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Transcholesterin Titers and Their Biological Significance in Experimental Atherogenesis in Rats* (33002)

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A previous report(1) described the development of a distinctive reaction line between components of human serum and cholesterol when subjected to immunoelectrophoresis in an agar gel. The specificity of the reaction was indicated by the fact that the line was neither elicited by cholesterol esters, close homologues, a variety of other steroids nor by the vehicle, Teepol "L". It was suggested that the reactive globulins, later found to be alpha-1 and alpha-2 globulins,[2] represent a natural cholesterol transport similar in principle to homeostatic mechanisms of transcortin, transferrins, haptoglobins, and natural antibodies; the term "transcholesterin" was proposed(1). Both the whole serum and lipoprotein-free serum demonstrated the reaction line in the regions of alpha globulins.[2] Furthermore, it was found that whole sera could be titrated in the transcholesterin assay with serial dilution of the sera in the same manner as antibodies are quantitated.

The studies of Neff *et al.*(2) on the development of atherosclerosis including coronary involvement in rats on an atherogenic diet and on various high-fat diets were repeated to provide serum samples for testing the reactivity of transcholesterin from such animals at different periods in the dietary and pathogenetic regimens.

The present paper reports the alterations in titers of transcholesterin and the total serum cholesterol values of rats on experimental diets in comparison with those on stock chow.

Materials and Methods. Sera of rats on experimental diets. Whole sera from young adult male Sprague-Dawley strain rats with an average weight of 200 gm on the four dietary regimens—high-fat(3) (peanut oil), high-fat(3) (butter), atherogenic(3,4), and stock chow, were assayed for transcholesterin titers without previous knowledge of their dietary regimens or their cholesterol levels. The numbers representing the serum samples were decoded after the procurement of the experimental values.

Total cholesterol determination. A modified Liebermann-Burchard procedure(5) utilizing the Bausch and Lomb Spectronic 505 spectrophotometer to read the density of the color at wavelength 640 mμ was adapted for cholesterol quantitation. The reproducibility of the analytical procedure was good with a standard deviation of ± 5%. The error was sometimes greater than ± 5% if the serum samples were thick and creamy (such as those obtained in rats during the later stages of

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TABLE I. A Comparison of Total Serum Cholesterol Levels and Transcholesterin Titers in Sera of Four Rats Bled at Different Time Intervals During High-Fat Peanut Oil Dietary Regimens.

	Rat no.	Predietary sera			Sera obtained at designated time (weeks) of dietary regimens							
					4	8	12	19	26	45	50	60
Cholesterol[a]			81	81	177	177	177					
Titer	3		80	80	200	200	200					
Cholesterol		68	68	67	77	90		89	89	99	150	159
Titer	7	80	100	100	160	160		160	200	200	200	200
Cholesterol			97	97	126	126	129					
Titer	11		100	100	100	100	200					
Cholesterol			100	100	112	160	161	133	180	150	160	
Titer	20		80	80	160	160	200	160	200	320	320	
Av cholesterol			84.2		123	138	156	111	135	125	155	159
Av titer			88.8		155	155	200	160	200	260	260	200

[a] (mg/100 ml).

feeding on high-fat butter and atherogenic diets).

Quantitation of transcholesterin titer. The serum samples were diluted 1:40, 1:80; 1:100; 1:120; 1:160; 1:200; 1:240; 1:320; 1:400; and 1:500 in isotonic saline. Immunoelectrophoresis(1,6) on agar gel of an aliquot of 0.01-ml sample of diluted serum was performed and the end point was read as the last dilution where a positive cholesterol–transcholesterin reaction line was still visible to the naked eye. The physical demonstration of the reaction line between cholesterol and serum globulin was presented in an earlier communication(1).

