Biochemical Pharmacology, Vol. 25, pp. 2125–2130. Pergamon Press. 1976. Printed in Great Britain.

EFFECT OF ETHANOL INTAKE ON THE INCORPORATION OF LABELLED AMINO ACIDS INTO LIVER PROTEIN*

JØRG MØRLAND† and ANNE E. SJETNAN
Department of Pharmacology, University of Oslo, Blindern, Oslo 3, Norway

(*Received* 8 *December* 1975; *Accepted* 7 *April* 1976)

Abstract—Male Wistar rats were given ethanol to provide approximately 30 per cent of the total calories consumed. Sucrose or lipids replaced ethanol isocalorically in controls. Ten per cent of the calories were usually provided by protein, except for in one experiment where both treated and control animals received 25 per cent of their calories from protein. The diets were given for periods up to 35 days. When ethanol replaced sucrose isocalorically, incorporation of labelled amino acids into liver cell protein was reduced after treatment for 30 days, but not after treatment for 1 or 3 days. The reduced incorporation was present both in the presence of ethanol and 24 hr after the last intake of alcohol. When ethanol replaced lipids isocalorically, the same reduction of protein labelling was found as when sucrose was replaced, after treatment for 35 days. When both ethanol-treated and controls were given a protein-rich diet for 35 days, ethanol treatment caused the same reduction of amino acid incorporation into protein, as when both groups were fed a low-protein diet. It is concluded that long-term ethanol treatment mediates a reduction of protein synthesis in the liver. This reduction requires some time to develop, is due to the inclusion of ethanol in the diet and is not counteracted by a high-protein diet.

The effects of ethanol on hepatic protein metabolism have been studied less extensively than the effects on other parts of liver biochemistry. This may appear strange since effects on protein synthesis seems to be of central importance in the action of a series of other hepatotoxins [1]. Some of the effects of ethanol on total protein metabolism reported so far are partly conflicting. A single dose of ethanol given either *in vivo* or *in vitro* have been reported either to reduce [2–6], or to increase [7–9], or usually not to influence [2, 6, 8, 10–14] the incorporation of labelled amino acids into stationary liver proteins. The chronic intake of ethanol has been followed by reduced incorporation in most instances [2, 14, 15–20], but increases [3] as well as no effects [13, 21, 22] have been reported. These contradictory results could have been due to differences among the various experiments with respect to animal type, strain and sex, to diets given, to the way of ethanol administration used, parameters which all could influence protein metabolism. We therefore wanted to test how the incorporation of labelled amino acids into liver protein was affected by ethanol given for various periods of time by the same mode of administration throughout all experiments at controlled dietary conditions.

MATERIALS AND METHODS

Animals. Male Wistar rats, 290–370 g final body wt, were offered an ordinary laboratory rat chow diet and water *ad lib.* for 7–14 days before start of the experiments. During this and the subsequent experimental period they were housed in separate plastic boxes, two animals per box at 22°, 60 per cent humidity on a 12 hr light–12 hr dark cycle from 7 p.m. to 7 a.m.

Ethanol treatment, diets and experimental design. We used a drinking model slightly modified from Porta and Gomez-Dumm [23]. This model allows the animals to choose between a liquid and a solid diet and omits the unphysiological administration of ethanol by either gastric tube or intraperitoneal injection. Five experiments were performed, designated A, B, C, D and E. All experiments included ethanol-treated (ethanol constituting approximately 30 per cent of the calories consumed) and control groups. The animals of all groups were offered both a solid synthetic diet *ad lib.* and in addition a liquid diet (Table 1) from small drinking bottles. Ethanol was always given in mixture with sucrose to ensure that a significant amount was consumed [23]. In experiments A, B, C and D all bottles were filled with isocaloric amounts of liquid food and the volumes consumed daily were recorded. In experiment E, the rats were pair-fed with one group of ethanol consuming animals as the leading.

The duration of each experiment, the kind of solid and liquid diets used are given in Table 1. It is seen that experiment A, B, C and D differred in duration only. Experiment D lasted for the same period of time as C, but the liquid diets of both experimental and control animals were replaced by water for the last 24 hr before sacrifice. In experiment E, lasting for 5 days more than D, another control group was included in which lipids instead of sucrose replaced ethanol. In this experiment we also introduced two groups of animals on a high protein diet. One of these groups received ethanol while sucrose replaced ethanol in the other. All diets in experiment E were replaced by water and the same solid diet (type I) 24 hr before decapitation of the rats.

