Biochemical Pharmacology, Vol. 27, pp. 1913–1918

0006-2952/78/0801-1913$02.00/0

INTERACTION OF GLYCINE WITH ETHANOL*

ARTHUR W. K. CHAN
Research Institute on Alcoholism, Buffalo, NY 14203, U.S.A.

(*Received* 28 *November* 1977; *accepted* 31 *January* 1978)

Abstract—It has been suggested by Blum *et al.* [*Science, N.Y.* **176**, 292 (1972)] that the enhancement of the ethanol sleep time by glycine in mice was due to an interaction of these compounds in the central nervous system. We have measured glycine levels in C57BL/6J mice after an ethanol injection (4 g/kg, i.p.) and found no significant alterations of glycine levels in four brain regions at 15, 45, 90 and 150 min after injection. An exception was in the medulla at 150 min, where a significant decrease (12 per cent) of glycine level was observed. The simultaneous injection of glycine (9 m-moles/kg) and ethanol (4 g/kg) resulted in a significant prolongation of the sleep onset time and a 20 per cent increase in the sleep time over controls, which were injected with saline–ethanol. When the same dose of glycine was administered 30 min before the ethanol injection, there was no significant change in the sleep duration but there was a prolongation of the sleep onset time. Plasma glycine levels were higher in mice injected simultaneously with glycine–ethanol than in those injected with glycine–saline. Brain glycine levels were only slightly elevated but not significantly different in these two groups. Gamma-aminobutyric acid (GABA) levels in the brain regions were not increased after glycine injection. The data indicate that the degree of interaction between glycine and ethanol was minimal, and that such an interaction was not a result of an alteration of the rate of ethanol metabolism. It was also unlikely to be a result of a glycine-induced elevation of GABA in the brain, as suggested by Blum *et al.*

Glycine fulfils almost all the necessary criteria to be identified as an inhibitory transmitter at the mammalian spinal motoneuron[1]. Other evidence suggests that it may also be an inhibitory transmitter in other areas of the central nervous system (CNS), particularly certain brain stem areas[1]. The effect of ethanol on the metabolism of glycine in the CNS has not been studied extensively. Häkkinen and Külonen[2] found no notable change in the concentration of glycine in the whole rat brain after an acute dose of ethanol.

It has been reported that glycine significantly enhanced the sleep time (loss of righting reflex) that was induced by ethanol in mice[3]. Blum *et al.*[3] concluded that the observed synergistic effect between ethanol and glycine was probably not related to an alteration of ethanol metabolism, but rather to an interaction of these compounds in the CNS. They also hypothesized that the potentiative effect observed between glycine and ethanol might have been due to the inhibitory action of glycine on glutamine metabolism, which in turn led to an increased production of γ-aminobutyric acid (GABA)[3]. However, plasma and brain concentrations of glycine or GABA were not determined in this study. In another paper, Blum *et al.*[4] have shown that glycine, when administered 30 min before an acute dose of ethanol, protected rats from ethanol-induced motor impairments. These workers suggested that the protective effect of glycine might be due to a suppression of the rate of absorption of ethanol from the gastrointestinal tract[4]. Similarly, Breglia *et al.*[5] reported that pretreatment of rats with glycine, L-lysine, L-arginine or L-ornithine, 30 min before ethanol administration, significantly prolonged the onset of ataxia and reduced the duration of sleeping time.

*Supported in part by NIAAA grant AA01858-02.

In light of the uneven distribution of glycine in the CNS[1, 6, 7], this paper examines the effect of an acute dose of ethanol on the contents of glycine in different regions of the mouse brain. We have also re-investigated the combined effect of ethanol–glycine administration on ethanol sleep time. Plasma and brain levels of glycine, as well as the effect of glycine injection on brain GABA concentrations, were also determined.

METHODS

Male C57BL/6J mice (90–95 days old) were purchased from the Jackson Laboratories, Bar Harbor, ME. They were housed four in a cage on a 12-hr light–dark cycle in a controlled temperature room (22–23°) and received Teklad mouse diet (Teklad Mills, Winfield, Iowa) and tap water *ad lib.* for at least 7 days before the beginning of an experiment.

Enzymes and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO).

