Biochimica et Biophysica Acta, 309 (1973) 271–279

BBA 66910

STUDIES ON THE STIMULATION OF PHENYLALANINE HYDROXYLASE ACTIVITY BY SHORT-CHAIN ALCOHOLS

DAVID A. BRASE* AND THOMAS C. WESTFALL

Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Va. 22903 (U.S.A.)

(Received November 21st, 1972)

SUMMARY

The activity of rat liver phenylalanine hydroxylase (EC 1.14.3.1) is enhanced *in vitro* by several short-chain alcohols. The stimulation is neither species- nor substrate-specific, and the degree of stimulation depends both upon alcohol concentration and chain length, substrate concentration, as well as the pH and temperature of the incubation medium. The stimulation at pH 7.4 by 7.5% ethanol (v/v) at 20, 30 and 37 °C is accompanied by a marked decrease in the apparent Michaelis constant for phenylalanine and, at 20 and 30 °C, by an increase in the maximum velocity. Ethanol (7.5%) also increases the apparent Michaelis constants for a synthetic pterin cofactor and for O_2. The relatively greater effect of ethanol at 20 °C than at higher temperatures suggests that it might act by lowering the activation energy for the hydroxylation reaction.

INTRODUCTION

There are several reports in the literature that short-chain alcohols stimulate the activity of certain enzymes *in vitro*, such as β-galactosidase[1] and adenyl cyclase[2]. Our observation that rat liver phenylalanine hydroxylase activity was increased by certain derivatives of vitamin E led to a screening of several water-insoluble compounds[3]. While employing ethanol as a vehicle for the addition of these compounds to the incubation mixture, it was observed that ethanol itself enhanced the hydroxylation of phenylalanine by rat liver preparations. As reported in a preliminary communication[4], this stimulation of tyrosine production is not specific for ethanol, but can also occur with other short-chain alcohols.

The present communication describes experiments performed during the investigation of the mechanism by which this stimulation occurs, using ethanol as a

Abbreviations: $MePteH_4$, 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine; Me_2PteH_4, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

* Present address: Department of Pharmacology, University of California, San Francisco, Calif. 94122, U.S.A.

representative stimulating agent. It will be shown that the degree of stimulation by ethanol depends upon various incubation conditions, and that the stimulation is accompanied by significant changes in the kinetic parameters of this enzyme for its substrates.

METHODS

Unless otherwise stated, the experiments were carried out with phenylalanine hydroxylase prepared from rat liver (male Sprague–Dawley, 200–350 g body wt) through the first $(NH_4)_2SO_4$ purification step as described by Kaufman[5]. The precipitate was stored 1–2 days at −70 °C before use. Residual $(NH_4)_2SO_4$ was removed by passage through a column of Sephadex G-25 equilibrated with 0.033 M potassium phosphate buffer, pH 7.4, after dissolving the precipitate in a small volume of the same buffer.

The standard incubation mixture (2.0 ml) contained, in μmoles: phosphate buffer (pH 7.4), 80; L-phenylalanine, 2.0; 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (Me_2PteH_4), 1.0; dithiothreitol, 6.0; and enzyme. All incubation solutions were prepared using 1.15% KCl to maintain approximate isotonicity. The incubations were carried out under air in a shaking water bath for 15 min at 30 °C. Blanks consisted of reaction mixtures prepared without phenylalanine or the pteridine cofactor and incubated under the same conditions. The reaction was stopped by the addition of 2.0 ml of 0.6 M trichloroacetic acid. After centrifugation, aliquots of the deproteinized mixtures were analyzed for tyrosine by the fluorometric method of Waalkes and Udenfriend[6] as modified by Ambrose and coworkers[7]. Protein was measured by the procedure of Lowry and coworkers[8], using crystalline bovine serum albumin (Sigma) as a standard.

The incubation medium for kinetic studies was the same as the standard medium above, except for phenylalanine as the fixed substrate (5.0 mM) when the concentration of the pteridine cofactor was varied. The apparent Michaelis constants (K_m) and maximum velocities (V) and their estimated standard errors were calculated from the data by the method of Wilkinson[9] with the aid of an Olivetti-Underwood Programma 101 computer.