* Some of the results were presented at the Sixth Congress of Pharmacology (July, 1975), Helsinki, Finland.

† Present address: Institute of Medical Biology, University of Tromsø, N-9001 Tromsø, Norway.

Table 1. Experimental design and mean daily intake of various diets*

Expt	Group	No. of rats	Duration of expt (days)	Solid diet (type)†	Liquid diet (type)‡	Liquid diet (ml/day)	Liquid diet (cal/day)	Total diet (% of cal) EtOH	Carboh.	Lipid	Protein
A	Control	6	1	I	IV	46	46	—	62	30	9
	Ethanol	4	1	I	V	28	43.4	33	26	32	9
B	Control	5	3	I	IV	46	46	—	62	30	9
	Ethanol	5	3	I	V	23	35.7	27	23	39	11
C	Control	5	30	I	IV	40	40		55	35	10
	Ethanol	5	30	I	V	23	35.7	27	23	39	11
D	Control	5	30 + 1	I	IV	39	39	-	54	36	10
	Ethanol	5	30 + 1	I	V	24	37.2	29	23	38	11
E	Control lipid	6	35 + 1	I	III	27	41.9	—	23	67	10
	Control	6	35 + 1	I	IV	41	41	—	56	34	10
	Ethanol	6	35 + 1	I	V	26	40.3	31	24	35	10
	Control high prot.	6	35 + 1	II	IV	41	41	—	56	20	24
	Ethanol high prot.	6	35 + 1	II	V	27	41.9	32	25	19	24

* Rats were given the various solid and liquid diets for different periods of time. An additional day without ethanol is designated by "+1". The daily intake of liquid diet in all groups varied less than ± 10 per cent from the figures given.

† The solid diets were supplemented with minerals, vitamins, choline, methionine [18] and 50 g cystine/kg, and contained (% of cal): Sucrose/starch (1:1) 10; Casein 20 (I) or 50 (II); Soybean oil/refined lard (2:3) 70 (I) or 40 (II).

‡ Containing (cal/ml): Total 1.55 (III and V) or 1.00 IV: as sucrose 0.55 (III), 1.00 (IV) or 0.60 (V); as soybean oil 1.00 (III); as ethanol 0.95 (V).

The daily intake of ethanol was about 13 g/kg body wt during the first week in all experiments. Since the rats drank an almost constant volume per day throughout the experiments, this figure declined to approximately 10 g/kg after 4–5 weeks due to the increase in body weight during this period.

It is seen from Table 1 that the rats from all groups received approximately 40 cal (35–46 cal) daily from liquid. The total daily intake of calories may roughly be estimated to 80 cal [24] by all rats since only minor variations of weight gain were recorded among the various groups at least during the last 3 weeks of long-term experiments (Fig. 1). Thus all rats covered approximately one half of their caloric demands by solid diet and the other half by liquid. The approximate caloric composition of the total diet calculated on this background is given in Table 1. Although this calculation may be misleading due to possible effects of ethanol on energy expenditure it appears that all rats received sufficient amounts of minerals, vitamins, lipotropes, protein and amino acids since the solid diets contained more than twice the recommended values [24].

Measurement of labelled and unlabelled amino acids and protein. All rats were given an i.p. injection of labelled amino acids 75 min prior to sacrifice. 1.25 μCi (200 μl) of [U-^{14}C]protein hydrolysate (CFB.25, 54 mCi/matom carbon, The Radiochemical Centre, Amersham) was injected per 100 g rat in experiments A, B, C and D, while 8 μCi (200 μl) of L-[4,5-^{3}H]leucine (TRK.170, 46 Ci/m-mole, The Radiochemical Centre, Amersham) per 100 g rat was given in experiment E. The rats were killed by decapitation, the liver was quickly excised, blotted, weighed and frozen in liquid nitrogen. In some experiments blood

