Electroanalysis, 3 (1991) 567–572

SHORT COMMUNICATION

A Potentiometric Approach to the Study of the Antagonism Between Acetazolamide and *l*-Carnitine Congeners on Carbonic Anhydrase Activity

Claudio Botré,[1] *Francesco Botré, and Cinzia Pranzoni*

Department of Studies of the Chemistry and Technology of Biologically Active Substances, "La Sapienza" University, Piazzale Aldo Moro, 5, 00185 Rome, Italy

Received May 1, 1990.

ABSTRACT

The in vitro interactions among carbonic anhydrase (CA), acetazolamide (ACTZ), and some short chain acylcarnitines (*l*- and *d*-isomers) were studied by means of a recently developed potentiometric method of analysis, based on a pCO_2 sensor. The *l*- and *d*-isomers of carnitine (C), acetylcarnitine (AC), propionylcarnitine (PC), and isobutyrrylcarnitine (iBC) were found not to affect CA catalytic activity in the absence of other compounds. Upon testing in the presence of ACTZ, the *l*-isomers of the carnitine congeners were shown to antagonize the chemical inhibition of carbonic anhydrase II from bovine erythrocytes, whereas in this case the *d*-isomers did not show any effect. The results obtained in these experiments, carried out at 25 and at 37°C, confirm the high reliability of this new approach and highlight the positive effect of *l*-carnitine congeners on CA catalytic activity. The potentiometric method should be suitable for monitoring the interaction of other enzymes and CA.

INTRODUCTION

Carbonic anhydrase (CA) is the enzyme that reversibly catalyzes the hydration of CO_2 to HCO_3^-. It was discovered simultaneously by Meldrum and Roughton [1] and by Stadie and O'Brien [2] almost 60 years ago, and studies on its structure and mechanism of action and on the biological functions of its different isozymes in living organisms continue to proliferate [3–5]. Acetazolamide (Fig. 1) is one of the most powerful CA inhibitors, and it has been employed both in the therapy [6] and in in vivo studies to evaluate the importance of CA in many different organs and tissues [7].

Carnitine (Fig. 2) is a β-OH-γ-amino acid that plays a direct role in a great variety of metabolic processes [8–10] and is being shown to participate in many biometabolic processes occurring in the mitochondrion [11, 12].

In the present work we tested the effect of some short chain *l*- and *d*-carnitine congeners on samples of CA II crystallized from bovine erythrocytes and inhibited by acetazolamide (ACTZ). We included in the study *l*- and *d*-carnitine (C), *l*- and *d*-acetylcarnitine (AC), *l*- and *d*-propionylcarnitine (PC), and *l*- and *d*-isobutyrrylcarnitine (iBC). Particularly, we studied the effect of different concentrations of *d*- and *l*-C, *d*- and *l*-AC, *d*- and *l*-PC, and *d*- and *l*-iBC on the catalytic power of carbonic anhydrase, in the presence of different concentrations of ACTZ.

The catalytic activity of CA and the extent of the interactions between ACTZ and carnitine congeners have been quantitatively evaluated according to a potentiometric procedure that allows operation under a wide range of experimental conditions. This procedure is based on the use of a commercial pCO_2 sensor, employed to monitor the speed of CO_2 diffusion that takes place from a buffered solution of sodium bicarbonate. Indeed, CA is capable, by transforming HCO_3^- to CO_2, to sharply enhance the speed of CO_2 diffusion from the reaction vessel to the external atmosphere, so that a higher CA catalytic activity corresponds to a greater CO_2 diffusion rate.

EXPERIMENTAL

The determination of CA activity was performed according to a recently described potentiometric technique [13], which measures the speed of CO_2 diffusion taking place from a buffered solution of sodium bicarbonate.

Commercially available pCO_2 electrodes (Orion 9502 or Ingold 15-232-3000) were used as pCO_2 sensors; the pH electrode was a glass combination Ross microelectrode (Orion 8102SC); the two-channel potentiometer, by which it is possible to monitor the pCO_2 and the pH values simultaneously, was an Orion 940EA Ionalyzer, connected to a Linseis L4100 two-channel recorder.

All chemicals were analytical grade. Doubly distilled water was used for the preparation of all reagent solutions.