Experiment 1: *Effect of acute ethanol administration on brain glycine levels*

Brain glycine levels were determined in animals which had been injected with ethanol (4 g/kg body wt) intraperitoneally (i.p.) as a 20 per cent (w/v) solution. Control animals received 0.9% NaCl. At selected intervals after the injection (15, 45, 90 and 150 min), the mice were killed by immersion in liquid N_2 and remained there for 2 min. The frozen mouse was wrapped in aluminum foil and stored in a stoppered jar at −80° until used. Procedures for the sampling of brain regions and for the preparation of tissue extracts have been described previously[8, 9]. Glycine was measured by the enzymatic method of Berger *et al.*[10]. Essentially, glycine was converted in two steps to glycolate

with D-amino acid oxidase (EC 1.4.3.3.), glyoxylate reductase (EC 1.1.1.26) and NADH. The NAD^+ generated was measured by an indirect fluorometric procedure [9, 10].

Experiment 2: *Effect of glycine on ethanol-induced sleep time*

We followed essentially the procedures employed by Blum *et al.* [3, 4].

(a) *Simultaneous injection of glycine and ethanol.* Mice were injected i.p. with a 0.9% NaCl solution containing glycine (3.4 g/100 ml) and ethanol (20%, w/v). The volume injected was 0.02 ml/g of body wt, which corresponds to doses of 9 m-moles/kg and 4 g/kg for glycine and ethanol respectively. The dose of glycine chosen was the same as that used by Blum *et al.* [3], in which they observed a synergistic effect with ethanol. Blum *et al.* [3] also used a lower dose of 4.5 m-moles/kg but did not observe any prolongation of the ethanol sleep time. Using this low glycine dose in our preliminary investigation, we also did not observe any prolongation of the ethanol sleep time. Therefore, we selected the above combination of glycine and ethanol. The dose of ethanol (4 g/kg) was lower than the 4.6 g/kg dose used by Blum *et al.* [3], but it is the same dose used in our previous investigations on ethanol sleep time [11] and metabolic effects of ethanol [8].

(b) *Pretreatment with glycine.* Mice were injected i.p. with a glycine solution in 0.9% NaCl (9 m-moles/kg from a 60 mg/ml solution) 30 min before injection of ethanol (4 g/kg). The time between injection and loss of the righting reflex (RR) was defined as the sleep onset time. The time between loss and subsequent recovery of the RR was defined as the sleep time. Mice were considered to have regained the RR when they were able to right themselves onto all four paws three consecutive times within 30 sec.

Experiment 3: *Plasma glycine levels after glycine injection*

In another experiment involving the simultaneous injection of glycine and ethanol or glycine and saline, blood was collected from the tip of the tail in heparinized capillary tubes at 0, 5, 7.5, 15, 30, 60 and 120 min after the injection. The plasma was separated after centrifugation. Perchloric acid extracts of the plasma samples were prepared as previously described [12]. Glycine was measured in these samples (aliquots of 2 to 7 μl were used [10]).

Experiment 4: *Blood ethanol levels after glycine–ethanol injection*

Blood from mice treated the same way as in Expt. 2 above was collected at 10, 30, 80 and 110 min after ethanol injection. Controls were injected with saline–ethanol. Blood ethanol was determined by the enzymatic method of Jones *et al.* [13], using the procedure described previously [8].

Experiment 5: *Effect of glycine injection on brain glycine and GABA levels*

Mice were injected simultaneously with saline and glycine or ethanol and glycine. The doses for ethanol and glycine were the same as those used in Expt. 2(a). The mice were killed at 30 min after injection by the same method as described in Expt. 1. Tissue extracts were prepared [8, 9] and they were analyzed for glycine [10] and GABA [8].

RESULTS

Experiment 1

It is seen from Table 1 that, among the four brain regions examined, the highest glycine level was in the medulla. Our values are somewhat higher than those reported for rats [6]. At 15, 45, 90 and 150 min after an acute dose of ethanol (4 g/kg), no significant change in glycine level was obser-

Table 1. Glycine content in regions of brain after an acute ethanol administration*

