Biochemical Pharmacology, Vol. 26, pp. 301–306, Pergamon Press, 1977. Printed in Great Britain.

EFFECT OF CLOFIBRATE ADMINISTRATION ON SEVERAL BIOCHEMICAL PARAMETERS OF NORMAL AND THYROIDECTOMIZED RATS

CARL R. MACKERER
Searle Laboratories, Chicago, IL 60680, U.S.A.

(*Received* 9 *June* 1976; *accepted* 23 *July* 1976)

Abstract—Clofibrate was administered in the diet (0.3%, w/w) for 7 days to normal and thyroidectomized male rats. At the end of the feeding period, the rats were killed by decapitation and several biochemical measurements were made. Inclusion of clofibrate in the diet did not affect weight gain or food consumption of either group of rats. Clofibrate lowered blood serum lipid levels of both normal and thyroidectomized rats, increased liver weight and kidney weight in normal rats and kidney weight in thyroidectomized rats, increased total liver lipid of both normal and thyroidectomized rats, decreased skeletal muscle O_2 consumption and increased liver O_2 consumption, and slightly decreased BMR of normal rats. The primary conclusions are: (1) the hypolipidemic effects of clofibrate do not require thyroxine but are more discernible when thyroxine is present, (2) the clofibrate-induced hypertrophy of liver but not of kidney is thyroxine dependent, and (3) clofibrate alters the O_2 consumption of rat tissues in a counterbalancing manner that results in a minimal effect on BMR.

Human patients who take clofibrate for prolonged periods of time exhibit an increase of body weight [1–3] that is not caused by fluid accumulation [2]. The cause of this side effect is not known but a possible explanation is that clofibrate alters the flux through basic metabolic pathways so that the balance favors weight gain. The actual internal organismic changes in such a situation could be quite large, but if the changes within organs occur in opposite directions, the effect on whole body parameters such as weight and BMR could be small or even negligible. Animal studies provide information about the metabolic shifts that clofibrate can produce and, in this regard, clofibrate has been shown to lower [4] or not affect [5] the BMR of rats while the O_2 consumption of liver is greatly enhanced [6]. These findings suggest that tissues of unknown identity must also have displayed decreased O_2 consumption. Our previous investigations [7] confirmed the marked increase of liver O_2 consumption evoked by clofibrate treatment but failed to show any alteration in kidney, intestine and adipose tissue. Skeletal muscle showed a slight decrease but the change was not statistically significant; however, it should be pointed out that even a small change of metabolic rate within this tissue is important because skeletal muscle in the normal control rat accounts for over half of the BMR [8].

In the present study, we have re-investigated the effects of clofibrate treatment on BMR and on O_2 consumption of several tissues including skeletal muscle from normal and thyroidectomized rats. Also, serum and liver lipid levels were determined and the values are reported and discussed.

METHODS

Animals and drug administration. Normal and thyroidectomized rats (CR–CD strain) were obtained from Charles River Breeding Laboratories and were allowed to stabilize for 1 and 6 weeks, respectively, prior to the beginning of the study. During this time the rats were individually housed and fed pellets of Rockland mouse/rat diet (complete), *ad lib.* Normal rats received tap water and thyroidectomized rats received Hank's solution [9]. After the stabilization period, the pelleted diet was replaced with powdered diet or powdered diet containing 0.3% (w/w) clofibrate. After 7 days of clofibrate treatment the rats were killed by decapitation, without prior fasting, between the hours of 8:00 and 11:00 a.m. Blood was collected from the wound and analyzed for levels of cholesterol [10], triglyceride [11] and thyroxine. Livers and kidneys were excised, weighed and utilized for several biochemical experiments as described below. In addition, a small piece of liver was frozen in liquid nitrogen and later analyzed for total lipid [12] and cholesterol [10].

All of the thyroidectomized rats had serum T_4 concentrations which were below detectable levels (i.e. < 1 μg/ml). The normal control rats had levels of approximately 6 μg/ml. In addition, the thyroidectomized rats had markedly decreased growth rate, and low BMR which was not increased by intraperitoneal injection of thyrotropin-releasing factor (TRF).