Carbonic anhydrase from bovine erythrocytes was supplied by Boeringer Mannheim (catalog no. C7500, 2500 Wilbur-Anderson U/mg). Acetazolamide (Diamox) was supplied by Sigma Chemical Company (catalog no.

[1] To whom correspondence should be addressed.

1040-0397/91/$3.50+.25

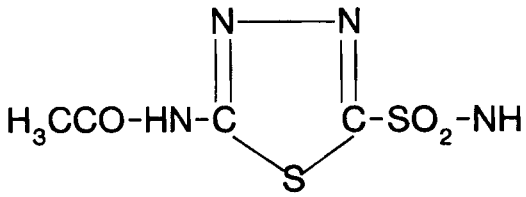


FIGURE 1. Molecular structure of acetazolamide (1,3,4,-tiadiazole-2-sulfonamide).

A6011). Carnitine congeners (C, AC, iPC, iBC) were supplied by Sigma Tau SpA, Pomezia (Rome).

An example of the potentiometric plots obtained by this method is given in Figure 3, where the analyses were performed under the following experimental conditions:

Phosphate buffer, 0.1 M, pH 7.00 ±0.01

Constant temperature, (25°C)

Constant magnetic stirring

If present, the carnitine congener and the acetazolamide were added to the buffer solution before the beginning of the assay.

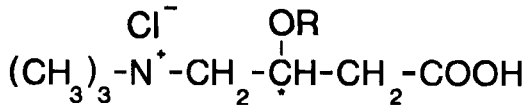
In Figure 3, curve a refers to the analysis performed under the preceding experimental conditions and in the presence of CA (20 mg/L); curve b refers to the same conditions, but in the presence of ACTZ (0.5 μM); curve c refers to the reference analysis (i.e., performed in the absence of both CA and ACTZ); and curve d refers to an analysis performed to check the potential influence of the compound under investigation (in this case ACTZ) on the speed of the uncatalyzed reaction (i.e., under the basic experimental conditions but in the absence of CA).

The slope of the final part of the curves of Figure 3—that is, the $\Delta E/\Delta t$ value (mV/min) recorded by the pCO_2 sensor—is proportional to the speed of CO_2 diffusion from the reaction vessel and, therefore, to the CA catalytic activity [13]. Consequently, the $\Delta E/\Delta t$ values for analyses performed in the presence of CA inhibitors can vary from the $\Delta E/\Delta t$ value of the curve a (no inhibition) to that of curve c (maximal inhibition). It follows that in the presence of a compound capable of antagonizing the effect of a CA inhibitor, the plot of the analysis is shifted back toward curve a.

RESULTS AND DISCUSSION

All the experiments, whose results are expressed as percentages of original CA catalytic activity, were performed

FIGURE 2. Molecular structure of carnitine congeners (* = asymmetric carbon): R = —H (carnitine), —$COCH_3$ (acetylcarnitine), —$COCH_2CH_3$ (propionylcarnitine), or —$COCH(CH_3)_2$ (isobutyrrylcarnitine).



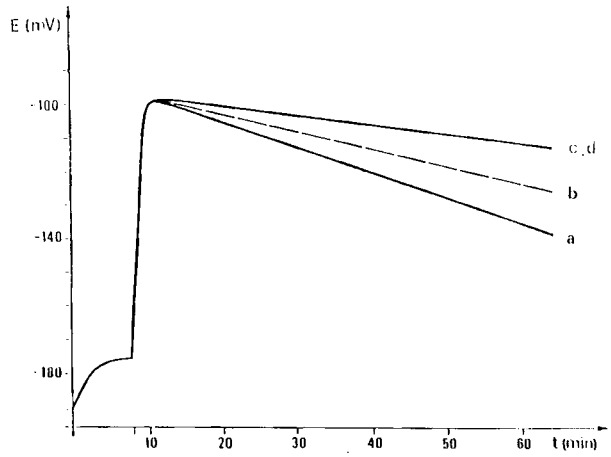


FIGURE 3. Potentiometric trends of enzymatic activity. The ordinate gives the value of the electromotive forces recorded by the pCO_2 sensor, while the corresponding times appear on the abscissa. The four plots were recorded under the following experimental conditions. **(a)**: 0.1 M phosphate buffer (pH 7.000), T = 25.0 ± 0.05°C, $NaHCO_3$ added to reach a final concentration of 25.5 mM; CA concentration, 20 mg/L (0.67 μM). The two arrows indicate the additions of the second aliquot of $NaHCO_3$ (the first being added at zero time) and of CA, respectively. **(b)** Same as in **a**, but in the presence of 0.5 μM acetazolamide. **(c)** Same as in **a**, but without CA. **(d)** Same as in **b**, but without CA.

with respect to assays carried out on CA II samples from bovine erythrocytes in a concentration of 20 mg/L.

