Biochemical Pharmacology, Vol. 21, pp. 811–819. Pergamon Press, 1972. Printed in Great Britain

EFFECT OF PYRAZOLE ON THE INDUCTION OF FATTY LIVER BY CHRONIC ADMINISTRATION OF ETHANOL

H. KALANT, J. M. KHANNA and G. O. BUSTOS

Department of Pharmacology, University of Toronto, Toronto, Canada

(*Received* 21 *June* 1971; *accepted* 1 *October* 1971)

Abstract—Groups of rats were pair-fed liquid diets containing ethanol or an equicaloric concentration of sucrose for a period of 3–4 weeks. Half of the animals on each diet also received by gavage a dose (37·5 mg/kg) of pyrazole every second day. On this treatment all animals maintained their weight and appeared healthy, but those receiving pyrazole plus ethanol had consistently higher blood ethanol levels than those receiving ethanol alone. At the end of the treatment period, the pyrazole–ethanol groups were found to have much higher hepatic triglyceride levels than those on ethanol alone, except when ethanol contributed only 20 per cent to the caloric intake. In contrast, animals receiving pyrazole plus sucrose had less hepatic triglyceride than those on sucrose alone, when fat contributed substantially to the diet. The findings suggest that pyrazole itself tends to reduce hepatic triglyceride by an as yet unknown mechanism, but enhances a direct hepatotoxic effect of ethanol by impairing its metabolism and prolonging the period of exposure of the liver to ethanol. However, other mechanisms of synergism between ethanol and pyrazole cannot be ruled out.

IT WAS recently reported[1] that pyrazole, an inhibitor of alcohol dehydrogenase activity both *in vitro* and *in vivo*,[2–4] given to fasted rats 10 min before gavage with ethanol, 4 g/kg, had no effect on the accumulation of hepatic triglycerides measured 16 hr after administration of the ethanol. The dose of pyrazole used, 272 mg/kg, however, prevented completely the disappearance of ethanol from the blood during the experimental period, as well as the reduction in hepatic $NAD/NADH_2$ ratio caused by ethanol alone. Therefore it was concluded that metabolism of ethanol is not required for the production of fatty liver by a large single dose of ethanol.

This acute fatty liver, however, may result from different mechanisms than those involved in the production of fatty liver in subjects consuming ethanol chronically.[5] It was therefore desirable to investigate the effect of pyrazole in the chronic model as well. Lelbach[6] reported that chronic administration of ethanol, either by provision of 15% (v/v) ethanol solution as the sole drinking fluid or by daily gavage with ethanol in a dose of 6·0–7·7 g/kg, produced liver cell necrosis in rats receiving pyrazole, 31 mg/kg, daily; biochemical studies on hepatic lipids were not included. He suggested that reduced activity of alcohol dehydrogenase resulted in a longer period of exposure of the liver to a direct hepatotoxic action of ethanol. Unfortunately, Lelbach's study did not include control groups receiving pyrazole without ethanol, nor others receiving appropriate diets to provide a caloric equivalent of the alcohol consumed.

The present report describes a study of hepatic triglyceride accumulation, in which

such controls have been included. The results indicate that pyrazole, under these conditions, can greatly enhance the accumulation of hepatic triglyceride caused by ethanol alone. Histological studies are currently in progress.