O_2 *consumption of mitochondria.* Mitochondria from liver and kidney were isolated by the method of Johnson and Lardy [13] and suspended in 0.25 M sucrose. Mitochondria from heart, vastus lateralis muscle, and diaphragm were isolated by the method of Tyler and Gonze [14] and suspended in 0.21 M mannitol, 0.075 M sucrose, and 0.1 mM EDTA.

O_2 consumption was measured polarographically as before [7, 15] at 30° in a Gilson Oxygraph (Gilson Medical Electronics Inc., Middleton, Wis.) equipped with a Clark oxygen electrode. The basic incubation medium (2 ml) was: 65 mM Tris buffer (pH 7.4), 75 mM KCl, 5 mM $MgCl_2$, 12 mM phosphate buffer (pH 7.4), and 1 mM EDTA.

Table 1. Effect of clofibrate on serum lipid concentrations of normal and thyroidectomized rats*

Rat treatment	Diet	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Normal	Control	84 ± 6 (9)	129 ± 14 (12)
	+0.3% Clofibrate	54 ± 2 (8)	68 ± 6 (15)
P		<0.001	<0.005
Thyroidectomized	Control	100 ± 6 (10)	62 ± 5 (10)
	+0.3% Clofibrate	80 ± 6 (10)	45 ± 3 (10)
P		<0.05	<0.01

* Values represent the means ± S. E. M. Statistical evaluations were done by Student's *t*-test. The numbers of rats for which values were obtained are given in parentheses.

O_2 consumption of tissues. O_2 consumption of several rat tissues was determined by a modification [7] of the procedure of Huston and Martin [8]. Slices of liver, kidney, rectus abdominus muscle, vastus lateralis muscle and heart left-ventricle were made with a Stadie–Riggs type of tissue slicer. Both tissue holder and blade were slightly moistened with 0.9% NaCl. After the blade was passed through the tissue, the resulting slice was removed with forceps and placed on a 2 cm square gauze pad which was premoistened with 0.9% NaCl. The diaphragm was excised, trimmed, cut into two pieces and spread on moist pads. Each pad was pushed into a 15-ml, single sac, Warburg flask without center well that contained: (a) 0.4 ml of 5% KOH (in the sac) for absorbing CO_2, (b) two drops of 0.9% NaCl (in the main chamber) to prevent dehydration of the tissues, and (c) a piece of 30-mesh, stainless steel, wire (1.4 × 3.0 cm) with the two short sides bent down 3 mm from the ends (in the main chamber) to prevent the pad from contacting the bottom of the flask.

The flasks were connected to a Gilson respirometer apparatus at 37.5° and gassed for 5 min with 100% O_2 which had been passed through a filter (molecular sieve, type 4A) and a hydrator. After a 15-min equilibration period, measurements of O_2 consumption were made at 5-min intervals up to 1 hr. After incubation, tissues were removed from the pads and weighed. It is important to note that in this procedure tissues were not incubated in aqueous media but were in direct contact with O_2.

Rat BMR. Rat O_2 consumption was measured at 24° in a glass desiccator that contained a concentrated solution of KOH to absorb CO_2. The desiccator outlet was connected to a vertically mounted 5-ml pipette. Rats were placed in the desiccator, the chamber was gassed for 5 min with 100% O_2, and the rats were allowed 30 min for equilibration. Readings were obtained by applying a drop of liquid detergent to the pipette tip and recording the length of time necessary for the bubble to drop from the 5 to the 0 ml mark. The value for each rat was the mean of ten successive determinations.

Suppliers. Normal and thyroidectomized rats were obtained from Charles River Breeding Laboratories, Wilmington, Mass. Total serum T_4 (test No. 1597) was determined by Mason–Barron Laboratories, Inc., Chicago, Ill. Organic reagents were from Sigma Chemical Co., St. Louis, Mo., and inorganic salts from Mallinckrodt Chemical Co., St. Louis, Mo.

RESULTS

Effect of clofibrate on lipid levels of blood serum and liver. The effect of clofibrate on serum lipid levels of normal and thyroidectomized rats is shown in Table 1. Clofibrate lowered the levels in both normal and thyroidectomized rats but the per cent decrease of triglyceride concentration was smaller in the thyroidectomized rats (−47 vs −27 per cent). However, the thyroidectomy itself decreased the level by 52 per cent.