L-Phenylalanine was purchased from Sigma Chemical Co. Me_2PteH_4 was purchased from Aldrich Chemical Co. D-L-p-Fluorophenylalanine was purchase from Nutritional Biochemical Co. 2-Amino-4-hydroxy-6-methyl-5,6,7,8-tetra-hydropteridine–HCl ($MePteH_4$) and dithiothreitol were purchased from Calbiochem.

RESULTS

Locus of the stimulation

In preliminary studies it was found that the rate of tyrosine formation was linear both in the absence and presence of ethanol over a 15-min incubation period, provided that the percentage conversion of phenylalanine to tyrosine was kept low (< 15%) by using an appropriate amount of the enzyme preparation.

Several possible explanations for the stimulation of tyrosine formation by ethanol were considered. One was that ethanol might stimulate a non-enzymatic hydroxylation of phenylalanine; however, boiled enzyme in the standard incubation

TABLE I

EFFECT OF *p*-CHLOROPHENYLALANINE TREATMENT ON ETHANOL STIMULATION OF TYROSINE FORMATION

Five rats were injected with a suspension of DL-*p*-chlorophenylalanine (*p*-Cl-Phe) (360 mg/kg, intraperitoneally) in 0.5% sodium carboxymethylcellulose and 5 control rats received the vehicle only. Phenylalanine hydroxylase was obtained 48 h later from the pooled livers of each group, and assayed as described in Methods.

	μmole tyrosine/mg protein per 15 min	
	No ethanol	*7.5% ethanol*
Control	0.831	1.856
p-Cl-Phe treated	0.062	0.177
% Inhibition	92.5	90.5

medium did not convert phenylalanine to tyrosine, either in the absence or presence of ethanol. A second possibility was that ethanol was participating in an enzymatic process that produced tyrosine, but did not involve phenylalanine hydroxylase, such as the peroxidase system described by Mason and coworkers[10]. The data in Table I, however, show that the net increase in the amount of tyrosine formed due to the presence of ethanol is greatly reduced by the pretreatment of rats with *p*-chlorophenylalanine, a specific irreversible inhibitor of phenylalanine[11] and tryptophan[12] hydroxylases, when administered *in vivo*. This suggested that the stimulation required intact phenylalanine hydroxylase.

Specificity studies

The stimulation of phenylalanine hydroxylase activity by ethanol is not limited to the rat liver enzyme. The activities of the phenylalanine hydroxylases from the livers of the rabbit, guinea pig, and cat (Table II), and from the rat kidney (Brase,

TABLE II

STIMULATION OF LIVER PHENYLALANINE HYDROXYLASE OF DIFFERENT SPECIES BY ETHANOL

Animals were anesthetized with 30–40 mg/kg of sodium pentobarbital before sacrifice. Standard incubations were carried out with 0.15-ml aliquots of the 10 000 × *g* supernatant fluid of 25% liver homogenates (in 1.15% KCl).

Ethanol (%)	*Hydroxylase activity (nmoles tyrosine/mg protein per 16 min.)*			
	Rat	*Rabbit*	*Guinea pig*	*Cat*
0	133	22.0	46.7	39.7
1.0	151	28.0	59.3	47.0
4.0	173	44.7	83.9	61.8
7.5	185	62.6	97.4	73.3

D. A. and Westfall, T. C., unpublished) are also increased by ethanol *in vitro*. The stimulation by ethanol is also not specific for phenylalanine and the dimethylpteridine cofactor as substrates. Stimulation of tyrosine production by ethanol also occurs when the monomethylpteridine cofactor ($MePteH_4$) is used or when *p*-fluorophenylalanine is used as the amino acid substrate (Table III). Other possible amino acid substrates and cofactors[5] were not studied.

TABLE III

THE SUBSTRATE SPECIFICITY OF THE ETHANOL STIMULATION OF RAT LIVER PHENYLALANINE HYDROXYLASE ACTIVITY

Standard incubations were carried out as described in Methods, with 1.0 mM L-phenylalanine or 2.0 mM DL-*p*-fluorophenylalanine (*p*-F-Phe) and 0.5 mM Me_2PteH_4 or $MePteH_4$. Values are means of duplicate incubations.

