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SULFINIC AND SULFONIC ANALOGS OF γ-AMINOBUTYRIC ACID AND SUCCINATE SEMIALDEHYDE, NEW SUBSTRATES FOR THE AMINOBUTYRATE AMINOTRANSFERASE AND THE SUCCINATE SEMIALDEHYDE DEHYDROGENASE OF *PSEUDOMONAS FLUORESCENS*

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SUMMARY

Homohypotaurine and homotaurine are transaminated by cell-free extracts of *Pseudomonas fluorescens* in the presence of α-ketoglutarate; the maximum rates of these transaminations are even higher than those observed for γ-aminobutyrate. The identity of the aminotransferase of homohypotaurine with aminobutyrate aminotransferase (EC 2.6.1.19) was demonstrated; equilibrium and kinetic constants were calculated for the different substrates.

Homohypotaurine and homotaurine in the same extracts induce a reduction of $NADP^+$ or NAD^+, in the presence of α-ketoglutarate. It is inferred from several results that the sulfinic and sulfonic aldehydes, resulting from the transamination step and characterized by thin-layer chromatography, are dehydrogenated in the presence of $NADP^+$ or NAD^+, most probably by the succinate semialdehyde dehydrogenase (EC 1.2.1.16), to form the corresponding sulfinic and sulfonic acids; in this reaction, the rate of formation of the postulated 3-sulfopropionic acid is low as compared to that of succinic acid or to that of the postulated 3-sulfinopropionic acid.

Neither taurine nor hypotaurine are substrates for the aminobutyrate aminotransferase of *Ps. fluorescens*.

INTRODUCTION

Aminobutyrate aminotransferase (EC 2.6.1.19)[1,2] and succinate semialdehyde dehydrogenase (EC 1.2.1.16)[3], when produced by *Pseudomonas fluorescens*, are enzymes with a very high specificity for their substrates; so far, only β-oxy-γ-aminobutyric acid has been found to act as a substrate in place of γ-aminobutyric acid with

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a comparatively slow rate of transamination[4] and neither α-ketoglutaric acid nor L-glutamic acid or succinate semialdehyde could be substituted[1,2]. Previous research from our laboratory has resulted in the synthesis and the isolation of sulfinic and sulfonic analogues of L-glutamic acid (homocysteinesulfinic acid) and γ-aminobutyric acid (homohypotaurine and homotaurine)[5,6]. Metabolic experiments have also provided evidence that homocysteinesulfinic acid, an oxidation product of homocysteine in mammals[7], acts as a substrate of several L-glutamic acid-specific enzymes[8–10]. These results stimulated interest in the development of this research to see if homohypotaurine and homotaurine could also act as substrates for the enzymes of γ-aminobutyrate metabolism in the same way, especially for the aminobutyrate aminotransferase and the succinate semialdehyde dehydrogenase of *Ps. fluorescens*. We report here the results of this research.

MATERIALS AND METHODS

Materials

Homohypotaurine and homotaurine were prepared according to Jollès-Bergeret[6]. Hypotaurine was purchased from Calbiochem and L-glutamate dehydrogenase of beef liver from Koch–Light. Lyophilysed strains of *Ps. fluorescens* (Pasteur Institute Collection 5690, 6326, 6913) were obtained from the Pasteur Institute, Paris.

Incubation mixtures

The composition of the incubation mixtures (0.2–0.3 ml) and the final concentration of the different components were the following ones, unless otherwise specified: buffer (0.1 M sodium pyrophosphate–HCl or 0.5 M Tris–HCl or universal buffer of Britton and Robinson, 0.15 M), substrates (amines and α-ketoglutarate) at a 0.05 M concentration, 0.025 M $NADP^+$ or NAD^+ and enzymatic preparations at different degrees of purification as specified for each experiment. Incubations were performed at 20 or 37 °C.

Purification of aminobutyrate aminotransferase

Aminobutyrate aminotransferase was purified from *Ps. fluorescens* (strain 5690), according to the method of Scott and Jakoby[2]. The purification involved the different steps summarized in Table II (sonication was carried out with an apparatus from Ultrasons Annemasse S.A., type 150 T.S.). Protein determination was performed by turbidimetry[11] in the first purification steps and then by ultraviolet absorption at 280 nm.

Identification, separation and assay of reaction products

The identification of the low molecular weight compounds present in the incubation mixture was realized by thin-layer chromatography (TLC aluminium sheets cellulose, Merck) in the following solvents, (v/v)[12]: phenol–water (8:2), Solvent A; *n*-butanol–acetic acid–water (50:25:25), Solvent B; ethyl acetate–acetic acid–water (60:25:15), Solvent C; *n*-butanol–ethanol–0.5 M ammonia (70:10:20), Solvent D. For the localization of the components on the chromatograms, the following reagents were used: for amino acids and α-keto acids, 0.1% ethanolic ninhydrin solution containing 2 drops of 2,6-lutidine for 100 ml of the reagent[13]; for aldehydes and ketones,

2,4-dinitrophenylhydrazine reagent[14]; for reducing sulfur compounds, iodoplatinate reagent[15], $FeCl_3$ (ref. 16), KI in the presence of HCl[16].