The effect of clofibrate on liver lipid level is summarized in Table 2. Based on the limited steady state data of this table, it appears that the effects of thyroidectomy and of clofibrate can be clearly differentiated with respect to effects on cholesterol level and on the levels of other lipids. Cholesterol level/g of liver tissue was increased by thyroidectomy and was moderately reduced, in the normal rat, by administration of clofibrate. In the thyroidectomized rat, clofibrate did not affect the cholesterol level. When the data were expressed as mg/liver/100 g body weight, the cholesterol level was not altered by either thyroidectomy or clofibrate administration.

Total lipid level (minus cholesterol)/g of liver tis-

Table 2. Effect of clofibrate on liver lipid levels*

Rat treatment	Diet	Cholesterol (mg/g)	Cholesterol (mg/100 g body wt)	Total lipid − cholesterol (mg/g)	Total lipid − cholesterol (mg/100 g body wt)
Normal	Control	2.06 ± 0.06 (9)	9.51 ± 0.24 (9)	36.3 ± 0.72 (9)	169 ± 4 (9)
	+0.3% Clofibrate	1.75 ± 0.05 (9)	9.98 ± 0.26 (9)	40.6 ± 0.76 (9)	233 ± 8 (9)
P		<0.005	NS	<0.005	<0.001
Thyroidectomized	Control	2.79 ± 0.31 (9)	8.44 ± 0.68 (9)	35.4 ± 0.96 (9)	110 ± 6 (9)
	+0.3% Clofibrate	3.07 ± 0.29 (11)	9.35 ± 0.74 (11)	43.8 ± 1.82 (11)	136 ± 9 (11)
P		NS	NS	<0.001	<0.05

* Values represent the mean ± S. E. M. Statistical evaluations were done by Student's *t*-test. The numbers of rats for which values were obtained are given in parentheses. NS = not significant.

Table 3. Effect of clofibrate on liver and kidney weight*

Rat treatment	Diet	Rat wt	Liver wt (g)	Liver wt (g/100 g body wt)	Kidney wt (g)	Kidney wt (g/100 g body wt)
Normal	Control	304 ± 4 (9)	14.0 ± 0.4 (9)	4.62 ± 0.072 (9)	2.49 ± 0.07 (9)	0.818 ± 0.019 (9)
	+0.3% Clofibrate	297 ± 4 (9)	17.0 ± 0.5 (9)	5.73 ± 0.134 (9)	2.77 ± 0.05 (9)	0.933 ± 0.012 (9)
P		NS	<0.001	<0.001	<0.005	<0.001
Thyroidectomized	Control	239 ± 12 (10)	7.31 ± 0.36 (10)	3.08 ± 0.12 (10)	1.23 ± 0.03 (6)	0.582 ± 0.004 (6)
	+0.3% Clofibrate	235 ± 11 (11)	7.35 ± 0.54 (11)	3.14 ± 0.20 (11)	1.35 ± 0.06 (6)	0.642 ± 0.018 (6)
P		NS	NS	NS	NS	<0.01

* Values represent the mean ± S. E. M. Statistical evaluations were done by Student's *t*-test. The numbers of rats for which values were obtained are given in parentheses. NS = not significant.

sue was not affected by thyroidectomy but was significantly elevated by clofibrate administration in both normal and thyroidectomized rats. However, when the data were expressed as mg/liver/100 g body weight, the thyroidectomized rat showed a markedly decreased lipid level which was only slightly raised by clofibrate administration. In the normal rat, this method of expressing the results further accentuated the hyperlipidic effect of clofibrate.

Effect of clofibrate on organ weights. The effects of clofibrate on liver weight, kidney weight and rat weight are presented in Table 3. Clofibrate did not affect weight gain ((data not shown) and, therefore, the values for rat weight at sacrifice were similar in the presence or absence of clofibrate. The thyroidectomized rats weighed considerably less than the normal rats and their liver and kidney weights, and corresponding liver and kidney weight: body weight ratios were much lower. Clofibrate increased both liver and kidney weight in the normal rat, but in the thyroidectomized rat only the kidney weight was increased.