Ethanol (%)	*Hydroxylase activity (μmoles tyrosine/mg protein per 15 min)*		
	Phenylalanine + Me_2PteH_4	*Phenylalanine + $MePteH_4$*	*p-F-Phe + Me_2PteH_4*
0	0.84	2.15	0.14
1.0	1.06	2.62	0.18
2.0	1.24	2.77	0.20
4.0	1.42	2.87	0.23
8.0	1.61	3.21	0.20

As mentioned earlier, the stimulation is not specific for ethanol. Fig. 1 shows the effect of concentration and alcohol carbon-chain length on the stimulation of phenylalanine hydroxylase activity by short-chain alcohols. At the higher concentrations of the alcohols, the enzyme activity was inversely related to the carbon chain-length of the alcohol. *n*-Butanol showed its maximum stimulatory effect at a concentration of about 2% (by vol.), *n*-propanol at about 4%, ethanol at about 8%, and methanol caused a concentration-dependent increase in activity up to 10% (by vol.), the highest concentration studied.

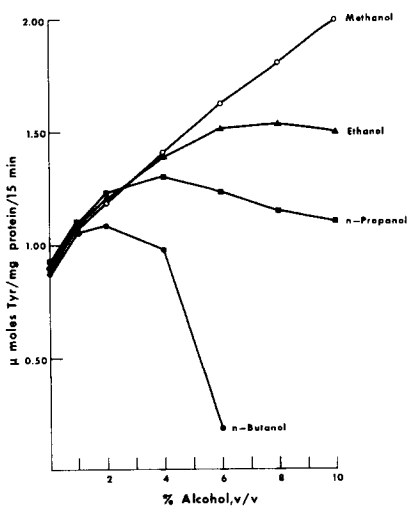


Fig. 1. Effect of chain length on the stimulation of phenylalanine hydroxylase activity by short-chain alcohols. Standard incubations were carried out as described in Methods.

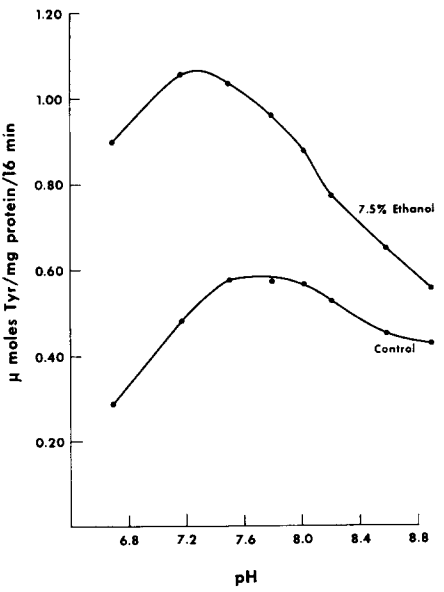


Fig. 2. The pH optimum curves of phenylalanine hydroxylase in the absence and presence of 7.5% ethanol. Standard incubations were carried out as described in Methods, except that 50 mM Tris–HCl buffer was used.

Effects of pH

Studies to determine whether ethanol stimulated enzyme activity by altering the pH optimum of phenylalanine hydroxylase were carried out using 50 mM Tris–HCl buffer. In the concentration of 7.5% (by vol.), ethanol did not change the pH of the incubation medium. In three separate experiments 7.5% ethanol caused an acidic shift in the pH optimum of approximately 0.5 pH unit as shown in the representative experiment illustrated in Fig. 2. This shift cannot account for the marked stimulation of activity by ethanol, because the maximum activity in ethanol was much higher than the maximum activity of the control curve. Fig. 2 also shows that as the pH decreases, the stimulation by ethanol becomes more marked when compared to enzyme activity in the absence of ethanol.