Table 1 refers to the results of CA activity determination performed in the presence and in the absence of different concentrations of ACTZ and of all the single

TABLE 1 Effect of ACTZ and All the Carnitine Congeners on the Catalytic Activity of a 20 mg/L Solution of CA[a]

Sample	*CA Catalytic Activity (%)*
CA	100
CA + ACTZ 10^{-7} M	81.0
CA + ACTZ 10^{-6} M	25.9
CA + ACTZ 10^{-5} M	12.1
CA + ACTZ 10^{-4} M	5.1
CA + ACTZ 10^{-3} M	3.4
CA + *d*-C 10^{-2} M	94.0
CA + *l*-C 10^{-2} M	97.0
CA + *d*-AC 10^{-2} M	96.0
CA + *l*-AC 10^{-2} M	99.0
CA + *d*-PC 10^{-2} M	97.0
CA + *l*-PC 10^{-2} M	102.0
CA + *d*-iBC 10^{-2} M	98.0
CA + *l*-iBC 10^{-2} M	97.0

[a] The enzymatic activity measurement was performed under the same experimental conditions described in connection with Figure 3.

carnitine congeners, tested separately. Clearly, while the loss of catalytic activity is proportional to ACTZ concentration, no significant difference was recorded between the CA catalytic activity in the presence and in the absence of any of the carnitine congeners.

In view of this preliminary set of results, two additional sets of experiments were carried out for each of the carnitine congeners: the former by testing, in the presence of different concentrations of ACTZ, a fixed concentration of carnitine congener, namely 10 mM, at 25 and 37°C (Figs. 4 and 5); the latter by testing, under the same experimental conditions, different concentrations of carnitine congener in the presence of a fixed concentration (0.1 μM) of ACTZ (Figs. 6 and 7).

More precisely, Figure 4 shows the antagonism between acetazolamide, present in a concentration varying from 10^{-7} to 10^{-3} M, and the *l*-carnitine congeners, present in a concentration of 10 mM, with respect to the CA catalytic activity at 25°C. It is evident that the effect is very pronounced for *l*-AC and, to a lesser extent, for *l*-C, if the ACTZ concentration remains within the range 10^{-7}–10^{-5} M. Above the limit of 10^{-5} M, such a protecting effect becomes negligible. As far as the *d*-isomers are concerned, none of them showed any kind of antagonism with ACTZ.

Consisting results are obtained at 37°C (Fig. 5), where an antagonism between ACTZ and *l*-PC and *l*-iBC is also recorded. Also in this case the *d*-isomers were not found to produce any effect.