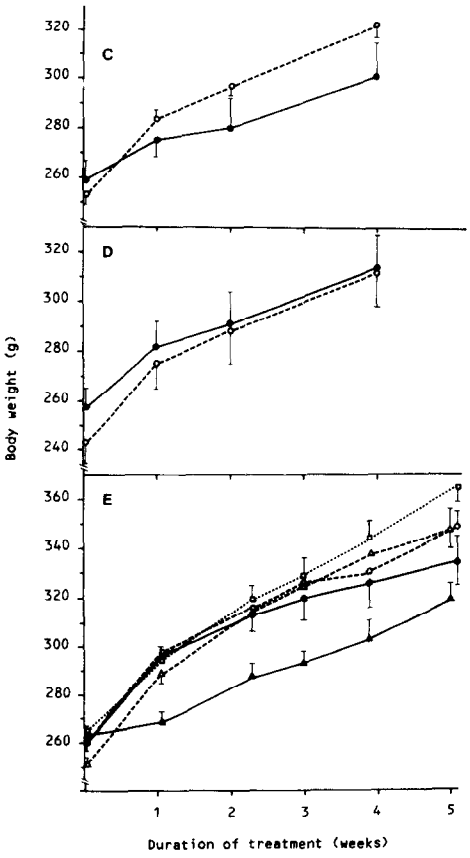

Fig. 1. Effect of diet on body weight in three different experiments (see also Table 1): ●, ethanol; ○, control; □, control lipid; △, control high protein; ▲, ethanol high protein. Vertical bars indicate S.E.M.

Table 2. Effect of duration of ethanol treatment on the incorporation of labelled amino acids into protein*

Expt	Control (dis/min/liver × 10^6)	Ethanol (dis/min/liver × 10^6)	(%of control)
A (1 day)	5.00 ± 0.31	4.86 ± 0.14	97.2
B (3 days)	5.05 ± 0.25	4.82 ± 0.46	95.6
C (30 days)	5.19 ± 0.27	4.24 ± 0.37†	81.5†
D (30 + 1 day)	4.50 ± 0.30	3.45 ± 0.22†	76.6†

* Rats were treated as described in Table 1. An additional day without ethanol is designated by "+1 day". 1.25 μCi of [U-^{14}C]protein hydrolysate per 100 g rat was injected i.p. 75 min before sacrifice. The results are expressed as mean values ± S.E.M.; the number of rats is given in Table 1.

† $\alpha < 0.05$ with respect to corresponding control.

from the neck vessels was collected, plasma was separated and frozen.

Samples of liver and plasma were homogenized in 20 vol 10% trichloroacetic acid. After centrifugation, 0.5 ml of the supernatant was mixed with scintillation liquid [25] and trichloroacetic acid-soluble radioactivity (representing free amino acid(s)) was determined [14]. In another supernatant sample, total free leucine was determined (Expt E) by methods described elsewhere [14]. The final protein pellet was prepared and the specific radioactivity measured as detailed elsewhere [18]. Internal standard, L-[4,5-^{3}H]leucine, was added to all radioactive samples and counted to check the quenching, which was found to be equal in all samples. Total protein was determined in liver and plasma by the methods of Lowry *et al.* [26] and Gornall *et al.* [27], respectively.

Additional procedures. Blood ethanol concentration was determined by gas chromatography [28]. Wilcoxon's test was used to determine the statistical significance of the results, differences accompanied by α-values above 0.05 were considered as insignificant.

RESULTS

The results of experiments A, B, C and D are summarized in Table 2. Voluntary consumption of ethanol for 1 or 3 days did not influence the incorporation of a mixture of labelled amino acids into liver protein. The rats used in these experiments weighed: 392.1 ± 8.2 g controls, 321.8 ± 10.4 g treated for one day; and 351.0 ± 6.8 g controls, 332.2 ± 7.5 g treated for 3 days. Treatment for 30 days reduced the incorporation significantly. Blood ethanol concentration at sacrifice varied between 0.5 and 19.8 mM in experiments A, B and C, the mean values were between 5 and 6 mM in all experiments. These variations probably represent differences in the daily pattern of ethanol consumption among different animals, since the amount consumed per single rat differed less than ± 10 per cent from the mean consumed per day. The omission of ethanol for one day before killing, after 30 days treatment (Expt D) did not significantly influence the reduced incorporation, indicating that the presence of the ethanol during the labelling period was unnecessary for the effect to occur. The weight of the rats used in experiments C and D are given in Fig. 1. The amount of trichloroacetic acid-soluble radioactivity was higher in livers from the ethanol-treated rats in all experiments, and the ratio between soluble counts from treated and control rats were: Expt A, 1.13; Expt B, 1.10; Expt C, 1.57; and Expt D, 1.09.

