example, the addition of 0.05ml. of a preparation containing 30 $units_{30}$ of L-serine dehydratase per ml. to the usual assay system resulted in the formation of 0.12μmole of pyruvate during 15 min. at 0°. On the other hand, 5.0 ml. of a freshly prepared one hundredth dilution (in 10mM tris-HCl buffer, pH 8.2) of the same preparation produced only 0.064μmole of pyruvate in 15min. under these conditions. A similar result was obtained even when precautions were taken to ensure that the buffer

Fig.12. Time courses for the formation of pyruvate from L-serine in two assay systems (1.0ml.) containing equal amounts of enzyme protein which had been added in the following different ways. To system 1 was added 0.04ml. of a cell-free extract and to system 2,0.80ml. ofafreshly prepared $^{1}/_{20}$ dilution (in 10mM-tris-HCl buffer, pH8.2) of the same extract. $MgCl_2$ was omitted from both systems. Following the addition of extract, pyruvate formation was followed (at 22°) by the $A_{315nm.}$ method. Values for the maximal rate of pyruvate formation in the two systems were 0.60µmoles/min. for number 1 and 0.57µmoles/min. for number 2.


0.40
0.35
0.30
0.25
0.20
0.15
$A_{315nm.}$ X 2.5
1
2
0
2
4
6
8
10
Time (min.)

used to dilute the enzyme preparation did not contain heavy metal ions which might have inhibited the enzyme. The diluent was prepared using water which had just been distilled twice in glass apparatus, and it contained, in addition to tris-HCl buffer, 0.5mM-EDTA to chelate any heavy metal ions present.

On following pyruvate formation continuously with time in this type of experiment, it became evident that diluting the enzyme prior to its addition to the assay system caused it to exhibit a more pronounced lag period (fig.12). On the other hand, diluting the enzyme at this point had no effect on the v_s attained.

The following experiment is taken to suggest that the reversible inactivation of the enzyme, which accompanies its dilution, is complete within 10-30 sec. of preparing the dilution at 0°. An enzyme preparation at 0° was diluted into buffer at this temperature and aliquots were removed from the dilute preparation at intervals measured from the time of dilution. The v_{15} values obtained for these aliquots were compared with those obtained simultaneously for the same amount of enzyme protein taken from the concentrated preparation. The decrease in v_{15} which accompanied dilution of the enzyme was complete at the time of taking the first aliquot, i.e. 10-30 sec. after preparing the dilution.

Effect of cations. At concentrations up to and exceeding 0.1 $units_{23}$/ml, the extracted enzyme produces little pyruvate in the first 15 min. of an assay when the assay system lacks an added cation. Any one of a variety of monovalent or divalent cations will, when added to the assay system, cause a large increase in the v_{15} observed under these conditions.

Table 16. Relative effectiveness of a variety of monovalent cations in stimulating the "activity" of L-serine dehydratase.

Assay systems (1.0ml.) contained 75μmoles of L-serine adjusted to pH8.0 with KOH, an extract of glycine-grown cells and 50μmoles of the compound supplying the cation (adjusted to pH8.0, where necessary, with HCl). Pyruvate formed after 15 min. incubation at 30° (v_{15}) was estimated as its DNPhydrazone. The pKa values were used to calculate the concentrations of the cationic species.

Source of cation	pKa	Concentration of cation (calculated) (mM)	Relative enzyme "activity" $\left[\frac{v_{15} \times 50}{v_{15} \text{ for KCl}}\right]$
KCl		50	50
Ethanolamine-HCl	9.5	49	48
NH_4Cl	9.25	47	44
Diethanolamine-HCl	8.9	45	38
Tris-HCl	8.08	27	29
Triethanolamine-HCl	7.80	19	23
Imidazole-HCl	6.95	4	7
None		0	2

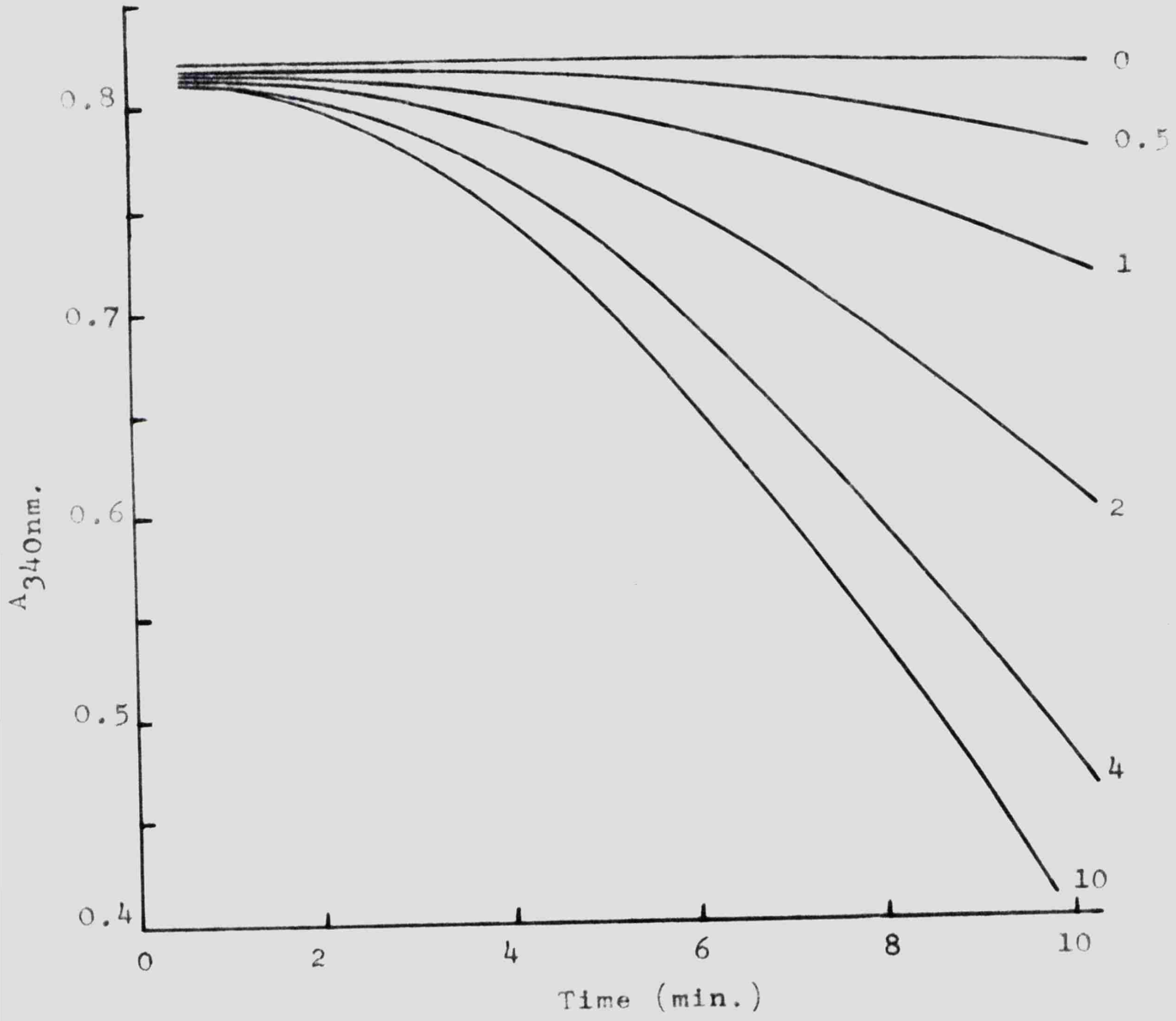
Table 17. Effect of K^+, Mg^{2+} and Ca^{2+} on the "activity" of L-serine dehydratase

The experimental conditions were as described in the legend to table 16. Cations were added to the assay systems at concentrations which produced a maximal v_{15}.

Cations added to assay system:			v_{15}
KCl (mM)	$MgCl_2$ (mM)	$CaCl_2$ (mM)	(μmoles/15min.)
-	-	-	0.001
150	-	-	0.059
-	15	-	0.064
-	-	15	0.068
150	15	-	0.061
150	-	15	0.068

Fig.13. Effect of Mg^{2+} concentration on the time course for pyruvate formation. Assay systems (1.0ml.) contained 50mM-L-serine adjusted to pH8.2 with KOH, as substrate and buffer, $MgCl_2$ at the concentrations (mM) indicated, a partially purified preparation of L-serine dehydratase ($0.07units_{23}$), which had been dialysed against 10mM-tris-HCl buffer, pH7.2, and the usual concentrations of lactate dehydrogenase and NADH. NADH oxidation was followed at 340nm; the incubation temperature was 23^{o}.

N.B. Zero on the abcissa only approximates to within ±0.5min. of the times of starting the assays.


0.8
0.7
0.6
0.5
0.4
$A_{340nm.}$
0
0.5
1
2
4
10
0
2
4
6
8
10
Time (min.)

A maximal v_{15} was obtained with a 15mM concentration of either Mg^{2+} or $Ca2^{+}$, or with a 150 mM concentration of K^{+}. Mn^{2+} was nearly as effective as Mg^{2+} when compared at a concentration of 1mM, whereas Fe^{2+} was without effect at this concentration. Other effective monovalent ions included Na^{+}, Li^{+} (either of which was about 60% as effective as K^{+} when compared at a concentration of 50mM), NH_4^{+} and the cationic forms of mono-, di- and triethanolamine, tris and imidazole. NH_4^{+} and the ions of the organic bases dissociate into uncharged species to a variable extent at the pH of the assay. When the degree of dissociation is taken into account, the cationic form of each of these substances is found to be approximately as effective as K^{+} in stimulating v_{15} (table 16). These comparisons assume that v_{15} is directly proportional to the concentration of each of the cations over the concentration range 0-50mM. This relationship has been established for K^{+}.

The simultaneous presence of monovalent and divalent cations, each at its optimal concentration, did not increase the v_{15} obtained with either alone (table 17).

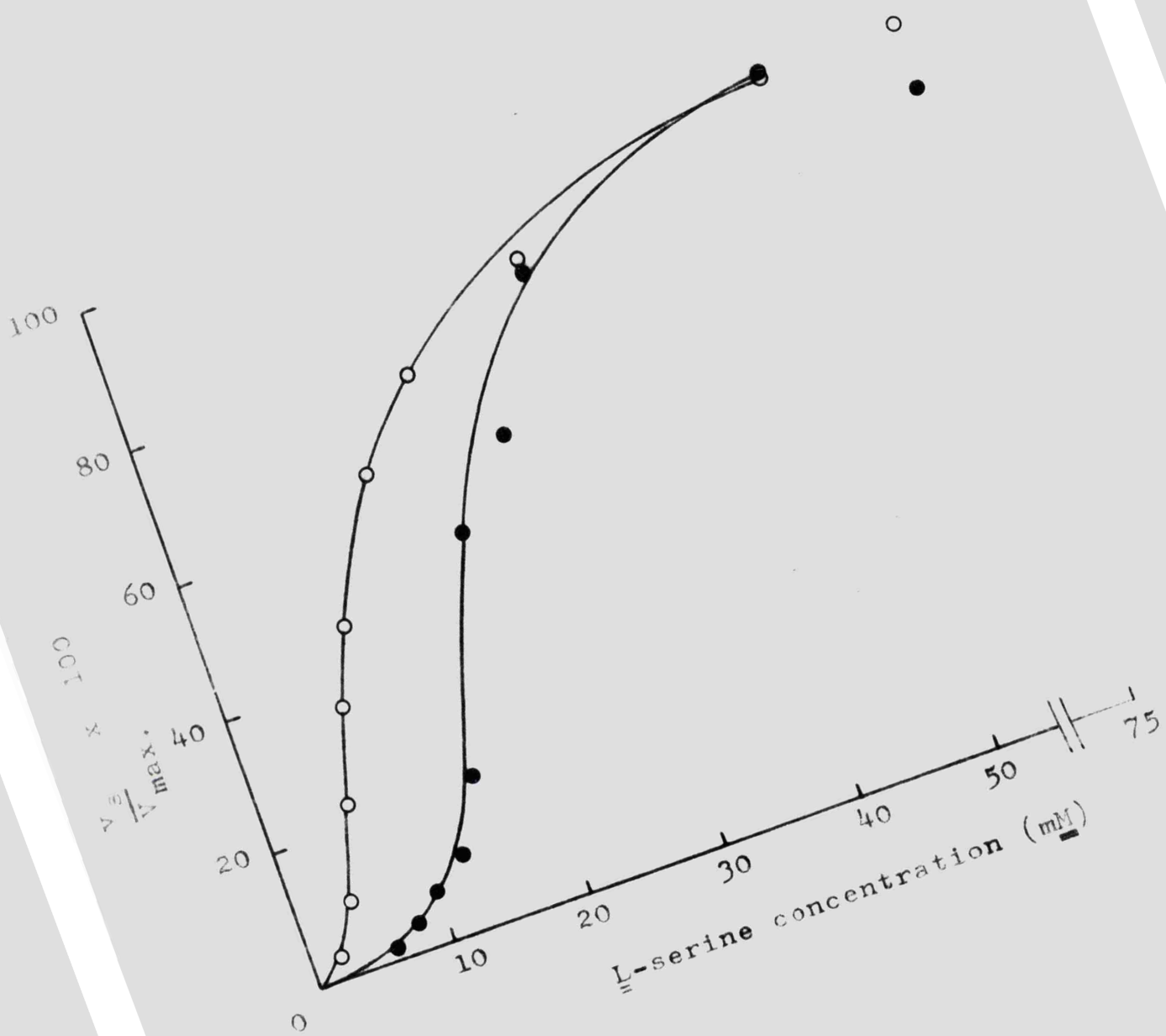
Mg^{2+} was chosen to satisfy the cation requirement in the standard assay system and for studies of the effect of a cation on the kinetics of pyruvate formation. The family of curves presented in figure 13 shows the effect of Mg^{2+} concentration on the time course for pyruvate formation. It can be seen that the amount of pyruvate formed over an initial period of, for example, 5 min. was progressively reduced by decreasing the Mg^{2+} concentration from 10mM to zero. On the other hand,

Table 18. Effect of Mg^{2+} concentration on v_s

The experimental details have been described in the legend to fig.13. Evidence is presented in the text which suggests that the rates of pyruvate formation (in square brackets) measured at 0.5 & 1.0mM concentrations of $MgCl_2$ were not true v_s values.

Concentration of $MgCl_2$ (mM)	v_s (μmoles/min.)
0.5	[0.008]
1.0	[0.011]
2.0	0.018
4.0	0.019
10	0.021

Fig.14. Curves for the saturation of L-serine dehydratase by L-serine at high (14units$_{23}$/ml.) (o) and low (0,035 units$_{23}$/ml.) (●) enzyme concentrations. The enzyme preparation employed had been partially purified. Maximal, linear rates of pyruvate formation (v_s) were estimated by the lactate dehyrogenase method at the low enzyme concentration, and by the $A_{315nm.}$ method at the high concentration. The incubation temperature was 23°. $V_{max.}$ was taken to be the maximal value observed experimentally for v_s. At both enzyme concentrations the value of v_s was not increased further by increasing the L-serine concentration above 75mM.


100
80
60
40
20
0
$v = \frac{V}{V_{max.}} \times 100$
10
20
30
40
50
75
L-serine concentration (mM)

v_s was very little affected by decreasing the Mg^{2+} concentration from 10mM to 2mM (table 18). It has been concluded from these results that the presence of Mg^{2+} diminishes the lag period without having any effect on v_s. The low values obtained for v_s at concentrations of Mg^{2+} lower than 2mM are not thought to be reliable. This is because it can be deduced (Chapter 12,p.224) that the lag period would have been so long under these conditions that the true v_s was not attained during the course of the assay.

Effect of L-serine concentration. The time course for pyruvate formation was not affected by L-serine concentration over the range 25 to 250mM.

A plot of v_s against L-serine concentration has a sigmoid form. The sigmoidicity of this type of plot was considerably reduced by increasing the enzyme concentration by four hundred fold, from 0.035 $units_{23}$/ml. to 14 $units_{23}$/ml.(fig.14).

Double reciprocal plots of these data (fig.20) are nonlinear; the departure from linearity is less marked at the higher enzyme concentration.

It will be seen from the saturation curves presented in figure 14 that the concentration of L-serine producing 50% of the maximal v_s, i.e. the K_m for L-serine, varies with enzyme concentration. At the lower enzyme concentration the K_m was approximately 21mM, whereas at the higher concentration it was approximately 11mM. Both of these values are rather high by comparison with the Km's of L-serine dehydratases from other sources. For example, a value of 2mM is reported for the enzyme from *Clostridium acidi-urici* (Benziman et al, 1960).

Fig.15. Effect of pH on the form of a curve relating % maximal v_{15} to L-serine concentration. Potassium phosphate buffer was used to obtain pH's 6.0 (o),6.5 (□), and 7.1 (●), and tris-HCl buffer to obtain pH's 8.0 (△) and 8.5 (▲). The concentration of cell-free extract employed at pH's 7.1,8.0 and 8.5 was increased by 1.3 and 2 fold for pH's 6.5 and 6.0, respectively. The amounts of pyruvate formed in 15min. at 30° (v_{15}) were determined in the usual way. At all pH's a maximal v_{15} was observed in the presence of either 50 or 100mM-L-serine.

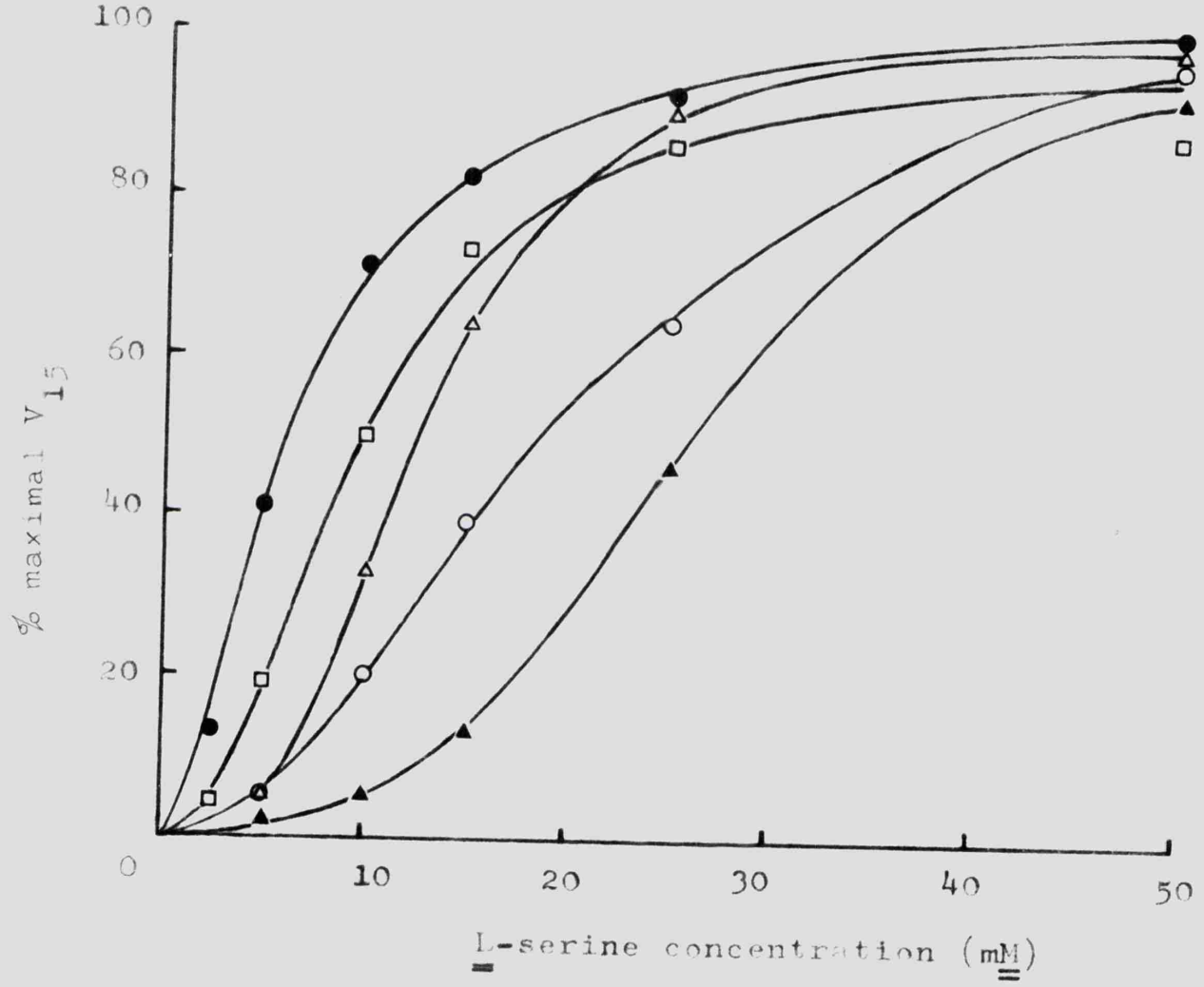

100
80
60
40
20
0
% maximal V_{15}
10
20
30
40
50
L-serine concentration (mM)

Fig.16. Effect of treating L-serine dehydratase with PHMB, at a partially inhibitory concentration, on the form of a curve relating % maximal v_{15} to L-serine concentration. A cell-free extract was preincubated for 5min. at 30° in the presence of 10^{-5}M PHMB and 50mM-tris-HCl buffer, pH8.2. This treatment caused approximately 50% inhibition of the L-serine dehydratase "activity" of the extract. Aliquots (0.10ml.) from the preincubation mixture were added to assay systems containing L-serine at a range of concentrations and the amounts of pyruvate formed in 15min. (v_{15}) determined (●). The second curve (o) was obtained using a similar concentration of the untreated extract.