Effect of clofibrate on rat BMR. As shown in Fig. 1, clofibrate produced a slight, marginally significant, decrease of O_2 consumption. In order to determine the locus of this effect, the O_2 consumption of tissue slices was determined. The effect of clofibrate on the O_2 consumption of liver, kidney, heart and diaphragm and skeletal muscle is shown in Table 4. O_2 consumption of kidney, heart and diaphragm was not altered by the clofibrate administration but liver O_2 consumption was enhanced. When expressed in terms of O_2 used/hr/100 g body weight, liver O_2 consumption was enhanced by 68 per cent and O_2 consumption of both rectus abdominus and vastus lateralis muscles was diminished by 14 per cent. Although this is only a small decrease when expressed on a gram of tissue basis, skeletal muscle represents about 44 per cent of the adult rat's body weight [8, 16], while liver represents only about 4.7 per cent of body weight. Slices of liver and vastus lateralis muscle from thyroidectomized rats (Table 5) showed reduced rates of O_2 consumption that were not altered by clofibrate administration.

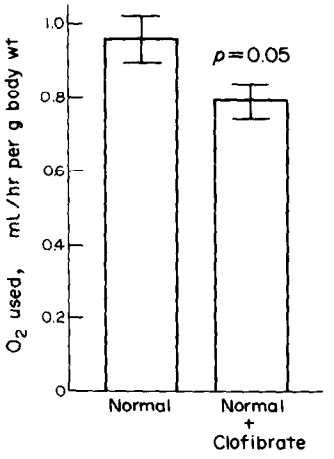

Fig. 1. Effects of clofibrate administration on the BMR of normal rats. Values represent the mean ± S. E. M. for twelve normal rats and twelve normal rats fed a diet containing 0.3% (w/w) clofibrate. The difference between the means was marginally significant according to Student's *t*-test (P = 0.05).

Effects of cholorophenoxyisobutrate (CPIB) on mitochondrial respiration. CPIB is rapidly produced *in vivo* via hydrolysis of clofibrate and is believed to be the active form of the drug [17]. CPIB added *in vitro* inhibits respiration and oxidative phosphorylation of rat liver mitochondria [7, 18] but this phenomenon does not appear to occur in liver *in vivo* since O_2 consumption of liver is increased [7] (see Table 4) rather than decreased. However, it is possible that the clofibrate-induced decrease of muscle respiration (see Table 4) was caused by direct inhibition at the mitochondrial level, perhaps because of a greater sensitivity of muscle mitochondria to the effects of CPIB. In order to investigate this possibility, we studied the effects of CPIB on respiration of mitochondria isolated from heart ventricle, vastus lateralis muscle, kidney cortex and liver. The results of this experiment are summarized in Fig. 2. Glutamate (20 mM) + malate (10 mM) was used as the substrate, and mitochondrial protein concentration was 0.75 mg/ml. The IC_{50} values for inhibiting mitochondrial state 3 respiration were: liver, 5.75 mM; heart, 6.25 mM; vastus lateralis muscle, 6.75 mM; and kidney, 8.75 mM. Similar results were obtained when the effects of CPIB were determined at a constant respiratory rate (500 natoms oxygen/min) rather than at a constant protein concentration.

DISCUSSION

The primary intent of the present investigation was to study O_2 consumption of rats and rat tissues to see if there were compensatory changes that would explain the findings of decreased [4] or unaltered BMR [5] and increased liver O_2 consumption [7] after clofibrate administration. In this regard, we

Table 4. Effect of clofibrate on respiration of several organs from normal rats*

Diet	Liver			Kidney			Heart			Diaphragm	Skeletal muscle	
	(g/100 g body wt)	(ml O_2/hr·g)	(ml O_2/hr·100 g body wt)	(g/100 g body wt)	(ml O_2·hr·g)	(ml O_2/hr·100 g body wt)	(g/100 g body wt)	(ml O_2·hr·g)	(ml O_2·hr·100 g body wt)	(ml O_2·hr·g)	Rectus Abdominus (ml O_2·hr·g)	Vastus lateralis (ml O_2·hr·g)
Control	4.04 ± 0.17	2.86 ± 0.13	11.5 ± 0.6 (7)	0.814 ± 0.023	4.25 ± 0.36	3.46 ± 0.29 (7)	0.283 ± 0.006	2.35 ± 0.12	0.665 ± 0.034 (7)	1.50 ± 0.04 (8)	1.67 ± 0.05 (7)	2.55 ± 0.11 (8)
±0.3% Clofibrate	5.93 ± 0.15	3.25 ± 0.09	19.3 ± 0.9 (7)	0.895 ± 0.020	4.09 ± 0.31	3.66 ± 0.28 (6)	0.284 ± 0.005	2.53 ± 0.08	0.718 ± 0.023 (7)	1.50 ± 0.06 (8)	1.43 ± 0.11 (6)	2.20 ± 0.09 (8)
P	<0.001	<0.05	<0.001	<0.05	NS	NS	NS	NS	NS	NS	<0.05	<0.05