In some cases, before the assay of the reaction products, aliquots of the reaction mixture were chromatographed on microcolumns containing 0.5 g of ion-exchange resins: Ag 1-X8 (formate form) for the separation of the glutamate formed by transamination of the amino acids initially present as γ-aminobutyrate, homohypotaurine or hypotaurine; glutamate was eluted from the resin with 1 M HCl. Dowex 50W X8 (H^+) was used either to retain the glutamate when homotaurine or taurine had been initially introduced, sulfonated amines not being retained on the resin and glutamate being eluted in this case with 1 M NaOH, or to separate the sulfinic aldehydes or carboxylic acids which originated from the reaction from homohypotaurine or hypotaurine.

Glutamate was assayed spectrophotometrically at 570 nm using a ninhydrin reagent according to Lee and Takahashi[17].

Sulfinic compounds were also determined spectrophotometrically at 500 nm, after reaction with the iodoplatinate reagent of Awwad and Adelstein[18], under the conditons described for sulfinic compounds[6].

Inorganic sulfite was determined directly in the reaction mixture by the method of Sörbo[19] using pararosaniline.

NADH and NADPH were assayed spectrophotometrically at 340 nm on a diluted aliquot of the incubation mixture (extinction coefficient, $6.22 \cdot 10^6$ cm^2/mol (ref. 20)).

Aldehydes were determined by their condensation products with 3,5-diaminobenzoic acid[21] by the method of Salvador and Albers[22] on aliquots of the incubation mixture, using a Farrand MK 1 fluorimeter.

RESULTS

1. Kinetics of the transaminations and of the subsequent dehydrogenations of sulfinic and sulfonic analogues of γ-aminobutyrate

Taurine, homotaurine, hypotaurine and homohypotaurine have been tested in the presence of α-ketoglutarate and $NADP^+$ as potential substrates for the γ-aminobutyrate enzymes found in the bacterial extract after sonication (Step I of the purification procedure). Simultaneous determinations of glutamate and NADPH have been realized; the results, summarized in Table I, indicate at first that at pH 8.6 where the aminobutyrate aminotransferase is the most active on γ-aminobutyrate[1] the only compounds other than γ-aminobutyrate which may be efficiently transaminated are homohypotaurine and homotaurine. Under our experimental conditions, the rates of transamination of these sulfur-containing amines were even higher than for γ-aminobutyrate, whereas hypotaurine and taurine were poor transamination substrates.

Hypotaurine and taurine did not induce any reduction of $NADP^+$ to NADPH in the presence of enzymatic extracts, but quite the reverse was noted for homohypotaurine and homotaurine and these results strongly suggest that the transamination products of these two amines can be enzymatically dehydrogenated just as is the case for succinate semialdehyde by extracts of *Ps. fluorescens*. However, as opposed to what was noted for homohypotaurine and γ-aminobutyrate, dehydroge-

TABLE I

TRANSAMINATIONS AND DEHYDROGENATIONS OF γ-AMINOBUTYRATE AND ITS SULFUR ANALOGUES IN *Ps. fluorescens*

Strain 5690; enzyme, Step I of the purification; buffer, Tris–HCl, pH 8.6; $5 \cdot 10^{-2}$ M α-ketoglutarate; 37 °C. Glutamate and NADPH were determined in two different experiments.

Amino acid present ($5 \cdot 10^{-2}$ *M*)	*µmoles glutamate/ min per mg protein*	*µmoles NADPH/ min per mg protein*	*% enzymatic activity*	
			Transamination	*Dehydrogenation*
γ-Aminobutyrate	0.620	0.373	100	100
Taurine	<0.010	0.000	<2	0
Hypotaurine	0.031	0.000	5	0
Homotaurine	0.795	0.096	128	26
Homohypotaurine	0.870	0.500	140	133

nation seemed to be, to a large extent, the limiting reaction when homotaurine was tested as a substrate, since in this case the amount of NADPH obtained is much lower than that of glutamate.

Neither hypotaurine nor homohypotaurine gave rise to any formation of inorganic sulfite in the incubation mixture.