Results. A. High-fat peanut oil diet (40% peanut oil supplement(3). All four rats fed high-fat peanut oil diet had elevated total serum cholesterol levels by the fourth week of the dietary regimen. Concurrent changes were found for the transcholesterin titers in three of them (Table I). These are represented in Fig. 1A and B as average total serum cholesterol and average serum transcholesterin titers versus duration of dietary regimen. However, the elevation in titers did not quantitatively correspond with the variations in total serum cholesterol values. There is a general tendency for a gradual increase in total serum cholesterol values as shown in Table I, during the 60 weeks of high-fat peanut oil diet. The transcholesterin titers followed a similar trend during this same span of dietary regimen. The variations in the levels observed in the different animals in this dietary group suggest that each had its own operational level or maintenance threshold. Rat no. 7 presented a comparatively low level before and during the dietary period, while rat no. 3 rose promptly to much higher values. It appears that the homeostatic transport mechanism is set at different levels. There is a suggestion that in the later stages both measurements were higher indicating perhaps that the continued load of dietary fat required further output of transcholesterin. Rat no. 20 (died in week 51) and no. 3 (sacrificed after 1 year) had subintimal lipid in coronary vessels.

B. High-fat butter diet (40% butter supplement(3). In the high-fat butter diet group of four rats shown in Table II, the total serum cholesterol values increased from predietary values of less than 100 mg/100 ml to as much as 250 mg/100 ml during the 60 weeks of high-fat butter diet. However, the increase of serum transcholesterin was considerably greater and earlier than the serum cholesterol rise. In 8 weeks of dietary regimen the serum transcholesterin titers of the four rats