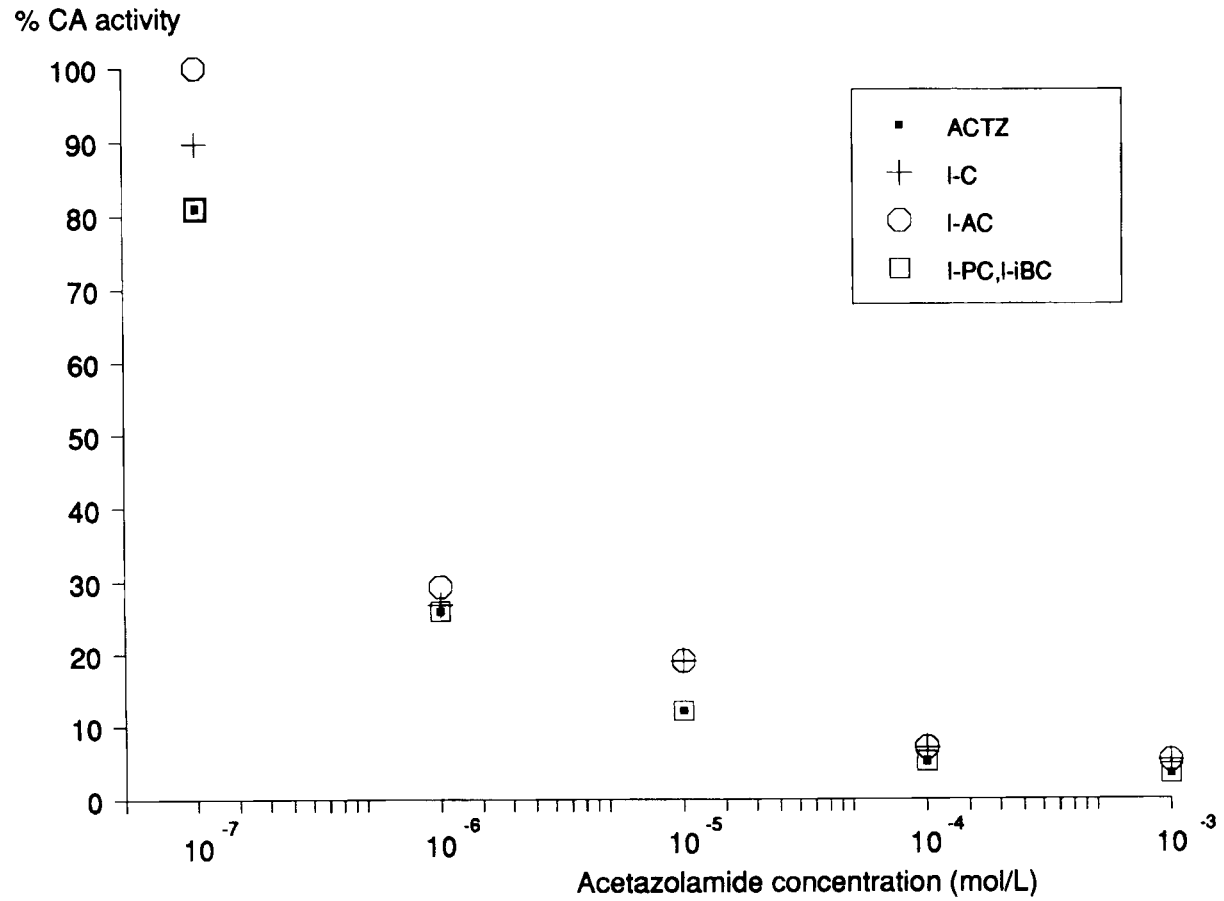
To investigate also the influence of *l*-carnitine congener concentration on the entity of the antagonism with ACTZ, additional experiments were performed by fixing the ACTZ concentration at the value of 10^{-7} M.

Figure 6 shows that the protecting effect of *l*-C and *l*-AC is proportional to their concentration, and that even at concentration values smaller than 10 mM, *l*-AC is still endowed with a protecting power greater than *l*-C.

Also in this case the results obtained at 25 and at 37°C (Fig. 7) substantially match.

Thus apart from slight differences probably due to the properties of the different side chains, the general molecular structure of *l*-carnitine congeners appears to

FIGURE 4. Effect of *l*-carnitine congeners, tested at a concentration of 10 mM, on the catalytic activity of bovine CA II, in the presence of different concentrations of acetazolamide. The analyses were performed under the experimental conditions of Figure 3, at 25°C, and with constant monitoring of pH values.