II. Transamination and dehydrogenation with homohypotaurine as substrate

A. Identification of the reaction products. In addition to the substrates introduced (homohypotaurine and α-ketoglutaric acid), it was possible to identify, in the reaction mixture, the following substances by thin-layer chromatography on cellulose: in the absence of $NADP^+$, glutamate and a sulfinic aldehyde; the R_F values of the latter were 0.23 in Solvent A, 0.33 in Solvent C and 0.20 in Solvent D; in Solvent B, two

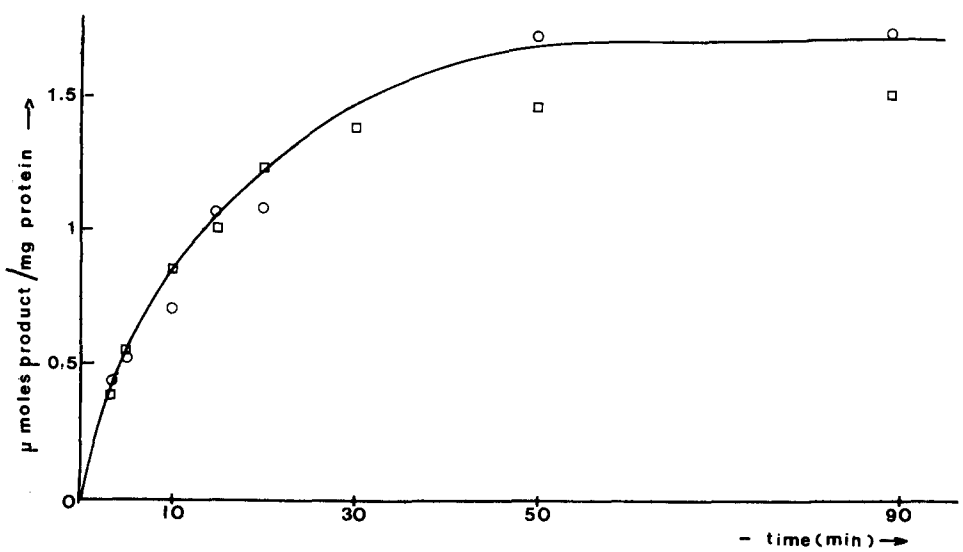

Fig. 1. Stoichiometry of the transamination reaction with homohypotaurine as substrate. Strain 5690; enzyme at Step I of the purification; buffer, Tris–HCl, pH 8.6; 37 °C. □—□, sulfinic aldehyde; ○—○, glutamate.

spots were noted at R_F values of 0.34 and 0.40. In the presence of $NADP^+$, glutamate and a sulfinic acid; the R_F values of the latter were 0.07 in Solvent A, 0.44 in Solvent B and 0.02 in Solvent D.

The occurrence of an aldehyde group, which was postulated on the chromatograms according to the positive reaction with the 2,4-dinitrophenylhydrazine reagent, could further be demonstrated by its reaction with 3,5-diaminobenzoic acid; a fluorescent compound was obtained with maximum excitation at 410 nm and maximum emission at 500 nm.

B. Stoichiometry of the reactions. Fig. 1 indicates that there is a good relationship between the determination of glutamate by the ninhydrin method and the sulfinic aldehyde by the iodoplatinate method during the transamination of hypotaurine. The formation of the sulfinic aldehyde could also be followed by fluorimetry[21,22]; however, the fluorescence of the condensation product obtained from the sulfinic aldehyde and 3,5-diaminobenzoic acid was less intense, for the same amount of newly produced glutamate, than when γ-aminobutyrate was used as a substrate.

The stoichiometry of the dehydrogenation reaction has been studied after addition of $NADP^+$ to the incubation mixtures. The results are compared with those obtained with γ-aminobutyrate (Fig. 2). These data confirm that in both cases transamination is the limiting factor and that, under our experimental conditions, the determination of NADPH can be used for the evaluation of the transamination reaction.

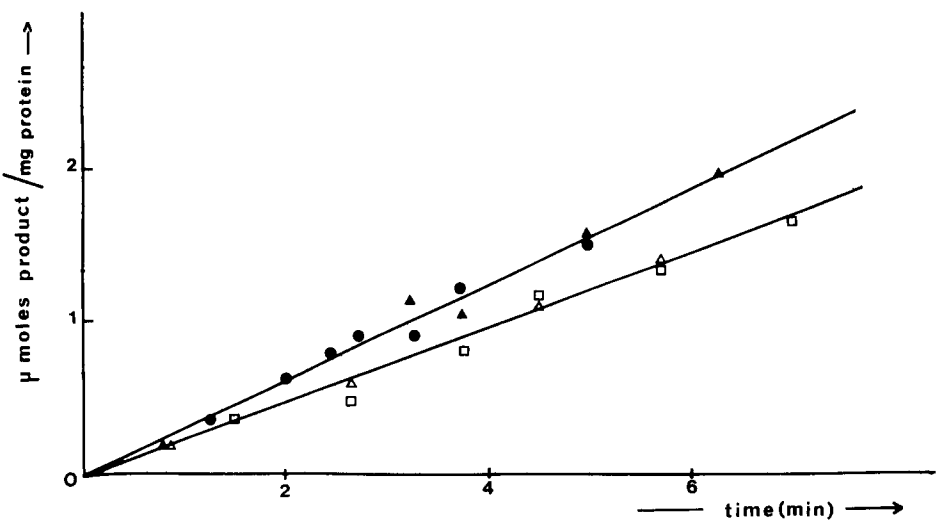

Fig. 2. Stoichiometry of the dehydrogenation reaction with γ-aminobutyrate and homohypotaurine as substrates. Strain 5690; enzyme at Step I of the purification; buffer, Tris–HCl, pH 8.6; 37 °C. ▲—▲, glutamate (homohypotaurine); △—△, glutamate (γ-aminobutyrate); ●—●, NADPH (homohypotaurine); □—□, NADPH (γ-aminobutyrate).