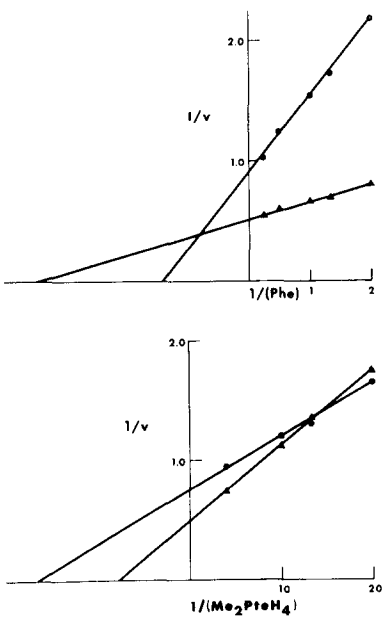
Kinetic studies with 7.5% ethanol

Further attempts to determine how ethanol stimulated phenylalanine hydroxylase activity included an investigation of the effects of ethanol on the kinetic parameters of the enzyme for its three substrates. The mean results ($\pm$ S.E.) of four experiments using three different batches of enzyme showed that ethanol decreased the apparent K_m value for phenylalanine approximately 3-fold (from 0.93 $\pm$ 0.14 to 0.30 $\pm$ 0.01 mM) and approximately doubled the apparent K_m value for the dimethylpteridine cofactor (from 61 $\pm$ 8 to 128 $\pm$ 14 μM. The control K_m values for phenylalanine and the dimethylpteridine cofactor are nearly identical to those reported by other investigators[5,13]. In addition, ethanol also increased V with either phenylalanine as the varied substrate (from 1.44 $\pm$ 0.22 to 1.98 $\pm$ 0.25 μmole tyrosine produced/mg protein per 15 min) or with the dimethylpteridine cofactor as the varied substrate (from 1.40 $\pm$ 0.26 to 2.33 $\pm$ 0.43 μmoles tyrosine/mg protein per 15 min. All of these changes induced by ethanol are significantly different from controls ($P < 0.0025$) using a t-test for paired data. Representative double-reciprocal plots with phenylalanine and the dimethylpteridine cofactor as the varied substrates are shown in Fig. 3. Ethanol also approximately doubled the apparent K_m value for O_2 (Fig. 4).

Although all four alcohols tested decreased the apparent K_m value for phenylalanine, attempts to correlate changes in the K_m values for phenylalanine or the dimethylpteridine cofactor with the alcohol chain length were only successful for the cofactor. The apparent K_m values for the cofactor in the presence of methanol, ethanol, *n*-propanol, and *n*-butanol, each at a concentration of 4.0% (by vol.), were 64, 89, 131, and 445 μM, respectively, compared to 49 μM for the control.

Effect of temperature

Experiments were carried out to determine the effects of the incubation temperature on the kinetic parameters for phenylalanine in the absence or presence of 7.5% ethanol (Table IV). The effects of ethanol on both the apparent K_m for phenylalanine and V were most marked at 20 °C, where the apparent K_m value for phenylalanine was decreased 5-fold by ethanol and V was increased 83%. Increasing the temperature from 20 to 30 °C decreased the apparent K_m value for phenylalanine either in the absence or presence of ethanol, but in the presence of ethanol the apparent K_m value for phenylalanine was decreased about 3-fold. Increasing the temperature further to 37 °C caused no further decrease in the apparent K_m for phenylalanine in



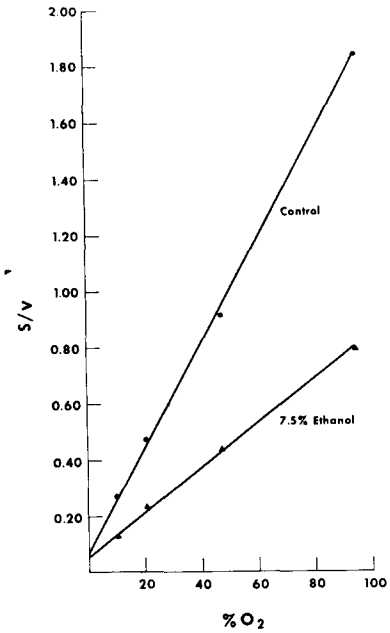


Fig. 3. The effects of 7.5% ethanol on double-reciprocal plots with phenylalanine or Me_2PteH_4 as the substrates varied. Control (circles) and ethanol (triangles) incubations were carried out as described in Methods. Abscissa units are mM^{-1} for both graphs, and velocity is expressed as μmoles tyrosine/mg protein per 15 min at 30 °C.

Fig. 4. The effect of 7.5% ethanol on the plot of S *vs* S/v with O_2 as the substrate varied. Incubations were carried out in capped weighing vials, each equilibrated for 45–50 s with the desired combination of 95% N_2, 5% CO_2 and 95% O_2, 5% CO_2 (total flow, 8–9 l/min) to achieve the O_2 concentrations of approximately 10.5%, 21%, 47.5%, and 95%. Velocity is expressed in μmoles tyrosine/mg protein per 15 min at 30 °C.