MATERIALS AND METHODS

Preliminary experiment. A preliminary experiment was carried out to determine the optimal dosage of pyrazole to be used in the chronic studies. Male rats of the Wistar strain, of about 200 g initial body weight, were obtained from Canadian Breeding Laboratories, Montreal. They were caged individually. Each rat was given 80 ml daily of homogenized liquid sucrose diet in graduated drinking tubes as the only source of food and water for a 1-week period to permit them to adapt to this form of feeding. The liquid diet was modified from that devised by Lieber *et al.*,[7] as described previously.[8] After 1 week, four groups of four rats each were set up. All four groups now received an ethanol-containing liquid diet in Richter drinking tubes. Ethanol provided 35 per cent of the total calories, protein hydrolysate 19 per cent, sucrose 5 per cent and fat 41 per cent. The amount of diet consumed by each rat was measured daily. The animals were weighed two or three times per week. Groups B, C and D also received pyrazole by intubation in doses of 25, 37·5 and 50 mg/kg respectively. No pyrazole was given to the animals of group A. For the first 4 days, these amounts of pyrazole were given daily, but with this treatment the rats on pyrazole reduced their intake of liquid diet substantially. They lost weight and one rat on the 37·5 mg/kg dose and two on 50 mg/kg died. No pyrazole was administered for the next 2 days. Thereafter the respective doses of pyrazole were given on alternate days. Tail vein blood samples of 0·05 ml were also taken two or three times per week from each rat, for measurement of ethanol concentration by the internal standard technique of gas–liquid chromatography.[9]

Experiment 1. Male Wistar rats of the same strain were given the sucrose liquid diet for 1 week. They were then divided into four groups, each containing seven animals, individually caged. The following four groups were set up: ethanol–pyrazole, ethanol, sucrose–pyrazole and sucrose.

The ethanol–pyrazole group received *ad lib.* the ethanol liquid diet in drinking tubes. The amount consumed by each animal was measured each day, and the same amount of the appropriate liquid diets was offered the following day to the corresponding pair-fed animals in the other three groups. In the sucrose–pyrazole and sucrose groups, ethanol was replaced isocalorically with sucrose. The ethanol–pyrazole and sucrose–pyrazole groups also received by intubation on alternate days 37·5 mg/kg of pyrazole. The diets used in this experiment were the same as reported in the preliminary experiment. For the first 2 weeks of the experimental period, tail vein blood samples were taken from each animal twice a day, once in the morning (around 11.00 a.m.) and the second time in the afternoon (around 3.00 p.m.). For the remaining 1 week of the experiment, only the afternoon sample was taken. Blood alcohol samples were taken before administration of pyrazole. No blood alcohol samples were taken on the weekend, but the diet and treatment were as usual. The body weights were taken three times per week.

Experiment 2. This experiment was similar to experiment 1, except that only 20 per cent of the calories were derived from ethanol and 25 per cent from fat. Morning and afternoon blood alcohol samples were taken throughout the experimental period.

Experiment 3. This experiment was similar to experiment 1, except that ethanol provided 25 per cent (group A) and 30 per cent (group B) of the total calories, protein hydrolysate 25 per cent, fat 10 per cent and the rest sucrose. The amount of lipotropic factors was increased by 50 per cent.

The composition of diets in the various experiments is shown in Table 1.

TABLE 1. COMPOSITION OF DIETS IN THE VARIOUS EXPERIMENTS*

	Ethanol and ethanol–pyrazole groups				Sucrose and sucrose–pyrazole groups		
Experiment	P	F	C	E	P	F	C
1	19	41	5	35	19	41	40
2	19	35	26	20	19	35	46
3A	25	10	40	25	25	10	65
3B	25	10	35	30	25	10	65

* Figures shown represent percentage of total calories contributed by P (protein), F (fat), C (carbohydrate) and E (ethanol). Lipotrope values were 73 in experiments 1 and 2, and 110 in experiment 3.

Triglyceride determination. At the end of the treatment period, the animals were killed by decapitation at about 11.00 a.m. The livers were removed and homogenized in 9 vol. of phosphate buffer 0·066 M, pH 7·0. Triglycerides were extracted as described by Butler *et al.*[10] and measured by the colorimetric method of Van Handel.[11] For inter-group comparisons within each experiment, the significance of differences was determined by calculating the ratios of triglyceride concentration in the liver of each animal in one group with that of its pair-fed mates in the other groups with which the first was being compared. The significance of ratios was calculated by the method of Bartlett and Kendall.[12]

RESULTS

Preliminary experiment. Figure 1 shows the growth curves and alcohol intakes of the four groups. In comparison with group A rats receiving no pyrazole, the daily administration of 25, 37·5 and 50 mg/kg of pyrazole markedly reduced the alcohol intake in groups B, C and D respectively. The reduction in body weight and alcohol intake was more marked in the animals receiving 50 mg/kg of pyrazole. Two rats on 50 mg/kg and one rat on 37·5 mg/kg pyrazole treatment died on the fifth day.

