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Studies on fatty liver induction by 4-aminopyrazolopyrimidine*

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SUMMARY

The effects of the adenine analogue, 4-aminopyrazolopyrimidine, on lipid metabolism in mouse liver have been studied in an attempt to ascertain the mechanism by which this drug causes fatty livers. Injection of 1 mg of APP caused an increase in the total liver lipid of 3- to 4-fold in 24 hr, which was almost entirely accounted for as neutral lipid. There was a small increase in the cholesterol content of treated livers, but no change in the amount of phospholipid. Treatment with APP inhibited the incorporation of acetate-C^{14} into lipids of liver slices *in vitro*, but had little effect on the oxidation of acetate-C^{14} and palmitate-C^{14} *in vitro*. The plasma lipid concentration was decreased by APP treatment, and this was caused by a drop in triglycerides and cholesterol, whereas phospholipids and FFA did not change. Palmitate-C^{14} was taken up less well by livers of mice treated with APP than by livers of control mice. Although APP caused a profound inhibition of incorporation of orotic acid-C^{14} into RNA, the incorporation of glycine-C^{14} into liver and plasma proteins was not diminished. The hyperlipemia normally elicited by Triton WR-1339 was inhibited by APP, which strongly suggests that this compound inhibits the secretion of triglycerides from the liver.

The adenine analogue, 4-aminopyrazolopyrimidine[1] is the only purine antimetabolite known to produce fatty livers in mice (1). Although APP has moderate carcinostatic activity in animals (2, 3), its use both in animals and man has been hampered by its hepatotoxicity (2, 3, 4). In view of its unique toxicity for a purine analogue, and in an attempt to investigate relationships between purine and lipid metabolism, the present study of the effects of APP on lipid metabolism in liver has been pursued.

The effect of APP on the synthesis of purines and on the incorporation of radioactive precursors into nucleic acids and proteins has been studied (5, 6, 7, 8), but its effects on lipid metabolism have not been examined. No satisfactory explanation of the mechanism of carcinostasis by APP has yet arisen from such work. In a study of the metabolism of APP in tumor-bearing mice, it was found that, of the tissues examined, liver contained both the highest concentration and the largest amount of the drug and its metabolites. The principal metabolites found were the mono-, di-, and triphosphoribonucleosides of APP (9).

A priori, there are four general mechanisms by which, singly or in combination, APP might increase the lipid concentration of liver: (a) through increased lipid synthesis, (b) through decreased fatty acid oxidation, (c) through increased uptake of blood lipids, or (d) through decreased secretion of lipids into the blood. In the present study, the effects of APP on each of these processes has been studied. The results suggest that the principal effect of this compound is to inhibit secretion of triglyceride from the liver, although the detailed mechanism of this action remains unclear.

MATERIALS AND METHODS

4-Aminopyrazolopyrimidine was obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute, and was dissolved in 0.15 M phosphate buffer, pH 7.4, for use. Triton WR-1339 (Winthrop Laboratories) was a gift of Dr. Rodolfc Paoletti, and was prepared for injection in 0.154 M sodium chloride immediately before use. Sodium acetate-1-C^{14} of specific activity 1.0 mc/mmole was

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[1] Abbreviations used: APP, 4-aminopyrazolopyrimidine; FFA, free fatty acids; UMP, uridylic acid; RNA, ribonucleic acid.

purchased from Tracerlab, Inc.; orotic acid-6-C^{14}, 2.26 mc/mmole, from Isotope Specialties, Inc.; glycine-2-C^{14}, 1.2 mc/mmole, from Volk Radiochemicals Co.; and palmitic acid-1-C^{14}, 23.1 mc/mmole, from Research Specialties Co. The latter was diluted with non-radioactive palmitic acid to a final specific activity of 5.8 mc/mmole. Albumin-bound palmitic acid-C^{14} was prepared by the method of Milstein and Driscoll (10) and contained 1 mg of palmitic acid per 90 mg of crystallized bovine serum albumin, purchased from Pentex Inc. Silicic acid (<325 mesh), prepared for use in the separation method of Hirsch and Ahrens (11), was purchased from California Corp. for Biochemical Research.

Total lipids were analyzed by the dichromate oxidation method of Bragdon (12), using as a standard mouse liver lipids measured gravimetrically. Cholesterol was analyzed by the method of Sperry and Webb (13); phosphate by a modification of the Fiske-Subbarow procedure (14); FFA by the method of Dole (15); and protein by the biuret procedure (16). Liver neutral lipid was determined by difference, as described by Vahouny *et al.* (17), and plasma triglycerides were determined by the method of Van Handel and Zilversmit (18, 19).