Catalytic amounts of $NADP^+$ could be used if the enzymatic system is coupled with a glutamate dehydrogenase in the presence of NH_4^+. Under these conditions, $NADP^+$ was continuously regenerated and the quantity of glutamate found was

about twice the quantity of the sulfinic acid produced; the equations are very likely the following ones:

$$R\text{–}CH_2NH_2(\gamma\text{-aminobutyrate or homohypotaurine}) + R'\text{–}COCOOH(\alpha\text{-ketoglutaric acid}) \rightleftharpoons R\text{–}CHO + R'\text{–}CH(NH_2)COOH \text{ (glutamic acid)}$$

$$R\text{–}CHO + NADP^+ + H_2O \rightarrow R\text{–}COOH + NADPH$$

$$R'\text{–}COCOOH + NADPH + NH_4^+ \rightleftharpoons R'\text{–}CH(NH_2)COOH + NADP^+ + H_2O$$

C. Optimum pH of the transamination reaction with homohypotaurine; relationship between activity and enzyme concentration. The optimum pH for the transamination of homohypotaurine with α-ketoglutaric acid was determined with Tris–HCl or universal buffer and found to be close to pH 8.6 for the three strains of *Ps. fluorescens*; this pH also represents the optimum pH for the transamination of γ-aminobutyrate[1].

Under our working conditions (initial rate calculated from the results obtained in 10 min), the production of glutamate or of sulfinic aldehyde is proportional to the concentration of enzyme (Step V of the purification), up to a value of 0.125 mg protein in 1 ml of incubation mixture.

III. Identity of the enzymes using γ-aminobutyrate and homohypotaurine as substrates

A. Thermal inactivation of aminotransferases. Aliquots of the enzyme preparation (strain 5690 of *Ps. fluorescens*, Step I of the purification) were incubated for 15 min at different temperatures and cooled at 0 °C; their activity on γ-aminobutyrate and homohypotaurine was then determined at 37 °C. The enzymatic activities, expressed as a percentage of the untreated blanks, are the same for the two sub-

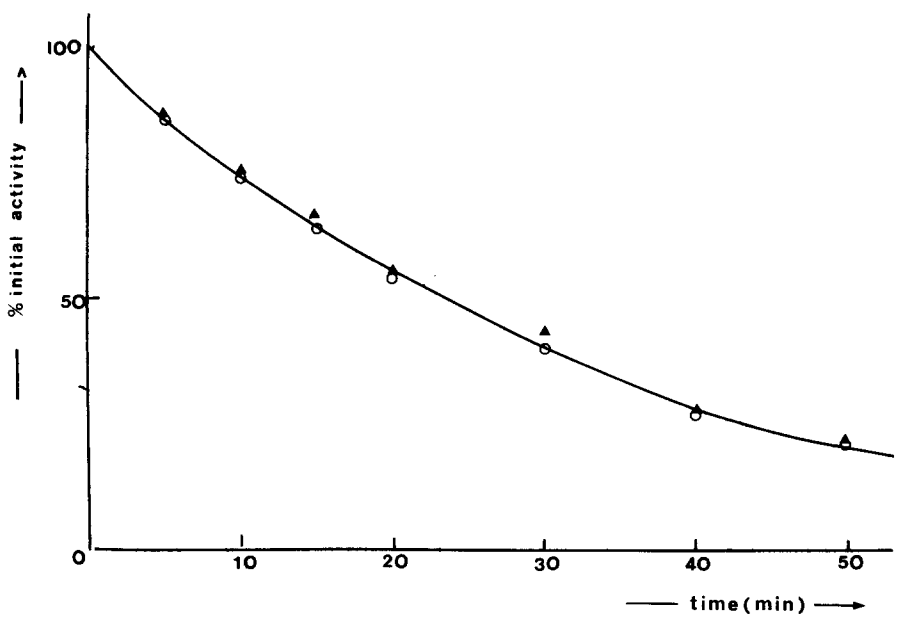

Fig. 3. Kinetics of thermal inactivation of aminobutyrate aminotransferase at 61 °C. Strain 5690; enzyme at Step I of the purification; buffer, Tris–HCl, pH 8.6. ▲—▲, γ-aminobutyrate; ○—○, homohypotaurine.

strates. The kinetic study of the thermal denaturation at the temperature of half-denaturation in 15 min (61 °C) indicates that, for incubation periods of 5–10 min, the loss of transaminating activity on the two substrates occurs at the same rate (Fig. 3).