When pyrazole was administered on alternate days, the rats began to increase their alcohol intake, although the intake was still lower than that of group A. The decrease in ethanol intake was proportional to the dose of pyrazole administered. There was no further loss in body weight, and the animals in group B actually rose to a little above their starting weight. None of the animals died on this new schedule.

Group A animals showed blood ethanols ranging from 55 to 155 mg/ml throughout the whole period. During the initial portion of the experiment when pyrazole was given daily, the alcohol intakes of groups B, C and D were so low that blood ethanol levels measured in the morning were the same as, or lower than, those of group A. When pyrazole treatment was decreased to every second day, ethanol intakes rose and the blood levels in groups B–D ranged from 240 to 436 mg/100 ml.

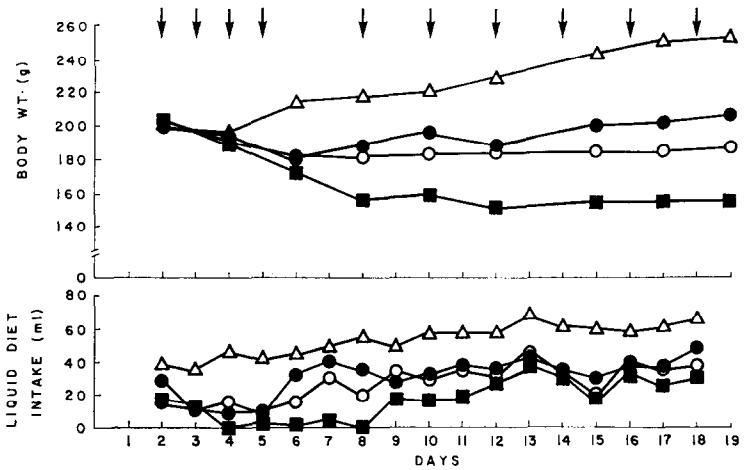

FIG. 1. Mean body weight curves and consumption of ethanol-containing diet by four groups in the preliminary experiment. Group A (△) received no pyrazole. Groups B (●), C (○) and D (■) received pyrazole by intubation in doses of 25, 37·5 and 50 mg/kg, respectively, on the days indicated by arrows.

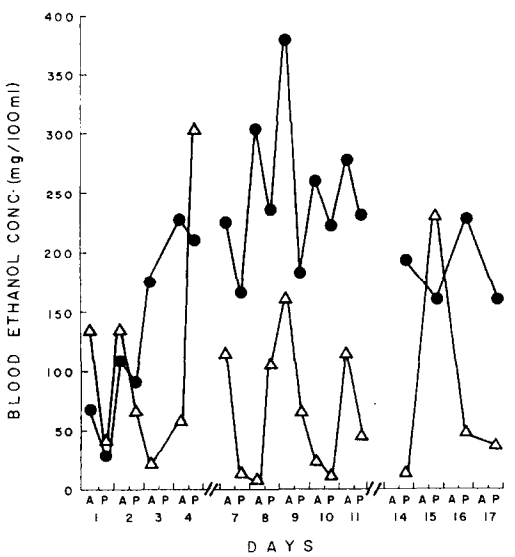

FIG. 2. Mean blood ethanol concentrations in the ethanol–pyrazole (●) and ethanol (△) groups in experiment 1. Diet and treatments are described in the text. A and P indicate blood sampling at 11.00 a.m. and 3.00 p.m. respectively.

On the basis of these preliminary results, the optimal pyrazole dosage chosen for the following experiments was 37·5 mg/kg every second day.

Experiment 1. The mean body weights of the four groups did not change during the experiment, remaining virtually constant at 250–255 g. All of the animals appeared healthy and there were no deaths.