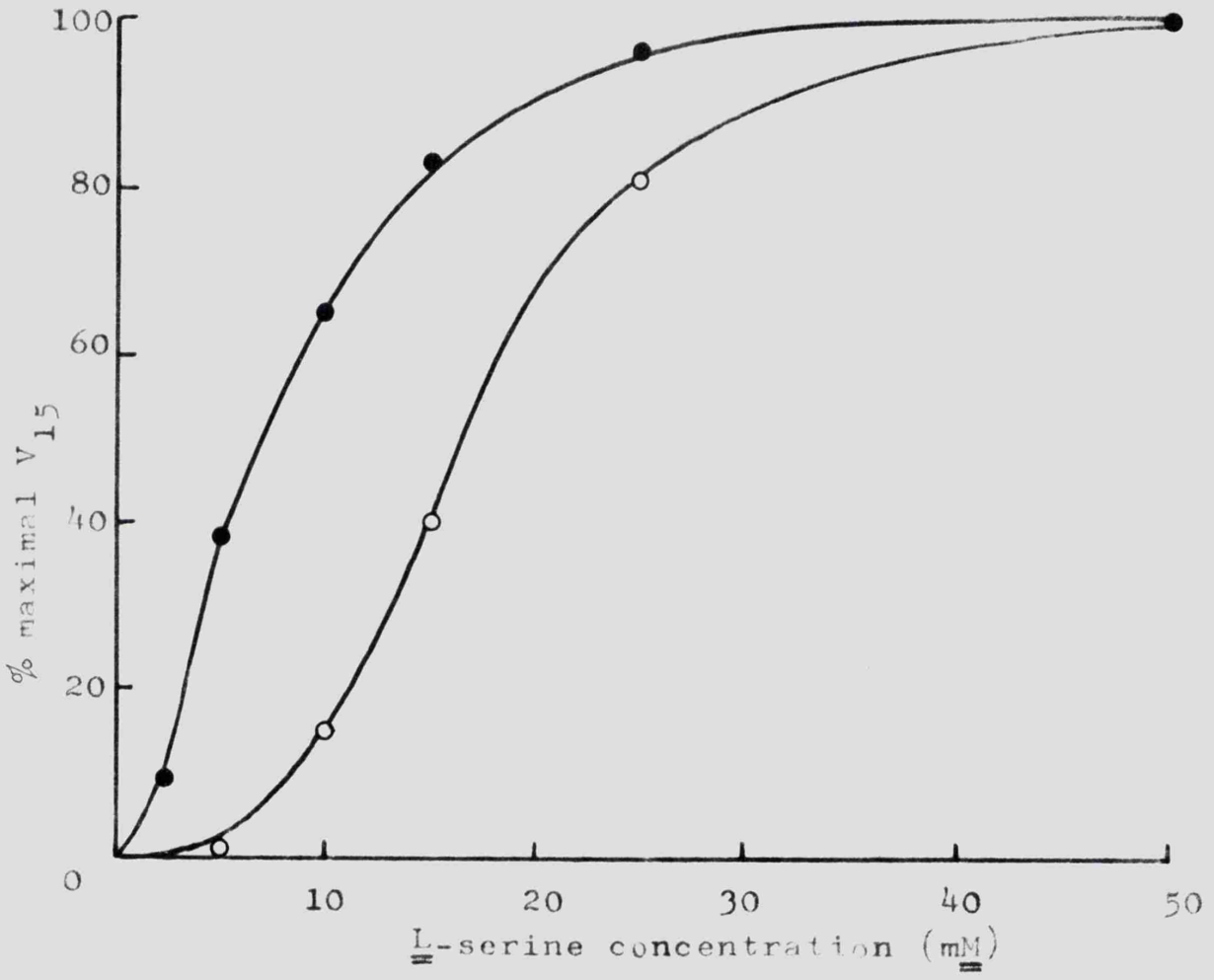
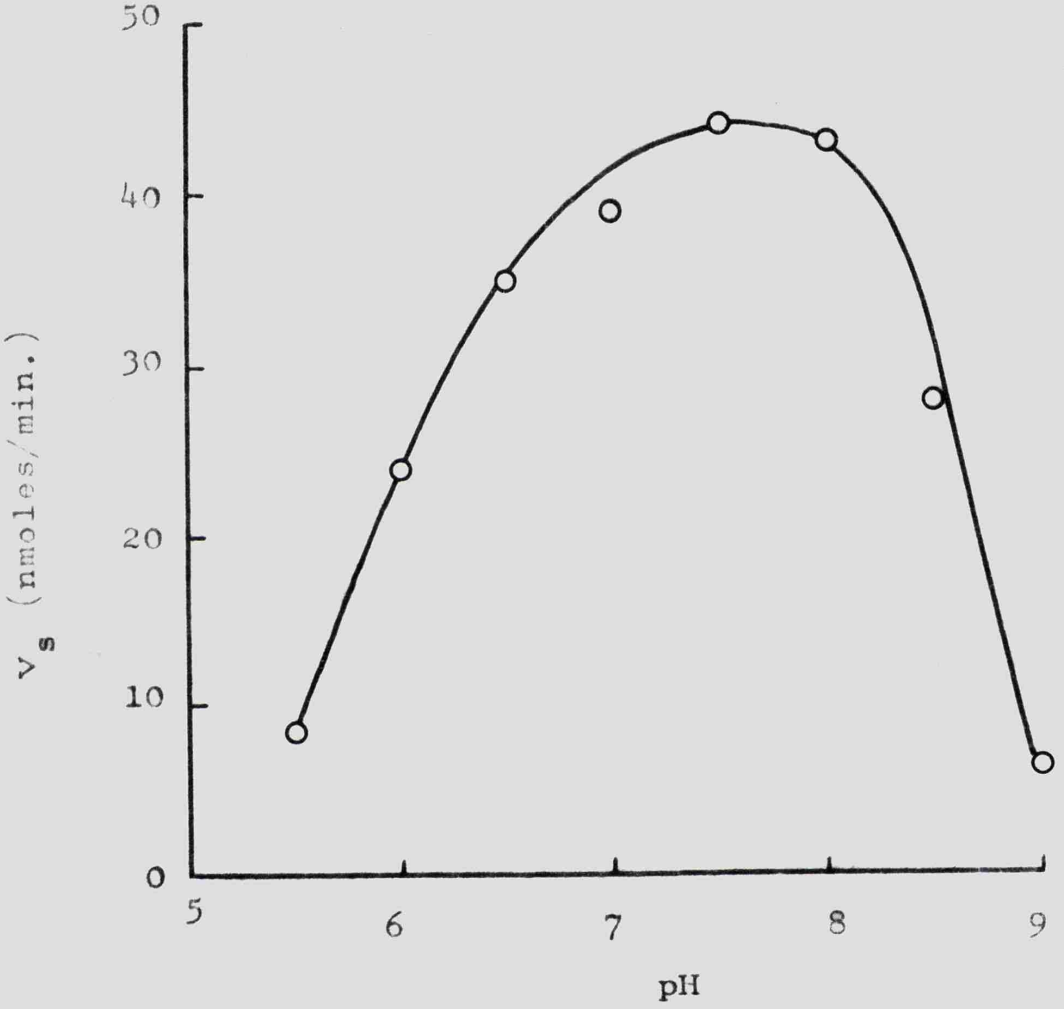

100
80
60
40
20
0
% maximal V_{15}
10
20
30
40
50
L-serine concentration (mM)

Fig.17. Effect of pH on the activity of L-serine dehydratase. Assay systems (1.0ml.) contained (μmoles): tris-maleate-NaOH buffer at the pH's indicated, 100; L-serine, 30; a partially purified preparation of enzyme, 0.05units$_{30}$; and other components in the usual amounts. Maximal, linear rates of pyruvate formation (v_s) were estimated by the lactate dehydrogenase method; the incubation temperature was 30°.


50
40
30
20
10
0
v_s (nmoles/min.)
5
6
7
8
9
pH

A curve relating v_{15} to L-serine concentration was also sigmoid. Its form was altered by changes in pH, and by the presence of partially inhibitory concentrations of mercuric compounds. At pH 7.1 a curve of this type was barely sigmoid, but as the pH was increased to 8.5 or decreased to 6.0 the curve became increasingly sigmoid (fig.15). At pH 8.2 the sigmoidicity of the curve was greatly reduced by the presence of p-hydroxymercuribenzoate (PHMB) (fig.16) or mercuric chloride (not shown), at concentrations causing approximately 50% inhibition.

Effect of pH on enzyme activity

The enzyme was active over a fairly broad pH range. Its pH optimum was around 7.5-8.0 (fig.17). This is similar to the pH optima reported for L-serine dehydratases from other sources (Selim and Greenberg, 1959; Benzimann et al, 1960).

Specificity towards L-serine.

Partially purified L-serine dehydratase was tested for the ability to form oxo-acid from D-serine, L-threonine, and L-cysteine. Its activity towards these amino acids was less than 2% of the activity shown towards L-serine. Cell-free extracts with L-serine dehydratase activity did not form detectable amounts of oxo-acid from DL-homoserine, DL-α-methyl-serine and L-serineamide. A small amount of oxo-acid was formed from DL-O-methylserine, but this was less than 5% of that formed from L-serine under the same conditions. Thus the enzyme is an L-serine-specific dehydratase like those from *Clostridium acidi-urici* (Benziman et al, 1960) and sheep liver

Table 19. Inhibition of L-serine dehydratase by carbonyl reagents.

An extract from glycine-grown cells was preincubated (at 30°) with the carbonyl reagent (neutralized where necessary with KOH) in the presence of the usual concentrations of tris-HCl buffer, pH8.2, and $MgCl_2$ (final volume, 0.85ml.) for 30 min., or 60 min. in the case of systems 1B and 2B. L-serine (0.15ml., 0.33 M) was then added, followed, after a further 15 min. incubation, by 0.2ml. 25% (w/v) perchloric acid. The assay systems were deproteinized as described in the legend to table 3 and aliquots (0.2ml.) from them were assayed for NH_3.

No.	Carbonyl reagent	Amount added (μmoles)	v_{15} (μmoles/ 15min.)	Inhibition (%)
1A	None	-	1.15	0
2A	Cyanide	1	1.15	0
3A	Hydroxylamine	1	0.56	52
4A	Phenylhydrazine	1	0.42	63
5A	Semicarbazide	10	1.22	0
1B	None	-	1.05	0
2B	Isoniazid	20	0.87	17

(Sayre and Greenberg, 1956).

Inhibition studies

Amino acids. The enzyme was inhibited approximately 50% by 20mM-D-serine when the concentration of L-serine was 50mM. L-cysteine was a potent inhibitor of the enzyme: a concentration of 1.7mM caused approximately 45% inhibition. On the other hand, L-cystine and L-S-methylcysteine, two compounds structurally related to L-cysteine but lacking a free thiol group, were not inhibitory at a concentration of 20mM. It should be noted that inhibition of pyridoxal phosphate enzymes by L-cysteine is not restricted to those enzymes whose substrates resemble it. It is thought to result from the formation of a stable thiazolidene ring by a condensation between the thiol and amino groups of L-cysteine and the aldehyde group of pyridoxal phosphate (Braunstein, 1960).

Carbonyl reagents. These inhibit pyridoxal phosphate enzymes generally, by reacting with the aldehyde group of the coenzyme. L-serine dehydratase was sensitive to inhibition by phenylhydrazine and hydroxylamine (table 19) but relatively insensitive to isoniazid, and completely insensitive to semicarbazide and cyanide at the concentrations employed.

Mercuric compounds. The enzyme was sensitive to inhibition by PHMB and Hg^{2+}. Its activity was approximately 70% inhibited following preincubation with 15μM-PHMB (diluted to 1.5μM in the assay system) or 0.2μM-$HgCl_2$ (diluted to 0.17μM in the assay system). At somewhat higher concentrations, these reagents are reported to cause inhibition of the L-threonine dehydratases from *E.coli* (Wood and Gunsalus, 1949) and Sheep liver (Nishimura and Greenberg, 1961).

Does the enzyme possess a pyridoxal phosphate coenzyme?

The sensitivity of the enzyme to inhibition by low concentrations of L-cysteine, phenylhydrazine and hydroxylamine is consistent with the probability that it has a pyridoxal phosphate coenzyme. Nevertheless, extracts which had lost L-serine dehydratase activity, as a result of either of the following treatments, did not regain any of this activity upon subsequent incubation with pyridoxal phosphate.

(i) Dialysis overnight against a buffer containing 20mM-hydroxylamine (Nishimura and Greenberg, 1961).

(ii) Prolonged dialysis against a buffer containing 20mM-L-cysteine, 20mM-mercaptoethanol and 50mM-potassium phosphate, pH 7.5 (renewed twice during a total period of 67 hr.). Bovine Serum albumin (final concentration 2mg./ml.) was added to the enzyme preparation before dialysis to stabilize any apoenzyme that might be formed (Dupourque, Newton and Snell, 1966). Before assaying the enzyme, it was passed through a column of G-25 sephadex to remove the L-cysteine and mercaptoethanol.

Finally, the activity of the partially purified enzyme was not stimulated by incubation with pyridoxal phosphate.

The failure to resolve the enzyme is probably evidence for the firmness with which the pyridoxal phosphate is bound to it, rather than evidence that it does not possess this coenzyme.

Table 20. Effect of temperature and concentration on the stability of L-serine dehydratase.

Temperature. Solutions containing an extract from glycine-grown cells (final concentration, 0.05-0.15mg. protein/ml.) and 70mM-tris-HCl buffer, pH8.0, were stored for 14 hr. at the temperatures indicated. Aliquots were removed before and after the period of storage and assayed for L-serine dehydratase by estimating the pyruvate formed in 15min. at 30° (v_{15}).

Storage temperature (°C)	v_{15} (μmoles/15min.)		% original "activity" remaining after 14hr.
	Initially	After 14hr.	
~ -20 (frozen solid)	0.28	0.24	86
0	0.28	0.16	57
4	0.28	0.13	46
20-23	0.28	0.033	12
30	0.28	0	0

Concentration. Solutions containing a range of concentrations of an extract from glycine-grown cells and 10mM-potassium phosphate buffer, pH7.5, were stored for 23hr. at 4°.

contd.....

Table 20. contd.

Concentration of extract during storage (mg.protein/ml.)	v_{15}(µmoles/15min.) Initially	After 23hr.	% original "activity" remaining after 23hr.
25	0.31	0.16	52
2.5	0.20	0.10	50
0.25	0.19	0.064	34
0.05	0.15	0.003	2

Stability

The enzyme was found to be relatively unstable, particularly in dilute solution and at temperatures higher than 0° (table 20). It was most stable at pH 7.0-7.5. The presence of 1% (w/v) bovine serum albumin or 0.5mM-L-cystine increased the L-serine dehydratase activity retained by a dilute extract, over a period of 24 hr. at 4°, from 24% to approximately 60% of the original activity.

Does the extraction procedure affect the properties of the enzyme?

The possibility was examined that the unusual properties described for the enzyme in ultrasonic extracts might be an artefact produced by the ultrasonic treatment. The finding that extracts prepared with the French Press or by the action of lysozyme showed the usual lag period in forming pyruvate from L-serine virtually rules out this possibility.

Properties of the enzyme in extracts from glucose-grown cells.

The relatively low L-serine dehydratase activity associated with extracts from glucose-grown cells exhibited a similar lag period in forming pyruvate as did extracts from glycine-grown cells.

DISCUSSION

A discussion of the results presented in this chapter, and in subsequent chapters, is delayed until Chapter 12 so that they can be considered together in terms of the model proposed for L-serine dehydratase.

SUMMARY

The L-serine dehydratase extracted from *A. globiformis* exhibits the following unusual properties:

(i) The time course for the appearance of pyruvate from L-serine is non-linear. The rate of pyruvate formation increases continuously at first, and eventually after a variable period, becomes constant (the constant rate is symbolized v_s).

(ii) The specific activity of the enzyme, as calculated from v_s, increases with increasing enzyme concentration, tending towards a limiting value at very high concentrations.

(iii) At relatively low enzyme concentrations, the amount of pyruvate formed during the first 15min. of an assay is greatly increased by the presence of relatively low concentrations of Mg^{2+}, Ca^{2+}, or Mn^{2+}, or by considerably greater concentrations of a variety of monovalent cations. The presence of Mg^{2+} has been shown to diminish the lag period without affecting v_s.

(iv). The enzyme exhibits a sigmoid saturation curve for L-serine. The sigmoidicity of this curve is decreased by increasing the enzyme concentration to high levels, with the consequence that the K_m for L-serine is dependent upon enzyme concentration.

On the other hand, the enzyme resembles L-serine dehydratases from other sources in its pH optimum, its high specificity for L-serine and in its sensitivity to inhibitors of pyridoxal phosphate enzymes, and to inhibition by mercuric compounds.

CHAPTER 9

PROPERTIES OF L-SERINE DEHYDRATASE IN TOLUENE-TREATED CELLS

INTRODUCTION

Toluene has been used as an "unmasking agent" in the assay of a variety of microbial enzymes, including β-galactosidase (EC3.2.1.23) (Monod, Cohen-Bazire and Cohn, 1951), alkaline phosphatase (EC 3.1.3.1)(Levinthal, Signer and Fetherolf, 1962), and isocitrate lyase (Ashworth, 1965) in *E. coli* and L-arabinose isomerase (EC 5.3.1.4) in *Pediococcus pentosaceus* (Dobrogosz and De Moss, 1963).

Studies with *E. coli* suggest that toluene renders cells freely permeable to small molecules. For example, Jackson and De Moss (1965) showed that the addition of toluene to cells of *E. coli* which had been allowed to accumulate [^{14}C]methyl-β-thiogalactoside

(an inducer of β-galactosidase) caused a rapid efflux of radioactivity from the cells. Silver and Wendt (1967) obtained a similar result with cells which had been allowed to accumulate $^{42}K^{+}$. Toluene also stopped the uptake of $^{42}K^{+}$ under these conditions.

Although cells of *E. coli* lose most of their RNA and 20-25% of their protein within 30 min. of adding toluene to them (at 37°), they retain large molecules such as β-galactosidase and they appear to remain intact as seen under the electron microscope (Jackson and De Moss, 1965). The effects of toluene on cells are irreversible and lead to a rapid loss of viability.

Results obtained in the present studies with cells of *A. globiformis* are taken to suggest that toluene-treatment allows L-serine and Mg^{2+} free access to L-serine dehydratase without releasing this enzyme from the cell. These tentative conclusions are drawn from the following properties of toluene-treated cells: (i) They appear quite normal as seen under the light microscope. (ii) When a toluene-treated cell suspension is subjected to centrifugation, L-serine dehydratase sediments with the cells. (iii) They differ from untreated cells in that they form pyruvate from L-serine under anaerobic conditions. Untreated cells are unable to metabolize pyruvate under these conditions and yet they do not accumulate pyruvate when supplied with L-serine. This is presumably because there is an energy dependent mechanism for

transporting L-serine into the cell*.

(iv) The L-serine dehydratase activity of toluene-treated cells is inhibited by low concentrations of Mg^{2+}.

The results presented in this chapter, with the obvious exception of those in the section on glucose-grown cells, came from studies with glycine-grown cells.

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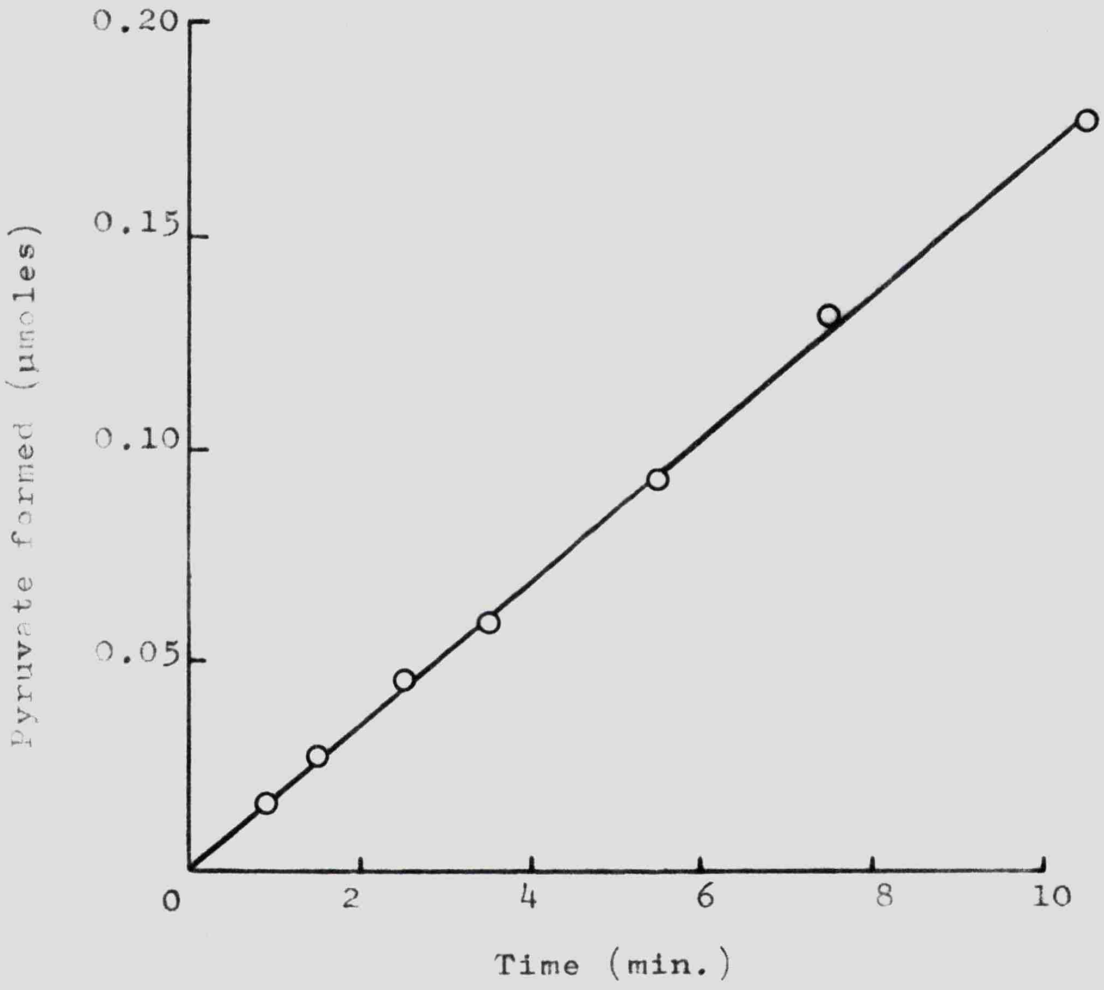
* This conclusion is also supported by the finding that, in the presence of 5mM-arsenite under aerobic conditions, a given quantity of whole cells forms pyruvate from L-serine at only one twentieth of the rate obtained for the same quantity of toluene-treated cells. Whereas 5mM-arsenite does not inhibit the L-serine dehydratase activity of extracts, it completely inhibits the pyruvate dehydrogenase activity of whole cells. This presumably restricts their energy supply, which in turn reduces the rate of serine uptake to the point where it limits the activity of intracellular L-serine dehydratase.