Sample	Time (min)	Saline (m-moles/kg wet wt)	Ethanol (m-moles/kg wet wt)
Cerebrum	15	1.42 ± 0.08	1.59 ± 0.09
	45	1.72 ± 0.08	1.90 ± 0.09
	90	1.74 ± 0.12	1.62 ± 0.07
	150	1.77 ± 0.10	1.59 ± 0.13
Cerebellum	15	1.44 ± 0.17	1.45 ± 0.05
	45	1.56 ± 0.06	1.75 ± 0.11
	90	1.60 ± 0.17	1.44 ± 0.05
	150	1.58 ± 0.11	1.38 ± 0.10
Thalamus-hypothalamus	15	2.61 ± 0.08	2.50 ± 0.07
	75	2.72 ± 0.06	2.53 ± 0.18
	90	2.61 ± 0.22	2.51 ± 0.06
	150	2.64 ± 0.09	2.38 ± 0.07
Medulla	15	7.08 ± 0.19	6.98 ± 0.14
	45	7.11 ± 0.36	6.70 ± 0.26
	90	7.87 ± 0.30	7.41 ± 0.54
	150	7.08 ± 0.14	6.03 ± 0.17†

*The results represent the means ± S. E. M. of eight to nine animals in each treatment group. Ethanol dose, 4 g/kg, i.p.

†$P \leq 0.005$.

ved, except in the medulla at 150 min, where a 12 per cent decrease ($P < 0.005$) occurred.

Experiment 2

The simultaneous injection of glycine (9 m-moles/kg) and ethanol (4 g/kg) caused a significant increase ($P < 0.05$) in the ethanol sleep onset time, and a prolongation (23 per cent, $P < 0.05$) of the sleep time was observed (Table 2). When glycine was injected 30 min before the injection of ethanol, there was also a significant increase in the sleep onset time, but no significant change in the sleep time was observed when the injections were simultaneous (Table 2).

Experiment 3

Concentrations of glycine in the plasma of mice injected simultaneously with ethanol–glycine were much higher than in those injected with saline–glycine (Fig. 1), especially during the first 60 min. During this period, the rate of elimination of glycine from the plasma was also faster in the glycine–ethanol group (Fig. 1).

Experiment 4

It is seen from Fig. 2 that the concentrations of ethanol in the blood after the simultaneous administration of saline and ethanol did not differ significantly from those obtained after the injection of glycine and ethanol. Similarly, mice injected with saline or glycine 30 min before ethanol administration did not differ in their blood ethanol concentrations (Fig. 3).

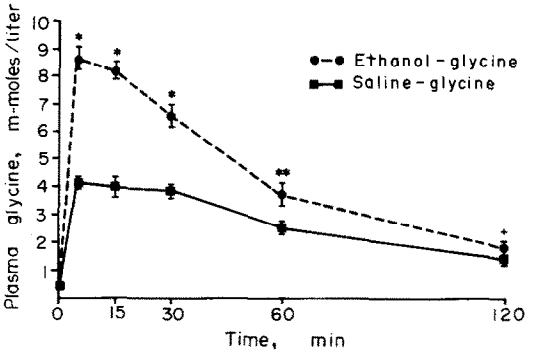

Fig. 1. Plasma glycine levels at selected intervals after the simultaneous injection (i.p.) of ethanol–glycine or saline–glycine. Results are expressed in means ± S. E. M. N = 8 in each group. Key: (*) $P < 0.001$; (**) $P < 0.005$; and (†)$P < 0.025$.

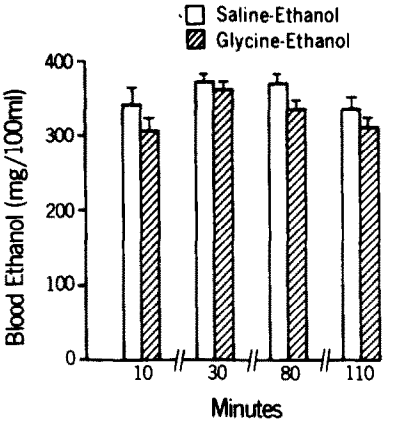

Fig. 2. Concentrations of ethanol in the blood after the simultaneous injection (i.p.) of saline and ethanol, or glycine and ethanol. Results are presented as means ± S. E. M. with eight mice represented in each group. Dosages: glycine, 9 m-moles/kg; ethanol, 4 g/kg.

Experiment 5

When compared with the data shown in Table 1, the glycine levels in the cerebellum and medulla at 30 min after glycine injection were slightly ele-

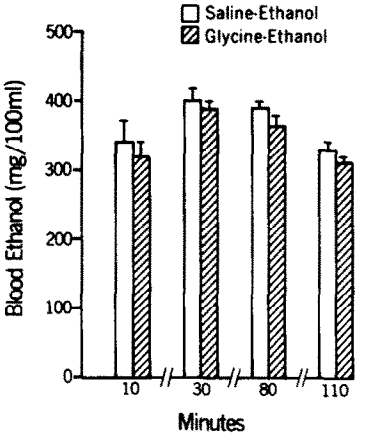

Fig. 3. Concentrations of ethanol in the blood of mice injected (i.p.) with ethanol 30 min after the injection of either saline or glycine. Dosages for glycine and ethanol were the same as those given in Fig. 2. Results are expressed in means ± S. E. M. N = 8 in each group.