B. Relative activities of enzyme preparations on γ-aminobutyrate and homohypotaurine during the purification of aminobutyrate aminotransferase. At each step of the purification of the enzyme, according to the method of Scott and Jakoby[2], the specific activities of the enzyme on γ-aminobutyrate and homohypotaurine have been determined. Table II indicates that the ratio of the specific activities on γ-aminobutyrate and homohypotaurine is the same at any step of the purification procedure (ratio, 1.46).

TABLE II

RATIOS OF SPECIFIC ACTIVITIES ON HOMOHYPOTAURINE AND ON γ-AMINOBUTYRATE AT DIFFERENT STEPS OF PURIFICATION OF AMINOBUTYRATE AMINOTRANSFERASE OF *Ps. fluorescens*

Step of purification	*Spec. act. on γ-aminobutyrate (μmoles/min per mg protein)*	*Spec. act. on homohypotaurine (μmoles/min per mg protein)*	*Ratio of spec. act. homohypotaurine/ γ-aminobutyrate*
I. Extract after sonication	0.59	0.86	1.44
II. Precipitation by ammonium sulfate	1.43	2.09	1.46
III. 1st precipitation by acetone	1.96	2.94	1.49
IV. 2nd precipitation by acetone	1.96	2.93	1.49
V. Supernatant of tricalcium phosphate gel	3.76	5.25	1.40

In our experiments, we obtained finally an enzyme with a specific activity of 3.76 on γ-aminobutyrate; this corresponds approximately to the value found by Scott and Jakoby[2]; however, in contrast to the findings of these authors, the inactive proteins were retained by the gel while the purified aminotransferase was found in the supernatant. This purified enzyme was rapidly inactivated even in the presence of 0.005% dithiothreitol. Many experiments were carried out with the enzyme purified until Step III; this enzyme preparation could be kept active at −15 °C for several months in the presence of dithiothreitol and 10^{-4} M pyridoxal phosphate.

C. Ratios of the dehydrogenation rates of succinate semialdehyde and the sulfinic aldehyde arising from homohypotaurine, with NAD⁺ or NADP⁺ as coenzymes. It is known that the succinate semialdehyde dehydrogenase of *Ps. fluorescens* can use either $NADP^+$ or NAD^+ as a coenzyme, but that $NADP^+$ is 8.2 times more active than NAD^+ when succinate semialdehyde is used as a substrate[1]. In the presence of $NADP^+$ it has already been seen that, for γ-aminobutyrate as for homohypotaurine, the rate of dehydrogenation was not limiting; if the same occurs in the presence of NAD^+, no difference will appear between the rate of formation of one or the other of the reduced coenzymes. If, however, the important reduction of the dehydrogenation rate due to the substitution of $NADP^+$ by NAD^+ implies that the reaction catalyzed by the dehydrogenase becomes limiting, a reduction in the rate of formation of the reduced coenzyme should be observed in the presence of NAD^+, and the ratio of the rates of

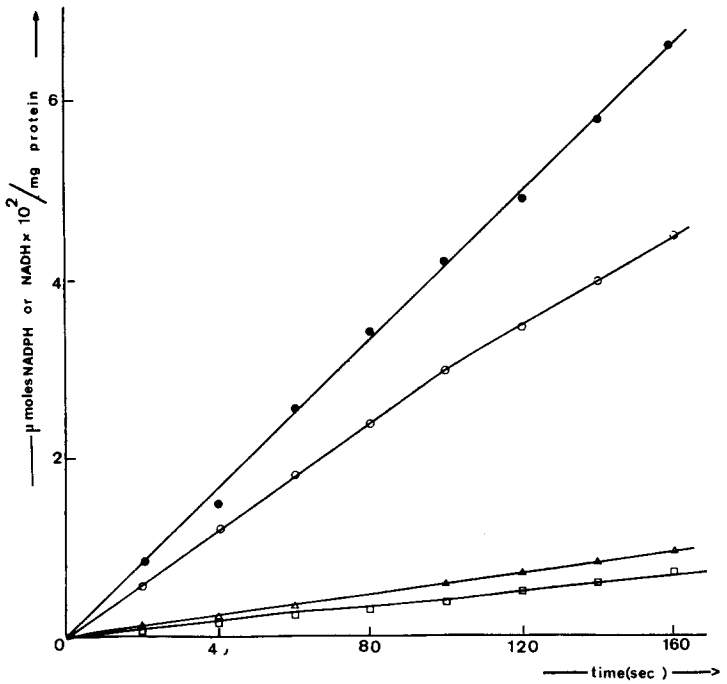

Fig. 4. Relative rates of NADPH and NADH formation with γ-aminobutyrate and homohypotaurine as substrates. Strain 6326; enzyme at Step I of the purification; buffer, Tris–HCl, pH 8.6; 20 °C. ●—●, homohypotaurine + NADP; ○—○, γ-aminobutyrate + NADP; △—△, homohypotaurine + NAD; □—□, γ-aminobutyrate + NAD.