Fig.18. Time course for the formation of pyruvate from L-serine by toluene-treated cells. The reaction was started by adding to the assay system (10ml.) a suspension of toluene-treated, glycine-grown cells. (0.30mg. dry weight). Aliquots (1.0ml.) were withdrawn at the times indicated and their pyruvate content estimated. The incubation temperature was 30°.


0.20
0.15
0.10
0.05
0
2
4
6
8
10
Pyruvate formed (μmoles)
Time (min.)

METHODS

Assay for L-serine dehydratase activity of toluene-treated cells.

The procedure followed was that employed with the extracted enzyme, except that $MgCl_2$ was omitted from the basal assay system (Chapter 8, p115). Pyruvate was estimated as its DNPhydrazone.

Toluene-treated cells formed pyruvate at a constant rate during the first 15 min. of an assay (fig.18). Also, the amount of pyruvate formed in this period was, for amounts up to 0.15μmole, directly proportional to the quantity of cells added to the assay system (fig.4). Thus the amount of pyruvate formed by toluene-treated cells during the first 15min. of an assay could be used as a direct measure of their L-serine dehydratase activity.

Treatment of cells with toluene

This procedure has been described in full in Chapter 4 (p.51).

RESULTS

Kinetics of pyruvate formation

In contrast to extracts, toluene-treated cells formed pyruvate from L-serine at a constant rate without a detectable lag period (fig.18).

Specific activity

The specific activity of L-serine dehydratase in toluene-treated, glycine-grown cells was 1.0-2.0μmoles of pyruvate/min./mg. of cellular protein at 30°. This is in agreement with the maximal specific activity obtained for the enzyme in extracts from glycine-grown cells, i.e. 1.0-1.4 $units_{30}$/mg.protein.

Table 21. Effect of $MgCl_2$ on the L-serine dehydratase activity of toluene-treated cells.

Assay systems (1.0ml.) contained $MgCl_2$ at the concentrations indicated, tris-HCl buffer, pH8.2, and L-serine at the usual concentrations, and a suspension of toluene-treated, glycine-grown cells. Systems were incubated at 30°for 15 min.

$MgCl_2$ concentration (mM)	L-serine dehydratase activity (nmoles/min.)
0	9.3
0.1	8.4
1.0	5.3
10	4.7

Fig.19. L-serine saturation curves for the L-serine dehydratase activity of toluene-treated cells grown on glycine (●) and glucose (o) . Assay systems (1.0ml) containing glycine-(0.040mg. dry weight) or glucose-grown cells (0.8mg. dry weight) were incubated for 15min. at 30°. Values for $V_{max.}$ were extrapolated from plots of $1/v$ (where v is the rate of pyruvate formation) against the reciprocal of the L-serine concentration.

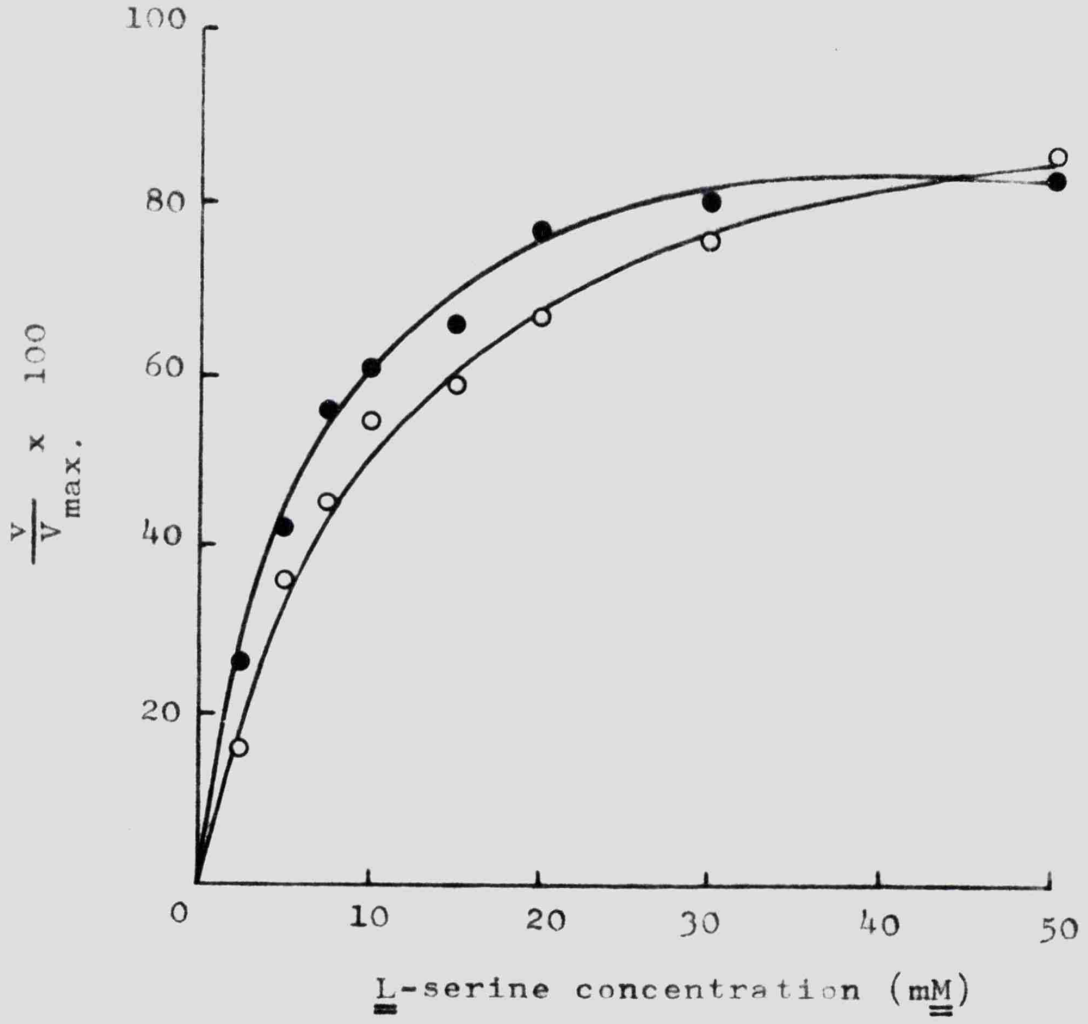
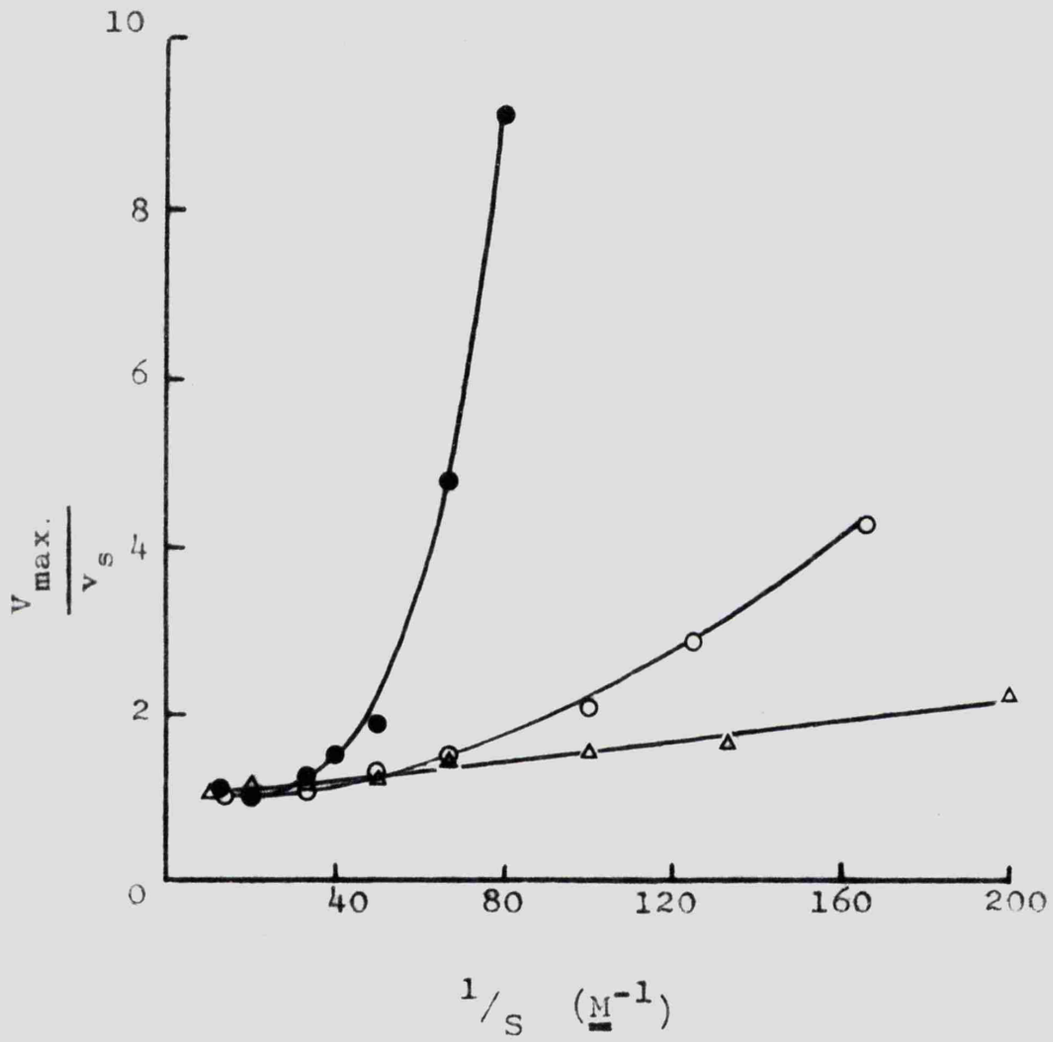

100
80
60
40
20
0
10
20
30
40
50
$\frac{v}{V_{max.}} \times 100$
L-serine concentration (mM)

Fig.20. Double reciprocal plots of the data for the saturation of L-serine dehydratase by L-serine. The data for the high (14units$_{23}$/ml.) (o) and low (0.035units$_{23}$/ml.) (●) concentrations of partially purified enzyme are taken from fig.14, and the data for toluene-treated,glycine-grown cells (△) from fig.19. S denotes L-serine concentration.


10
8
6
4
2
0
40
80
120
160
200
$\frac{V_{max.}}{v_s}$
$^1/_S$ ($\underline{M}^{-1}$)

The latter estimate was calculated from v_s at high enzyme concentrations.

Effect of magnesium ion.

The L-serine dehydratase activity of toluene-treated cells was slightly inhibited by the addition of Mg^{2+} to the assay system (table 21). This result is in complete contrast to the stimulating effect which Mg^{2+} has on the 'activity' of the extracted enzyme.

Effect of substrate concentration

The L-serine saturation curve obtained for the dehydratase activity of toluene-treated cells has the form of a rectangular hyperbola (fig.19). A double reciprocal plot of these data is linear (fig.20). These results contrast with the sigmoid, L-serine-saturation curves and non-linear, double reciprocal plots obtained for the extracted enzyme.

In the case of toluene-treated cells, the K_m for L-serine can be extrapolated from the linear, double reciprocal plot. A value of 7mM was obtained for cells grown on glycine. This is somewhat lower than the value of 11mM obtained for a high concentration of the enzyme extracted from glycine-grown cells. The latter value was determined by interpolation from the saturation curve.

Effect of pH.

The curve relating L-serine dehydratase activity to pH, for toluene-treated cells, was very similar to that obtained for the extracted enzyme.

Glucose-grown cells

The L-serine dehydratase activity of toluene-treated, glucose-grown cells, though present at a much lower level than the corresponding activity of glycine-grown cells, resembled it in the following ways:

(i) It formed pyruvate from L-serine at a constant rate without a detectable lag period.

(ii) It was not stimulated by the presence of added Mg^{2+}.

(iii) The saturation curve for L-serine was a rectangular hyperbola (fig. 19), from which a linear, double reciprocal plot was obtained. A value of 9mM was obtained for the Km for L-serine, by extrapolation from the double reciprocal plot.

Properties of L-serine dehydratase extracted from toluene-treated cells.

When toluene-treated cells were lysed with lysozyme (or disrupted by ultrasonic vibrations), the L-serine dehydratase liberated from them was identical in the following respects with that extracted from untreated cells:

(i) It exhibited a definite lag period in forming pyruvate from L-serine.

(ii) The amount of pyruvate it formed in a 15 min. assay was greatly increased by the addition of Mg^{2+} to the assay system.

(iii) It exhibited a sigmoid saturation curve for L-serine.

DISCUSSION

The properties of the enzyme extracted from toluene-treated cells are, so far as is known, identical with those of the enzyme extracted from untreated cells. Thus the differences between the properties of the extracted enzyme and those of the enzyme in toluene-treated cells are unlikely to result from some direct

effect of toluene on the enzyme itself. The change in properties undergone by the enzyme when it is liberated from the toluene-treated cell, and presumably from the untreated cell also, suggest that it becomes fundamentally altered in the process.

SUMMARY

1. The L-serine dehydratase activity of toluene-treated cells differs from that of cell-free extracts in the following ways:

a. It forms pyruvate from L-serine at a constant rate without a detectable lag period.

b. It is slightly inhibited by the presence of Mg^{2+}.

c. The saturation curve for L-serine has the form of a rectangular hyperbola; a double reciprocal plot of these data is linear.

2. Upon extracting the enzyme from toluene-treated cells, its properties become so altered as to be indistinguishable from those of the enzyme extracted from untreated cells.

CHAPTER 10

AN INVESTIGATION OF THE CAUSE FOR THE LAG PERIOD

INTRODUCTION

The lag period observed in assays of the extracted enzyme has been detected by three independent assay methods, two of which measured pyruvate formation continuously with time. It is known that this lag period did not result from a rise in temperature or change in pH of the assay system during incubation. It may therefore be assumed that the lag period results from a genuine property of the enzyme when extracted from *A. globiformis*.

Of the possible causes for the lag period, the following are considered to be the most likely:

1. Activation of the enzyme by a product of L-serine metabolism, e.g. pyruvate or ammonia.
2. Accumulation of a hypothetical intermediate which is necessarily involved in the conversion of L-serine into pyruvate and ammonia.
3. Slow activation of the enzyme by a cofactor present in the enzyme preparation or assay reagents.
4. Slow destruction of an inhibitor of L-serine dehydratase by an enzyme from the L-serine dehydratase preparation.
5. Slow activation of the enzyme by its substrate, L-serine.

METHODS

Estimation of radioactivity in [^{14}C]-pyruvate from assay systems containing L-[3^{14}C]-serine.

Assay systems in which [^{14}C]-pyruvate had been formed from L-[3^{14}C]-serine were deproteinized with TCA. To an aliquot of the deproteinized supernatant was added 98μmoles of carrier pyruvate and excess DNPhydrazine in 2M-HCl. This solution was left overnight at 30° to allow complete conversion of pyruvate to its DNPhydrazone, and then stood at 0° for several days to allow complete precipitation of the pyruvate DNPhydrazone. The precipitate was collected on a paper disc (2.5 cm.diameter, Whatman No.1) by vacuum filtration and rinsed thoroughly with distilled water. At this point the precipitate should be evenly

distributed over the surface of the filter disc. The filter disc was allowed to dry and then mounted on an aluminium disc with glue. The radioactivity of the precipitate was determined with an end window counter. The result obtained is the radioactivity of the pyruvate DNPhydrazone at"infinite thickness."

RESULTS

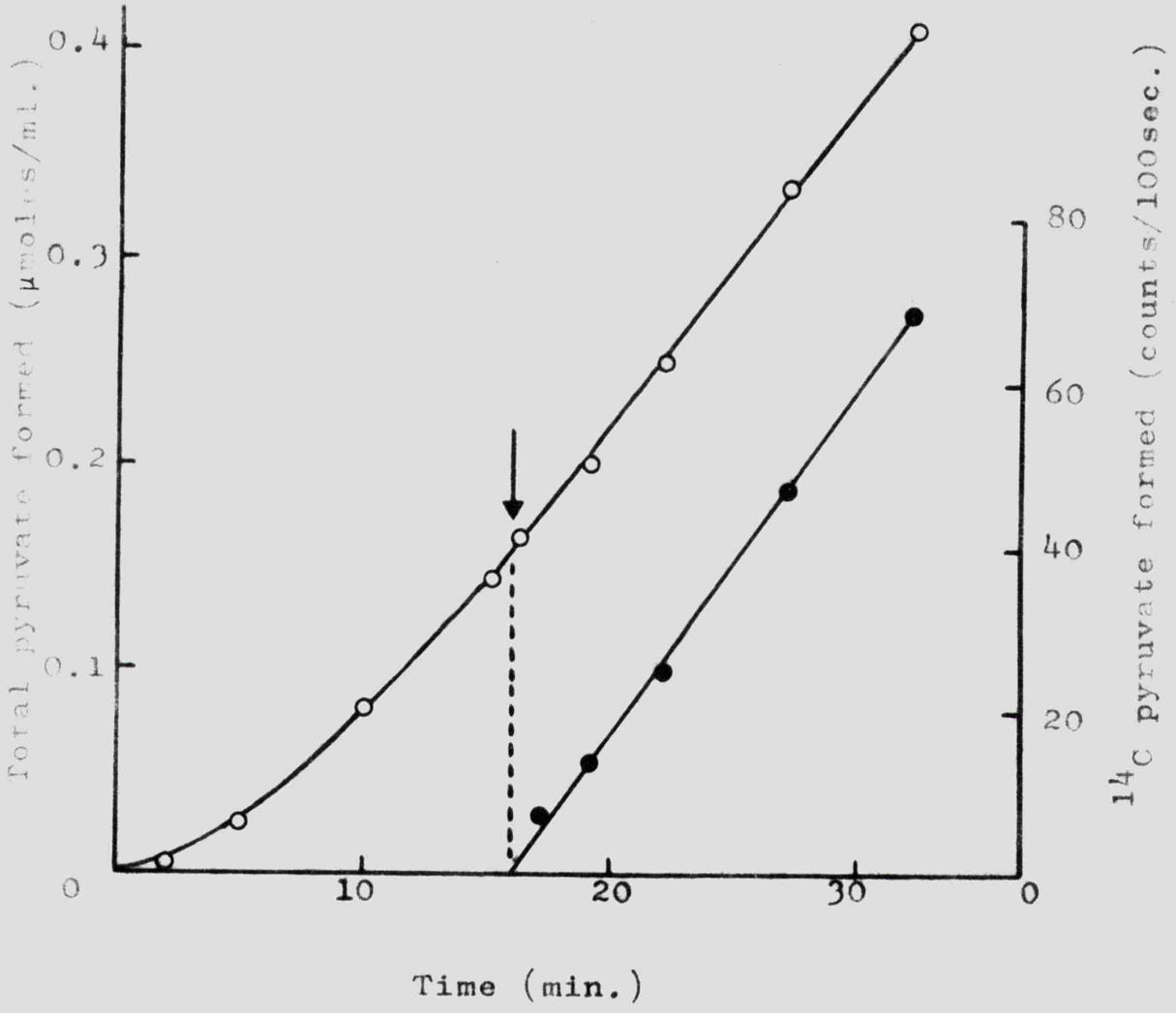
An investigation of the possible causes for the lag period.

The most likely causes for the lag period will be considered in the order in which they were outlined in the introduction.

1. Activation by a product. Pyruvate and ammonia were the only quantititatively important products formed from L-serine under the conditions of the assay (tables 3 and 4). The results of the following experiment rule out the possibility that the enzyme is activated by either pyruvate or ammonia. A little extract was preincubated for 15min. with 1mM sodium pyruvate, 1mM ammonium chloride and the usual concentrations of tris-HCl buffer, pH 8.2 and magnesium chloride. Then L-serine was added to the system and the formation of pyruvate followed with time. The time course obtained was found to be identical to that observed for a control system, from which pyruvate and ammonium chloride had been omitted.

2. Accumulation of a hypothetical intermediate. The lag period might represent the time taken for a hypothetical intermediate, which is necessarily involved in the conversion of L-serine into pyruvate and ammonia, to accumulate to a steady state level. This possibility can be tested in the following way. In an assay system which is

Fig.21. Time course for the formation of [^{14}C] pyruvate (●) following the addition of L-[3-^{14}C] serine to an assay system in which the formation of pyruvate (o) from L-serine had previously attained a constant rate. The assay system (11.0ml.) initially contained (μmoles): potassium phosphate buffer, pH7.1, 550; L-serine, 220; $MgCl_2$ 220; and a cell-free extract. The reaction was started by the addition of the serine. Samples (1ml.) were withdrawn and added, at the times indicated, to 20% ($^{w}/v$) TCA (1ml.). After 16min. incubation (at 30°) 6μmoles of a solution (0.15ml.) of L-[3-^{14}C] serine (4.7μc/μmole) was added to the system and further samples withdrawn at the times indicated. An aliquot (0.5ml.) was taken from each sample for estimation of their pyruvate content. The remainder of each sample was assayed for [^{14}C] pyruvate as described in the text.