Figure 2 shows the blood alcohol concentrations in the ethanol–pyrazole and ethanol groups. It is evident that pyrazole effectively decreased the rate of ethanol metabolism; the blood ethanol concentrations were consistently higher, both in the morning and afternoon samples, in the ethanol–pyrazole group than in the ethanol group.

The results of hepatic triglyceride analysis at the end of 18–21 days of administration of the ethanol and sucrose diets, with and without pyrazole, are shown in Table 2.

TABLE 2. HEPATIC TRIGLYCERIDE CONCENTRATIONS IN RATS CONSUMING LIQUID DIETS CONTAINING ETHANOL OR ISOCALORIC SUCROSE IN PRESENCE AND ABSENCE OF PYRAZOLE*

Experiment	Ethanol plus pyrazole (mg/g liver wet wt.)	Ethanol (mg/g liver wet wt.)	Sucrose plus pyrazole (mg/g liver wet wt.)	Sucrose (mg/g liver wet wt.)
1	136·0 ± 13·1† (7)	28·2 ± 5·0‡ (7)	6·0 ± 1·6§ (7)	10·9 ± 1·5 (7)
2	32·6 ± 5·4 (6)	24·4 ± 1·5 (6)	11·5 ± 0·7§ (6)	27·8 ± 2·3 (6)
3A	63·4 ± 21·3† (4)	19·9 ± 1·3 (4)	18·0 ± 6·3 (4)	20·6 ± 4·9 (4)
3B	84·6 ± 11·3† (4)	18·9 ± 1·9 (4)	20·5 ± 6·9 (4)	14·0 ± 3·4 (4)

* Values shown are mean ± S.E.M., with number of animals per group in parentheses. The significance of difference between groups was estimated from the ratios of triglyceride concentrations in the corresponding pair-fed animals in the groups being compared.[12]

† Significantly higher than values for other groups; in experiment 1, $P < 0.001$ in each case; in experiment 3A, $P < 0.05$, $0.1 > P > 0.05$ and $P < 0.025$ respectively; in experiment 3B $P < 0.005$, $P < 0.05$ and $P < 0.005$ respectively.

‡ Significantly higher than values in corresponding sucrose group; $P < 0.001$.

§ Significantly lower than values in corresponding sucrose group; $P < 0.05$ in experiment 1, and $P < 0.001$ in experiment 2.

Ethanol rats exhibited a 2·5-fold increase of hepatic triglycerides as compared with the sucrose rats. However, the hepatic triglyceride values in the ethanol–pyrazole group were much higher than in the other three groups, the differences being highly significant in all cases. In contrast, pyrazole administration appeared to decrease the level of triglyceride in animals receiving the sucrose diet ($P < 0.05$).

Experiment 2. In contrast with experiment 1, the administration of liquid diet providing only 20 per cent of calories from ethanol resulted in very low blood ethanol levels in the ethanol group. Pyrazole administration did elevate the blood ethanol concentrations in the ethanol–pyrazole group, but the levels were still markedly lower than in experiment 1 (Fig. 3).

There was no significant difference in hepatic triglyceride levels among the ethanol–pyrazole, ethanol and sucrose groups, but all three were significantly higher than the sucrose–pyrazole group (Table 2). Thus, the tendency of pyrazole to reduce the accumulation of triglycerides in animals receiving the sucrose diet, which was suggested in experiment 1, was clearly evident in experiment 2.

Experiment 3. Administration of liquid diets containing 25 and 30 per cent calories from ethanol resulted in blood ethanol levels which were intermediate between those found in experiments 1 and 2 (Fig. 4). Thus the blood ethanol levels achieved depend upon the percentage of ethanol calories in the diet. The administration of pyrazole again elevated the blood ethanol levels, and more so in the group on 30 per cent ethanol than in those on 25 per cent ethanol.