formation of NADPH and NADH, ranging between 1 and 8.2, could be specific for a given dehydrogenase. Therefore, we have determined the rates of formation of NADPH and NADH with the enzymatic extract (Step I of the purification), in the presence of γ-aminobutyrate and homohypotaurine. Fig. 4 indicates the results obtained: the ratio of the rates of formation of the reduced coenzymes, calculated from these curves, was found to be equal to 7.0 for both substrates. This result, though not a proof, is a good argument in favor of the identity between the enzyme acting on the sulfinic aldehyde and the succinate semialdehyde dehydrogenase.

IV. Comparative study of the action of aminobutyrate aminotransferase on its substrates

A. Equilibrium constants. From preliminary determinations, using data obtained at the equilibrium when homohypotaurine or homotaurine are substrates together with α-ketoglutaric acid, we calculated a value of 0.11 for the equilibrium constant, K, of homohypotaurine and homotaurine transamination. Under the same conditions, a value of 0.20 ($K = 0.10$ see ref. 2) was found when γ-aminobutyrate was the substrate.

B. Kinetic constants. The apparent affinity constants of aminobutyrate aminotransferase for homohypotaurine, γ-aminobutyrate and α-ketoglutarate, as well as the maximum catalytic rates per min per mg protein (enzyme at Step III of the purification) obtained for homohypotaurine and γ-aminobutyrate, have been calculated

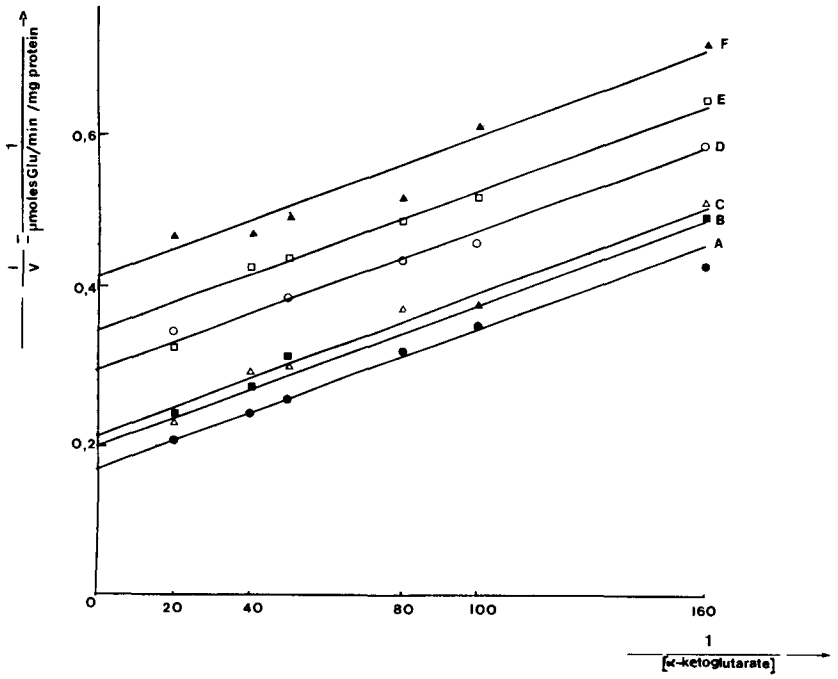

Fig. 5. Relations between initial rate and α-ketoglutarate concentration in the presence of various concentrations of homohypotaurine. Strain 5690; enzyme at Step III of the purification; buffer, pyrophosphate–HCl, pH 8.6; 37 °C. Homohypotaurine: A, $5 \cdot 10^{-2}$ M; B, $2.5 \cdot 10^{-2}$ M; C, $2 \cdot 10^{-2}$ M; D, $1.25 \cdot 10^{-2}$ M; E, 10^{-2} M; F, $6.25 \cdot 10^{-3}$ M.

from the curves of Figs 5 and 6 (and similar ones for γ-aminobutyrate). Figs 7 and 8 where $1/v$ was related to $1/[S_1]$ at infinite concentrations of $[S_2]$, gave the values of K_m and V which are listed in Table III. The K_m values found for γ-aminobutyrate and α-ketoglutarate are identical to those indicated by Scott and Jakoby[2]. It can further be seen from Table III that the affinity of aminobutyrate aminotransferase for homohypotaurine is about 3 times lower than for γ-aminobutyrate, whereas homohypotaurine is transaminated more rapidly than γ-aminobutyrate, as already described.