In experiment E we found that the reduction of labelled L-leucine incorporation after ethanol was of the same order of magnitude as the reduction of incorporation found in experiment D in which a mixture of labelled amino acids were used as precursors. It was further found that the effect of ethanol was present also when compared to a diet in which lipids replaced ethanol isocalorically (3 upper lines, Table 3). The possibility existed that the reduction of incorporation so far reported could at least partly have been due to the low protein content of the diet (about 10 per cent of the total calories consumed, Table 1). From Table 3 (2 lower lines) it appears that ethanol reduced the incorporation of labelled L-leucine into protein also when a protein-rich diet had been given. There was a consistent tendency towards decreased incorporation when the protein content of the diet had been raised (compare line 2 and 4, or 3 and 5 in Table 3). In experiment E there was no more radioactivity in the trichloroacetic acid-soluble fraction of treated livers than in control livers (Table 3). When the specific activity of trichloroacetic acid-soluble L-leucine was calculated, no significant differences were found among the various groups in experiment E (Table 3). This showed that neither ethanol nor other dietary changes exerted major effects on the hepatic leucine pool. Ethanol consumption did further not significantly reduce hepatic protein content as found and discussed earlier [19].

We finally wanted to test if the labelling of circulating proteins was affected in the same manner as stationary liver proteins. Table 4 presents the results obtained in experiment E. It is seen that the incorporation into circulating proteins was reduced to the same degree as found for fixed liver proteins. The effect of ethanol treatment on plasma protein levels, however, was much less pronounced and was significant for rats on high protein diet only.

DISCUSSION

The present reduced incorporation found after long-term ethanol treatment represents some alteration of amino acid or protein metabolism.

The level of trichloroacetic acid-soluble radioactivity in the liver was not decreased due to ethanol administration when measured 75 min after the injection of labelled amino acids. This was also the case when trichloroacetic acid-soluble radioactivity was

Table 3. Effect of different diets and ethanol treatment for 35 days on liver composition, hepatic free leucine and on incorporation of labelled leucine into liver protein*

Group	Liver			Trichloroacetic acid precipitable		Trichloroacetic acid-soluble	
	wt (g)	rel. wt (g/100 g)	protein content (g)	(dis/min/liver $\times 10^5$)	(dis/min/mg protein)	(dis/min/mg liver)	(dis/min/μmoles leucine)
Control lipid	11.95 ± 0.37	3.32 ± 0.08	2.50 ± 0.05	10.4 ± 0.7	417 ± 32	15.4 ± 0.9	60.6 ± 6.3
Control (sucrose)	12.12 ± 0.29	3.47 ± 0.04	2.59 ± 0.07	10.6 ± 0.5	409 ± 26	14.4 ± 1.1	61.6 ± 7.1
Ethanol	11.37 ± 0.44	3.39 ± 0.04	2.50 ± 0.17	8.2 ± 0.6†	331 ± 25†	14.8 ± 1.0	64.4 ± 6.8
Control high protein	13.28 ± 0.58	3.89 ± 0.12	2.78 ± 0.08	9.1 ± 0.8	327 ± 31	15.5 ± 0.8	62.1 ± 6.0
Ethanol high protein	11.90 ± 0.57	3.78 ± 0.10	2.54 ± 0.09	6.9 ± 0.6†	273 ± 30	14.7 ± 0.8	58.4 ± 7.3

* Rats were treated as described in Table 1. Expt E. 8 μCi of L-[4,5-^{3}H]leucine per 100 g rat were injected i.p. 75 min before sacrifice. The results are expressed as mean values ± S.E.M.; the number of rats is given in Table 1.

† $\alpha < 0.05$ with respect to corresponding control(s).