* Values represent the mean ± S. E. M. Statistical evaluations were done by Student's *t*-test. The numbers of rats for which values were obtained are given in parentheses. NS = not significant.

Table 5. Effect of clofibrate on respiration of slices from liver and vastus lateralis muscle of thyroidectomized rats*

Diet	Liver (g/100 g body wt)	Liver (ml O_2/hr/g)	Vastus lateralis muscle (ml O_2/hr/ 100 g body wt)	Vastus lateralis muscle (ml O_2/hr/g)
Control	3.02 ± 0.17 (6)	2.24 ± 0.087 (6)	6.76 ± 0.46 (6)	1.79 ± 0.086 (6)
+0.3% Clofibrate	3.17 ± 0.32 (7)	2.32 ± 0.065 (7)	7.35 ± 0.77 (7)	1.73 ± 0.099 (6)
P	NS	NS	NS	NS

* Values represent the mean ± S. E. M. Statistical evaluations were done by Student's *t*-test. The numbers of rats for which values were obtained are given in parentheses. NS = not significant.

found that clofibrate treatment did not affect the respiratory rates of kidney, diaphragm and heart (see Table 4) or of adipose tissue and intestine [7]. However, slices of rectus abdominus and vastus lateralis skeletal muscles showed a small but statistically significant decrease of O_2 consumption. Thus, inhibition of O_2 consumption in skeletal muscle offsets the increased O_2 consumption in liver.

The reasons for these changes are not understood but a mechanism involving indirect effects elicited through the actions of endogenous thyroxine is a possibility. In this regard, clofibrate causes some thyroxine-like effects such as enhanced levels of mitochondrial α-glycerolphosphate dehydrogenase [6, 19], enhanced O_2 consumption of liver tissue [20], increased number of mitochondria [21, 22], depletion of liver glycogen [23], and increased liver protein synthesis [23]. These findings when taken together suggest that clofibrate causes a hyperthyroid condition in the liver.

In agreement with this suggestion, we have found (see Tables 4 and 5) that clofibrate increased the O_2 consumption of liver tissue from normal rats but not from thyroidectomized rats. Also, clofibrate did not inhibit O_2 consumption of skeletal muscle slices from thyroidectomized rats, suggesting that perhaps clofibrate causes a slight hypothyroid condition in the muscle of the normal rat. However, it is quite possible

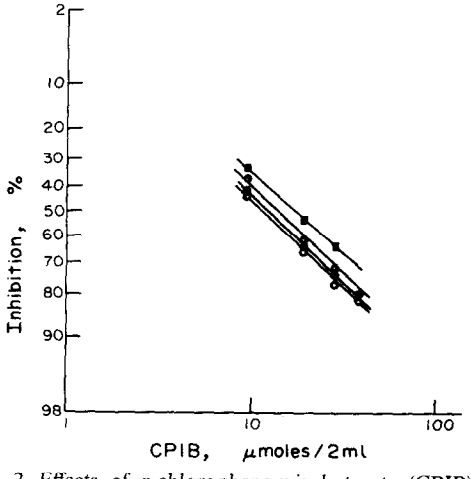

Fig. 2. Effects of *p*-chlorophenoxyisobutyrate (CPIB) on oxygen consumption of mitochondria respiring in state 3. Mitochondrial protein concentration was constant at 0.75 mg/ml. Glutamate (20 mM) + malate (10 mM) was the substrate. Key: ■, kidney cortex mitochondria; ◑, vastus lateralis muscle mitochondria; ●, heart ventricle mitochondria; and ⊙, liver mitochondria. Values are the medians of three replicate determinations.

that the greatly reduced skeletal muscle respiration seen after thyroidectomy obscured any additional effects of clofibrate.