DISCUSSION

The preceding data indicate that homohypotaurine and homotaurine are transaminated by cell-free extracts of *Ps. fluorescens* in the presence of α-ketoglutarate at a slightly higher rate than γ-aminobutyrate; several types of results lead to the conclusion that the aminotransferase which is involved is identical to the aminobutyrate aminotransferase (EC 2.6.1.19), for which the only known aminated substrates, in addition to glutamate, have so far been γ-aminobutyrate and β-oxy-γ-aminobutyric acid, the rate of transamination being much lower for the latter compound than for γ-aminobutyrate[4]; neither β-alanine[2] nor hypotaurine or taurine are appreciably transaminated by the enzyme of *Ps. fluorescens* which has also no action on

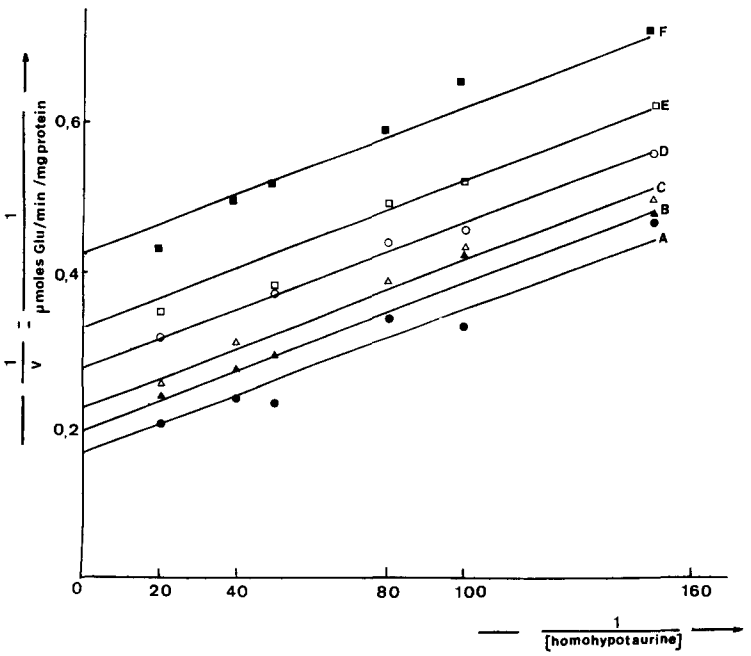

Fig. 6. Relations between initial rate and homohypotaurine concentration in the presence of various concentrations of α-ketoglutarate. Same conditions as for Fig. 7. α-Ketoglutarate: A, $5 \cdot 10^{-2}$ M; B, $2.5 \cdot 10^{-2}$ M; C, $2 \cdot 10^{-2}$ M; D, $1.25 \cdot 10^{-2}$ M; E, 10^{-2} M; F, $6.25 \cdot 10^{-3}$ M.

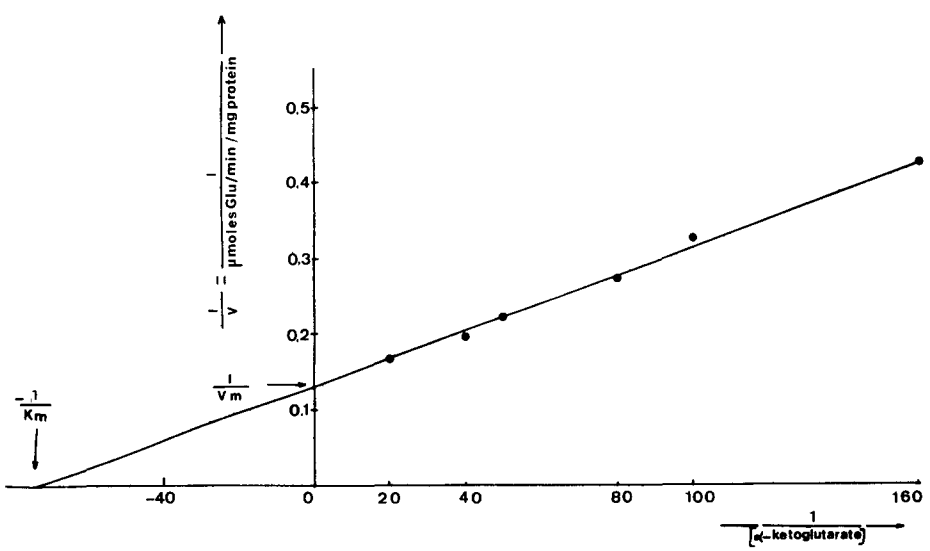

Fig. 7. Determinations of K_m for α-ketoglutarate. $1/v$ points are the intercepts on the $1/v$ axis in Fig .5.