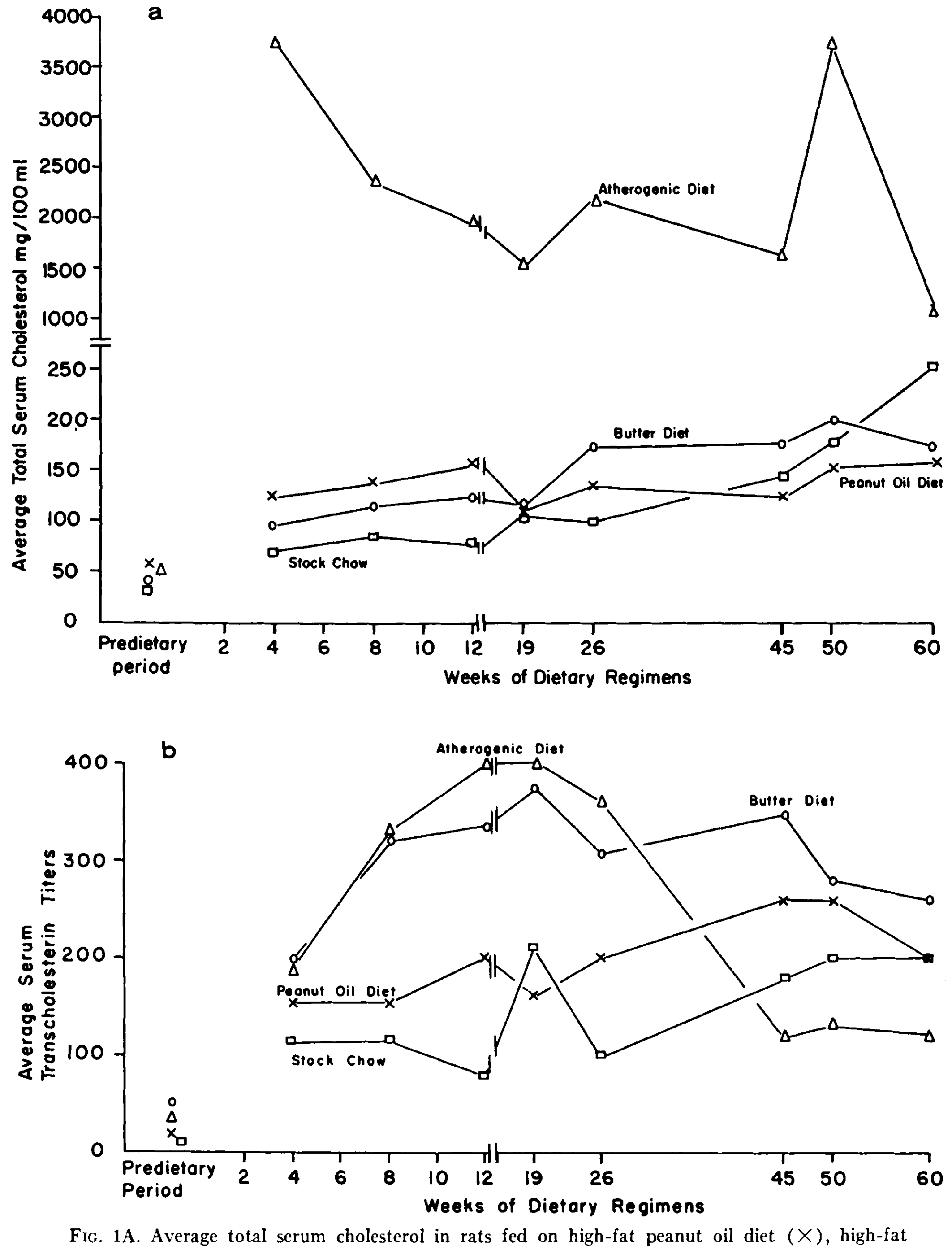

FIG. 1A. Average total serum cholesterol in rats fed on high-fat peanut oil diet (×), high-fat butter diet (○), atherogenic diet (△), and stock chow (□). B. Average serum transcholesterin titers in rats fed on high-fat peanut oil diet (×), high-fat butter diet (○), atherogenic diet (△), and stock chow (□).

TABLE II. A Comparison of Total Serum Cholesterol Levels and Transcholesterin Titers in Sera of Four Rats Bled at Different Time Intervals During High-Fat Butter Dietary Regimens.

	Rat no.	Predietary sera			Sera obtained at designated time (weeks) of dietary regimens							
					4	8	12	19	26	45	50	60
Cholesterol[a]			84	97	112	110	113	110	250	200	200	
Titer	31		100	100	200	320	200	320	320	400	320	
Cholesterol			84	97	107	129	145	100	150	160	200	150
Titer	33		200	200	200	320	400	400	200	320	200	200
Cholesterol		48	49	48	70	72		129	121	174	200	200
Titer	36	100	100	100	200	320		400	400	320	320	320
Cholesterol			97	65	97	145	113					
Titer	38		100	100	200	320	400					
Av cholesterol			74.3		96	114	124	113	174	178	200	175
Av titer			122.1		200	320	333	373	307	347	280	260

[a] (mg/100 ml).

reached 320 and were maintained between 200 and 400 until the end of the 60-week period. Again individual variations are seen in the predietary and subsequent levels of total serum cholesterol as well as in the height of transcholesterin responses. In general the measurements of total serum cholesterol levels and transcholesterin titers were higher in the high-fat butter group than those in the group fed high-fat peanut-oil diet (Fig. 1A and B).

Rat no. 38 was sacrificed after 60 weeks on high-fat butter diet because this animal was losing weight for lack of appetite. Histopathological changes of focal myocardial damage, endocardial lipids, thrombus in atrial appendage, lipid infiltration of coronary vessel walls, and lipid degeneration of renal tubule cells were observed in this rat.

A significant observation in high-fat diet groups was the striking degree of obesity exhibited by the two groups of animals on either the high-fat, peanut-oil diet or the high-fat, butter diet.

C. Atherogenic diet(3,4) (supplemented with 5.0% cholesterol, 0.15–0.30% thiouracil, and 2.0% sodium cholate). The data in Table III show, after four weeks of feeding, sharply elevated total serum cholesterol levels in the four rats: 4260 mg/100 ml for two rats (nos. 56 and 61) 3060 mg/100 ml of rat no. 55 and 2720 mg/100 ml for rat no. 60. The serum transcholesterin titer also doubled. High total serum cholesterol levels over 1000 mg/100 ml were maintained until week 50 of the dietary regime. Moreover, the transcholesterin rose to the highest measureable titers of 400. It is interesting to note that they remained at high levels of 320 and 400 between 8 and 26 weeks on atherogenic diet and then dropped off to levels of 100 and 160 at 45–60 weeks. This was in contrast to the titers of rats fed either high-fat peanut oil or high-fat butter diet which maintained their titers at 200 or more. At the same time interval the rats on atherogenic diet had extremely high total serum cholesterol in contrast to the relatively low values obtained in rats fed the high-fat diets (Fig. 1A and B).