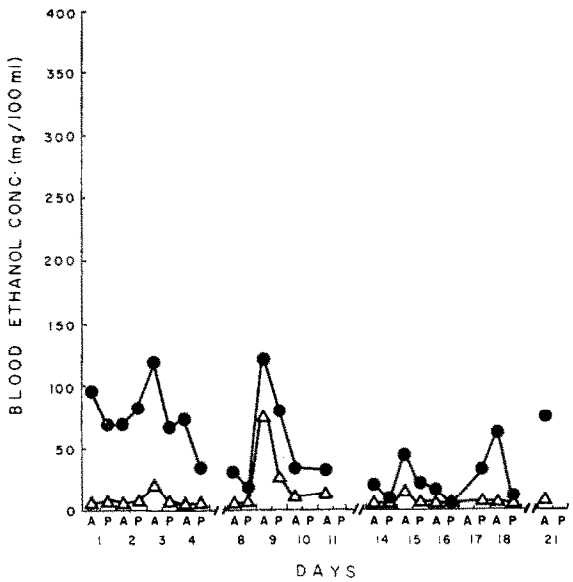

FIG. 3. Mean blood ethanol concentrations in the ethanol–pyrazole (●) and ethanol (△) groups in experiment 2. Diet and treatments are described in the text. A and P as in Fig. 2.

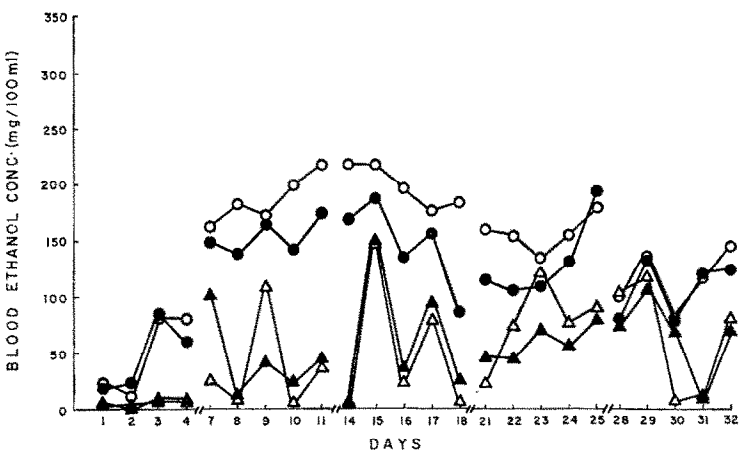

FIG. 4. Mean blood ethanol concentrations in experiment 3: circles indicate ethanol–pyrazole groups and triangles indicate ethanol groups; solid symbols for experiment 3A, open symbols for 3B. Diets and treatments are described in the text. All samples were taken at 11.00 a.m.

There was no significant difference in triglyceride values in ethanol, sucrose–pyrazole and sucrose groups, but all three were significantly lower than the ethanol–pyrazole group (Table 2). This was true with both diets, providing either 25 or 30 per cent of calories from ethanol. Pyrazole, however, did not decrease the hepatic triglyceride levels in the corresponding sucrose groups.

DISCUSSION

In acute studies it is possible to distinguish direct pharmacologic effects of ethanol from indirect consequences of its metabolism by using a dose of pyrazole large enough

to inhibit ethanol metabolism completely. Obviously, this cannot be done in chronic studies. If the animal continued to ingest ethanol, the concentration in the blood would rise rapidly. If, as in the preliminary experiment, it stopped ingesting the ethanol diet, it would rapidly lose weight (Fig. 1). In either case the regimen would soon prove fatal. Moreover, pyrazole itself can have toxic effects, as shown by ultramicroscopic changes in hepatocellular structure.[13] It was therefore important to select a dose of pyrazole which would cause as great a reduction in the rate of ethanol metabolism as possible without leading to progressive accumulation, and which would also have minimal toxic effect of its own.