Total lipids were extracted by homogenizing perfused tissues in 5–8 volumes of 3:1 ethanol–diethyl ether in a loose-fitting Potter-Elvehjem homogenizer, followed by heating to incipient boiling (20). The extraction was repeated twice with fresh portions of solvent. Trial experiments revealed that further extraction under these conditions did not yield any more lipid. This extract was evaporated to dryness *in vacuo* and the residue dissolved in petroleum ether. Aliquots of this extract were taken for chemical analysis. Plasma and whole blood were extracted without prior homogenization, and incubation media from *in vitro* experiments were removed by centrifugation before extraction of tissue slices.

Neutral lipids were separated from phospholipids by the batch silicic acid procedure of Marks *et al.* (21). Phosphate analysis demonstrated complete separation of phospholipid from neutral lipid, and extended extraction with diethyl ether did not yield more neutral lipids. The resulting diethyl ether extracts containing the neutral lipids (and FFA) and the methanol extracts containing the phospholipids were evaporated to dryness *in vacuo*. In some experiments, the lipids were redissolved in petroleum ether and aliquots taken directly for radioactivity measurements. In other experiments, the lipids were redissolved and saponified in methanolic 2 N NaOH for 3 hr at 80° (20). After acidification, the lipids were extracted with petroleum ether. The solution of fatty acids derived from the phospholipids was evaporated to dryness *in vacuo*. The fatty acids were redissolved in petroleum ether and an aliquot taken for radioactivity measurements. The extract of the saponified neutral lipid fraction was evaporated to dryness, redissolved in 2:1 acetone–ethanol, and sterol precipitated as the digitonide (13). The supernatant fraction was evaporated to dryness, the fatty acids redissolved in petroleum ether, and aliquots taken for radioactivity measurements. The sterol digitonide was dissociated with pyridine (22), and an aliquot of the sterol-containing solution was taken for radioactivity measurements.

Protein was prepared from the perfused livers and from plasma following extraction with cold 0.2 M perchloric acid and hydrolysis of nucleic acids by 0.4 M perchloric acid at 100° for 1 hr (23). The protein residue was washed with acetone, dried, and its radioactivity measured. Uridylic acid was isolated from the hot 0.4 M perchloric acid hydrolysate by chromatography on Dowex-1-formate (24) and repurified by paper chromatography in isopropanol–HCl (25).

In the *in vitro* experiments, liver slices (500 mg) were incubated in 25-ml Erlenmeyer flasks containing 3.6 ml of calcium-free Krebs-Ringer phosphate medium, pH 7.4, and α-ketoglutarate, 5.5 mM. Air was the gas phase. The flasks were shaken at 37° for 1 hr, and carbon dioxide collected, when necessary, in 0.4 ml of 7.7 N KOH in plastic cups cemented inside the flasks. Barium carbonate was prepared for radioactivity measurements by standard procedures.

Radioactivity measurements were made in a windowless gas-flow counter. All such measurements were corrected to infinite thinness and were made such that the error was less than 10%.

Female ICR Swiss mice, 20–25 g, were obtained from Laboratory Animals, Inc., and maintained on Purina mouse chow and water *ad libitum*.

In order to minimize variations arising from diurnal variation in metabolism and food intake, all experiments were done at the same time of day. When animals were to be treated for different lengths of time, treatment was scheduled so that all animals could be sacrificed at the same time of day.

RESULTS

The first two tables show the characteristics of the liver response to APP administration. Total lipid content following intraperitoneal injection of several doses of APP on various schedules are shown in Table 1. There was a definite dose-response relationship up to the 1000-μg level, at which dose the total lipid content

TABLE 1. EFFECT OF APP ON THE LIPID CONTENT OF LIVER*

	APP Dose	No. of Injections†	Time after Last Injection	Total Lipid
	μg		*hr*	*mg*
A.	0	1	24	34.0 ± 5.5‡
	250	1	24	42.4 ± 5.4
	250	2	24	49.8 ± 8.6
	250	1	48	37.2 ± 4.6
	500	1	24	54.8 ± 4.0
	500	2	24	73.2 ± 18.6
	500	1	48	40.0 ± 6.4
	1,000	1	24	90.9 ± 8.8
	1,000	2	24	119.9 ± 12.6
	1,000	1	48	55.6 ± 5.3
	2,000	1	24	45.6 ± 6.4
	2,000	2	24	57.2 ± 4.9
B.	0	1	3	38.0 ± 4.6
	1,000	1	3	49.0 ± 5.2
	1,000	1	6	51.2 ± 4.9

* Swiss mice, 20–25 g., each received intraperitoneal injections of APP in 0.15 M phosphate buffer, pH 7.4. Each injection contained the amount of APP indicated. Each value is a mean of separate measurements on a total of 12 animals in three experiments. Control mice received injections of the buffer.