Table 2. Effect of glycine on the ethanol-induced sleep time*

Injection scheme	Substance injected	Sleep onset time ± S. E. M. (min)	Sleep time ± S. E. M. (min)
Simultaneous	Saline–ethanol	1.23 ± 0.07	102.83 ± 6.62
Simultaneous	Glycine–ethanol	1.48 ± 0.06	125.22 ± 8.78
		$P < 0.05$	$P < 0.05$
Saline preinjection†	Saline–ethanol	1.40 ± 0.06	82.88 ± 4.69
Glycine preinjection†	Glycine–ethanol	1.77 ± 0.04	78.63 ± 2.36
		$P < 0.001$	NS

*Dosages: ethanol, 4 g/kg; glycine, 9 m-moles/kg. N = 9 for each group. NS = not significant.

†Injected i.p. 30 min before ethanol injection.

vated (group A, Table 3). The increases were relatively small when compared with the increases seen in plasma (Fig. 1). There was no significant difference in the glycine content in the mice injected with saline–glycine (group A) and in those injected with ethanol–glycine (group B). At 150 min, the glycine concentrations in the two brain regions of both groups A and B returned to the same levels as those shown in Table 1 for saline-treated mice. GABA levels in the medulla were essentially the same in both groups A and B (Table 3). These values agree fairly well with those we reported previously[8] for the same strain of mice injected with saline or ethanol. In the cerebellum the GABA level after saline–glycine injection (Table 3, group A) was slightly lower than that reported previously by us for mice 15 min after saline injection (1.36 ± 0.05 m-moles/kg wet wt)[8]. A significant increase in GABA concentration was observed in the same brain region after ethanol–glycine injection (Table 3, group B). This elevated level was comparable to our previously reported data for mice at 15 and 45 min after ethanol injection (1.57 ± 0.03 and 1.65 ± 0.05 m-moles/kg wet wt respectively)[8].

DISCUSSION

Our results show that glycine, when injected either simultaneously with or before the injection of ethanol, significantly prolonged sleep onset time, but a significant increase (25 per cent) in sleep time was noted only when both substances were injected simultaneously (Table 2). This effect was in the same direction as that reported by Blum *et al.*[3]. However, these investigators reported that a combination dose of ethanol (4.6 g/kg) and glycine (9 m-moles/kg) caused a duration of sleep approximately ten times the sum of the sleep time when ethanol was given alone. This very large increase may be related to the unusually low sleep time (less than 10 min) reported by these workers in mice injected with saline–ethanol. While differences in ethanol sleep time for a given dose of ethanol in different strains of mice are not uncommon[14], a dose of 4.6 g/kg, in general, would be expected to produce a sleep time of much more than 10 min. Blum *et al.*[3] used Swiss–Webster mice from a Texas source.* According to Gessner,† the ED_{50} for ethanol in Swiss–Webster mice that would maintain a sleep time of longer than 25 min was 3.6 g/kg. For the short-sleep strain (SS) of mice, a strain selectively bred for low sensitivity to the acute effect of ethanol[15], the mean sleep time after an ethanol dose of 4.1 g/kg was reported to be about 20 min[16]. Therefore, it appears that the difference in magnitude of the glycine-induced prolongation of ethanol sleep time between this study and that of Blum *et al.*[3] is due mainly to the abnormally low sleep time in the control animals used by these investigators.

Blum *et al.*[3] reported that equivalent concentrations of ethanol in the blood and brain were obtained from mice treated simultaneously with saline–ethanol and glycine–ethanol. This led to their suggestion that a direct interaction of glycine and ethanol might exist in the CNS[3]. These workers also reported that, when a higher dose of glycine (20 m-moles/kg) was given orally to rats 30 min before ethanol, the blood-alcohol levels at 30 min and 2 hr post-ethanol were about $\frac{1}{5}$ and $\frac{1}{2}$ of the levels in rats treated similarly with water and ethanol respectively[4]. These results suggested that glycine, being more pronounced in the early phase, suppressed the rate of absorption of ethanol. Since in our experiments glycine and ethanol were injected intraperitoneally, the results cannot be compared directly to those of Blum *et al.*[4]. Nevertheless, our observation that ethanol sleep onset time was prolonged when glycine was injected either simultaneously with or before ethanol injection seems to indicate that at least the initial absorption of ethanol might have been suppressed by the presence of glycine. If this actually happened, the inhibition must have been transient, since our determinations of the blood-alcohol levels, as early as 10 min, did not reveal any significant difference between the saline–ethanol and glycine–ethanol groups (Figs. 2 and 3).