TABLE IV

TEMPERATURE DEPENDENCE OF THE KINETIC EFFECTS OF ETHANOL

Incubations were carried out as described in Methods with a range in phenylalanine concentrations of 0.5–4.0 mM.

Temperature (°C)	*7.5% ethanol*	*Phenylalanine K_m (10^{-3} M)*	*V (μmole tyrosine/mg protein per 15 min)*
20	—	2.17 ± 0.27	0.77 ± 0.05
20	+	0.42 ± 0.01	1.41 ± 0.01
30	—	0.76 ± 0.06	1.47 ± 0.04
30	+	0.26 ± 0.04	1.63 ± 0.04
37	—	0.75 ± 0.02	1.71 ± 0.01
37	+	0.28 ± 0.01	1.68 ± 0.01

the absence or presence of ethanol. At 37 °C ethanol still lowered the apparent K_m value for phenylalanine (about 3-fold), but did not change V.

The lack of an increase in V in the presence of ethanol at 37 °C may be due to a possible partial inactivation of the enzyme by the relatively high concentration of ethanol at this temperature or to a change in the rate-limiting component of the

reaction in going from 30 to 37 °C, such that the rate-limiting component at 37 °C is not affected by ethanol. Neither of these possibilities was explored.

DISCUSSION

The results above have demonstrated that several short-chain alcohols in certain concentrations can increase the rate of conversion of phenylalanine to tyrosine by mammalian phenylalanine hydroxylase *in vitro*. They also show that the magnitude of the stimulation observed depends upon a large variety of factors which include the concentrations of the substrates, the structure and concentration of the alcohol, the pH of the incubation medium, and the incubation temperature.

These studies do not rule out a possible effect of ethanol on dihydropteridine reductase, which functions to regenerate the tetrahydro form of the cofactor in the presence of NADH or NADPH[14]. The finding that ethanol greatly stimulates tyrosine production when NADH is not included in the incubation medium (Table I), strongly suggests that ethanol has a stimulatory effect on the enzyme, phenylalanine hydroxylase. The assay system for phenylalanine hydroxylase used in this study is completely independent of dihydropteridine reductase activity, because dithiothreitol is used to regenerate the tetrahydro form of the cofactor chemically and also to protect the enzyme from inactivation by H_2O_2 produced by air oxidation of the cofactor[14].

The possibility was considered that ethanol was causing only an apparent increase in activity by stabilizing phenylalanine hydroxylase. Enzyme stabilization by ethanol has been reported for other oxygenases such as metapyrocatechase[15] and protocatechuate 4,5-dioxygenase[16]. However, the rate of tyrosine formation was found to be linear with time in the absence (or presence) of ethanol; thus, phenylalanine hydroxylase appeared to be stable during the usual 15-min incubation period at 30 °C.

The increased activity of phenylalanine hydroxylase caused by ethanol is presumed to be at least partially reversible, because the enzyme is exposed to concentrations of ethanol as high as 21% (by vol.) during the purification procedure[5]. Purification is not a prerequisite for stimulation, since the activity of cruder preparations is also enhanced by ethanol (Table II). In addition, the stimulation appears to be a fairly general phenomenon which occurs with the enzyme from several species and with all of the alternate substrates that were tested.

The primary kinetic effects of ethanol which would be consistent with a stimulation of enzyme activity are a lowering of the apparent Michaelis constant for phenylalanine and an increase in V. The increases in the apparent K_m values for the pteridine cofactor and O_2 caused by ethanol would tend to antagonize the stimulatory effect of ethanol. Increasing the concentration of a particular alcohol will probably increase the amount of tyrosine produced until the concentration is reached where its effect on the interaction of the pteridine cofactor and/or O_2 with the enzyme (increased K_m values) over-rides its effect on the interaction of phenylalanine (decreased K_m) with the enzyme. The finding that the increase in the apparent K_m for the pteridine cofactor becomes greater with increasing alcohol chain length is consistent with the chain-length effect shown in Fig. 1. It has been reported that ethanol in the concentrations used in the present study does not affect the solubility of O_2 in water[17].