Two rats (nos. 60 and 61) died in weeks 54 and 63 respectively. Rat no. 56 was on atherogenic diet for 50 weeks and was sacrificed later. Rat no. 55 was on the experimental diet for 82 weeks and died 10 weeks later while on stock diet. The common postmortem histopathology was *cholesterol clefts* in the aortic wall, atherosclerosis in coronary arteries, large calcified subintimal plaques in the aorta, and lipid infiltration with patchy foam cell areas in the intima. The final weights of the dead animals were 350–450 gm.

TABLE III. A Comparison of Total Serum Cholesterol Levels and Transcholesterin Titers in Sera of Four Rats Bled at Different Time Intervals During Atherogenic Dietary Regimens.

	Rat no.	Predietary sera			Sera obtained at designated time (weeks) of dietary regimens							
					4	8	12	19	26	45	50	60
Cholesterol[a]			58	64	3060	1450	2350	1600		1000		1450
Titer	55		100	100	200	320	400	400		80		80
Cholesterol			65	68	4260	2080	2070	1300	1003		3800	792
Titer	56		100	100	200	400	400	400	400		100	160
Cholesterol		91	90	90	2720	2720		1696	3400	2300	3630	
Titer	60	100	100	100	160	200		400	320	160	160	
Cholesterol			81	98	4260	3060	1450					
Titer	61		80	160	200	400	400					
Av cholesterol			78.3		3875	2327	1957	1532	2201	1650	3715	1121
Av titer			104.3		190	330	400	400	360	120	130	120

[a] (mg/100 ml).

TABLE IV. A Comparison of Total Serum Cholesterol Levels and Transcholesterin Titers in Sera of Four Rats Bled at Different Time Intervals During Stock Chow Dietary Regimens.

	Rat no.	Predietary sera		Sera obtained at designated time (weeks) of dietary regimens							
				4	8	12	19	26	45	50	60
Cholesterol[a]		65	81	48	71	71					
Titer	64	80	80	80	80	80					
Cholesterol		50	50	80	100		110	100	190	150	210
Titer	65	80	80	100	100		100	100	160	200	200
Cholesterol		68	68	90	100		100		105	209	300
Titer	70	100	100	200	200		320		200	200	200
Cholesterol		97	71	68	65	81					
Titer	71	80	80	80	80	80					
Av cholesterol		68.7		71	84	76	105	100	147	179	255
Av titer		85.0		115	115	80	210	100	180	200	200

[a] (mg/100 ml).

D. Stock chow (Purina standard rat chow). All the 16 Sprague-Dawley rats (Tables I–IV) had serum total cholesterol values ranging from 48 to 100 mg/100 ml while on the predietary stock chow. The transcholesterin titers, determined for the same sera, were in the 80–100 range with the exception of two rats, nos. 33 and 61, were higher titers of 160 and 200.

The serum transcholesterin titers of three rats (nos. 64, 65, and 71) on stock chow remained between 80 and 100 after 12 weeks of dietary regimentation and the total serum cholesterol levels fluctuated between 50 and 97 mg/100 ml. The data are presented in Table IV. There was a tendency for a gradual increase in total serum cholesterol levels in this group of rats where maximum values of 210 mg/100 ml and 300 mg/100 ml were obtained in rats nos. 65 and 70, respectively, after 60 weeks. However, for rat no. 70 the transcholesterin titers attained the value of 200 much quicker than rat no. 65. Rat no. 70 reached that at the end of 4 weeks of feeding and maintained it until 60 weeks. Again the average total serum cholesterol levels and the average serum transcholesterin titers for rats given stock chow were compared with those of the other rats representing three other dietary regimens (Fig. 1A and B).

It is shown that there was a sudden increase of total serum cholesterol values in rats nos. 65 and 70 after feeding on stock chow for 45 and 50 weeks, respectively. Rat no. 65 showed concurrent rise in transcholesterin titer. However, rat no. 70 was odd in terms of its transcholesterin titers which went up to 200 on the fourth and eighth weeks, and leveled off at 200 towards weeks 45 and 60 on stock chow. Perhaps the elevation of total serum cholesterol at the later stages of life of rats could be due to aging; old rats had been shown by Thomas *et al.*(7) and Humphreys(8) to exhibit spontaneous atherosclerosis in the coronary arteries similar to the proliferative lesions found in rats fed hypercholesterolemic diets.