Total pyruvate formed (μmoles/ml.)
0.4
0.3
0.2
0.1
0
10
20
30
0
20
40
60
80
^{14}C pyruvate formed (counts/100sec.)
Time (min.)

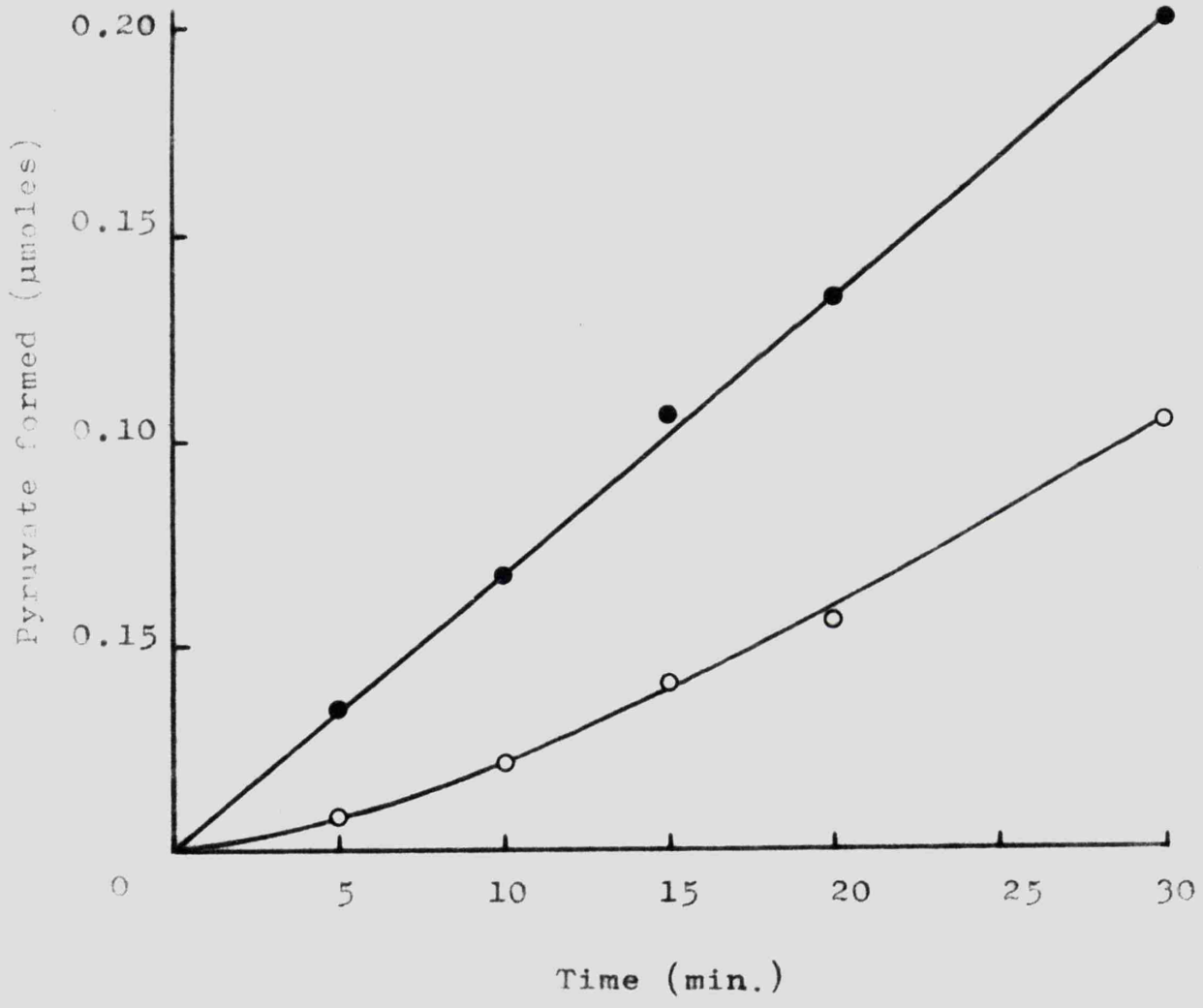
forming pyruvate at a constant rate, having passed the lag period, it would be expected that the hypothetical intermediate would have attained a constant level. The addition, at this point, of a small amount (relative to the amount of non-radioactive L-serine already present) of L-[^{14}C]-serine should have no effect on the overall rate of pyruvate formation. However, if there is present in the assay system a significant amount of an intermediate between L-serine and pyruvate, the rate of appearance of radioactivity in pyruvate would be expected to show a lag period, similar to that observed initially for total pyruvate formation. This lag period would correspond to the period during which the level of radioactivity in the intermediate is building up to the point where its specific activity is equal to that of the serine in the assay system. If no such intermediate is present, the rate of formation of [^{14}C]-pyruvate would be constant from the instant of adding the L-[^{14}C] serine.

In an experiment of this type, L-[3^{14}C]-serine was added to an assay system at a point where pyruvate formation had attained a constant rate, having passed a definite lag period. From this instant, the time courses for the formation of total pyruvate and [^{14}C]-pyruvate (i.e. radioactivity incorporated into pyruvate) were both linear, and the latter one extrapolated to zero at the time of adding the L-[3^{14}C]-serine (fig.21). The absence of a lag period in the appearance of [^{14}C]-pyruvate in this experiment shows that the initial lag period observed in the appearance of pyruvate was not caused by the accumulation of an intermediate.

3. Slow activation by a cofactor. It is possible that the lag period results from a slow activation of the enzyme by a cofactor present either in the enzyme preparation itself, e.g. pyridoxal phosphate, or in one of the assay reagents. This possibility was tested by preincubating an extract for 15 min. with tris-HCl buffer and magnesium chloride alone, and together with either an ultrafiltrate of concentrated extract or pyridoxal phosphate. L-serine was then added and pyruvate formation followed. In each case the time course obtained was identical with that observed for a control system of identical composition, in which the extract had been added after the L-serine. Therefore it is unlikely that the lag period results from a slow activation of the enzyme by tris-HCl buffer, magesium chloride, pyridoxal phosphate, or by a cofactor present in the enzyme preparation itself.

4. Slow enzymic destruction of an inhibitor. The lag period could represent the time taken for an inhibitor of L-serine dehydratase to be metabolized to a sub-inhibitory level by an enzyme in the L-serine dehydratase preparation. Since preincubating the L-serine dehydratase preparation with all the components of the assay system other than L-serine did not eliminate the lag period, it must be assumed that L-serine contains the hypothetical inhibitor. It would be expected from this explanation that L-serine dehydratase taken from an assay system in which pyruvate is being formed at a constant rate, that is to say where the hypothetical inhibitor has been metabolized

Fig.22. Effect of preincubating L-serine dehydratase with L-serine on the time course for pyruvate formation. A cell-free extract was preincubated for 15min. (at 30°) together with 42mM-tris-HCl buffer, pH8.2, and 17mM-$MgCl_2$, both in the presence of 60mM-L-serine (●) and in its absence (o). Samples (0.5ml.) from each system were then diluted into fresh assay systems (final volume, 10 ml.) and incubation was continued. Aliquots (1.0ml.) were removed from both assay systems at the times indicated and their pyruvate content estimated. The results presented for the system containing the extract which had been preincubated with L-serine have been corrected for the pyruvate content of the aliquot withdrawn at zero time.


0.20
0.15
0.10
0.15
Pyruvate formed (µmoles)
0
5
10
15
20
25
30
Time (min.)

to a subinhibitory level, would, when transferred to a fresh assay system, again exhibit a lag period.

In an experiment where extract was incubated with tris-HCl buffer, pH8.2, magnesium chloride and L-serine for 15 min., and an aliquot of this system then diluted twenty fold into a fresh assay system, the rate of pyruvate formation was constant from the time of transfer (fig.22). For a control experiment the extract was pre-incubated for 15 min. with tris-HCl buffer and magnesium chloride in the absence of L-serine. In this case a definite lag period was observed when an aliquot of the control system was transferred to a fresh assay system. It is evident from these results that the assay system does not contain an inhibitor of L-serine dehydratase which is slowly metabolized during the lag period.

5. Slow activation by L-serine. The experiment described in the previous section shows that the lag period can be eliminated by pre-incubating the enzyme with L-serine in the presence of tris-HCl buffer and magnesium chloride. It had previously been shown that the lag period cannot be eliminated by preincubating the enzyme with pyruvate and ammonia, both of which would have been present in addition to L-serine during the preincubation period in the experiment above. Furthermore, it is known that the lag period does not result from the accumulation of an intermediate between L-serine and pyruvate. Therefore it must be assumed that the elimination of the lag period in the experiment above is the result of preincubating the enzyme with L-serine itself.

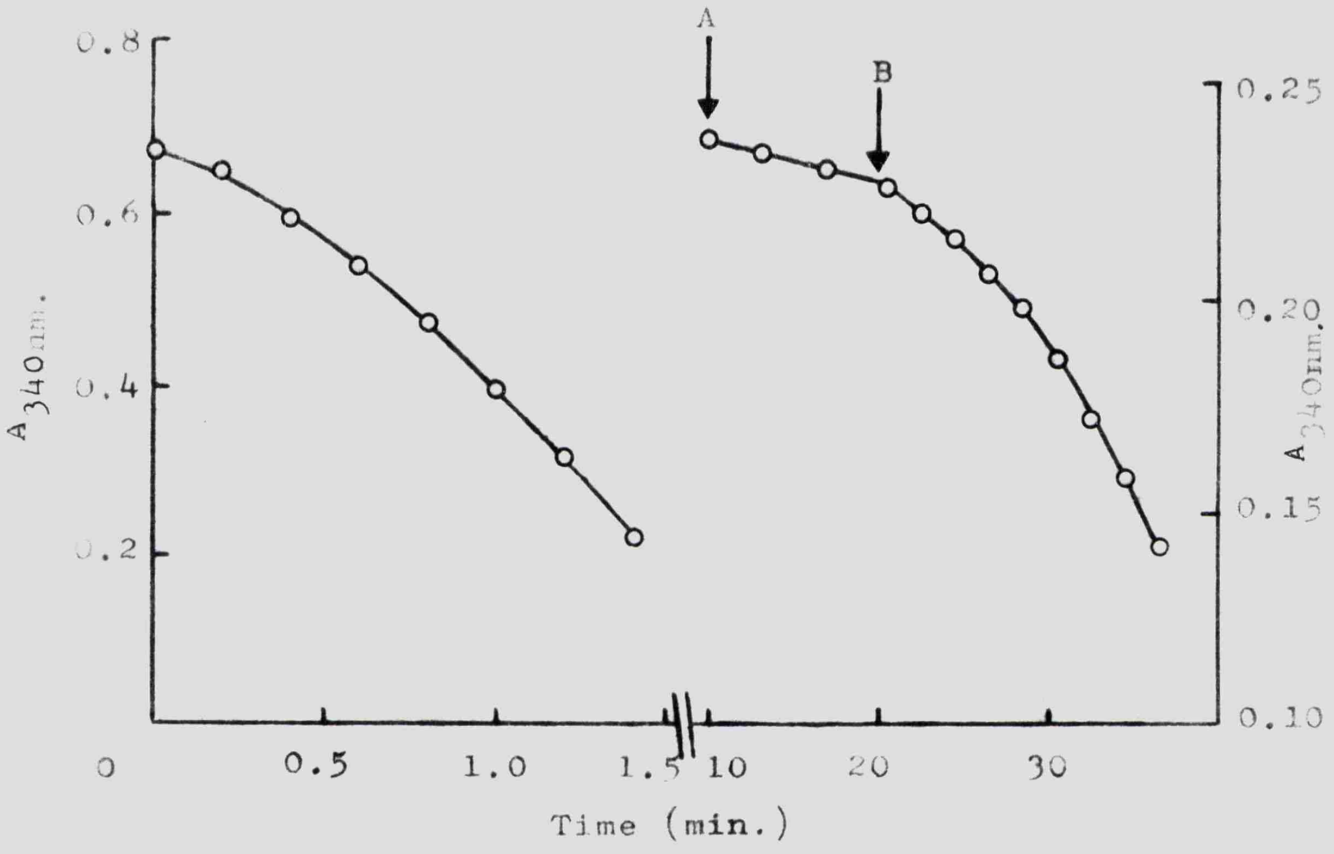
It follows from this assumption that the lag period is indicative of a slow, L-serine-induced activation of the enzyme.

Some features of the activation process

Specificity for L-serine. A range of substances structurally and, or, metabolically related to L-serine were tested in the following manner for the ability to activate L-serine dehydratase. A little extract was preincubated for 15 min. with the substance to be tested (unless stated otherwise, at a concentration of 20mM), tris-HCl buffer, pH 8.2, and magnesium chloride. L-serine was then added and pyruvate formation followed. The marked lag period observed under these conditions when preincubation was carried out in the absence of L-serine, or of a substance related to it, was completely eliminated by including 20mM-L-serine in the preincubation system. On the other hand, the lag period was not diminished in the slightest by substituting for L-serine the following substances: ethanolamine, glycine, L-alanine, L-cysteine (0.5-5mM), calcium DL-glycerate, D-serine, L-serineamide, L-aspartate, DL-homoserine, DL-hydroxyaspartate (erythro- or threo-), DL-α-methylserine, DL-O-methylserine, or L-threonine. During the course of the 15 min. preincubation period, there was, with the exception of DL-O-methylserine (see Chapter 8, p.147), no detectable formation of oxo-acid from these substances.

Thus it appears that activation of the enzyme by L-serine involves a stereospecific process.

Fig.23. Demonstration that activation of L-serine dehydratase by L-serine is reversed by diluting the L-serine to a low level. Pyruvate formation was followed continuously by the lactate dehydrogenase method in an assay system (1.0ml.) containing approximately 0.2units$_{30}$ of partially purified L-serine dehydratase, 20mM-L-serine and the usual concentrations of other components . When the lag period (left hand curve) was passed and a linear rate had been maintained for 7min., 0.05ml. of this system was diluted (point A) into a fresh assay system (final volume,1.0ml.), from which L-serine had been omitted. Pyruvate formation was followed in the latter system for 10min. At this point (B) L-serine was added to the system to give a final concentration of 20mM and pyruvate formation followed for a further 16min. The incubation temperature throughout was 30°. The left hand ordinate scale refers to the first assay system and the right hand scale to the second one.


A
B
0.8
0.6
0.4
0.2
$A_{340nm.}$
0.25
0.20
0.15
0.10
$A_{340nm.}$
0
0.5
1.0
1.5
10
20
30
Time (min.)

Reversibility. The L-serine-induced activation of the enzyme was reversed when the enzyme was freed from L-serine, or the concentration of L-serine reduced sufficiently by dilution. This is demonstrated by the following two experiments.

Partially purified L-serine dehydratase was incubated with 20mM-L-serine, tris-HCl buffer, pH 8.2, and magnesium chloride until the lag period was past and the rate of pyruvate formation had remained approximately constant for about 5 min. At this point the enzyme was judged to be fully activated under the conditions obtaining. An aliquot was diluted twenty fold into a fresh assay system from which L-serine had been omitted. The dilute enzyme continued to form pyruvate at a constant, though much reduced rate. After a further 10 min. incubation, L-serine was added to the latter system to produce a final concentration of 20mM, as in the former system. For a second time, the enzyme exhibited a definite lag period in forming pyruvate (fig.2 3).

In a similar experiment, the fully activated enzyme was freed from L-serine by chromatography on a column of G-25 sephadex at 4°. When a sample of the enzyme eluted from the column was added to a fresh assay system, containing 20mM-L-serine, it exhibited a lag period for the second time.

SUMMARY

1. The lag period which is observed in the time course for pyruvate formation results from a slow activation of the enzyme by L-serine.

This conclusion was reached after a number of other possible explanations had been eliminated by experiment.

2. Of a variety of amino acids and other substances tested, only L-serine is able to cause activation of the enzyme.

3. Activation of the enzyme by L-serine is reversed when the enzyme is freed from L-serine, or the concentration of L-serine lowered sufficiently by dilution.

CHAPTER 11

THE EFFECT OF L-SERINE ON SOME MOLECULAR PROPERTIES OF THE ENZYME

INTRODUCTION

The activation of L-serine dehydratase by L-serine is unusual because it occurs slowly, and also because L-serine is both activator and substrate for the enzyme. Relatively few cases have been reported where the activation of an enzyme by a low molecular weight substance occurs slowly. In at least two of these cases the activator was found to cause a slow association of inactive enzyme subunits. The cases in point are the activation of acetyl CoA carboxylase (EC 6.4.1.2) from rat adipose tissue by citrate (Vagelos, Alberts and Martin, 1963) and of L-tartaric acid dehydratase from

Pseudomonas putida by Fe^{2+} plus either cysteine or reduced glutathione (Hurlbert and Jakoby, 1965). In view of these findings it was of interest to determine whether the slow activation of L-serine dehydratase is accompanied by a change in its quaternary structure.

METHODS

The techniques of gel filtration and sucrose gradient centrifugation both have the advantage that they can be employed to study molecular properties of enzymes using impure preparations. The gel filtration or sedimentation rate of the enzyme being studied is compared with that of one or more "standard" proteins of known molecular weights and sedimentation constants.

Gelfiltration

The procedure followed was based on that of Andrews (1964).

Preparation of columns. Columns of G-100 sephadex (1.6cm. diameter x 50cm.) were equilibrated with the elution buffer for at least 24 hr. at a flow rate of 30ml/hr. This buffer contained 50mM-tris-HCl, pH 7.5, 0.15M-KCl and, where noted in the text, L-serine or a related compound at the appropriate concentration. Columns were repoured after using once or twice.

Gel filtration runs. All gel filtration runs were carried out at 4°. L-serine dehydratase (generally 2-4 $units_{23}$) which had been freed from NADH oxidase activity was mixed with lactate dehydrogenase (9 units), bovine serum albumin (15mg.), cytochrome c (1-2mg.) and blue dextran (1mg.). The protein mixture was made up to

0.5ml. with the elution buffer, dialysed against this buffer for 2hr. and applied in its entirety to the column. The column effluent was collected in 3.0ml. fractions at a rate of approximately 10 fractions/hr.

Analysis of fractions. Cytochrome c and blue dextran were estimated by their absorbance at 408nm. and 650nm., respectively, using the whole of each fraction. Aliquots were taken from the fractions to estimate bovine serum albumin (0.20ml.), by the method of Lowry et al (1951); L-serine dehydratase (0.5ml.), by the lactate dehydrogenase method; and lactate dehydrogenase (0.05ml.), by the method described in Chapter 2 (p.32).

Estimation of elution volume, "peak volume", R_{SA} and molecular weight. V_e, the elution volume (in ml.) for a substance, was estimated by drawing tangents to its elution profile. The effluent volume at which the tangents intersect (fig.24) was taken as V_e. (V_e was equal to zero at the point where the substance first began to enter the surface of the gel).

The tangents to the elution profile of a substance can be extended to intersect the abcissa at separate points. The volume of effluent (in ml.) falling between these two points will be termed the "peak volume".

Gel filtration rates of proteins were compared by reference to bovine serum albumin (Andrews, 1964). Rates are expressed as

R_{SA} values:

$$R_{SA} = \frac{\text{migration rate of protein}}{\text{migration rate of serum albumin}}$$

$$= \frac{V_e \text{ for serum albumin}}{V_e \text{ for protein}}$$

Molecular weights were estimated by interpolation from a plot of V_e against log [molecular weight] , which was prepared from the data obtained for cytochrome c, bovine serum albumen and lactate dehydrogenase. Andrews (1964, 1965) has studied the gel filtration behaviour of a variety of proteins on columns of G-100 sephadex. For proteins with approximately spherical molecules which are substantially free from carbohydrate, he showed that a plot of V_e against log [molecular weight] is very nearly linear over the molecular weight range 1×10^4 to 7×10^4. At higher molecular weights the exclusion limit for the gel is approached and the plot becomes increasingly curved.

In the present studies molecular weight estimations were based on the assumption that Cytochrome c (molecular weight, 1.24×10^4) and bovine serum albumin (molecular weight of monomer, $6.5\text{-}7.0 \times 10^4$) both fell on a linear portion of this plot. Estimates of molecular weights greater than 7×10^4, but lower than 13.2×10^4 (lactate dehydrogenase), were obtained as follows. A maximal estimate was obtained by interpolation from the line joining the points for bovine serum albumin and lactate dehydrogenase. A minimal

estimate was obtained by extrapolation of the line joining the points for cytochrome c and bovine serum albumin.

Sucrose gradient centrifugation

The procedure followed was that described by Martin and Ames (1961).

Preparation and Centrifugation of gradients. The gradients (of volume, 4.0ml) were prepared from 5% and 20% (w/v) solutions of sucrose in 0.1M-potassium phosphate buffer, pH 7.5. Gradients containing L-serine were prepared by dissolving this in each of the sucrose solutions at the required concentration. The L-serine dehydratase used in these studies had been partially purified and dialysed overnight against 10mM-potassium phosphate buffer, pH 7.5. The standard proteins, lactate dehydrogenase, malate dehydrogenase and cytochrome c, were mixed in the proportions given below and freed from ammonium sulphate by passing the mixture through a column of G-25 sephadex which had been previously equilibrated with 10mM-potassium phosphate buffer, pH 7.5. The solution (0.10ml) which was layered on the surface of the gradient contained: L-serine dehydratase, approximately 0.6 $units_{30}$; lactate dehydrogenase, 16 units; malate dehydrogenase, 16 units; and cytochrome c, 0.26mg.