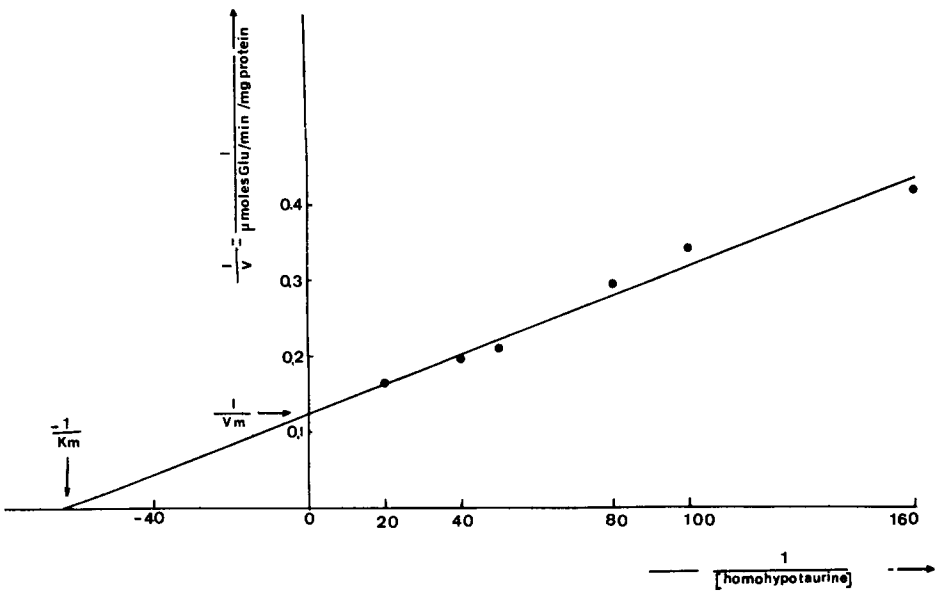

Fig. 8. Determinations of K_m for homohypotaurine. $1/v$ points are the intercepts on the $1/v$ axis in Fig. 6.

TABLE III

DETERMINATION OF KINETIC CONSTANTS FOR AMINOBUTYRATE AMINOTRANSFERASE

Ps. fluorescens strain 5690; enzyme, Step III of the purification procedure; buffer, pyrophosphate–HCl, pH 8.6; 37 °C.

Substrate	K_m *(M)*	*V (moles/min per mg protein)*
Homohypotaurine	$1.5 \cdot 10^{-2}$	$8.0 \cdot 10^{-6}$
γ-Aminobutyrate	$5.0 \cdot 10^{-3}$	$5.0 \cdot 10^{-6}$
α-ketoglutarate	$1.4 \cdot 10^{-2}$	—

C_5 and C_6 ω-amino acids[2]. Our results confirm that, for the aminobutyrate aminotransferase of *Ps. fluorescens*, the length of the chain of the ω-amino acids is of outstanding importance[4]. They further demonstrate that the nature of the acid group of the molecule (carboxylic, sulfinic or sulfonic group) is only of minor importance and introduces relatively slight variations in the affinity and in the reaction rate.

Furthermore, our data indicate that the sulfinic and sulfonic aldehydes produced by the transamination of homohypotaurine and of homotaurine can be dehydrogenated in the presence of $NADP^+$ or NAD^+ by extracts of *Ps. fluorescens*. From the fact that the ratios of the rates of formation of NADPH and NADH in the presence of γ-aminobutyrate and homohypotaurine are identical, one can presume the identity between the succinate semialdehyde dehydrogenase (EC 1.2.1.16) and the dehydrogenase of the transamination product of homohypotaurine (and probably of homotaurine). The isolation of the sulfinic and sulfonic aldehydes and acids resulting from the action of aminobutyrate aminotransferase on homohypotaurine and homotaurine is presently in progress in our laboratory. We feel that, in view of the

preceding data, it is possible to postulate that, in *Ps. fluorescens*, homohypotaurine and homotaurine are transaminated and dehydrogenated by aminobutyrate aminotransferase and succinate semialdehyde dehydrogenase in the same way as already described for γ-aminobutyrate:

Homohypotaurine (homotaurine) + α-ketoglutaric acid $\rightleftharpoons$ 3-sulfinopropionaldehyde (3-sulfopropionaldehyde) + glutamic acid (I)

3-sulfinopropionaldehyde (3-sulfopropionaldehyde) + $NADP^+$ (NAD^+) $\rightleftharpoons$ 3-sulfinopropionic acid (3-sulfopropionic acid) + NADPH (NADH) (II)

For homohypotaurine as for γ-aminobutyrate, the limiting reaction is Reaction I (transamination) whereas, with homotaurine as the initial substrate, Reaction II (dehydrogenation) becomes the limiting one and this indicates a much lower rate of formation of the sulfonic acid from the corresponding aldehyde than for succinic acid from γ-aminobutyrate or for the sulfinic acid from hypotaurine. In this second dehydrogenation reaction, the nature of the acid group in the molecule seems to be critical, and the relationship between the carboxylic compounds and their sulfinic analogues is therefore demonstrated once more[9,10].

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