Rats no. 64, 70, and 71 were sacrificed 73, 74, and 94 weeks, respectively, after starting on the "control" diet. Postmortem examinations of these rats revealed only gross changes in kidneys similar to chronic obstructive nephropathy which was also reported by Wilgram and Ingle(9) for aging female breeder rats. No histopathology was performed on tissues of the four rats. Wilgram(10) had observed several kinds of spontaneous vascular lesions in aging rats during the second year of life. Also, he pointed out that the lesions were different from those induced experimentally by the feeding of atherogenic diets and that the latter were more infiltrated with lipid.

The appearance of the sera from rats on atherogenic diet was definitely lipemic. Those sera from rats on high-fat butter diet were slightly lipemic, and those from rats on high-fat peanut oil and stock chow were not visually lipemic.

Discussion. Several observations reported here with rats fed atherogenic diets and high-fat diets are worthy of note. Rats sustained on stock chow and on high-fat peanut oil regimens demonstrated slightly elevated total serum cholesterol and transcholesterin titers. However, the increase in titers was more marked in the animals fed on high-fat butter meals. The rats on butter diets had lipemic sera, high transcholesterin titers but only moderate increase of serum total cholesterol in the first 5 months. It is interesting that the butter diet has been reported to promote thrombosis and myocardial infarction(11) in rats. The animals from both high-fat dietary groups became grossly obese. Rats fed on atherogenic regimens yielded hyperlipemic sera, high transcholesterin titers and extreme heights of serum total cholesterol during 60 weeks.

The prompt rise of transcholesterin titers in response to increase in levels of serum total cholesterol in the sera of rats fed on stock chow and high-fat diets would represent the adequacy of transcholesterin function in the homeostatic control of cholesterol in the blood. The transcholesterin production tends to remain active as the animals progress towards old age. The elevation of total serum cholesterol levels at the later stages of the life of rats on regular stock chow in the present study may be attributed to aging. In contrast, those animals on an atherogenic diet demonstrated extreme heights of serum total cholesterol early and subsequent increase in transcholesterin titers followed by fall in serum total cholesterol after the peak of transcholesterin titers, then a final decline in the titers and rise in cholesterol levels.

However, the rise and fall of serum total cholesterol levels (Table III and Fig. 1A) do not correspond numerically with the transcholesterin titers. Hence, the transcholesterin titration is not necessarily another measurement for serum total cholesterol. The explanation for the rapid increase in transcholesterin titers can be attributed to a homeostatic mechanism which is operating at the level of cholesterol transport by transcholesterin (i.e., its mobilization to meet the demands). The time required for the system to be under excessive metabolic stress (i.e., beyond its capacity for normal body metabolism and transport of cholesterol) would appear to be between weeks 26 and 50 of feeding on an atherogenic diet. The striking decrease in serum transcholesterin titers while serum cholesterol is high during the later stages may represent an exhaustion of the transcholesterin mechanism. These shifts of the two reactants reveal the dynamic nature of the mechanism of meeting cholesterol stress. In contrast, in rats on thrombogenic diet (high-fat butter), the transcholesterin titers remained high, between 260 and 373, even up to week 60 of dieting (Table II and Fig. 1B).

Since serum lipoproteins have been reported to be synthesized by rat liver slices(12) and by rat liver ribosomes(13); thus the adaptive biosynthesis of transcholesterin in liver counteracts the abnormally high blood cholesterol resulting from the feeding of atherogenic diet in experimental rats. The striking specificity(1) of transcholesterin to bind only cholesterol and dihydrocholesterol would also suggest the physiological importance of the transcholesterin in the biological transport of free cholesterol. It can be seen in the data obtained from rats fed atherogenic diet that the homeostatic control of cholesterol pool levels is under metabolic stress, i.e., the system is overloaded beyond its capacity for normal body metabolism and transport of cholesterol. Table III shows some definite evidences supporting a control mechanism at work. Organ damage such as fatty liver(10,14) and spleen(14) infiltrated with lipids (including cholesterol esters) in rats fed atherogenic diets which interferes with normal physiological function may constitute the mode of action of excessive cholesterol in tissues thereby affecting the homeostatic control mechanism of transcholesterin.