† Twenty-four-hour interval between injections.

‡ Standard deviation.

was increased 3- to 4-fold. The effect of APP was reversible, however, the liver lipid levels being less at 48 hr after injection than at 24 hr after injection. The proportional effect of 2000 μg was much less, and it was observed that the liver in these cases was greatly distended and had hemorrhagic margins, phenomena never noticed at smaller doses. Wet, defatted, dry liver weights were not significantly changed. In part B, it is seen that 1000 μg of APP caused an increase in total lipid even 3 hr after administration.

The liver lipids were fractionated following administration of single and double injections of 500 and 1000 μg of APP to ascertain which component(s) increased. These data are given in Table 2. No significant alterations in the phospholipid content were noted, while there was a definite but small increase in the cholesterol content. The principal effect of administration of APP, however, was to increase the neutral lipid content of the liver. Whereas the content of total lipid was increased only 3-fold by a single injection of 1000 μg of APP, the level of neutral lipid was increased over 10-fold under these conditions. This dose, 1000 μg, was used for subsequent experiments because of the speed and magnitude of the response to it.

The effect of APP on lipid synthesis from acetate-C^{14} is shown in Table 3. Liver slices were incubated *in vitro* with sodium acetate-C^{14}, and the total incorporation of radioactivity into sterol, fatty acids from phospholipids, and the fatty acids from triglycerides plus sterol esters was measured. Incorporation of acetate-C^{14} into all fractions measured was inhibited to about the same extent in slices from livers exposed to APP for 3 hr. Thereafter, the inhibition declined, although not as the same rate for all three fractions. This difference in recovery rates suggests that these results are at least not entirely due to changes in acetate pool sizes.

Table 4 shows the effects of APP on the oxidation of both acetate-C^{14} and palmitate-C^{14} *in vitro*. Mice were treated and liver slices prepared and incubated as described in the preceding paragraph. Oxidation of both substrates was inhibited to a small extent at the 24-hr period. This might be due either to a specific drug action or to dilution of the radioactive substrates by the increased concentration of neutral lipids in the liver. The different time course of the effects of APP on incorporation of acetate into lipids and on oxidation of acetate and palmitate is unexplained.

The effects of APP on the uptake of radioactive

TABLE 2. EFFECT OF APP ON THE COMPOSITION OF LIVER LIPID*

APP Dose	No. of Injections†	Time after Last Injection	Cholesterol	Phospholipid	Neutral Lipid	Total Lipid
μg		*hr*	*mg*	*mg*	*mg*	*mg*
0	1	24	2.6 ± 0.56‡	26.8 ± 3.8	5.6 ± 0.62	34.0 ± 5.5
500	1	24	2.9 ± 0.42	32 0 ± 4.2	15.9 ± 2.9	54.8 ± 4.0
500	2	24	3.85 ± 0.52	31.0 ± 2.9	36.4 ± 5.2	73.2 ± 18.6
1,000	1	24	3.3 ± 0 64	28.5 ± 3.2	59.1 ± 4.2	90.9 ± 8.8
1,000	2	24	4.05 ± 0.48	24.0 ± 3.9	91.8 ± 9.2	119.9 ± 12.6

* Swiss mice, 20–25 g, each received intraperitoneal injections of APP in 0.15 M phosphate buffer, pH 7.4. Control mice received injections of the buffer. Each value is the mean of separate measurements on a total of 12 animals in 3 experiments.

† Twenty-four-hour interval between injections.

‡ Standard deviation.