Table 4. Effect of different diets and ethanol treatment for 35 days on the incorporation of labelled leucine into plasma proteins*

Group	Trichloroacetic acid-precipitable (dis/min/ml plasma × 10^4)	Plasma protein (mg/ml plasma)
Control lipid	4.76 ± 0.36	63.4 ± 3.7
Control (sucrose)	4.25 ± 0.30	58.6 ± 4.1
Ethanol	3.64 ± 0.28†	62.8 ± 3.2
Control high protein	4.09 ± 0.28	67.3 ± 4.1
Ethanol high protein	2.66 ± 0.21†	58.2 ± 3.5†

* Experimental details are given in Table 3.
† $\alpha < 0.05$ with respect to corresponding control(s).

determined 10 and 40 min after the administration of label (J. Mørland, unpublished observations). Nor was the specific radioactivity of the precursor changed. Although we were not able to measure the specific radioactivity of leucine at the site of protein synthesis [29], our results did not indicate any changes of amino acid metabolism which could explain the reduced incorporation found. Since ethanol treatment previously has been found not to effect the rate of degradation of newly synthesized proteins [18], and since an increased release of labelled proteins to the blood was unlikely [18] (Table 4), reduced protein synthesis appears to be the most likely explanation of the present results. With the reservation discussed here in mind, we will further refer to the reduced incorporation as an expression of a reduced rate of synthesis of at least some liver proteins. The reduced incorporation of amino acids into circulating proteins could, however, also represent a decreased secretion of labelled proteins by the liver or other organs.

Our experiments showed a significant reduction of the synthesis of total stationary liver protein, caused by chronic ethanol treatment. This reduction was independent of gross nutritional deficiencies, of great reductions in growth rate and occurred when ethanol was consumed voluntarily replacing either sucrose or lipids isocalorically in a controlled way (Table 3). Ethanol feeding may reduce the concentration of amino acids in the liver due to decreased amino acid uptake, transport and other mechanisms, for discussion see [4, 14, 16, 30, 31]. The effect of ethanol on protein synthesis in animals given a low-protein diet could therefore have been due to a reduction of the tissue concentration of some free amino acids below a critical limit, leading to reduced protein synthesis, since it has been shown that the rate of hepatic protein synthesis is correlated to the tissue level of certain free amino acids [32, 33]. This was however not likely in the present experiments, since reduced protein synthesis was found also in rats receiving a high protein diet. Since increased intake of protein during the feeding period did not stimulate hepatic protein synthesis, the low-protein diet seemed nutritionally sufficient with respect to protein synthesis.

Some results obtained in less strictly controlled experiments also revealed reduced incorporation of amino acids into fixed hepatic proteins [2, 14–20] after long-term ethanol treatment. Other reports do not show any reduction of protein synthesis [3, 13, 21, 22]. In three of the latter reports [3, 13, 21], however, incorporation was tested before ethanol treatment had lasted for 14 days. In the final report [22] the lack of isocaloric feeding might have contributed to the lack of effect.

The effect of chronic consumption of ethanol on protein synthesis was found to be independent of the presence of ethanol during the period of incorporation of label (Table 2). This effect was therefore different from the effect of ethanol on albumin synthesis [34–36]. The reduction was thus not probably directly linked to the redox changes accompanying ethanol metabolism, but was rather due to effects of a more permanent character.

Since our animals received ethanol in the same manner both in acute and chronic experiments, the present results indicate a progressive reduction of hepatic protein synthesis with time when ethanol is consumed daily. Few comparisons between acute and chronic effects of ethanol on protein metabolism have been reported in earlier literature [3, 13]. These results are further difficult to interpret in relation to the present ones since the same mode of ethanol administration was not used in acute and chronic experiments, and since the consumption of ethanol lasted for only 14 days. Recently Burke *et al.* [20] reported an inhibition of hepatic mitochondrial protein synthesis which appeared after ethanol treatment of miniature swine for 6 weeks, but which was not present after 3 weeks, supporting the concept of time-dependent inhibition of total protein synthesis described in this paper.

The biological importance of this time-dependent reduction of protein biosynthesis is unclear. It may be of some importance to notice that this reduction is present in livers where no triglycerides have accumulated and no other major changes of either biochemical or morphological nature are present [18, 19]. The present results may have a bearing at the well-known difference between short-term and long-term exposure to ethanol with respect to damaging potential to the liver, if inhibition of protein synthesis is considered as a hepatotoxic event.

Acknowledgements—Dr. H. Prydz, University of Tromsø, is gratefully acknowledged for the determination of free hepatic leucine concentration.

REFERENCES

1. E. Faber, in *International symposium on hepatotoxicity* (Eds. M. Eliakim, J. Eshchar and H. J. Zimmerman) p. 3. Academic Press, New York and London (1975).