The finding in the present study that the stimulatory effect of ethanol on phenylalanine hydroxylase activity is relatively greater when the incubation temperature is decreased suggests that ethanol may be acting by lowering the energy of activation (ΔH^*) of the enzymatically catalyzed reaction. The findings that ethanol decreases the apparent K_m value for phenylalanine but increases the apparent K_m values for the pteridine cofactor and O_2 suggest that the effect of ethanol may be primarily on lowering the energy for the activation of phenylalanine or for the reaction of phenylalanine with activated O_2. Storm and Kaufman[18] have suggested that the activated O_2 in the phenylalanine hydroxylase reaction is at the oxidation level of peroxide. Studies with highly purified phenylalanine hydroxylase will be required for the determination of the actual values of ΔH^* in the absence and presence of ethanol and for determining whether the effect of ethanol involves changes in the tertiary of quarternary structure of the enzyme protein.

While the present studies were in progress, Sullivan and coworkers[19] reported that the hydroxylation of tryptophan by rat liver preparations was stimulated up to 10-fold by 3% propanol at low concentrations of tryptophan. More recently, Fisher and Kaufman[20] have reported that propanol, butanol, and sodium dodecyl sulfate stimulate the activity of a 90% pure preparation of rat liver phenylalanine hydroxylase. The activity was also increased by certain phospholipids including lysolecithin, sphingomyelin, phosphatidylserine and lysophosphatidylserine, particularly when the naturally-occurring cofactor tetrahydrobiopterin was used[20]. Although the effect of varying the incubation temperature on the stimulation by phospholipids was not studied, the effects of (1 mM) lysolecithin on the K_m for phenylalanine and on V with Me_2PteH_4 as the cofactor[20] are similar to those reported for 7.5% ethanol in the present study. It seems possible, therefore, that alcohols and phospholipids might be stimulating activity by the same or a similar mechanism.

ACKNOWLEDGEMENTS

The gift of DL-*p*-chlorophenylalanine by Chas. Pfizer and Co., Inc., is gratefully acknowledged. We also thank Dr Joseph Larner for his helpful discussions. This study was supported by a United States Public Health Service Predoctoral Fellowship (5 Fo1 GM 4483).

REFERENCES

1 Shifrin, S. and Hunn, G. (1969) *Arch. Biochem. Biophys.* 130, 530–535
2 Gorman, R. E. and Bitensky, M. W. (1970) *Endocrinology* 87, 1075–1081
3 Brase, D. A. and Westfall, T. C. (1972) *Biochem. Biophys. Res. Commun.* 48, 1185–1191
4 Brase, D. A. and Westfall, T. C. (1971) *Pharmacologist* 13, 193
5 Kaufman, S. (1970) *Methods Enzymol.* 17A, 603–609
6 Waalkes, T. P. and Udenfried, S. (1957) *J. Lab. Clin. Med.* 50, 733–736
7 Ambrose, J. A., Sullivan, P., Ingerson, A. and Brown, R. L. (1969) *Clin. Chem.* 15, 611–620
8 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R .J. (1951) *J. Biol. Chem.* 193, 265–275
9 Wilkinson, G. N. (1961) *Biochem. J.* 80, 324–332
10 Mason, H. S., Onopryenko, I. and Buhler, D. (1957) *Biochim. Biophys. Acta* 24, 225–226
11 Guroff, G. (1969) *Arch. Biochem. Biophys.* 134, 610–611
12 Jequier, E., Lovenberg, W. and Sjoerdsma, A. (1967) *Mol. Pharmacol.* 3, 274–278
13 Bublitz, C. (1969) *Biochim. Biophys. Acta* 191, 249–256

14 Kaufman, S. (1963) *Proc. Natl. Acad. Sci. U.S.* 50, 1085–1093
15 Nozaki, M., Ono, K., Nakazawa, T., Kotani, S. and Hayaishi, O. (1968) *J. Biol. Chem.* 243, 2682–2690
16 Ono, K., Nozaki, M. and Hayaishi, O. (1970) *Biochim. Biophys. Acta* 220, 224–238
17 Nogami, H. and Kato, Y. (1959) (Chem. Abstr. 53, 19526f) *Zakugaku Zasshi* 79, 958–960
18 Storm, C. B. and Kaufman, S. (1968) *Biochem. Biophys. Res. Commun.* 32, 788–793
19 Sullivan, P. T., Kester, M. and Norton, S. J. (1971) *Fed. Proc.* 30, 1067
20 Fisher, D. B. and Kaufman, S. (1972) *J. Biol. Chem.* 247, 2250–2252