TABLE 3. EFFECT OF APP ON INCORPORATION OF ACETATE-1-C^{14} *in vitro**

Time after APP	Total Fatty Acids plus Cholesterol	Neutral Lipid Fatty Acids	Phospholipid Fatty Acids	Sterol
hr	*cpm*	*cpm*	*cpm*	*cpm*
0	19,400 ± 2,100†	11,900 ± 974	5,900 ± 437	3,320 ± 333
3	4,230 ± 398	3,010 ± 276	1,030 ± 98	792 ± 59
6	7,710 ± 673	3,050 ± 243	3,260 ± 276	1,030 ± 98
24	16,290 ± 1,710	11,300 ± 1,010	4,140 ± 459	1,850 ± 201

* Liver slices, 500 mg, prepared from mice injected with 1,000 μg APP 3, 6, and 24 hr previously, were incubated for 1 hr at 37° in 3.6 ml of calcium-free Krebs-Ringer phosphate medium, pH 7.4, containing 5.5 mM α-ketoglutarate and 6 μmoles sodium acetate-C^{14}, with air as the gas phase. Each figure is a mean of values from separate analysis of a total of four flasks in two experiments.

† Standard deviation.

palmitate (as the albumin complex) is shown in Fig. 1. APP-treated livers took up less radioactive palmitate than did the controls, and this depression of uptake increased with the time of APP exposure. To determine if these results were due to differences in the distribution of the injected radioactivity, the total blood lipid radioactivity of each animal was determined. No significant differences were found between control and treated animals at any time. For example, the total blood radioactivity at 5, 10, 15, and 30 min was 2860, 3210, 1950, and 976 cpm for control animals, and 2580, 3440, 2040, and 1070 cpm for 24-hr treated animals.

Plasma lipids were determined in an attempt to measure both the availability of fatty acids to the liver and the secretion of lipids by liver (Table 5). Administration of APP was followed by a rapid and prolonged decrease in the concentration of total plasma lipids, which was due almost entirely to a decrease in the triglyceride and cholesterol fractions. Plasma phospholipids underwent only a small change in concentration. The level of plasma FFA was not altered by APP treatment, suggesting that abnormal release of depot fatty acids is not involved in this type of fatty liver. To study this point further, mice were injected with 12.5 mg/kg of phenoxybenzamine (Dibenzyline), an agent known to prevent release of FFA from adipose tissue (26), and also with 1000 μg of APP after 1 hr. After 24 hr, the levels of total liver lipid were the same in mice that had received both APP and phenoxy-

TABLE 4. EFFECT OF APP ON OXIDATION OF PALMITATE-1-C^{14} AND ACETATE-1-C^{14} *in vitro**

Time after APP	Substrate	$BaCO_3$	
hr		*mg*	*cpm/mg*
0	Acetate-C^{14}	6.3 ± 0.5	8,799 ± 902†
3	"	5.5 ± 0.4	7,725 ± 643
24	"	6.0 ± 0.6	5,189 ± 502
0	Palmitate-C^{14}	4.9 ± 0.3	262 ± 24
3	"	4.8 ± 0.4	236 ± 31
24	"	5.2 ± 0.4	191 ± 14

* Liver slices, 500 mg, prepared from mice injected with 1,000 μg APP 3, 6, and 24 hr previously, were incubated for 1 hr at 37° in 3.6 ml of calcium-free Krebs-Ringer phosphate medium, pH 7.4, containing 5.5 mM α-ketoglutarate and either 6 μmoles sodium acetate-C^{14} (0.25 mc/mmole in this experiment) or 0.5 μmoles of palmitic acid-C^{14}, with air as the gas phase. Each figure is a mean of values from separate analyses of a total of four flasks in two experiments.

† Standard deviation.