During the period that the mice lost the righting reflex, ethanol did not cause any change in the glycine concentrations in various brain regions (Table 1). Blum *et al.*[3] hypothesized that the

*Information provided by a reviewer of this paper.

†Dr. Peter Gessner, Dept. of Pharmacology, SUNYAB, Buffalo, NY, personal communication.

Table 3. Glycine and GABA levels in the cerebellum and medulla after glycine–ethanol injection*

Brain region	Group	Glycine (m-moles/kg wet wt)	GABA (m-moles/kg wet wt)
Cerebellum	A†	2.15 ± 0.16	1.15 ± 0.03
	B‡	1.98 ± 0.10	1.53 ± 0.06§
Medulla	A†	8.30 ± 0.18	1.81 ± 0.07
	B‡	8.01 ± 0.36	1.90 ± 0.04

*Mice were sacrificed at 30 min after injection. Values expressed were means ± S. E. M. (N = 8 in each group).

†Mice injected (i.p.) with saline and glycine (9 m-moles/kg) simultaneously.

‡Mice injected with ethanol (4 g/kg) and glycine (9 m-moles/kg) simultaneously.

§Significantly different from group A, $P < 0.001$.

potentiation observed between glycine and ethanol might be due to the inhibitory action of glycine on glutamine metabolism, which in turn led to an increased production of GABA. Our results indicate that, despite the large increase in plasma glycine levels after glycine injection, brain glycine levels were not greatly elevated (Table 3). At least part of the elevated glycine levels observed in the cerebellum and medulla (Table 3) was in the extracellular space, due to the high plasma glycine levels. Moreover, there was no significant difference in brain glycine content in the mice injected with saline–glycine and in those injected with ethanol–glycine, indicating that the blood–brain barrier to glycine was not altered by ethanol. We measured glycine levels in the plasma and brain only in the case of simultaneous injection of both glycine and ethanol, because, in this instance, plasma glycine concentrations would be higher (compared to glycine preinjection) when the blood-ethanol level was appreciable. Also, a prolongation of ethanol sleep time was observed only with this treatment. A significant ($P < 0.001$) increase in GABA content was observed in the cerebellum in mice injected with ethanol–glycine compared to those injected with saline–glycine (Table 3). However, this elevated GABA level was essentially similar to that reported previously by us for mice injected with ethanol alone[8]. Therefore, a combination of ethanol and glycine did not result in a higher cerebellar GABA concentration than that which resulted from the injection of ethanol alone.

It is of interest to note that higher plasma glycine levels were obtained in mice injected (i.p.) simultaneously with glycine–ethanol than in those injected with glycine–saline (Fig. 1). This phenomenon was consistently observed when the experiment was repeated. These results were the opposite of those expected when the substances were administered orally, since Chang *et al.*[17] and Israel *et al.*[18] have demonstrated that ethanol inhibited the active transport of a number of amino acids in the intestine. We cannot readily explain the observed higher plasma glycine levels in the case of the intraperitoneal injection of glycine–ethanol. This effect may be related to an increase in blood flow by ethanol, thereby increasing the absorption of glycine from the abdominal cavity. There is a paucity of information concerning the effect of ethanol on the peripheral vasculature[19]. Reports concerning the circulatory status of alcohol-treated subjects have been conflicting, some claiming an increase in plasma and blood volume[20, 21], whereas others report a decrease[22] or no change[23–25]. Other factors, such as differences in the distribution and rate of elimination of glycine, might also contribute to the observed difference. After the initial higher plasma glycine level was attained (within 5 min, Fig. 1) in the mice injected with ethanol glycine, a much faster disappearance of glycine from the plasma followed. This could be due to an increased excretion of glycine, or a redistribution of glycine in other tissues. It has been reported[26] that ethanol inhibited *in vivo* the uptake of amino acids by the liver and kidney in rats.