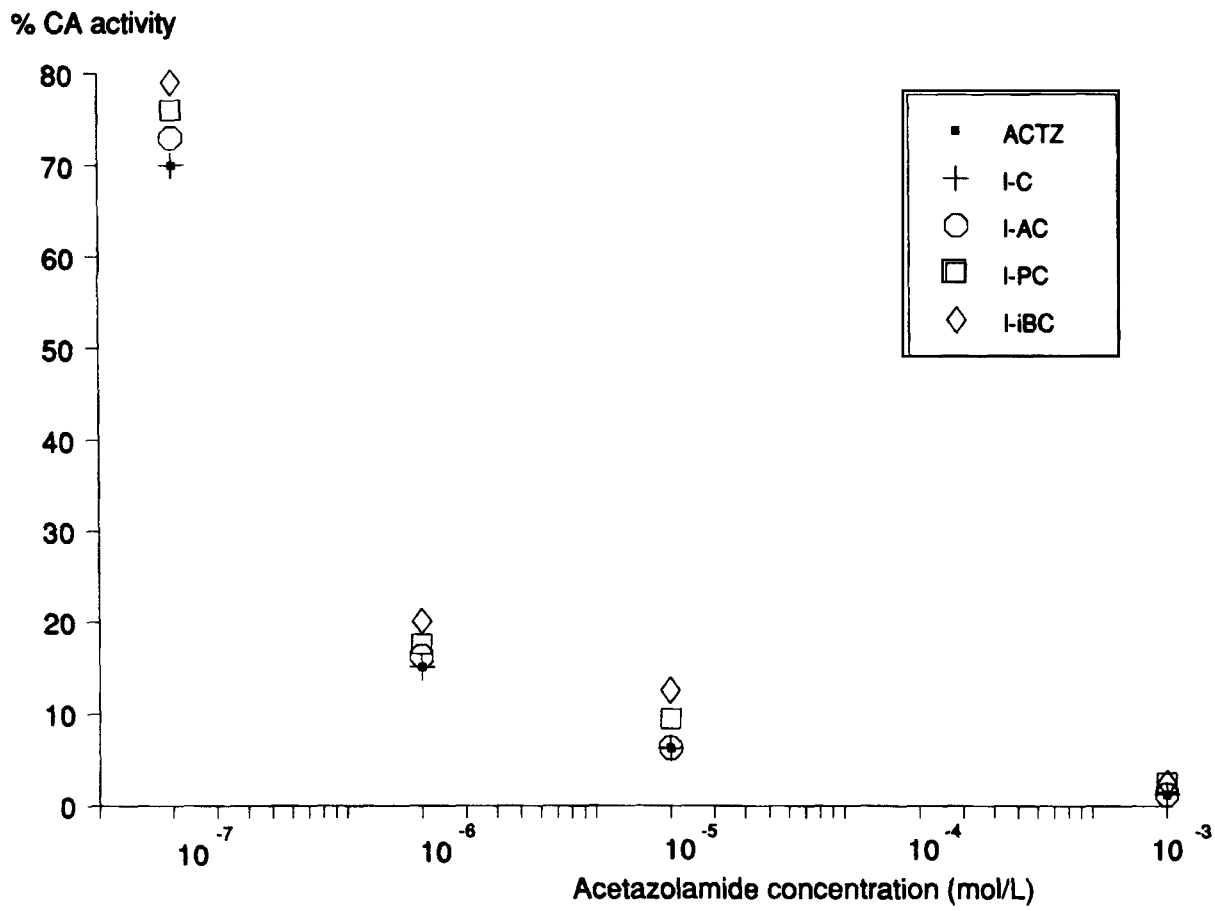


FIGURE 5. Effect of *l*-carnitine congeners, tested at a concentration of 10 mM, on the catalytic activity of bovine CA II, in the presence of different concentrations of acetazolamide. The analyses were performed under the experimental conditions of Figure 4, but at 37°C.

be capable of interacting with CA, producing a sort of reactivating or protecting effect. Moreover, it is worthwhile to highlight that only the *l*-isomers of carnitine congeners are endowed with such a relevant feature, while the *d*-isomers do not affect the speed of the reaction catalyzed by CA. This evidence is even more relevant if related to the results obtained in the study of a new class of sulpiride analogues, where the inhibitory effect of the *l*-isomers of different molecular structures was stronger than the one of the corresponding *d*-isomers [14, 15].

The difference in terms of the effect on CA catalytic power, recorded between *l*-isomers and *d*-isomers of carnitine congeners, also represents the best evidence of the specificity of the interactions with CA and, therefore, of the reliability of the results obtained.

In conclusion, the method we have applied can allow the determination in vitro not only of the catalytic activity of carbonic anhydrase, but also of the effect of the interactions among different compounds. Among the main advantages of such a technique are the following.

1. The analyses can be performed in a wide range of experimental conditions, including the physiological.
2. Different compounds, as well as different enzymatic systems, can be tested at the same time and in fixed and controlled experimental conditions.
3. The equipment required for the assays is very inexpensive, and the experimental procedure is extremely simple.