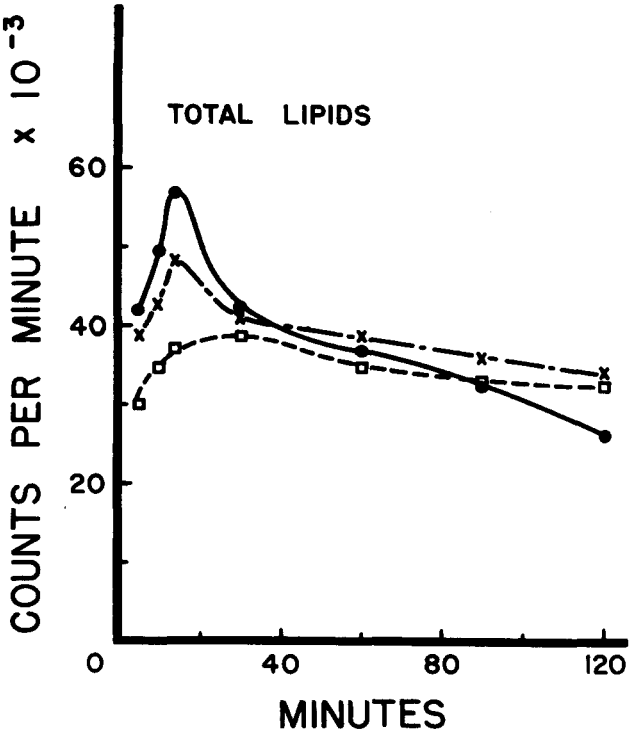

FIG. 1. Effect of APP on palmitic acid-1-C^{14} incorporation into total liver lipids. Mice each received an intraperitoneal injection of 1000 μg APP, and 0, 3, and 24 hr later received an intravenous injection of palmitate-albumin complex containing 30 μg of palmitic acid-C^{14} (673,000 cpm). O——O, control; x—x, 3 hr; □------□, 24 hr.

TABLE 5. EFFECT OF APP ON PLASMA LIPIDS*

Time after APP	Total Lipids	Cholesterol	Phospholipid	Triglycerides	FFA
hr	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μEq/ml*
0	2,000 ± 249†	340 ± 40	650 ± 72	1,100 ± 120	8.1 ± 0.7
1	1,310 ± 146	260 ± 32	595 ± 62	455 ± 52	8.2 ± 0.6
3	1,260 ± 109	240 ± 24	583 ± 54	437 ± 42	8.0 ± 0.9
6	1,250 ± 123	180 ± 17	596 ± 48	474 ± 49	7.9 ± 0.9
24	1,020 ± 127	100 ± 8	618 ± 73	302 ± 37	8.1 ± 0.6

* Mice were each injected intraperitoneally with 1,000 μg APP. Each figure is a mean of separate analyses of samples from eight mice in two experiments.

† Standard deviation.

benzamine as in those that had received only APP.

These results suggested that the principal effect of APP on the metabolism of liver lipids that results in fat accumulation might be inhibition of triglyceride and cholesterol secretion from the liver. To test this possibility more directly, control and APP-treated mice were injected intravenously by tail vein with Triton WR-1339, an agent known to produce massive hyperlipemia by preventing the removal of triglycerides from the plasma (27, 28, 29). Table 6 shows that the normal accumulation of lipid in the plasma following Triton administration was markedly reduced in mice that had received APP 3 hr previously, indicating that lipids were not entering the blood at their normal rate.

Although little is known about the process of lipid secretion from the liver, lipids are believed to be secreted as lipoprotein complexes, the form in which they appear in the plasma (30, 31, 32). While there is some question as to the relative stoichiometry of lipid and lipoprotein synthesis in the liver (30), it seemed that inhibition of the synthesis of the protein moiety of these lipoproteins by APP was a possible site of APP action in view of the report of inhibition of protein synthesis by APP in ascites tumor cells by Booth and Sartorelli (6). The effect of APP on the incorporation of glycine-C^{14} into liver and plasma protein, and of orotic acid into the UMP in liver RNA is shown in Table 7. The specific activity of UMP was used as a measure of RNA synthesis, which was here measured because of the intimate relationship between RNA and protein synthesis. APP administration was followed immediately by a 70–80% inhibition of orotic acid incorporation, which returned to normal (or above) by 24 hr. However, the incorporation of glycine into both liver and plasma proteins was not diminished by drug treatment.

TABLE 6. EFFECT OF APP ON TRITON WR-1339-INDUCED HYPERLIPEMIA*

Time after Triton	Plasma Triglycerides	
	Control	APP
hr	*μg/ml*	*μg/ml*
0	1,178 ± 182†	512 ± 46
1	4,325 ± 426	692 ± 73
2	7,498 ± 673	842 ± 89
3	10,800 ± 986	1,020 ± 120

* Triton WR-1339 (200 mg/kg) dissolved in 0.154 M sodium chloride was injected intravenously into control mice and into mice injected intraperitoneally 3 hr previously with 1,000 μg of APP. Each figure is a mean of values from separate analyses of a total of 4 mice in 2 experiments.

† Standard deviation.