Gradients were centrifuged two or three at a time in the swinging bucket rotor (SW-39) of a Beckman, Model L-2 centrifuge (Beckman Instruments, Inc., Palo Alto, California, U.S.A.) at 106000g (36000r.p.m.) for 11.5 - 14.5 hr. The internal

temperature of the rotor was estimated to be 0-2°.

Fractionation and analysis of gradients. After centrifugation the tube containing the gradient was pierced and the contents collected in 32 fractions of 0.13ml. each. Using an Abbé refractometer to estimate the refractive index of fractions obtained from a sample gradient, the gradient was shown to be a linear one.

Aliquots were taken from the fractions to assay L-serine dehydratase (0.04ml.), by the lactate dehydrogenase method; lactate dehydrogenase (0.02ml.), and malate dehydrogenase (0.02ml.), both as described in Chapter 2 (p.32); and cytochrome c (0.02ml.). The cytochrome c was diluted to 1.0 ml. before measuring its absorbance at 408nm.

Estimation of $S_{20,w}$ and molecular weight. Martin and Ames (1961) showed that the rates of sedimentation of proteins through the linear sucrose gradient employed in this method are very nearly constant over a period of 17 hr. at 3°. For proteins with the same partial specific volume, the following relationship applies:

$$R = \frac{\text{distance travelled from meniscus by protein}}{\text{distance travelled from meniscus by standard}}$$

$$= \frac{S_{20,w} \text{ of protein}}{S_{20,w} \text{ of standard}}$$

where $S_{20,w}$ denotes the sedimentation constant in water at 20°. Most proteins have partial specific volumes of between 0.70 and 0.75 $cm.^3/g.$ (Edsall, 1953); variation within these limits does not significantly affect the validity of the above equation (Martin and Ames, 1961).

The relative distance travelled from the meniscus by a protein was calculated as follows. Tangents were drawn to the sedimentation profile of the protein to obtain the exact position of the peak (fig. 25) as a fraction number. This number was subtracted from the total number of fractions collected from the gradient to obtain a relative measure of the distance travelled from the meniscus by the protein.

An estimate for the molecular weight of a protein can be obtained from a knowledge of its sedimentation rate relative to that of another protein of known molecular weight through application of the following equation (Schachman, 1959):

$$R = \frac{S_1}{S_2} = \left[\frac{MW_1}{MW_2}\right]^{2/3}$$

where S denotes sedimentation constant and MW, molecular weight. This equation is based on the assumption that the two proteins being compared have spherical molecules. The molecules of most globular proteins are only roughly spherical, but nevertheless the equation is found to be a reasonable approximation (Schachman, 1959). It should be noted that the molecular weight estimate, because it relies upon this additional assumption, is less reliable than the $S_{20,w}$ estimate.

Molecular weights and sedimentation constants of standard proteins

Bovine serum albumin (monomer) is taken to have a molecular weight of 65 - 70 x 10^3 (Phelps & Putnam, 1960; Hughes & Dintzis, 1964). The values taken for the sedimentation constants ($S_{20,w}$) and molecular weights of lactate and malate dehydrogenases and cytochrome c are given

Fig 24. Profiles for the elution of L-serine dehydratase from a column of G-100 sephadex in the presence of 50mM-L-serine (●) and in its absence (o). The positions of the peaks of the proteins used as standards, i.e. lactate dehydrogenase (▵) and cytochrome c (□), were unaffected by the presence of L-serine in the elution buffer. L-serine dehydratase and lactate dehydrogenase were both assayed by spectrophotometric measurement of rates of NADH oxidation (ΔA_{340nm}). Cytochrome c was estimated by its absorbance at 408nm. Details of the procedure are given in the text.

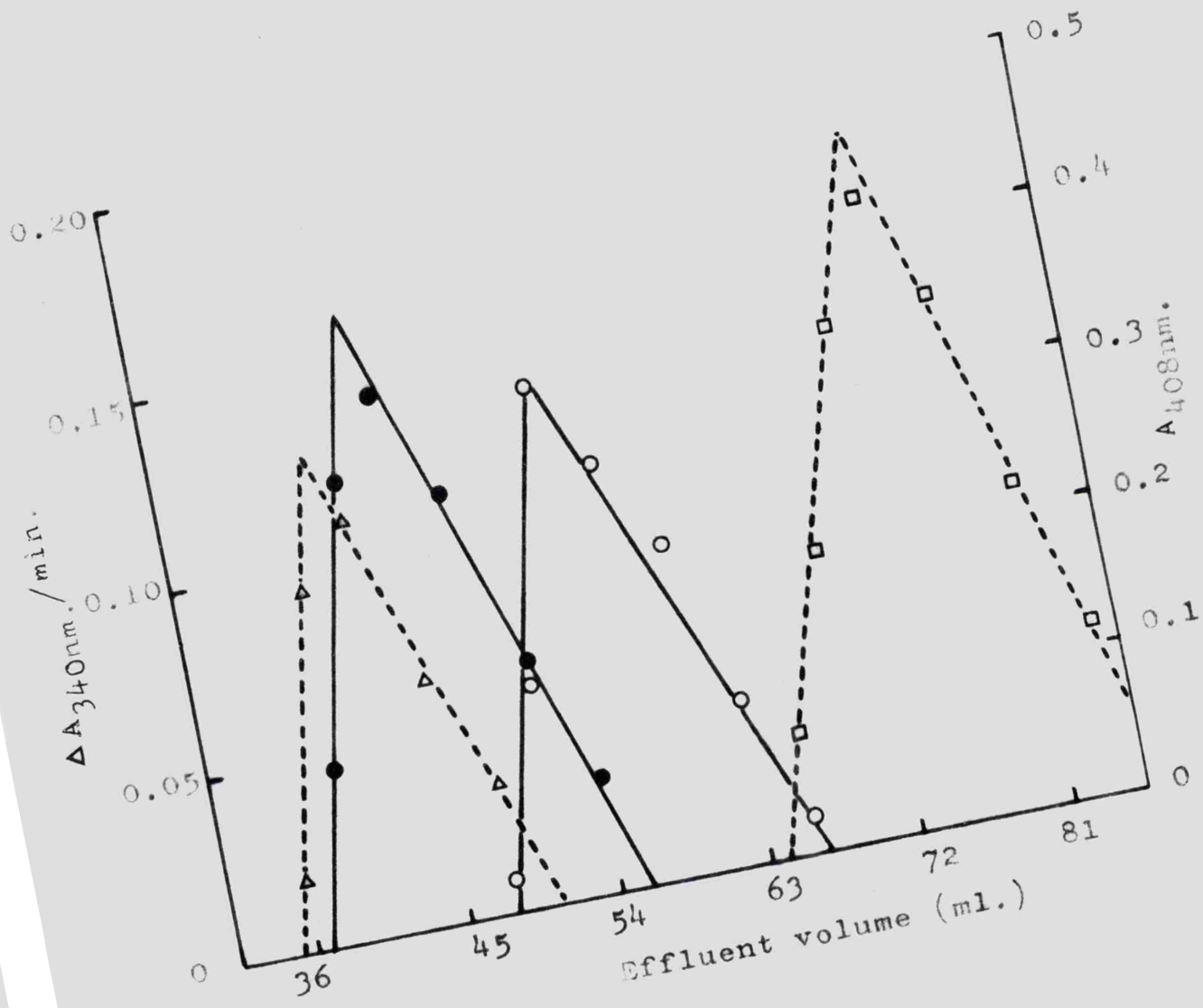

0.20
0.15
0.10
0.05
0
$\Delta A_{340nm.}$/min.
0.5
0.4
0.3
0.2
0.1
0
$A_{408nm.}$
36
45
54
63
72
81
Effluent volume (ml.)

Table 22. Effect of L-serine on the gel filtration behaviour of L-serine dehydratase.

L-serine was added to the tris-KCl buffer used for elution at the concentrations indicated. The R_{SA} of L-serine dehydratase is the ratio of its migration rate during gel filtration to that of bovine serum albumin. The figures in parentheses are the number of determinations on which the results are based. Further details are given in the text.

Concentration of L-serine (mM)	R_{SA} of L-SD	"peak volume" of L-SD / "peak colume" of Cyt.c
0	0.87-0.88(3)	0.7-0.9(3)
4	0.88 (1)	-
9	0.88 (1)	0.9 (1)
12	0.90-0.94(3)	0.9-1.0(3)
15	0.92-0.97(3)	1.0-1.4(3)
20	1.02-1.04(2)	1.1-1.3(2)
30	1.04 (2)	-
50	1.07-1.09(4)	0.8-0.9(3)
100	1.10 (1)	0.9 (1)

L-SD denotes L-serine dehydratase and Cyt.c, cytochrome. c.

in table 24.

RESULTS

Gel filtration studies

Effect of L-serine. When subjected to gel filtration in the absence of L-serine, L-serine dehydratase was eluted after bovine serum albumin, in a single peak, with an R_{SA} of 0.87 - 0.88 and an estimated molecular weight of 45,000 ± 4,500. In the presence of 50 or 100 mM-L-serine, the L-serine dehydratase was now eluted before bovine serum albumin ~~(fig. 24)~~, with an R_{SA} of 1.07 - 1.10 and an estimated molecular weight of between 80,000 and 96,000 (fig. 24). In the latter instance the highest concentration of pyruvate detected in the column effluent was 0.8 mM. This shows that the concentration of L-serine in the eluting buffer remained high enough to saturate L-serine dehydratase throughout the course of gel filtration. The R_{SA} values obtained for lactate dehydrogenase and cytochrome c were unaffected by the presence of L-serine in the elution buffer.

When gel filtration was carried out in the presence of L-serine at concentrations which are partially saturating for L-serine dehydratase, the R_{SA} values obtained for the enzyme were intermediate between the value obtained in the presence of 50 or 100 mM - L - serine and that obtained in its absence (table 22). The R_{SA} for the enzyme showed no increase for L-serine concentrations up to 9 mM; it increased sharply over the concentration range 9 to 20 mM and approached a limiting value at higher concentrations.

Table 23. Effect of L-serine, glycine, L-threonine and ethanolamine on the gel filtration rate of L-serine dehydratase.

L-serine, glycine, L-threonine or ethanolamine (all at a final concentration of 50mM) was added, where indicated, to the tris-KCl buffer used for elution. Gel filtration rates are given as R_{SA} values (see table 22.) Estimates for the molecular weight of L-serine dehydratase (L-SD) are based on the known molecular weights of the proteins used as standards, i.e. lactate dehydrogenase (LDH) bovine serum albumin and cytochrome c (cyt.c)

Substance added to the elution buffer	R_{SA}			Molecular weight of L-SD x 10^{-3}
	L-SD	LDH	Cyt.c	
None	0.87-0.88	1.16-1.20	0.62-0.63	44-46
L-serine	1.07-1.09	1.16-1.20	0.62-0.63	80-98
Glycine	0.85	1.16	0.63	40
L-threonine	0.86	1.14	0.62	42
Ethanolamine	0.87	1.15	0.63	44

The volume occupied by the L-serine dehydratase peak after elution from the column, i.e. its "peak volume", was also influenced by the concentration of L-serine in the elution buffer, whereas the "peak volumes" of the proteins included as standards were not. The ratio: ["peak volume" of L-serine dehydratase] / ["peak volume" of cytochrome c] had a maximal value when the elution buffer contained 15 to 20 mM - L - serine. Its value was minimal when elution was carried out either in the absence of L-serine, or in the presence of a 50 mM concentration (table 22).

Effect of substances structurally related to L-serine. When either glycine, L-threonine, or ethanolamine was added to the elution buffer (all at a concentration of 50 mM) in place of L-serine, the R_{SA} value observed for L-serine dehydratase was very similar to that observed for the enzyme in the absence of L-serine (Table 23).

Effect of enzyme concentration. In the absence of L-serine, the gel filtration rate of L-serine dehydratase was not changed by increasing the enzyme's concentration during gel filtration by over a hundred fold. The concentration of this enzyme in the sample applied to the column was increased from approximately 2 units $_{20}$/ml. (0.5 ml. applied) to 240 units $_{20}$/ml. (2.0 ml. applied). The corresponding maximal concentrations of the enzyme in the effluent were approximately 0.2 units $_{20}$/ml. and 63 units $_{20}$/ml.

Reversal of the L-serine effect. The following experiment demonstrates that the L-serine-induced increase in the gel filtration rate of the enzyme can be reversed by subjecting the enzyme to gel filtration a second time in the absence of L-serine. First, the enzyme was

Fig.25. Profiles for the sedimentation of L-serine dehydratase through a sucrose gradient in the presence (●) and absence (○) of L-serine (initial concentration, 50mM). The positions of the peaks of the proteins used as standards,ie. lactate dehydrogenase (△) and cytochrome c (□), were unaffected by the presence of L-serine in the gradient. L-serine dehydratase was assayed by the DNPhydrazine method (v_{15}) in fractions from the gradient lacking L-serine, and by the lactate dehydrogenase method ($\Delta A_{340nm/min.}$) in fractions from the gradient containing L-serine. Lactate dehydrogenase was assayed by spectrophotometric measurement of rates of NADH oxidation ($\Delta A_{340}/min.$) and cytochrome c by its absorbance at 408nm. Details of the procedure are given in the text.

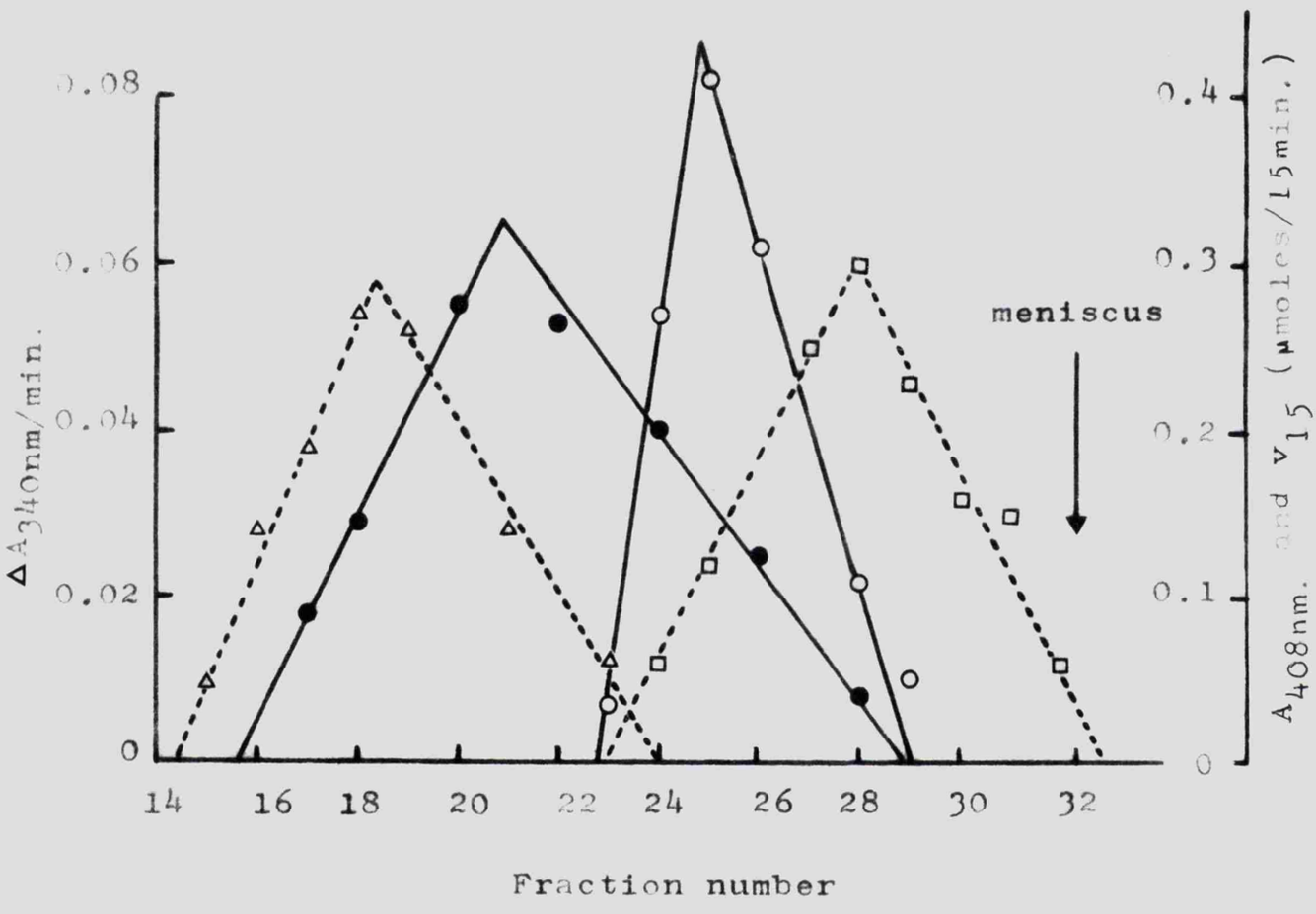

0.08
0.06
0.04
0.02
0
ΔA_{340nm}/min.
0.4
0.3
0.2
0.1
0
A_{408nm}. and v_{15} (μmoles/15min.)
meniscus
14
16
18
20
22
24
26
28
30
32
Fraction number

Table 24. Effect of L-serine on the sedimentation rate of L-serine dehydratase.

Where indicated, L-serine was incorporated in the sucrose gradient at a final concentration of 50mM. The distance sedimented by each protein is given in terms of the number of fractions falling between the meniscus of the gradient and the protein peak. In two of the sedimentation runs, 32 fractions of 0.13ml. each were collected from the gradients. In a third run, for which the data is given in parentheses, 44.6 fractions of 0.10ml. each were collected. The $S_{20,w}$ and molecular weight estimates for L-serine dehydratase (L-SD) are based on the data presented below for the proteins used as standards.

Distance sedimented (in fractions):	L-serine absent	L-serine present
L-SD	6.9(12.1)	11.0
Cyt. c	4.2(7.1)	4.3
MDH	- (13.0)	-
LDH	13.2(20.7)	13.2

contd....

Table 24 contd.

$S_{20,w}$ of L-SD:	L-serine absent	L-serine present
Cyt.c as standard	3.1(3.2)	4.8
MDH " "	- (4.0)	-
LDH " "	3.9(4.3)	6.2
Molecular weight of L-SD x 10^{-3}:		
Cyt.c as standard	26 (28)	50
MDH " "	- (49-63)	-
LDH " "	48 (60)	100

Data for standard proteins

Protein	$S_{20,w}$	Molecular weight (x 10^{-3})
Cytochrome c (cyt.c)	1.89 (Svedberg & Pederson,1940)	12.4 (Margoliash, 1962)
Malate dehydrogenase (MDH)	4.32 (Thorne,1962)	55-70 (Thorne & Kaplan,1963)
Lactate dehydrogenase (LDH)	7.43 (Fromm,1963)	132 (Fromm,1963)

subjected to gel filtration in the presence of 50 mM-L-serine, under which conditions an R_{SA} of 1.07 was obtained for it. Then a sample of the enzyme was taken from the effluent fraction containing the highest activity and applied, without delay, to a second G-100 sephadex column. This time the enzyme was eluted with an L-serine-free buffer. The enzyme now exhibited an R_{SA} value of 0.88, which is in accordance with the value normally obtained for it under these conditions.

Sedimentation studies

In the absence of L-serine, L-serine dehydratase sedimented through a linear sucrose gradient a little less rapidly than malate dehydrogenase (fig. 25), with an estimated $S_{20,w}$ of 3.9 - 4.3. This estimate was based on the known $S_{20,w}$'s of lactate and malate dehydrogenases (table 24). When the gradient contained 50 mM-L-serine, L-serine dehydratase now sedimented more rapidly than malate dehydrogenase, with an estimated $S_{20,w}$ of 6.2. Although the initial concentration of L-serine in the gradient was 50 mM, after centrifugation most of the L-serine in the zone through which L-serine dehydratase had sedimented had been converted to pyruvate. The sedimentation rates of the standard proteins were unaffected by the presence of L-serine in the gradient.

The ratio: [molecular weight of L-serine dehydratase in the presence of L-serine]/[molecular weight of L-serine dehydratase in its absence] was calculated to be 1.97 - 2.02 from the ratio of the

distances travelled by the enzyme in the presence and absence of L-serine, respectively. Estimates for the molecular weight of the enzyme (table 24) under each of these conditions, based on the known molecular weights of lactate and malate dehydrogenases, were in fair agreement with the estimates obtained from gel filtration rates. When cytochrome c was taken as the standard, considerably lower estimates were obtained for both the molecular weight and the sedimentation constant of L-serine dehydratase, both in the presence and absence of L-serine.

DISCUSSION

L-serine could increase the rate of sedimentation of L-serine dehydratase either by causing it to polymerize, or by causing the molecules to become more compact through altering their conformation or degree of hydration. The fact that the gel filtration rate of the enzyme is increased in the presence of L-serine indicates that the enzyme molecules enter the gel particles less readily under these conditions. This in turn suggests that the presence of L-serine causes the enzyme molecules to become more bulky. Therefore it seems likely, from a consideration of both the sedimentation and gel filtration behaviour of the enzyme, that L-serine causes it to polymerize. The sedimentation rates of the enzyme in the presence and absence of L-serine correspond precisely to molecular weights in the ratio of two to one respectively. A comparable, though less precise, value for this ratio is obtained from the corresponding gel filtration rates of the enzyme. It is therefore concluded that L-serine induces the

enzyme to dimerize.

Gel filtration studies have shown that the L-serine-induced dimerization of the enzyme involves a fairly specific process in that it is not brought about by substances structurally related to L-serine. It has also been shown that the removal of L-serine from the dimeric form of the enzyme causes a reversal of dimerization.