Finally, it must be noted that this basic potentiometric approach can be adopted successfully to investigate also the possibility of interactions between CA and other enzymatic systems: promising results in this field have already been reached with respect to the urease system [16] and are now being extended to other decarboxylating enzymes.

ACKNOWLEDGMENT

The authors thank Dr. Franco Gaetani (Sigma Tau SpA, Pomezia, Rome) for supplying all the samples of carnitine congeners.

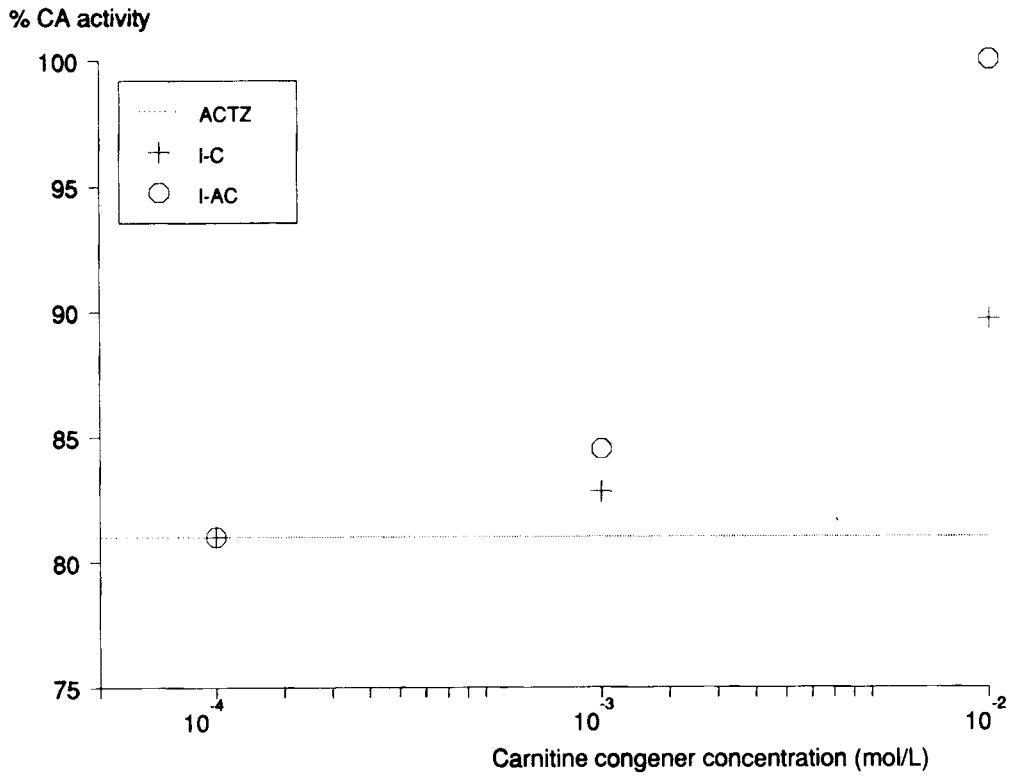


FIGURE 6. Effect of different concentrations of *l*-carnitine congeners on the catalytic activity of bovine CA II, in the presence of a fixed concentration of acetazolamide (10^{-7} M). The analyses were performed under the experimental conditions described in Figure 3, at 25°C.