Aside from the multitude of possible indirect mechanisms that might be proposed for the clofibrate-induced inhibition of skeletal muscle respiration, one must also consider the likelihood of direct effects. Clofibrate [7, 15] and CPIB [18] are known to inhibit respiration and oxidative phosphorylation of rat liver mitochondria, *in vitro*, and the possibility existed that skeletal muscle mitochondria might be even more sensitive to these inhibitory effects. The possibility of direct inhibition of mitochondrial respiration as an explanation of the decreased skeletal muscle respiration is discounted, however, by the data of Fig. 2, which show liver and skeletal muscle mitochondria to be equally sensitive to the inhibitory effects of CPIB. Liver mitochondria are not inhibited *in vivo* since liver respiration is increased rather than decreased by clofibrate administration.

Clofibrate has been shown to increase the weight of both liver [24] and kidney [25, 26] in the normal rat. This hepatomegaly is inhibited by thyroidectomy [27], suggesting that the effect requires thyroxine although thyroxine does not cause liver hypertrophy in either normal or thyroidectomized rats [5]. In agreement with these findings, we observed both liver and kidney hypertrophy (see Table 3) and found that the hepatomegaly was completely prevented by thyroidectomy. However, the kidney hypertrophy was not inhibited by thyroidectomy, indicating that endogenous thyroxine was not required for this effect.

It is commonly assumed that the hypolipidemic effect of clofibrate in rats requires the presence of thyroxine but this does not appear to be well established in the literature. Best and Duncan [24] reported that clofibrate lowered the serum cholesterol levels of both normal and thyroidectomized rats but lowered triglyceride levels in only the normal rats. It was noted, however, that thyroidectomy *per se* markedly lowered the triglyceride levels, perhaps to a physiologic minimum, thereby obscuring additional effects of clofibrate. On the other hand, Platt and Thorp [23] and Azarnoff and Svoboda [28] reported that the effect of clofibrate on serum cholesterol is entirely abolished by thyroidectomy. In contrast to the results of these studies, we have found that clofibrate produces a statistically significant reduction of serum cholesterol and triglyceride in both normal and thyroidectomized rats (see Table 1). The hypotriglyceridemic effect is less pronounced, however, in the thyroidectomized rat because of the low baseline concentration of triglycerides. Thus, our data show that the hypolipidemic effects of clofibrate do not require

thyroxine but are more easily discerned when thyroxine is present.

The effects of clofibrate on the measured liver lipids could be crudely divided into two classes: (1) effects on cholesterol and (2) effects on total lipids minus cholesterol (primarily phospholipid and triglyceride). When expressed as mg cholesterol/g of liver, the cholesterol levels were decreased by clofibrate in the normal rat [29] (see Table 2) and were increased by thyroidectomy (see Table 2). However, clofibrate did not change the levels in the thyroidectomized rat (see Table 2).

It appears, in this study, that the cholesterol levels varied with the relative liver weight:body weight ratios, which were dependent upon endogenous thyoxine. When cholesterol levels were expressed as mg cholesterol/liver/100 g body weight, levels were unchanged regardless of treatment.

The total lipid fraction (minus cholesterol) of the liver, when expressed as mg lipid/g of liver, was increased to a similar degree by clofibrate in both normal and thyroidectomized rats (see Table 2), indicating that thyroxine is not required for this effect. It may be that these lipids increase to a self-limiting concentration during clofibrate therapy and when liver is permitted to grow larger, as in the presence of endogenous thyroxine, the amount of these lipids in the liver is further increased.

Several conclusions can be drawn from the results of this study: (1) clofibrate causes hypertrophy of both liver and kidney but only the liver growth is mediated by thyroxine, (2) clofibrate alters O_2 consuming metabolic pathways in a counterbalancing manner (i.e. increased O_2 consumption in liver and decreased O_2 consumption in skeletal muscle) so that effects on BMR are minimal, (3) the decreased skeletal muscle O_2 consumption is not caused by a peculiar sensitivity of skeletal muscle mitochondria to respiratory chain inhibition by CPIB, (4) the hypotriglyceridemic and hypocholesterolemic effects of clofibrate do not require thyroxine, (5) total cholesterol/liver/100 g body weight is unaffected by clofibrate administration or by thyroidectomy, and (6) total liver lipid (minus cholesterol) levels are elevated by clofibrate via a mechanism that does not require thyroxine; however, this effect is enhanced by the presence of endogenous thyroxine because of increased liver growth.