SUMMARY

1.a. The gel filtration rates observed for L-serine dehydratase in the presence of 50 or 100 mM-L-serine, and in its absence, correspond to molecular weights of 45,000 ± 4,500 and 80 - 96 x 10^3, respectively.

b. Intermediate gel filtration rates were observed for the enzyme at L-serine concentrations in the range 9 to 20 mM.

c. The effect of L-serine on the gel filtration rate of the enzyme was reversed by removing the L-serine.

d. The gel filtration rate of the enzyme was not affected by the presence of either glycine, L-threonine, or ethanolamine in the elution buffer.

2. The sedimentation rates observed for the enzyme in the presence and absence of L-serine, respectively, corresponded to molecular weights in the ratio of 1.97 - 2.02 : 1.

3. It is concluded from these results that L-serine induces a reversible dimerization of the enzyme.

CHAPTER 12.

A MODEL FOR L-SERINE DEHYDRATASE

INTRODUCTION

L-serine both activates and dimerizes L-serine dehydratase. Each of these effects is caused specifically by L-serine, and is reversed by removing it. It therefore seems reasonable to suggest that activation and dimerization may be two aspects of a single phenomenon. Implicit in this suggestion are the postulates that the dimeric species of the enzyme is enzymically more active than the monomeric one and that dimerization occurs slowly. It will be further postulated that the dimer is the more active species because it possesses greater affinity for L-serine. This would provide a possible explanation for the role played by L-serine in dimerization (or activation). Thus, if the enzyme normally exists as an equilibrium

mixture of monomer and dimer, the addition of L-serine to it will displace the equilibrium in favour of dimer, because L-serine will bind preferentially to this species.

The explanation developed above is really an extension of the model proposed by Monod et al. (1965) for allosteric proteins. This model offers primarily an explanation for "homotropic, co-operative interactions". A sigmoid saturation curve, such as L-serine dehydratase exhibits for L-serine, is indicative of these interactions. "Homotropic" is used to describe interactions taking place between identical ligand molecules. Co-operativity implies that the binding of a small amount of ligand to a protein facilitates the binding of more. The capacity to mediate interactions of this type has been considered by Monod et al. to characterize allosteric proteins. Since L-serine dehydratase appears to possess this capacity, it can be regarded as an allosteric protein, even though no effector which could be described as "allosteric" has yet been found for it. In these respects it resembles haemoglobin.

The Monod-Wyman-Changeux model offers the following explanation for homotropic,co-operative interactions. It postulates that allosteric proteins are oligomers comprising two or more identical subunits, or protomers. It further postulates that an allosteric protein is capable of existing in at least two conformational states, designated R and T states*, which are in reversible equilibrium with

* In the case of an enzyme where the two states possess different affinities for the substrate, it is the R state which possesses the greater affinity.

one another. It follows from these postulates that any ligand which possesses different affinities for the two states of a protein will, when added to it, cause a displacement of the equilibrium in favour of the state for which it has greater affinity. Take, for example, a ligand which preferentially binds to the R state of a protein. At low concentrations of ligand co-operative binding results because several new R-type sites (the exact number will depend on the number of protomers in the protein molecule) are created for every molecule of ligand bound to an otherwise unoccupied molecule of protein in the R state. The model predicts that, when the R and T states of a protein possess very different affinities for a ligand, a curve relating the fractional saturation of the protein (both states included) to the concentration of the ligand will have a sigmoid form.

Two principal extensions have been introduced to the Monod-Wyman-Changeux model in applying it to L-serine dehydratase. First, the R and T states are represented by the dimeric and monomeric forms of the enzyme, respectively, and therefore contain different numbers of subunits. Thus the allosteric transition involves a reversible association of enzyme subunits. Secondly, in the direction leading from monomer to dimer the transition occurs slowly. There are a number of precedents for an allosteric enzyme which exists as an equilibrium mixture of monomeric and oligomeric species. Thus the activation of the catabolic L-threonine dehydratase from *E. coli* by AMP is accompanied by the dimerization of relatively inactive subunits (Phillips & Wood, 1964). Other examples are provided by

homoserine dehydrogenase (EC 1.1.1.3) from Rhodospirillum rubrum (Datta, Gest & Segal, 1964), deoxycytidylate deaminase from chick embryo (Maley & Maley, 1965) and acetyl CoA carboxylase from rat adipose tissue (Vagelos et al., 1963). The latter enzyme also provides a precedent for postulating a slow allosteric transition. When acetyl CoA carboxylase is incubated with citrate, its activity increases over a period of 10 - 30 min. Under these conditions inactive enzyme subunits aggregate to form an active oligomer.

A number of other theories which have been advanced to explain properties of individual allosteric enzymes make the common assumption that these enzymes possess two discrete types of substrate-binding site, namely catalytic and regulatory sites (Atkinson, 1966; Stadtman, 1966). These theories, which will be referred to collectively as the "two-site hypothesis" (Stadtman, 1966), explain co-operative interactions between substrate molecules in the following way. The binding of a substrate molecule to a regulatory site is assumed to induce a conformational alteration in the enzyme molecule such that the affinity of the catalytic sites for substrate is increased. This hypothesis differs from that of Monod et al., not only in postulating two types of substrate-binding site, but also in postulating that an enzyme molecule undergoes a conformational alteration as a direct result of binding a substrate molecule.

The two-site hypothesis does not provide so simple an explanation for the properties of L-serine dehydratase as does the extended Monod-Wyman-Changeux model.

$$M + M \overset{\text{cation}}{\rightleftharpoons} D$$

$$D + S \rightleftharpoons D_{s1}$$

$$D_{s1} + S \rightleftharpoons D_{s2}$$

$$- - - - - - - - - - - - - -$$

$$D_{s(n-1)} + S \rightleftharpoons D_{sn}$$

Fig. 26. A model for L-serine dehydratase based on the Monod-Wyman-Changeux model for allosteric proteins. M denotes monomer (molecular weight, approximately 45,000), D,dimer; S, L-serine; D_{sn}, a complex containing one molecule of dimer and n molecules of L-serine; and n the number of L-serine-specific binding sites on the dimer.

FORMAL DESCRIPTION OF THE MODEL

The model which is presented schematically in figure 26 is described by the following statements:

1. In solution the enzyme exists as an equilibrium mixture of monomer* and dimer molecules.

2. The association of monomers in pairs to form dimers occurs slowly relative to the rates of the reactions involved in the deamination of L-serine, and in the interconversion of the various L-serine-dimer complexes.

3. The rate of association of monomers is increased by the presence of Mg^{2+} and probably by other cations.

4. The L-serine-specific binding sites on the dimer have a very much greater affinity for L-serine than does the corresponding site (or sites) on the monomer, to the extent that the monomer can be assumed to possess negligible enzymic activity.

---

* It should be noted that, according to the terminology employed by Monod et al., "monomer" describes an enzyme subunit possessing only one substrate-binding site. In the present context, however, the term monomer is used simply to describe the smaller molecular species of L-serine dehydratase (of molecular weight, approximately 45,000); it does not carry any implication concerning the number of L-serine-specific binding sites possessed by that species.

It follows from this model that the addition of L-serine to a solution of the enzyme will lead to the removal of dimer through the formation of a series of complexes with L-serine. These complexes will be designated D_{s1}, D_{s2}--D_{sn}, where the subscript refers to the number of molecules of L-serine bound to a single molecule of dimer. The removal of free dimer in this way will displace the equilibrium between monomer and dimer, causing monomers to associate to form more dimer. This will continue slowly until steady state conditions are established. During this period of adjustment there will be a continuous increase in the rate of L-serine deamination, because enzymically active dimer is being formed from inactive monomer. This will account for the lag period observed in the time course for pyruvate formation.

The relationship between the rate of L-serine deamination and the rate of dimerization will now be considered. In the presence of a saturating concentration of L-serine, that fraction of the enzyme which is dimeric will exist almost entirely as D_{sn}, i.e. the complex in which all L-serine-binding sites are occupied. Under these conditions it will therefore be assumed that the sum of the concentrations of other dimeric species, D, D_{s1}--$D_{s(n-1)}$, is negligible by comparison with the concentration of D_{sn}. Therefore, at any instant, the rate of product formation (pyruvate or ammonia) will be directly proportional to the concentration of D_{sn}. This may be formulated

as follows:

$$dP/dt \propto D_{sn} \qquad (1)$$

where P denotes the concentration of either product and t, time. It follows that

$$\frac{d^2P}{dt^2} \propto \frac{dD_{sn}}{dt} \qquad (2)$$

Thus, at any instant during the period of attainment of steady state conditions, the rate of increase in the rate of product formation, which will be called the rate of activation of the enzyme, will be directly proportional to the rate of dimer formation when the L-serine concentration is saturating. This provides a means for studying the kinetics of dimerization.

EVIDENCE IN SUPPORT OF THE MODEL

Activation of the enzyme involves its dimerization

According to the model the rate limiting reaction in the formation of D_{sn} is the association of monomers. Therefore, for a saturating concentration of L-serine,

$$\frac{dD_{sn}}{dt} \propto M^2$$

where M is the concentration of monomer. Then, substituting from relationship (2),

$$\frac{d^2P}{dt^2} \propto M^2 \qquad (3)$$

Now let "c" be the total concentration of enzyme (in arbitrary units) reckoned as dimer, i.e. the concentration of dimer under hypothetical conditions where the enzyme exists entirely as this species. Let "a" be the fraction of dimer which has dissociated into monomer under specified, real conditions. Then the total concentration of dimeric species will be c (1-a), and the concentration of monomer, 2 ca.

In dilute solution, in the absence of L-serine, the enzyme is known to exist almost entirely as monomer from its gel filtration behaviour. Under these conditions

$$a \doteqdot 1$$

and $$M \doteqdot 2c$$

Therefore, for a short period following the addition of a saturating concentration of L-serine to a dilute solution of enzyme, i.e. while "a" still approximates to 1,

$$\frac{d^2P}{dt^2} \propto C^2$$

$$\text{and } \log\left[\frac{d^2P}{dt^2}\right] \propto 2 \log c \qquad (4)$$

It follows from the latter relationship that a plot of $\log\left[\frac{d^2P}{dt^2}\right]$ against log c will be linear, with a slope of 2.

This prediction was tested experimentally by determining the rate of activation at a range of enzyme concentrations from time

Fig.27. Time courses for the activation of L-serine dehydratase at a range of enzyme concentrations (μg. protein/ml.) The values for $\Delta P/\Delta t$ were calculated from the slopes of tangents drawn at regular time intervals to plots of pyruvate formation ($A_{340nm.}$) against time (t) including those presented in fig.10.

N.B. Zero on the abcissa only approximates to within ±0.5min. of the times of starting the assays.

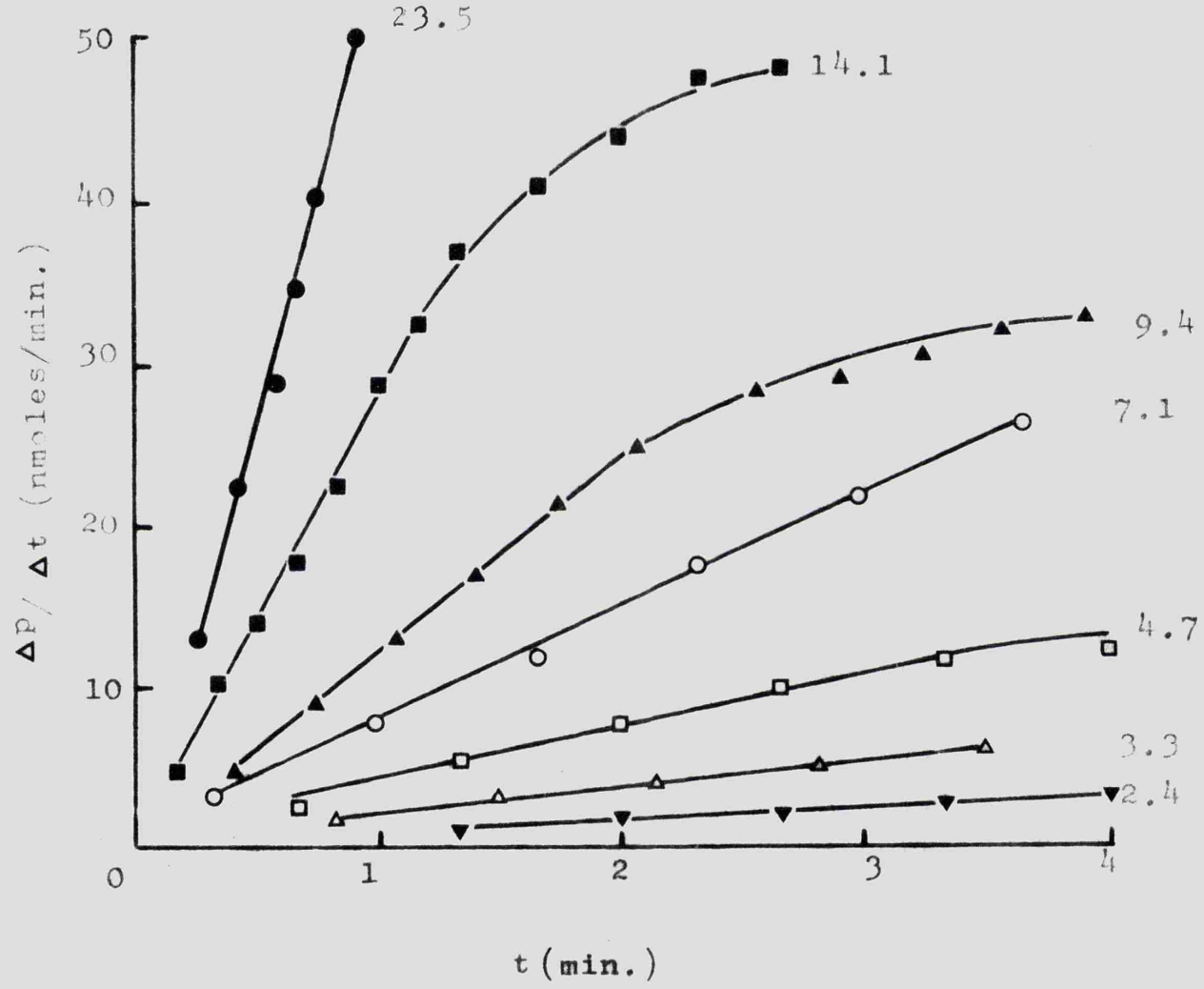
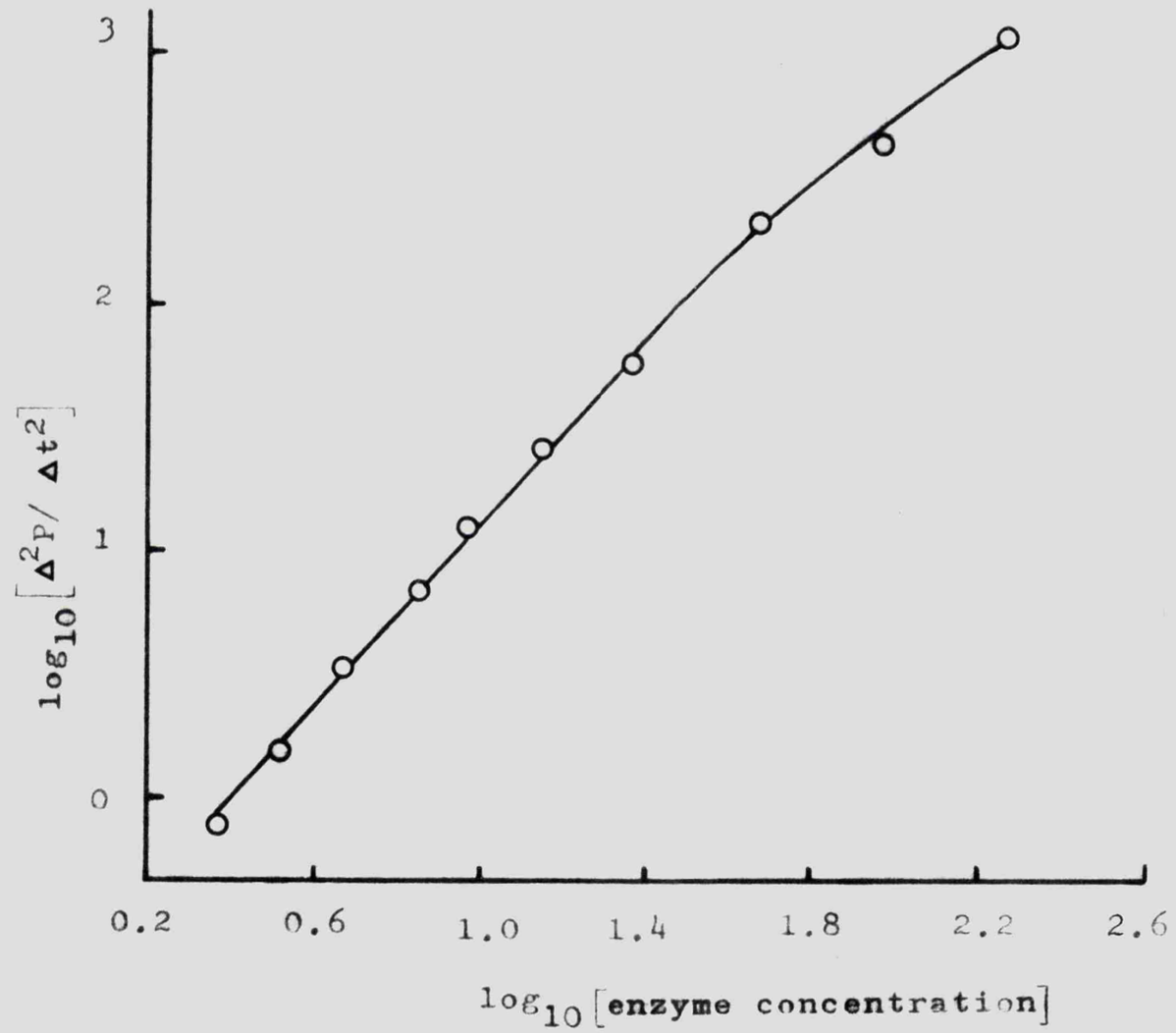

23.5
14.1
9.4
7.1
4.7
3.3
2.4
50
40
30
20
10
0
1
2
3
4
ΔP/ Δt (nmoles/min.)
t (min.)

Fig.28. Determination of the Kinetic order of the activation reaction with respect to L-serine dehydratase concentration. Values for $\Delta^2P/\ \Delta t^2$, at a range of enzyme concentrations (μg. protein/ml.), were calculated from the initial slopes of plots of $\Delta P/\ \Delta t$ (nmoles/min.) against t, including those presented in fig.27. The slope of the curve in the figure opposite is 1.9


3
2
1
0
$\log_{10}[\Delta^2 P/\Delta t^2]$
0.2
0.6
1.0
1.4
1.8
2.2
2.6
$\log_{10}$[enzyme concentration]

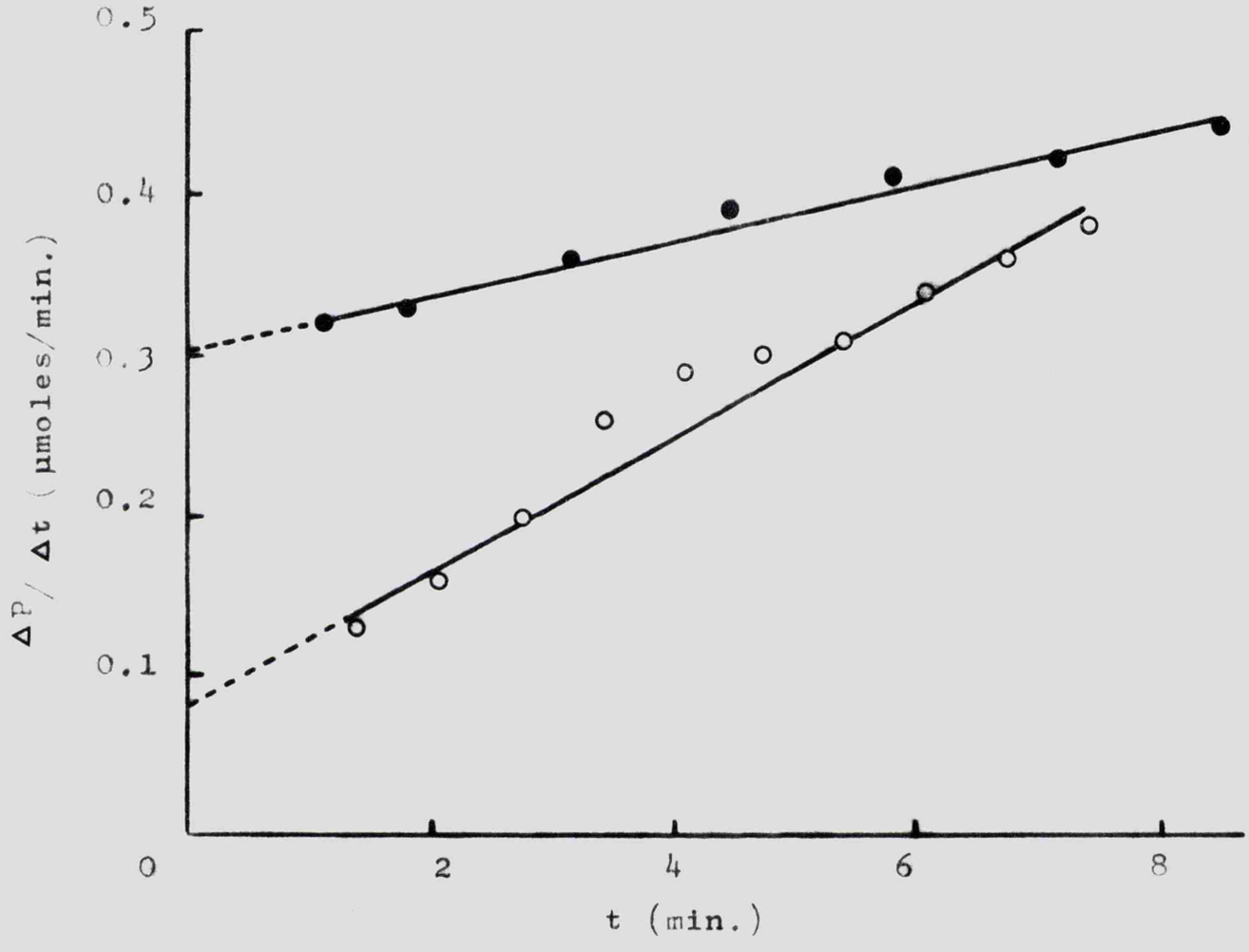
courses for pyruvate formation (including those presented in figure 10). Tangents were drawn at frequent intervals to each of the plots of $A_{340nm.}$ against t (in min.). The slope of these tangents, $\Delta P/\Delta t$ (in μmoles pyruvate/min.), was plotted against t for each enzyme concentration (in µg. protein/ml.). The plots thus obtained were linear over an initial period (fig. 27), but, as is to be expected, $\Delta P/\Delta t$ approached a limiting value over longer periods. $\Delta^2P/\Delta t^2$ was determined for each concentration of enzyme by taking the slope of the initial, linear portion of each of these plots. A plot of $\log_{10} [\Delta^2P/\Delta t^2]$ against $\log_{10}$ [enzyme concentration] was linear over most of its range and had a slope of 1.9 (fig. 28). The good agreement between this result and the predicted value of 2 substantiates the assumption on which the prediction was based: that activation of the enzyme involves its dimerization as a rate limiting reaction.