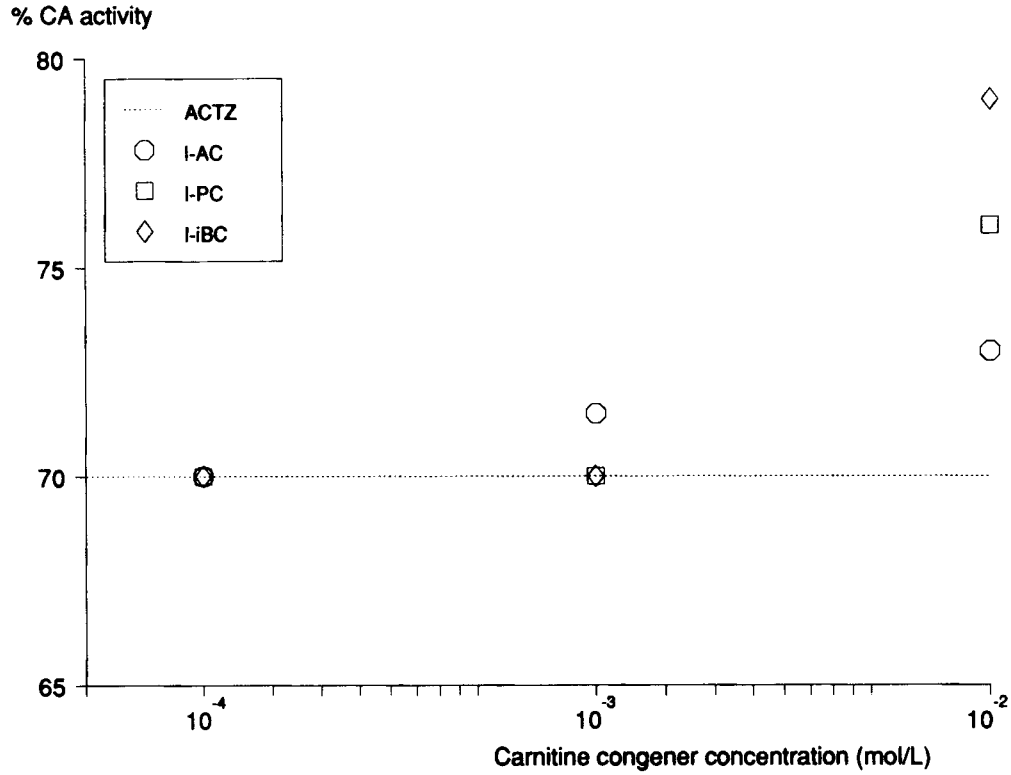


FIGURE 7. Effect of different concentrations of *l*-carnitine congeners on the catalytic activity of bovine CA II in the presence of a fixed concentration of acetazolamide (10^{-7} M). The analyses were performed under the experimental conditions described in Figure 6, but at 37°C.

REFERENCES

1. N. U. Meldrum and F. J. W. Roughton, *J. Physiol. (London) 75* (1933) 15.
2. W. C. Stadie and H. O'Brien, *Biochem. Z. 237* (1933) 521.
3. T. H. Maren, *Physiol. Rev. 47* (1967) 595.
4. Y. Pocker and S. Sarkanen, *Adv. Enzymol. Relat. Areas Mol. Biol. 47* (1978) 149.
5. R. E. Tashian and D. Hewett-Emmett, in *Biology and Chemistry of the Carbonic Anhydrases*, R. E. Toshian and D. Hewett-Emmett, Eds. [*Ann. N.Y. Acad. Sci.* (1984) 429].
6. T. H. Maren, E. Mayer, and B. C. Wadsworth, *Bull. Johns Hopkins Hosp. 95* (1954) 199.
7. T. H. Maren, "The General Physiology of Reactions Catalyzed by Carbonic Anhydrase and Their Inhibition by Sulfonamides," in *Biology and Chemistry of the Carbonic Anhydrases*, R. E. Tashian and D. Hewett-Emmett, Eds. [*Ann. N.Y. Acad. Sci. 429* (1984) 568].
8. O. Fanelli, *Life Sci., 23* (1978) 2563.
9. J. Bremer, *Physiol. Rev. 63* (1983) 1420.
10. P. R. Borum, *Clinical Aspects of Human Carnitine Deficiency*, Pergamon Press, Oxford, 1986.
11. R. G. Hansford, *Biochim. Biophys. Acta, 726* (1983) 41.
12. R. F. Villa, L. Turpeenoja, G. Benzi, and A. M. Giuffrida Stella, *Neurochem. Res. 13* (1988) 909.
13. C. Botré and F. Botré, *Anal. Biochem. 185* (1990) 254.
14. C. Botré, F. Botré, G. Jommi, and R. Signorini, *J. Med. Chem. 29* (1986) 1814.
15. C. Botré, F. Botré, and C. Rosati, *Eur. Rev. Med. Pharmacol. Sci. 12* (1990) 169.
16. C. Botré and F. Botré, *Biochim. Biophys. Acta, 997* (1989) 111.