The first requirement appears to have been met by the dose selected on the basis of the preliminary experiment. Measured ethanol levels in the blood were elevated in the pyrazole-treated animals, in both morning and afternoon samples (Figs. 2 and 3). This agrees well with the recent report by Reynier[14] that pyrazole in a dose of 40 mg/kg produced a 36 per cent reduction in the rate of ethanol metabolism *in vivo*. Yet all the ethanol consumed must have been metabolized during each 24-hr period since no accumulation occurred. In part, this reflects a reduction in ethanol intake by the pyrazole group, to a level which they could metabolize (Fig. 1). The four treatment groups in each experiment did not differ from each other in body weight, so that the ethanol calories must have been utilized as effectively as those derived from sucrose. Therefore the net effect of the pyrazole treatment must have been to prolong the period during which ethanol was present in the body by reducing the rate, but not the total extent, of its metabolism.

It is less certain that the second requirement was met, since indices of hepatotoxicity other than lipid accumulation were not studied in this work. Indeed, the reduction in hepatic triglyceride levels in the sucrose plus pyrazole groups of experiments 1 and 2 might conceivably be an indication of hepatotoxicity. The general state of the animals in all groups, however, as well as their stability of body weight, suggest that toxicity was not prominent with the dose of pyrazole used. It is worth noting that the dose of pyrazole used by Lieber *et al.*[13] in their chronic study was approximately twice that used in the present work, and was given daily rather than every second day.

In the groups receiving the ethanol diet alone, hepatic fat accumulation occurred when the diet provided 35 per cent of calories as ethanol, 41 per cent as fat and 19 per cent as protein hydrolysate, but not when ethanol was reduced to 20 per cent or protein increased to 25 per cent. These findings are in good agreement with those of Lieber *et al.*,[15] as well as those of Porta *et al.*[16] They are consistent with the hypotheses relating hepatic triglyceride accumulation both to the nucleotide changes resulting from ethanol metabolism[5,17] and to a relative deficiency of protein and lipotropic factors.[16] In addition, the reduction in dietary fat in experiment 3 may have played a role in preventing hepatic triglyceride accumulation in the ethanol group.

However, the marked increase in liver triglycerides which occurred when pyrazole treatment was combined with the ethanol diet in experiments 1, 3A and 3B suggests an additional mechanism independent of the metabolism of ethanol. Since pyrazole was shown to elevate the blood ethanol levels (Figs. 2–4) by reducing the rate of ethanol metabolism,[14] it can be inferred that it was also decreasing the ethanol effect on $NAD/NADH_2$ ratio, as in the acute study[1] but to a less marked degree. Rubin *et al.*[18] have presented evidence that pyrazole may affect not only alcohol dehydrogenase but also microsomal systems which have been shown to oxidize ethanol under conditions

in vitro.[19] In relation to the present work, the important point is that pyrazole impairs ethanol oxidation, regardless of which enzyme system is involved. Therefore the additional accumulation of hepatic triglyceride in the pyrazole groups correlates with a longer exposure to higher ethanol concentrations, and is consistent with a direct pharmacological effect of ethanol on the liver cell, as suggested by Lelbach.[6]

In the absence of ethanol, pyrazole appeared to lower the hepatic triglyceride level in the sucrose–pyrazole groups in experiments 1 and 2, but not in experiment 3. One possible explanation is that pyrazole, like some of its analogues,[20,21] might act to decrease fatty acid mobilization from adipose tissue. In experiments 1 and 2 the diets were relatively high in fat, so that synthesis of fatty acids in the liver would be low.[22] Under these conditions impairment of fatty acid mobilization might explain a low hepatic triglyceride level several hours after the end of the normal nocturnal feeding period in the rat. In contrast, the low dietary fat content in experiment 3 would stimulate hepatic synthesis of fatty acids, so that inhibition of mobilization should have little effect. An alternative explanation may be that pyrazole independently tends to inhibit triglyceride synthesis in the liver.[23] The lack of difference between the sucrose and sucrose plus pyrazole groups in experiment 3 may then be related to some protective effect of increased dietary protein rather than to a reduction of dietary fat.