DISCUSSION

Three general classes of fatty livers have been distinguished biochemically. One large class appears to be caused by a stimulation of FFA release from adipose tissue and often appears to be associated with stress. Intact pituitary and adrenals, or at least circulating corticosteroids, are required (references in [33]). Ethanol (26, 33, 34), phosphorus (35), pituitary

TABLE 7. EFFECT OF APP ON PROTEIN AND RNA SYNTHESIS *in vivo**

Time after APP	Liver		Plasma Protein	
	UMP	Protein		
hr	*cpm/μmole*	*cpm/mg*	*mg/ml*	*cpm/mg*
0	659 ± 71†	597 ± 62	5.7 ± 0.6	450 ± 46
1	180 ± 14	794 ± 89	5.3 ± 0.6	569 ± 61
3	139 ± 15	671 ± 65	5.4 ± 0.4	545 ± 59
6	268 ± 29	613 ± 54	5.6 ± 0.5	524 ± 48
24	709 ± 81	752 ± 82	5.8 ± 0.6	486 ± 50

* Mice were each injected intraperitoneally with 1,000 μg APP, and either 1, 3, 6, or 24 hours later were injected simultaneously with 2 μmoles of glycine-C^{14} and 0.5 μmoles of orotic acid-C^{14}. The mice were killed 1 hr after the second injection. Each figure is a mean of values from separate analyses of a total of six mice in two experiments.

† Standard deviation.

extracts (35, 36, 37, 38), and fasting (36) are representative inducers of this class of fatty liver. The normal values for plasma FFA observed here do not by themselves rule out this mechanism. However, increased utilization of FFA by liver was not observed, as measured by incorporation of palmitate-C^{14}. The lack of effect of phenoxybenzamine is also a strong argument against the direct involvement of FFA.

Increased synthesis of lipid in the liver may be involved in the fatty livers described after feeding excess cystine (38) or orotic acid (39). Recently, Yoshida and Harper (40) have ascribed the effects of choline deficiency to increased lipid synthesis also, although earlier work had suggested a defect in lipid transport (38, 41). Again, there is no evidence that APP acts in this manner. The decrease in fat synthesis observed after APP treatment may be related to a feedback inhibition such as that described by Siperstein and Guest for cholesterol synthesis (42).

Diminished secretion of lipid from the liver has been associated with carbon tetrachloride toxicity (43, 44) and with choline deficiency (38), and the available evidence suggests that APP also causes fatty livers in this way. The diminished levels of plasma triglycerides and cholesterol are one piece of direct evidence. Triton WR-1339 has been used as a tool to study triglyceride secretion from liver by Byers and Friedman (29) and to establish the inhibition of this process by carbon tetrachloride by Recknagel et al. (43). The observed inhibition of Triton-induced hyperlipemia by APP strongly suggests that this purine analogue inhibits the secretion of triglyceride from the liver.

The fourth theoretically possible cause of fatty liver formation, decreased fatty acid oxidation, appears never to have been associated with actual fatty liver formation. Although APP treatment results in a slight depression in this process, decreased oxidation of fatty acid appears to be a relatively minor contribution to fatty liver formation.

Because the concentration of phospholipid in both liver and plasma was essentially unchanged by APP treatment, it is considered unlikely that the conversion of triglycerides to phospholipids, which normally occurs in liver (45), is the site of action of APP.

Modes of interference with secretion of lipid from the liver are as little understood as the process of secretion itself. The recent work of several investigators (29, 46, 47) has shown that liver is the major (but not the sole) source of plasma triglycerides, and that this class of lipids is secreted at a very rapid rate. Bates (48) has also reported that the turnover rate of plasma triglycerides was much faster than that of plasma phospholipids, which are also secreted by the liver (49), but which also return to a large extent to the liver (50). The possible association of the endoplasmic reticulum with triglyceride secretion has been shown by the demonstration of damage to this cell fraction by carbon tetrachloride (44), possibly by its solvent action. Presumably, inhibition of synthesis of the protein moiety of these lipoproteins could cause the same result. Circulating lipoproteins are known to have a much faster turnover rate than the remaining serum proteins, considered as a group (51). Whereas the experiments reported have apparently disproved this possibility, there is still some question on this point because of the marked inhibition of protein synthesis in Ehrlich ascites tumor cells by APP (6), and the depression of synthesis of liver RNA, which is believed to be intimately connected with protein synthesis. The possibilities that the radioactive precursor was incorporated into an acid-insoluble form that was not a complete protein, or that the synthesis of only specific proteins was inhibited, have not been ruled out. The possibility also exists that purine nucleotide co-factors are required for the secretion process in some manner as yet unknown, and that the APP ribonucleotides, which are found in considerable quantities in liver (9), may interfere with their function.

Although the present study has not clearly demonstrated the mechanism of inhibition of triglyceride secretion by the purine analogue, APP, it is hoped that future studies on the mechanism of triglyceride secretion from liver will shed further light on this matter. In view of the speed and magnitude of the APP effect, this compound might prove to be a useful tool for such studies.

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