2. E. Rubin, D. S. Beattie and C. S. Lieber, *Lab. Invest.* **23**, 620 (1970).
3. K. Kuriyama, P. Y. Sze and G. E. Rauscher, *Life Sci. II* **10**, 181 (1971).
4. J. W. Chambers and V. J. Piccirillo, *Q. J. Stud. Alc.* **34**, 707 (1973).
5. A. Perrin, G. Scalabrino, A. Sessa and A. Arnaboldi, *Biochim. biophys. Acta* **366**, 101 (1974).
6. K. N. Jeejeebhoy, A. Bruce-Robertson, J. Ho and U. Sodtke, in *Alcohol and Abnormal Protein Biosynthesis* (Eds. M. A. Rothschild, M. Oratz and S. S. Schreiber) p. 373. Pergamon Press, Oxford (1975).
7. K. J. Isselbacher and N. J. Greenberger, *New Engl. J. Med.* **270**, 402 (1964).
8. J. Jarlstedt and A. Hamberger, *J. Neurochem.* **19**, 2299 (1972).
9. J. W. Chambers and V. J. Piccirillo, *Fedn Proc.* **33**, 554 (1974).
10. A. Seakins and D. S. Robinson, *Biochem. J.* **92**, 308 (1964).
11. C. F. Johnson, *Texas Med.* **62**, 63 (1966).
12. S. Mookerjea and A. Chow, *Biochim. biophys. Acta* **184**, 83 (1969).
13. F. Tobon and E. Mezey, *J. Lab. clin. Med.* **77**, 110 (1971).
14. J. Mørland, *Biochem. Pharmac.* **24**, 439 (1975).
15. A. Albertini, E. Bonera and V. Zambotti, *FEBS Meeting, Prague*, Abstr. 655 (1968).
16. W. L. Banks Jr., E. S. Kline and E. S. Higgins, *J. Nutr.* **100**, 581 (1970).
17. E. P. Noble and S. Tewari, *Ann. N.Y. Acad. Sci.* **215**, 333 (1973).
18. J. Mørland, *Biochem. Pharmac.* **23**, 21 (1974).
19. J. Mørland, *Acta pharm. tox.* **35**, 155 (1974).
20. J. P. Burke, M. E. Tumbleson, K. W. Hicklin and R. B. Wilson, *Proc. Soc. exp. Biol. Med.* **148**, 1051 (1975).
21. E. Baraona and C. S. Lieber, *J. clin. Invest.* **49**, 769 (1970).
22. J. Jarlstedt, *J. Neurochem.* **19**, 603 (1972).
23. E. A. Porta and C. L. A. Gomez-Dumm, *Lab. Invest* **18**, 352 (1968).
24. R. G. Warner, in *Nutrient Requirements of Domestic Animals*, nr. 10-Laboratory Animals (Pub. 990) p. 51. National Academy of Sciences- National Research Council, Washington (1962).
25. J. B. Osnes, T. Christoffersen, J. Mørland and I. Oye, *J. Chromatog.* **67**, 139 (1972).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
28. I. Sunshine and N. Hodnett, in *The Biology of Alcoholism* (Eds. B. Kissin and H. Begleiter), Vol. 1, p. 545. Plenum Press, New York-London (1971).
29. J. Ilan and M. Singer, *J. molec. Biol.* **91**, 39 (1975).
30. J. M. Orten and V. M. Sardesai, in *The Biology of Alcoholism* (Eds. B. Kissin and H. Begleiter) Vol. 1, p. 229. Plenum Press, New York-London (1971).
31. H. A. Krebs, R. Hems and P. Lund, *Biochem. J.* **134**, 697 (1973).
32. L. S. Jefferson and A. Korner, *Biochem. J.* **111**, 703 (1969).
33. R. W. Wannemacher Jr., C. F. Wannemacher and M. B. Yatvin, *Biochem. J.* **124**, 385 (1971).
34. M. A. Rothschild, M. Oratz, J. Mongelli and S. S. Schreiber, *J. clin. Invest.* **50**, 1812 (1971).
35. K. N. Jeejeebhoy, M. J. Phillips, A. Bruce-Robertson, J. Ho and U. Sodtke, *Biochem. J.* **126**, 1111 (1972).
36. G.-S. D. Nadkarni, *Biochem. Pharmac.* **23**, 389 (1974).