Some evidences supporting the idea of active cholesterol transport by serum protein fractions have been provided by Bailey(15,16). He has shown that mammalian cells grown in tissue culture take up radioactive ^{14}C-labeled cholesterol intracellularly from the growth medium. Upon transferring to unlabeled medium, the excretion of ^{14}C-labeled cholesterol extracellularly was enhanced by the addition of isolated serum alpha globulins. By comparison with the electrophoretically fractionated serum alpha globulins which gave positive transcholesterin test[2] it is assumed that the alpha globulins active in the excretion of cholesterol extracellularly by mammalian cells are similar to transcholesterin.

Summary. The rise in titer of transcholesterin in the sera of rats on different high-fat or atherogenic diets was demonstrated. The reactive component is a serum alpha globulin which appears to combine specifically with cholesterol in agar gel immunoelectrophoresis. The serum cholesterol levels rise in various degrees in relation to diets but they do not correlate directly with transcholesterin titers. The observations suggest that transcholesterin functions as a homeostatic mechanism to meet the excess demands in disposal of cholesterol. The prompt rise of serum transcholesterin titer in rats on the high-fat diets and the moderate hypercholesterolemia may represent the adequacy of transcholesterin function in these animals. On an atherogenic diet, extreme heights of serum total cholesterol are reached early, again with mobilization of transcholesterin, after which the latter declines suggesting the homeostatic effort is approaching exhaustion or that the transcholesterin has been so fully combined that little remains free for further combining. The fall in total serum cholesterol after the peak of transcholesterin titers would be in keeping with the idea that this serum protein (or proteins) has a transport or clearing function for excessive cholesterol. The failure of cholesterol levels to be reduced to a normal range while transcholesterin titers decline is in accord with the concept of homeostatic failure. There was observed further the probable effect of aging upon total serum cholesterol accumulation in some animals on the control diet; this, too, was accompanied by moderate increase in serum transcholesterin titers.

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Studies of Glucuronidation. IV. Evidences of Different Processes for *o*-Aminophenol and *p*-Nitrophenol* (33003)

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In recent years several authors have suggested that there may be more than one, perhaps several, hepatic glucuronyl transferases. Isselbacher *et al.*(1,2) reported that an enzyme solubilized from rabbit liver microsomes readily conjugated ether and ester aglycones, but not aniline. The Gunn rat, which has an inherited inability to conjugate bilirubin, has been found to conjugate aniline(3) and *p*-nitrophenol (PNP)(4), but not *o*-aminophenol (OAP)(3,5,6,7). Certain differences between OAP and PNP have been found in their reactions to various inhibitors of glucuronidation(8), and in their rates of development in the immature mouse(9).

The studies reported herein provide additional evidence that OAP and PNP are conjugated with glucuronic acid by different means, probably involving different enzymes.

Materials and Methods. The basic procedures were as described elsewhere(10). "Adult" Holtzman rats, Gunn rats, Hartley guinea pigs and postmortem human liver samples were used. The rats ranged in age from 3 to 6 months, and the guinea pigs 2–4 months. Animals were decapitated, after being stunned by a blow to the head, and their livers were excised and washed in cold (approximately 4°C) 0.15 *M* KCl containing 3.2×10^{-4} *M* $KHCO_3$, pH 7.0. Human postmortem liver specimens were obtained as soon after death as possible (usually several hours) and then were frozen immediately until used.

Portions of liver were weighed, placed in five volumes of the cold KCl–$KHCO_3$ solution and homogenized using a motor-driven Teflon homogenizer in an ice bath. This homogenate was then used in the "whole homogenate" assays for glucuronyl transferase activity. The "microsomal fraction" was obtained by centrifuging the whole homogenate at 8500*g* for 20 min and then centrifuging the supernatant at 109,000*g* for 30 min. The sedimented fraction, resuspended in 5 volumes of the KCl–$KHCO_3$ solution, was used for assaying microsomal activity.

Except where stated otherwise, the incubation mixture contained liver homogenate or microsomes equivalent to 200 mg (wet weight) of liver, 0.1 ml of 0.5 *M* Tris buffer at pH 7.4, 0.15 *M* $MgCl_2$, .07 m*M* OAP or

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