The plot of $\log_{10} [\Delta^2P/\Delta t^2]$ against $\log_{10}$ [enzyme concentration] became non-linear, the slope of the plot decreasing below 1.9, at enzyme concentrations greater than 0.6 $units_{30}$/ml. This probably indicates that the enzyme can no longer be considered to exist entirely as monomer at these concentrations, an assumption made in predicting the bimolecular kinetics.

Evidence for the existence of dimer in the absence of L-serine

The kinetic data just discussed suggest that even in the absence of L-serine a significant proportion of the enzyme may exist as dimer when it is relatively concentrated. The following

Fig.29. Time courses for the activation of L-serine dehydratase in two assay systems containing identical amounts of enzyme protein taken from a concentrated preparation (●) and from a freshly prepared dilution of that preparation (o). Values for $\Delta P / \Delta t$ were calculated from the data presented in fig. 12 by the procedure described in the legend to fig.27.


0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
ΔP/ Δt (μmoles/min.)
t (min.)

evidence supports this view also.

When a plot of $\Delta P/\Delta t$ against t for a relatively high enzyme concentration is extrapolated to t = 0, it is found to intersect the ordinate at a positive value for $\Delta P/\Delta t$. This suggests that the enzyme preparation contained an enzymically active species before its addition to the assay system. In an experiment (Chapter 8, p.131) where the final enzyme concentration was held constant, the value obtained for $\Delta P/\Delta t$ at t = 0 was reduced by more than half when the enzyme preparation (approximately 14 $units_{22}$/ml.) was diluted twenty-fold just prior to its addition to the assay system (fig. 29). Diluting the enzyme at this point had no effect on v_s (fig. 12). This experiment is taken to indicate that the concentration of the enzymically active species originally present in the concentrated enzyme preparation, presumably the dimer, was decreased by more than half by dilution. In the same experiment, diluting the enzyme prior to its addition to the assay system was observed to increase the initial slope of the plot of $\Delta P/\Delta t$ against t, i.e. the initial rate of activation. Since the rate of activation is dependent upon the monomer concentration squared, this observation is consistent with the expectation that diluting the enzyme preparation will increase its monomer content.

If, in the experiment above, it could be assumed that all the monomer had been converted to dimer at the point where v_s was attained, then it would be possible to estimate the proportion of dimer originally present in the concentrated enzyme preparation. However, evidence to

be presented later (p.228) suggests that, even in the presence of a saturating concentration of L-serine, the conversion of monomer to dimer probably only approaches completion at enzyme concentrations much higher than the one employed in this experiment.

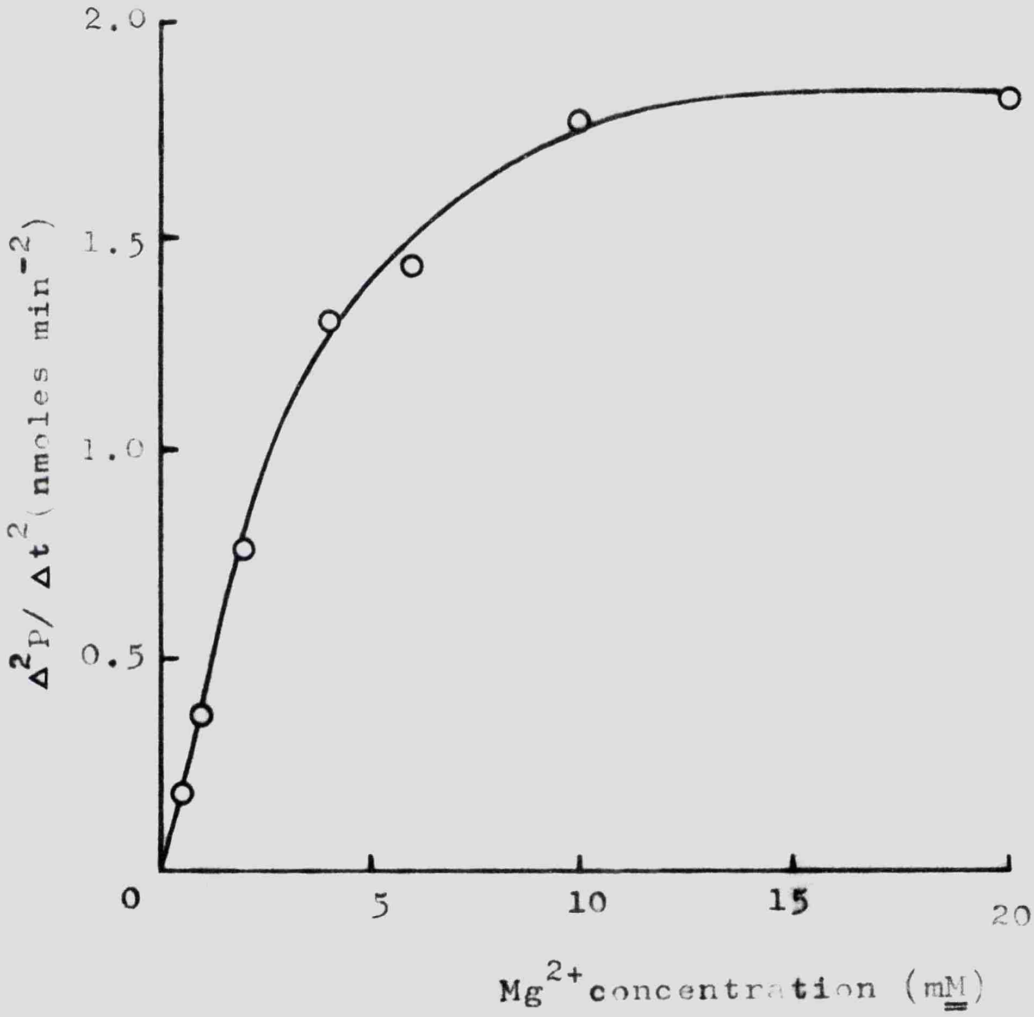
It has been noted elsewhere (Chapter 8, p.131) that the reversible inactivation of the enzyme which accompanies its dilution is complete within 10 - 30 sec. of preparing the dilution at 0^{o}. Therefore, if the dissociation of dimer does account for this inactivation, then it occurs much more rapidly than the formation of dimer by the reverse reaction.

Despite this indirect evidence for the existence of dimer in relatively concentrated, L-serine-free solutions of enzyme, it has not proved possible to detect the presence of dimer under similar conditions by gel filtration (Chapter 11, p.196). This technique would require that some 10% or more of the enzyme should be dimer for its presence to affect the overall mobility of the enzyme peak. On the other hand, some of the results presented in favour of the existence of dimer in relatively concentrated enzyme preparations, while possibly indicating that this species may have accounted for somewhat more than 10% of the enzyme, would nevertheless have revealed the presence of only a few per cent of dimer in these preparations.

Mg^{2+} increases the rate of activation

The relationship between the rate of activation, $\Delta^2P/\Delta t^2$, and Mg^{2+} concentration was investigated on the basis of the data presented in figure 13. A plot of $\Delta^2P/\Delta t^2$ against Mg^{2+} concentration has the

Fig.30. Rate of activation of L-serine dehydratase as a function of Mg^{2+} concentration. Assay systems (1.0ml.) contained 0.07units$_{22}$ of partially purified L-serine dehydratase which had been dialysed against 10mM-tris-HCl buffer, pH8.2, 50mM-L-serine adjusted to pH8.2 with KOH, serving as substrate and buffer, and $MgCl_2$ at the concentrations indicated. Pyruvate formation was followed continuously (at 23°), by the lactate dehydrogenase method. Initial rates of activation ($\Delta^2P/\ \Delta t^2$) were calculated from the time courses for pyruvate formation as described in the text.


2.0
1.5
1.0
0.5
0
5
10
15
20
$\Delta^2 P / \Delta t^2$ (nmoles min^{-2})
Mg^{2+} concentration (mM)

form of a rectangular hyperbola (fig. 30). Activation follows first order kinetics with respect to Mg^{2+} concentration for concentrations up to approximately 2mM. At higher Mg^{2+} concentrations the kinetic order tends to zero.

The following evidence suggests that Mg^{2+} is not directly involved in the deamination of L-serine by this enzyme. For Mg^{2+} concentrations greater than 2 mM, v_s was very nearly independent of Mg^{2+} concentration (table 18). At lower Mg^{2+} concentrations, a plot of $\Delta^2 p/\Delta t^2$ against t revealed that activation took place so slowly that it was not complete during the course of the assay, and so the true v_s was not measured. A second line of evidence is provided by the observation that the deamination of L-serine by toluene-treated cells is not stimulated by added Mg^{2+}.

The kinetics of activation with respect to Mg^{2+} concentration could be explained by postulating that Mg^{2+} reacts rapidly with monomer to form a Mg^{2+}- monomer complex which is the active species in the subsequent rate limiting dimerization reaction. The concentration of the Mg^{2+}-monomer complex will be increased by increasing the Mg^{2+} concentration to the point where effectively all the monomer will exist in this form.

It is considered likely that other cations which have been shown to stimulate the amount of pyruvate formed in a 15 min. assay can probably substitute effectively for Mg^{2+} in the activation process. It might be speculated that cations facilitate the association of monomers by neutralizing excess negative charge on their surface.

This possibility would explain the lack of specificity shown towards the cation, and the observation that divalent cations, with their greater charge density, are much more effective at low concentrations than monovalent ones.

It should be noted that cations, and especially divalent ones, have quite frequently been reported to influence the association and dissociation of proteins (Reithel, 1963; Schachman, 1959; Hurlbert & Jakoby, 1965). Of particular interest in the present context is a report (Schlesinger, 1964) that the inactive subunits which are formed by dissociating alkaline phosphatase (EC3.1.3.1.) from E.coli slowly dimerize to give the active enzyme in the presence of Zn^{2+}, or any one of several other divalent cations.

The rate of activation is independent of L-serine concentration

The model predicts that the rate of activation of the enzyme will be independent of L-serine concentration because L-serine is not a reactant in the rate limiting dimerization reaction. Experimentally, a test of this can only be made at saturating concentrations of L-serine. At sub-saturating concentrations it can no longer be assumed that D_{sn} is the only quantitatively important dimeric species, and so under these conditions $\Delta^2p/\Delta t^2$ is not a direct measure of the rate of dimer formation. It should be emphasized that if L-serine were a reactant in the dimerization reaction, the rate of activation would be expected to increase with increasing L-serine concentration, even over a concentration range which is saturating for the deamination of L-serine.

Table 25. Effect of L-serine concentration on the rate of activation of L-serine dehydratase.

Assay systems (1.0ml.) contained 0.05units$_{30}$ of partially purified L-serine dehydratase, L-serine (adjusted to pH8.2 with KOH) at the concentrations indicated and other components at the usual concentrations. Pyruvate formation was followed continuously with time by the lactate dehydrogenase method. The initial rate of activation of the enzyme, $\Delta^2P/\ \Delta t^2$ (where P denotes the amount of pyruvate formed, in nmoles, and t, the incubation time in min.), was calculated at each L-serine concentration from the corresponding time course for pyruvate formation, as described in the text.

L-serine concentration (mM)	$\Delta^2P\ /\ \Delta t^2$ (nmoles min.$^{-2}$)
25	5.5
50	5.8
75	5.5
100	5.6
150	4.8
200	6.2
250	5.6

The rate of activation, $\Delta^2P/\Delta t^2$, was found by experiment to be independent of L-serine concentration over the range 25-250mM (table 25). It is therefore concluded that L-serine is not a reactant in the rate limiting reaction of activation.

AN EXPLANATION OF EXPERIMENTAL RESULTS ON THE BASIS OF THE MODEL

Effect of enzyme concentration on v_S

According to the model, for a saturating concentration of L-serine,

$$v_S \propto D'_{sn} \text{ (from relationship (1) p.211)}$$

where D'_{sn} denotes the steady state concentration of D_{sn}. If the fraction of enzyme (reckoned as dimer) existing as monomer under these steady state conditions is denoted by a_s, then

$$D'_{sn} \doteq c\,(1-a_S)$$

where c retains its original meaning (p.212).

Therefore $v_S \propto c\,(1-a_S)$ (5)

or $v_S/c = K\,(1-a_s)$

where K is the proportionality constant. At very high concentrations of enzyme a_s will tend to zero.

Hence $\lim_{a_s \to 0} v_S/c = K$

These predictions are borne out experimentally. As is to be expected from relationship (5) above, a plot of v_S against enzyme concentration is non-linear, the departure from linearity being most pronounced at low enzyme concentrations (fig. 11). When the specific activity of the enzyme (v_S/enzyme concentration) was calculated from the same data,

it was found to increase with increasing enzyme concentration until it approached a limiting value at very high concentrations (fig. 11).

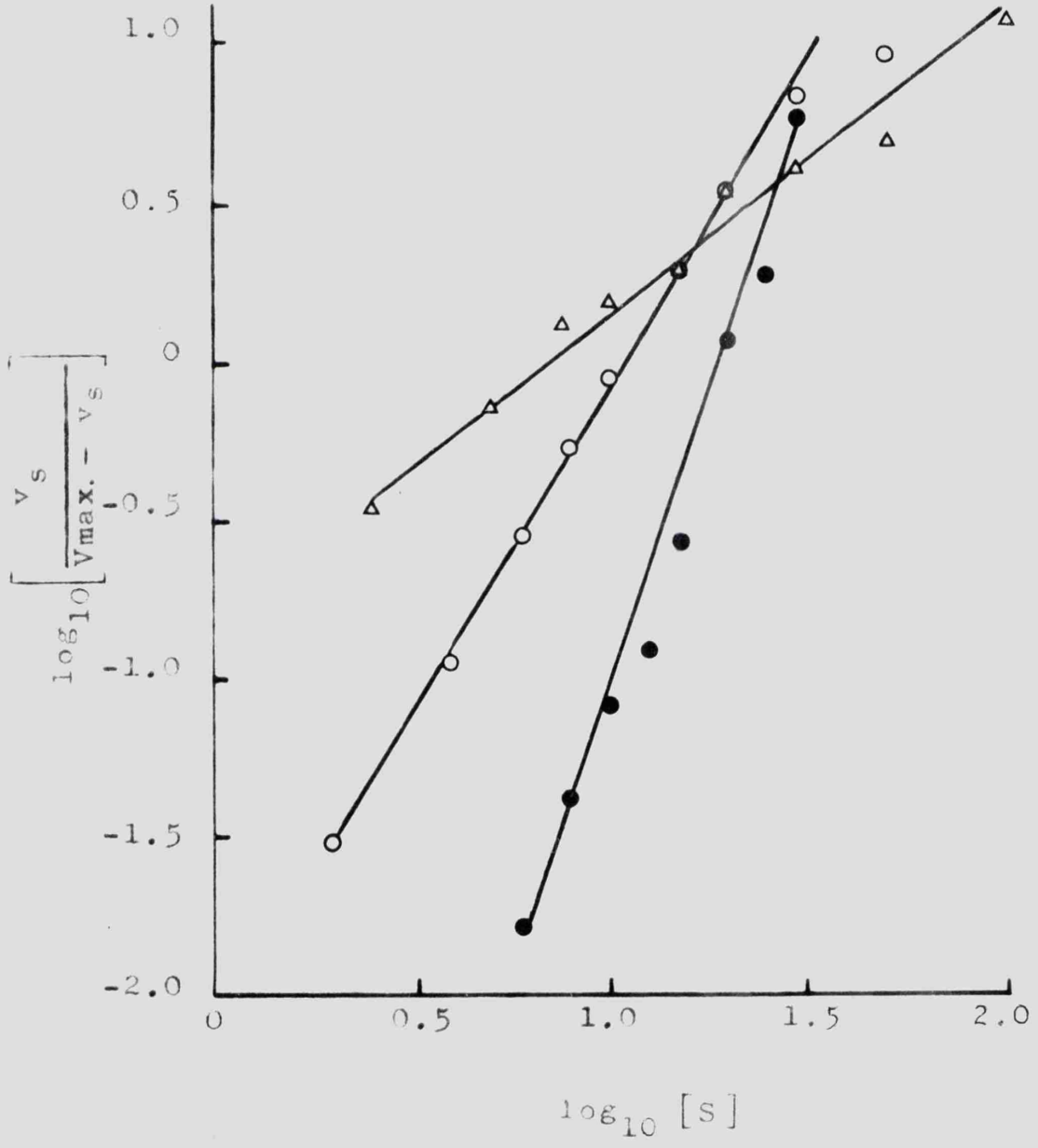
Assuming this explanation for the results to be correct, an estimate can be obtained from the data presented in figure 11 for the proportion of enzyme existing as dimer at a saturating concentration of L-serine, under steady state conditions. It is estimated that only 50% of the enzyme was present as dimer under these conditions when the enzyme concentration was 0.07 - 0.14 $units_{23}$/ml. However, the gel filtration behaviour of the enzyme provides no evidence for the presence of monomer under conditions very similar to these (Chapter 11 p. 194). The inconsistency between these results and the prediction above has not been satisfactorily explained.

Effect of L-serine concentration on v_s

Providing the quaternary structure of the dimer obeys certain symmetry restrictions which have been described by Monod et al. (1965), the model proposed for L-serine dehydratase is, for the purpose of explaining the observed co-operative binding of L-serine, essentially equivalent to the Monod-Wyman-Changeux model. The model proposed for L-serine dehydratase differs from the latter model in one interesting respect: the position of equilibrium between monomer and dimer is influenced by the overall concentration of the enzyme. The Monod-Wyman-Changeux model assumes that the R and T states of an allosteric protein each comprise the same number of protomers and so the relative proportions of the two states is not affected by the overall concentration of the protein.

The model for L-serine dehydratase predicts that the addition of a particular, sub-saturating concentration of L-serine to a concentrated solution of the enzyme will cause a smaller increase in the proportion of enzyme existing as dimer than it will when added to a dilute solution. This is because the concentrated enzyme solution will contain a relatively high proportion of dimer even before the L-serine is added to it. In other words, the binding of L-serine by the enzyme is predicted to become less co-operative as the enzyme concentration is increased to the point where the proportion of dimer becomes appreciable even in the absence of L-serine. The decrease observed in the sigmoidicity of the substrate saturation curve at high enzyme concentrations (fig. 14) is consistent with this prediction. A confirmation of the prediction is obtained from the same data by calculating the Hill coefficient, which has been used as a measure of the strength of co-operative interactions (Monod et al., 1965). This is determined from a plot of $\log \left[v/(Vmax-v) \right]$ against log [S], where v is the velocity of the enzymic reaction at a substrate concentration S, and Vmax. its velocity at a saturating substrate concentration. The Hill coefficient is the slope of this plot at the point where $v/(Vmax-v) = 1$ (Atkinson, 1966). It is a function of both the number of interacting substrate binding sites per enzyme molecule, and of the strength of interaction (Atkinson, Hathaway and Smith, 1965). Where intersite interactions do not exist, the Hill coefficient has a value of 1 regardless of the number of interacting sites. As the strength of interactions increases the Hill coefficient approaches a limiting value equal to

Fig.31. Hill plots for high (14units$_{23}$/ml.) (o) and low (0.035units$_{23}$/ml.) (●) concentrations of partially purified L-serine dehydratase and for the enzymic activity of toluene-treated, glycine-grown cells (▵). These data are taken from figs.14 and 19. S denotes L-serine concentration (mM). The Hill coefficients obtained from the slopes of these plots are 3.6 for the low concentration of extracted enzyme, 2.1 for the high concentration and 1.0 for the enzymic activity of toluene-treated, glycine-grown cells.


1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
0
0.5
1.0
1.5
2.0
$\log_{10}\left[\frac{v_s}{V_{max.} - v_s}\right]$
$\log_{10}[S]$

the number of interacting sites.

Plots of $\log_{10}\left[v_s/(V_{max}-v_s)\right]$ against $\log_{10}$ (S) for L-serine dehydratase were linear (fig. 31), and had slopes of 3.6 and 2.1 for enzyme concentrations of 0.035 and 14 $units_{23}$/ml., respectively. Thus the Hill coefficient, and hence the co-operativity of binding of L-serine, was reduced, as predicted, by increasing the enzyme concentration to a high level. The value of 3.6 obtained for the Hill coefficient at the lower enzyme concentration might be taken to suggest that the dimer molecule possesses more than two substrate binding sites.