The net effect of pyrazole in the alcohol-fed animals appears therefore to reflect the balance of two opposite actions on hepatic triglyceride levels, viz. a reduction due to an independent effect of pyrazole and an increase due to its prolongation of the period of ethanol exposure. In experiment 2, when ethanol provided only 20 per cent of calories, the first effect was relatively more important, so that the sucrose–pyrazole group showed a marked reduction of hepatic triglyceride while the ethanol–pyrazole group did not show a significant increase over the ethanol and sucrose groups. In the other three experiments, the second effect predominated, the increase in triglycerides being greater with increasing proportion of ethanol in the diet.

It is not possible to rule out a synergism between some other subthreshold hepatotoxic effect of pyrazole and that of ethanol. However, the present results lend at least tentative support to the concept that chronic ethanol exposure has a direct hepatotoxic effect which is independent of the consequences of its metabolism. Further investigation of this possibility is warranted.

Acknowledgements—We are indebted to Miss J. Saksun, Mr. A. Hui, Mr. G. Donkin and Mr. G. Ho for technical assistance. This work was supported by the Alcoholism and Drug Addiction Research Foundation of Ontario.

REFERENCES

1. G. O. BUSTOS, H. KALANT, J. M. KHANNA and J. LOTH, *Science, N.Y.* **168,** 1598 (1970).
2. H. THEORELL and T. YONETANI, *Biochem. Z.* **338,** 537 (1963).
3. D. LESTER, W. Z. KEOKOSKY and F. FELZENBERG, *Q. Jl Stud. Alcohol* **29,** 449 (1968).
4. L. GOLDBERG and U. RYDBERG, *Biochem. Pharmac.* **18,** 1749 (1969).
5. C. S. LIEBER, *Adv. intern. Med.* **14,** 151 (1968).
6. W. K. LELBACH, *Experientia* **25,** 16 (1969).
7. C. S. LIEBER, D. P. JONES, J. MENDELSON and L. M. DE CARLI, *Trans. Ass. Am. Phycns* **76,** 289 (1963).
8. J. M. KHANNA, H. KALANT and G. O. BUSTOS, *Can. J. Physiol. Pharmac.* **45,** 777 (1967).
9. A. E. LEBLANC, *Can. J. Physiol. Pharmac.* **46,** 665 (1968).
10. W. H. BUTLER, H. M. MALING, M. G. HORNING and B. B. BRODIE, *J. Lipid Res.* **2,** 95 (1961).
11. E. VAN HANDEL, *Clin. Chem.* **7,** 249 (1961).
12. M. S. BARTLETT and D. G. KENDALL, *Jl R. statist. Soc.* suppl. **7,** 113 (1946).

13. C. S. Lieber, E. Rubin, L. M. De Carli, P. Misra and H. Gang, *Lab. Invest.* **22,** 615 (1970).
14. M. Reynier, *Agressologie* **11,** 401 (1970).
15. C. S. Lieber, D. P. Jones and L. M. De Carli, *J. clin. Invest.* **44,** 1009 (1965).
16. E. A. Porta, O. R. Koch, C. L. A. Gomez-Dumm and W. S. Hartroft, *J. Nutr.* **94,** 437 (1968).
17. H. Kalant, J. M. Khanna and J. Loth, *Can. J. Physiol. Pharmac.* **48,** 542 (1970).
18. E. Rubin, H. Gang and C. S. Lieber, *Biochem. biophys. Res. Commun.* **42,** 1 (1971).
19. C. S. Lieber and L. M. De Carli, *J. biol. Chem.* **245,** 2505 (1970).
20. A. Bizzi, M. T. Tacconi, E. Veneroni and S. Garattini, *Nature, Lond.* **209,** 1025 (1966).
21. R. Carmena and F. Grande, *Biochem. Pharmac.* **19,** 1838 (1970).
22. E. J. Masoro, *J. Lipid Res.* **3,** 149 (1962).
23. A. V. Prancan and J. Nakano, *Pharmacologist* **13,** 276 (1971).