Acknowledgements—The author appreciates the contribution of the Searle Laboratories Bioanalytical Laboratory, under the direction of Dr. T. C. Hutsell, for performing the serum and liver lipid determinations.

REFERENCES

1. M. F. Oliver, *J. Atheroscler. Res.* **3**, 427 (1963).
2. D. C. Macmillan, M. F. Oliver, J. D. Simpson and P. Tothill, *Lancet* **2**, 924 (1965).
3. Veterans Administration Cooperative Study Group, *Stroke* **4**, 684 (1973).
4. J. M. Thorp, *J. Atheroscler. Res.* **3**, 351 (1963).
5. W. R. Ruegamer, N. T. Ryan, D. A. Richert and W. W. Westerfeld, *Biochem. Pharmac.* **18**, 613 (1969).
6. W. W. Westerfeld, D. A. Richert and W. R. Rueganmer, *Biochem. Pharmac.* **17**, 1003 (1968).
7. C. R. Mackerer and J. R. Haettinger, *Biochem. Pharmac.* **23**, 3331 (1974).
8. M. J. Huston and A. W. Martin, *Proc. Soc. exp. Biol. Med.* **86**, 103 (1954).
9. J. H. Hanks and R. E. Wallace, *Proc. Soc. exp. Biol. Med.* **71**, 196 (1949).
10. W. D. Block, K. J. Janett and J. B. Levine, in *Automation in Analytical Chemistry* (Ed. L. T. Skeggs, Jr.), p. 345. Mediad, New York (1966).
11. R. P. Nobel and F. M. Cambell, *Clin. Chem.* **16**, 166 (1970).
12. J. Folch, M. Lees and G. A. Sloan-Stanley, *J. biol. Chem.* **226**, 497 (1957).
13. D. Johnson and H. A. Lardy, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 10, p. 94. Academic Press, New York (1967).
14. D. D. Tyler and J. Gonze, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 10, p. 75. Academic Press, New York (1967).
15. C. R. Mackerer, J. R. Haettinger and T. C. Hutsell, *Biochem. Pharmac.* **22**, 513 (1973).
16. H. H. Donaldson, *The Rat, Data and Reference Tables for the Albino Rat and the Norway Rat, Memoirs of the Wistar Institute*, pp. 1–469. The Wistar Institute of Anatomy and Biology, Philadelphia (1924).
17. J. M. Thorp, *Lancet* **1**, 1323 (1962).
18. S. R. Panini, *Biochem. J.* **128**, 50P (1972).
19. R. Hess, W. Staubli and W. Reiss, *Nature, Lond.* **208**, 856 (1965).
20. M. T. Kähönen, R. H. Ylikahri and I. Hassinen, *Life Sci.* **10**, 661 (1971).
21. D. Osario, K. W. Walton, C. H. W. Browne, D. West and P. Whystock, *Biochem. Pharmac.* **14**, 1479 (1965).
22. C. K. R. Kurup, H. N. Aithal and T. Ramasarma, *Biochem. J.* **116**, 773 (1970).
23. D. S. Platt and J. M. Thorp, *Biochem. Pharmac.* **15**, 915 (1966).
24. M. M. Best and C. H. Duncan, *J. Lab. clin. Med.* **64**, 634 (1964).
25. T. P. Krishnakantha and C. K. R. Kurup, *Indian J. biochem. Biophys.* **10**, 224 (1973).
26. S. Miyazawa, T. Sakurai, M. Imura and T. Hashimoto, *J. Biochem., Tokyo* **78**, 1171 (1975).
27. D. A. Richert and W. W. Westerfeld, *Endocrinology* **87**, 1274 (1970).
28. D. L. Azarnoff and D. J. Svoboda, *Archs int. Pharmacodyn. Thér.* **181**, 386 (1969).
29. C. Dalton, W. C. Hope, H. R. Hope and H. Sheppard, *Biochem. Pharmac.* **23**, 685 (1974).