In conclusion, we have observed a small degree of interaction between glycine and ethanol which was reflected in a prolongation of ethanol sleep time in mice infected simultaneously with the two substances. This was in contrast to the results of Blum *et al.*[3], who reported a 10-fold increase in sleep time. Our results indicate that the observed glycine–ethanol interaction was not a result of an alteration of the rate of ethanol metabolism; also, it was unlikely to be a result of a glycine-induced elevation of GABA in the brain, as suggested by Blum *et al.*[3]. We cannot exclude the possibility that there might be localized accumulations of glycine in discrete areas of the brain as a result of increased plasma glycine concentrations. In these areas the physiological response to the inhibitory actions of glycine might be altered by ethanol. A combination of these factors might contribute to the observed potentiation of ethanol sleep time.

Acknowledgements—I thank Dr. C. M. Smith and Dr. C. B. Moffett for helpful criticism of this manuscript. The skilful technical assistance of Mrs. R. Ghosh, Ms. Donna Schanley and Cynthia Cercone is gratefully acknowledged.

REFERENCES

1. N. Davidson, in *Neurotransmitter Amino Acids*, pp. 39–56. Academic Press, New York (1976).
2. H. M. Häkkinen and E. Külonen, *Biochem. J.* **78**, 588 (1961).
3. K. Blum, J. E. Wallace and I. Geller, *Science, N.Y.* **176**, 292 (1972).
4. K. Blum, J. E. Wallace and R. N. Friedman, *Life Sci.* **14**, 557 (1974).
5. R. J. Breglia, C. O. Ward and C. I. Jarowski, *J. pharm. Sci.* **62**, 49 (1973).
6. M. H. Aprison, R. P. Shank, R. A. Davidoff and R. Werman, *Life Sci.* **7**, 583 (1968).
7. M. L. Uhr and M. K. Sneddon, *J. Neurochem.* **12**, 231 (1972).
8. A. W. K. Chan, *Life Sci.* **19**, 597 (1976).
9. O. H. Lowry and J. V. Passonneau, in *A Flexible System of Enzymatic Analysis.* Academic Press, New York (1972).
10. S. J. Berger, J. A. Carter and O. H. Lowry, *Analyt. Biochem.* **65**, 232 (1975).
11. A. J. Siemens and A. W. K. Chan, *Life Sci.* **19**, 581 (1976).
12. A. W. K. Chan, H. B. Burch, T. R. Alvey and O. H. Lowry, *Am. J. Physiol.* **229**, 1034 (1975).
13. D. Jones, L. P. Gerber and W. Drell, *Clin. Chem.* **16**, 402 (1970).
14. R. A. Deitrich and A. C. Collins, in *Alcohol and Opiates* (Ed. K. Blum), p. 109. Academic Press, New York (1977).
15. G. E. McClearn and R. Kakihana, *Behav. Genet.* **3**, 409 (1973).
16. W. D. W. Heston, V. G. Erwin, S. M. Anderson and H. Robbins, *Life Sci.* **14**, 365 (1974).
17. T. Chang, J. Lewis and A. Glazko, *Biochem. biophys. Acta* **135**, 1000 (1967).
18. Y. Israel, I. Salazar and E. Rosenmann, *J. Nutr.* **96**, 499 (1968).
19. D. H. Knott and J. D. Beard, in *The Biology of Alcoholism* (Eds. B. Kissin and H. Begleiter), Vol. 2, p. 345. Plenum Press, New York (1972).
20. W. M. Nicholson and H. M. Taylor, *Q. Jl Stud. Alcohol* **1**, 472 (1940).
21. J. D. Beard, G. Barlow and R. R. Overman, *J. Pharmac. exp. Ther.* **148**, 348 (1965).

22. D. H. Knott, G. Barlow and J. D. Beard, *New Engl. J. Med.* **269**, 292 (1963).
23. M. B. Strauss, J. D. Rosenbaum and W. P. Nelson, *J. clin. Invest.* **29**, 1053 (1950).
24. M. E. Rubini, C. R. Kleeman and E. Lamdin, *J. clin. Invest.* **34**, 439 (1955).
25. J. G. Reves and W. H. Newman, *Toxic. appl. Pharmac.* **18**, 660 (1971).
26. J. W. Chambers and V. J. Piccirillo, *Q. Jl Stud. Alcohol* **34**, 707 (1973).