Values of 1.0 and 1.1 were obtained for the Hill coefficient of the enzyme in toluene-treated cells, grown on glycine and glucose, respectively. This is taken to indicate that the binding of L-serine shows little or no co-operativity under these conditions.

Effect of pH and mercuric compounds on the sigmoidicity of a plot of v_{15} against L-serine concentration

The value obtained for v_{15} under a particular set of conditions is dependent both on the rate of activation and on v_s. Since the rate of activation is independent of L-serine concentration, the effect of L-serine concentration on v_{15} at any particular enzyme concentration should be comparable with its effect on v_s.

The sigmoidicity of a plot of v_{15} against L-serine concentration was observed to increase at pH's extreme from 7.1 (fig. 15). This result could be explained by postulating that the equilibrium between monomer and dimer is displaced in favour of monomer at the pH's extreme

from 7.1.

On the other hand, no simple explanation has been found for the observation that partially inhibitory concentrations of PHMB (fig. 16), or Hg^{2+}, decrease the sigmoidicity of a plot of v_{15} against L-serine concentration. However, it should be noted that enzyme which has been partially inhibited with PHMB exhibits the usual lag period in forming pyruvate from L-serine.

Results of gel filtration and sedimentation studies

Systems comprising an oligomeric protein in reversible equilibrium with its monomeric form behave in a characteristic fashion when sedimented in the ultracentrifuge (Schachman, 1959), or subjected to gel filtration (Ackers & Thompson, 1965; Winzor & Scheraga, 1963). So long as the attainment of equilibrium is rapid compared with the hypothetical rate of separation of monomer from oligomer, the protein migrates as a single peak with a weight average mobility. When the ratio of monomer to oligomer is of the order of one, the protein peak is characteristically broader than when either species is in great excess.

L-serine dehydratase behaved in this way when subjected to gel filtration in the presence of partially saturating concentrations of L-serine. Since the enzyme always eluted as a single peak it appears that the equilibrium between monomer and dimer was maintained throughout gel filtration. Therefore the intermediate gel filtration rates observed for the enzyme at partially saturating concentrations of L-serine (table 22) will be weight average values. The increase

observed in the breadth of the enzyme peak under these conditions was greatest at 15 and 20 mM concentrations of L-serine (table 22). At these concentrations the gel filtration rates of the enzyme indicated that the ratio of monomer to dimer was close to one.

Comparable results have been reported by Phillips & Wood (1964) for the catabolic L-threonine dehydratase from E.coli. AMP, an allosteric activator of this enzyme, also causes its dimerization. When subjected to sucrose gradient centrifugation in the presence of a partially saturating concentration of AMP, the enzyme sedimented in a single peak. Its sedimentation rate in this instance was intermediate between the rates obtained in the presence of a saturating concentration of AMP, and in its absence. A pronounced broadening of the enzyme peak at partially saturating concentrations of AMP was also reported.

Properties of the enzyme in toluene-treated cells

It is concluded, from a consideration of its following properties, that the enzyme in toluene-treated cells exists very largely as dimer.

1. It forms pyruvate at a constant rate without a detectable lag period.
2. It binds L-serine in a non-cooperative manner.
3. The specific activity of the enzyme in glycine-grown cells is comparable with that of the enzyme extracted from these cells when the latter enzyme is assayed under conditions where it is known to exist largely as dimer.

The high intracellular concentration of the enzyme may in itself

be sufficient to explain the high proportion of enzyme existing as dimer in the cell. If the intracellular concentration of the enzyme were known, this possibility could probably be tested by in vitro experiments, using similar concentrations of purified enzyme.

On the assumption that L-serine dehydratase is uniformly distributed throughout the cytoplasm, a minimal estimate for its intracellular concentration can be calculated. The overall concentration of soluble protein in cells of A. globiformis is estimated to be between 100 and 150 mg./ml.* This is comparable with an estimate of 75 - 105 mg./ml.† for E.coli. The estimate for A. globiformis was multiplied by the specific activities of L-serine dehydratase in toluene-treated cells to obtain minimal estimates of the intracellular concentrations of the enzyme. The minimal estimates obtained are 100 - 300 $units_{30}$/ml., for glycine-grown cells, and 1.5 - 4.5 $units_{30}$/ml., for glucose-grown cells.

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* A centrifugally packed pellet of A.globiformis contained 0.084 - 0.108 g. protein/g. wet weight. Making allowance for intercellular water, which will account for approximately 10 - 20% of the wet weight, and taking a value of 1.1g/ml. for the average density of bacterial cells (Luria, 1960) the estimate above is obtained.

† This figure is based on the following data for E.coli, and on a value of 1.1g./ml. for the average density of bacterial cells. Intracellular water accounts for 73 - 78% of the mass of washed, packed, wet cells, and soluble protein for 30 - 35% of their dry weight (Luria, 1960).

At a concentration somewhat greater (approximately 14 $units_{23}$/ml.) than the minimal estimate for glucose-grown cells, the enzyme extracted from glycine-grown cells, and purified, exhibited the following properties. When Mg^{2+} was omitted from the assay system it exhibited a definite lag period in forming pyruvate. It exhibited a sigmoid saturation curve for L-serine, from which data a Hill coefficient of 2.1 was obtained. Moreover, even at concentrations somewhat greater than 14 $units_{23}$/ml., the enzyme still behaved as monomer when subjected to gel filtration.

The fact that these properties are inconsistent with those exhibited by the enzyme when it is inside the toluene-treated cell suggests two possibilities. The minimal estimate for the concentration of the enzyme within the glucose-grown cell may be far too low, as, for example, would be the case if the enzyme were localized within a cellular compartment. Alternatively, some factor other than the enzyme's concentration, that is to say a factor which has not been realized *in vitro*, may favour the existence of a high proportion of dimer within the cell. For example, the enzyme may be loosely bound to a cellular membrane.

AN APPRAISAL OF THE MODEL

The model is able to explain and to relate, one to another, properties of the enzyme which had hitherto seemed quite unrelated. It has been used as a working hypothesis, from which accurate predictions have been made concerning the kinetics of pyruvate formation with respect to the concentrations of enzyme, L-serine and Mg^{2+} .

There are just two instances where the experimental results are at variance with the predictions of the model. Gel filtration studies provided no evidence for the predicted existence of (a) dimer in concentrated enzyme solutions in the absence of L-serine, and (b) monomer in dilute enzyme solutions in the presence of a saturating concentration of L-serine.

The model might be placed on a firmer experimental basis if the time course for dimerization could be followed and shown to correspond exactly with the time course for activation. It might be possible to follow the course of dimerization directly, by light scattering measurements on purified preparations of the enzyme. For this purpose the enzyme would probably not need to be completely pure, especially if it were free from molecules larger than the monomer.

Any other model which is proposed for the enzyme has to account for the observed kinetics of activation with respect to the concentrations of enzyme, L-serine and Mg^{2+}. These kinetics require that a model should incorporate, as the rate limiting step in the formation of active enzyme, a reaction which involves two molecules of an inactive form of the enzyme, and which does not involve L-serine as a reactant.

An alternative model must also account for the fact that activation and dimerization of the enzyme are both specifically induced by its substrate. It should also explain the observed co-operative binding of substrate.

The two-site hypothesis (see p.207) does not fulfill these conditions without considerable modification, and so does not provide

a satisfactory basis for an alternative model. The following model has been devised specifically for L-serine dehydratase, on the basis of the conditions set out above. It is summarized by the reactions

$$2M + 2S \rightleftharpoons 2\ M{\cdot}S \overset{\text{cation}}{\rightleftharpoons} D{\cdot}S_2$$

where M denotes free monomer; S, L-serine; M·S an L-serine-monomer complex; and $D{\cdot}S_2$, an L-serine-dimer complex containing two L-serine molecules bound to one of dimer. It is assumed that the substrate binding sites on the monomer, although possessing affinity for substrate, have negligible catalytic activity. The corresponding sites on the dimer possess both affinity for substrate and catalytic activity. The L-serine-dimer complex is assumed to dimerize more readily than the free monomer, and dimerization to occur slowly relative to the other reactions.

To explain the co-operative binding of substrate on the basis of this model, it would be necessary to make the additional assumption that the sites on the dimer have greater affinity for substrate than those on the monomer. This model is unnecessarily more complex and somewhat less plausible than the model currently favoured.

The extended Monod-Wyman-Changeux model for L-serine dehydratase is preferred to other models at the present time, because it provides the simplest explanation known of most of the results currently available.

GENERAL CONCLUSIONS

If L-serine dehydratase were to exist as an equilibrium mixture of monomer and dimer *in vivo*, then it would possess most of the properties required of a regulatory enzyme. Any metabolite which were to possess different affinities for monomer and dimer, as does L-serine, would influence the position of the equilibrium between them, and thereby regulate the enzyme's activity. However, a range of coenzymes and substances metabolically related to L-serine have been tested without one having been found which causes significant activation or inhibition of the enzyme. Moreover, considering the role played by L-serine dehydratase in *A. globiformis* growing on glycine, it is difficult to imagine what physiological function would be served by the regulation of this enzyme's activity.

If, on the other hand, an allosteric effector does not exist for the enzyme, then its *in vitro* properties may be of no physiological significance. Thus, it might be speculated that the enzyme exists very largely as dimer *in vivo*, but that it dissociates into monomer upon being extracted from the cell as a result of the accompanying dilution. There are many precedents for proteins dissociating in this way (Reithel, 1963; Schachman, 1959, 1960).

Nevertheless, if the *in vitro* properties of the enzyme have no physiological significance, they may be relevant to allosteric transitions in general. L-serine dehydratase seems to represent a special case of the generalized, allosteric system described in the Monod-Wyman-Changeux model. In this instance the allosteric transition,

at least in one direction, occurs slowly, and involves a reversible association of enzyme subunits.

Acetyl CoA carboxylase is the only other example known to the author of an allosteric system of this type. In the latter case, it is an allosteric effector, and not the substrate, which induces a slow aggregation of inactive enzyme subunits (Vagelos et al., 1963). The fact that L-serine dehydratase has these special features, to which should also be added the involvement of a cation in dimer formation, recommends it as a system worthy of further study.

SUMMARY

A model has been proposed for L-serine dehydratase which is based on the Monod-Wyman-Changeux model for allosteric proteins. It is postulated that the extracted enzyme always exists as an equilibrium mixture of monomer and dimer, and that the monomer possesses negligible affinity for L-serine by comparison with the dimer. The rate of dimerization is assumed to be slow relative to the rates of other reactions involved in L-serine deamination, and to be increased by the presence of Mg^{2+} and probably by other cations.

The following predictions made on the basis of this model have been verified by experiment.

1. The rate of activation, $\Delta^2 P / \Delta t^2$ (where P. denotes the concentration of pyruvate or ammonia; and t, time), will be:

a. Directly proportional to the enzyme concentration squared, for low enzyme concentrations.

b. Independent of L-serine concentration, for saturating concentrations of L-serine.

c. A function of Mg^{2+} concentration.

2. The binding of L-serine by the enzyme will become less co-operative as the enzyme concentration is increased to a high level. Some indirect evidence has been obtained for the predicted existence of dimer in concentrated, L-serine-free preparations of the enzyme, but this is in conflict with the results of gel filtration studies. The model also accounts for the observed gel filtration behaviour of the enzyme at partially saturating concentrations of L-serine.

The properties exhibited by the enzyme in toluene-treated cells are taken to suggest that the enzyme exists very largely as dimer under these conditions.

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ABSTRACT

Some aspects of glycine metabolism in Arthrobacter globiformis,

by E.S. Bridgeland

Arthrobacter globiformis is able to utilize glycine as sole carbon and energy source for growth. It has been shown that the glycine is first converted to pyruvate by way of the serine pathway. Two of the enzymes in this pathway, namely the tetrahydropteroylglutamate-dependent glycine cleavage system and L-serine dehydratase, increase in activity as the bacterium adapts to growth on glycine.

A. globiformis possesses a pyruvate carboxylase and a functioning tricarboxylic acid cycle. Therefore the pyruvate formed from glycine should serve both as a source of energy and of biosynthetic precursors. The pyruvate carboxylase shows an absolute requirement for catalytic amounts of acetyl CoA. It is cold labile and protected from cold inactivation by acetyl CoA.

A. globiformis possesses a phosphoenolpyruvate carboxykinase which is considerably more active with inosine or guanosine nucleotides than with adenosine nucleotides. During growth on glycine and other substrates not degraded to phosphoenolpyruvate, the level of this enzyme is relatively high, suggesting that its function is probably to provide the phosphoenolpyruvate required under these growth conditions for gluconeogenesis and other biosynthetic purposes.

The L-serine dehydratase extracted from A. globiformis is slowly activated by its substrate, L-serine, for which it exhibits a

sigmoid saturation curve. The rate of activation of the enzyme is increased by the presence of Mg^{2+}, and, most probably, by that of any one of a variety of other cations. The enzyme has been shown to undergo a reversible dimerization in the presence of L-serine. Kinetic studies suggest that dimerization of the enzyme is the rate limiting reaction in its activation by L-serine.

In contrast to the extracted enzyme, the enzyme in toluene-treated cells shows no evidence of substrate activation.

Most of the unusual properties of the extracted enzyme have been explained in terms of an extended form of the Monod-Wyman-Changeux model for allosteric proteins.

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The Metabolism of Glycine by *Arthrobacter globiformis*

By K. M. Jones and E. S. Bridgeland. (*Department of Biochemistry, University of Leicester*)

Two routes for the metabolism of glycine have been demonstrated in micro-organisms growing on this amino acid as sole source of carbon. One pathway, found in *Pseudomonas* sp., is initiated by the conversion of glycine into glyoxylate, which is metabolized via tartronic semialdehyde to intermediates of the glycolytic pathway (Callely, Trudgill & Dagley, 1961). In the other, demonstrated in the anaerobe *Peptococcus glycinophilus*, glycine is oxidized to carbon dioxide, ammonia and methylenetetrahydrofolic acid, which then reacts with a second molecule of glycine to form serine. The serine is deaminated to yield pyruvate (Sagers & Gunsalus, 1961). It is the purpose of this communication to show that the aerobic soil bacterium, *Arthrobacter globiformis*, metabolizes glycine via serine and not via glyoxylate.

When cultures of *A. globiformis* growing on glycine are given [2-^{14}C]glycine, label appears first in serine and shortly thereafter in citrate and aspartate. Radioactivity can also be detected in pyruvate, but not in glyoxylate. Washed suspensions and extracts of glycine-grown organisms oxidise glycine, L-serine and pyruvate, but not glyoxylate or formate. The formation of pyruvate, but not of glyoxylate, can be demonstrated in extracts metabolizing glycine.

Three enzyme-catalysed reactions, which are together capable of converting glycine into pyruvate, have been detected in extracts of glycine-grown *A. globiformis*. They are: (1) the tetrahydropteroylglutamic acid-dependent cleavage of glycine to methylenetetrahydropteroylglutamic acid, carbon dioxide and ammonia; (2) the reversible reaction between glycine and methylenetetrahydropteroylglutamic acid to form L-serine and tetrahydropteroylglutamic acid, catalysed by serine hydroxymethylase; and (3) the deamination of L-serine to pyruvate and ammonia, catalysed by L-serine dehydratase (Bridgeland & Jones, 1965).

The glycine cleavage system and L-serine dehydratase are found at low levels in extracts of glucose-grown cells, but at high levels in cells grown on glycine. The levels of these enzymes rise sharply during the lag period of 2–4hr. which ensues when organisms grown on glucose are transferred to glycine medium. The level of serine hydroxymethylase is not markedly affected by the nature of the growth substrate.

Glyoxylate carboligase, tartronic semialdehyde reductase, isocitrate lyase, malate synthase and the enzymes of the β-hydroxyaspartate pathway (Kornberg & Morris, 1963, 1965) are all absent from, or present at insignificant activities in, glycine-grown *A. globiformis*.

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L-Serine Dehydratase of *Arthrobacter globiformis*

By E. S. BRIDGELAND and K. M. JONES. (*Department of Biochemistry, University of Leicester, Leicester*)

The conversion of L-serine into pyruvate is thought to be a key step in the utilization of glycine as sole source of carbon and nitrogen for growth by *Arthrobacter globiformis* (Morris, 1963). The enzyme catalysing this reaction, L-serine dehydratase (E.C. 4.2.1.13) (Yanofsky & Reissig, 1953; Greenberg, 1961), is present in high activity in glycine-grown *A. globiformis*.

When studied in toluene-treated cells the enzyme showed the following properties.

(*a*) Pyruvate production proceeded linearly with time.

(*b*) No requirement for cations was observed.

(*c*) The L-serine saturation curve deviated only very slightly from the usual Michaelis–Menten form.

However, when extracts were prepared, either from toluenized cells by lysozyme treatment or from fresh cells by lysozyme treatment or ultrasonic vibrations, the enzyme exhibited markedly different properties.

(*a*) There was a lag in pyruvate formation. The rate of the reaction increased over the first 10–30 min. and then became constant. The lag could be overcome by preincubating the extract with L-serine and a suitable cation, but pyruvate, NH_4^+ and a number of compounds structurally related to serine were ineffective. When an extract, preincubated with L-serine, was passed through a column of Sephadex G-25, the lag in pyruvate formation reappeared. It seems unlikely that the lag is due to the accumulation of a hypothetical intermediate between L-serine and pyruvate, since when L-[3-^{14}C]serine was added to an incubation mixture in which pyruvate formation was proceeding at a constant rate, the appearance of radioactivity in pyruvate showed no lag.

(*b*) The enzyme showed a cation requirement which could be fully satisfied by Mg^{2+}, Ca^{2+}, K^+ or NH_4^+, though the divalent ions were effective at one-tenth of the concentration of the monovalent ions.

(*c*) The serine saturation curve of the enzyme had a markedly sigmoid shape, though the saturation curve for K^+ had the classical Michaelis–Menten form. Treatment of the extract with Hg^{2+} (2×10^{-7} M) or *p*-hydroxymercuribenzoate (10^{-5} M) led to a more nearly normal serine saturation curve.

These results are consistent with the view that extracts of *A. globiformis* contain an L-serine dehydratase which is slowly activated by its substrate.

This work was performed during the tenure by E. S. B. of a D.S.I.R. Research Studentship.

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Formation of Dicarboxylic Acids and Phosphoenolpyruvate in *Arthrobacter globiformis*

By E. S. BRIDGELAND and K. M. JONES. (*Department of Biochemistry, University of Leicester*)

Arthrobacter globiformis metabolizes glycine to pyruvate (Jones & Bridgeland, 1966). In order to grow on glycine the organism must be able to convert pyruvate into the C_4 dicarboxylic acids necessary to maintain the tricarboxylic acid cycle and into phosphoenolpyruvate (PEP) which serves as a precursor of carbohydrates and related materials (Kornberg, 1966).

These interrelated conversions are brought about, in *A. globiformis*, by the action of pyruvate carboxylase (EC 6.4.1.1), which carboxylates pyruvate to oxaloacetate, and of PEP carboxykinase (EC 4.1.1.32), which converts oxaloacetate into PEP. The levels of these enzymes in cells of glycine-grown *A. globiformis* are sufficient to account for the observed rate of growth, while other enzymes which might serve these functions [PEP synthase (Cooper & Kornberg, 1965), PEP carboxylase (EC 4.1.1.31; Cánovas & Kornberg, 1966), PEP carboxytransphosphorylase (EC 4.1.1.38) and malic enzyme (EC 4.1.1.40)] are absent. Further, the levels of PEP carboxykinase in cells grown on glycine or lactate are very much higher than those found in cells grown on glycerol or glucose.

The pyruvate carboxylase in extracts of *A. globiformis* catalyses the reaction between pyruvate and bicarbonate to yield oxaloacetate in the presence of ATP, Mg^{2+} ions, and acetyl-CoA. The reaction is inhibited by incubating the extract with avidin. Using a partially purified preparation, free from citrate synthase activity, there is an absolute requirement for acetyl-CoA, which cannot be replaced by CoA. Acetyl-CoA acts catalytically in the pyruvate carboxylase reaction; the formation of 0·22 μmole of oxaloacetate occurred in the presence of 0·10 μmole of acetyl-CoA, of which 0·07 μmole was recovered unchanged after the reaction was complete. The enzyme is markedly cold-labile and can be protected against cold by 0·5 mM-acetyl-CoA or by M-sucrose, but not by CoA. In its dependence upon acetyl-CoA, the pyruvate carboxylase of *A. globiformis* resembles more closely the enzyme from chicken liver (Keech & Utter, 1963) than those from microbial sources, which either show activity in the absence of acetyl-CoA, though stimulated by its presence (Ruiz-Amil, de Torrontegui, Palacián, Catalina & Losada, 1965; Cooper & Benedict, 1966), or are unaffected by acetyl-CoA (Seubert & Remberger, 1961; Bloom & Johnson, 1962).

PEP carboxykinase catalyses the reaction:

$$\text{Oxaloacetate} + \text{GTP (or ITP)} \rightleftharpoons \text{PEP} + CO_2 + \text{GDP (or IDP)}$$

The enzyme from *A. globiformis*, assayed by measuring the PEP-dependent fixation of carbon dioxide, shows approximately the same activity with either GDP or IDP as nucleotide, but only one-tenth of this activity with ADP. This nucleotide specificity is similar to that of the analogous enzyme from higher animals (Kurahashi, Pennington & Utter, 1957; Chang & Lane, 1966), and differs from that of the enzyme in yeast (Cannata & Stoppani, 1963) and other micro-organisms (Baugh, Lanham & Surgalla, 1964; Theodore & Engelsberg, 1964; L. C. Sage, unpublished observations), which require adenine nucleotides.

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