SUSTAINED HYPERLIPEMIA INDUCED IN RABBITS BY MEANS OF INTRAVENOUSLY INJECTED SURFACE-ACTIVE AGENTS*

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The amount of cholesterol and phospholipid in the blood serum of mammals remains remarkably constant during health, as detailed studies in a number of species have shown (1–4), though it is well known that hyperlipemia of varying degrees often accompanies certain disease states, for example hypothyroidism diabetes, nephrosis, and xanthomatosis. No method has heretofore been devised whereby sustained elevations of the major blood lipids of mammals can be readily brought about, though elevations of blood cholesterol and to a lesser extent of blood phospholipid have been produced with considerable difficulty in experimental animals by such procedures as feeding cholesterol or cholesterol and thiouracil during prolonged periods of time (5, 6), the production of alloxan diabetes (7) or nephrotoxic nephritis (8), and repeated massive bleedings (9). The injection of lipid emulsions directly into the blood stream has resulted merely in transitory increases in the level of the particular lipid injected (10). In the work now to be described striking and sustained elevations of blood cholesterol, phospholipid, and neutral fat have regularly followed the intravenous injection into rabbits of two surface-active agents of relatively low toxicity.[1] Besides their interest in connection with the problems of the intermediary metabolism of blood and tissue lipids, the findings provide a means for further studies on the pathogenesis of experimental atherosclerosis; the latter are given in an associated paper (11).

Materials, Methods, and Control Observations

Surface-Active Agents.—Tween 80[2] and Triton A20[3] were the surface-active agents employed in these studies. Tween 80 is a polyoxyalkylene derivative of sorbitan monooleate,

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[1] For preliminary notes see *Fed. Proc.*, 1949, **8**, 359 and *Am. J. Path.*, 1950, **26**, 732.

[2] Manufactured by the Atlas Powder Co., Wilmington.

[3] Manufactured by Rohm and Haas, Inc., Philadelphia.

and Triton A20 is an arylalkylpolyether of phenol. Both are non-ionic surface-active agents, miscible in all proportions with water. Solutions for injection were made in 0.9 per cent saline and buffered at pH 7.3 with McIlvaine's buffer solution. Tween 80 was used in a concentration of 20 per cent, the commercial product being diluted 1 to 5. Commercial Triton A20 is a 25 per cent solution, and for injection the commercial product was diluted with equal parts of saline to make a 12.5 per cent solution.

Animals and Diet.—The rabbits used in these experiments were market-bought animals of mixed breeds weighing between 2400 and 3500 gm. In each experiment equal numbers of males and females were employed. The animals were fed a stock diet of Rockland rabbit pellets supplemented by occasional feedings of green vegetables. The diet can be considered to be free of cholesterol, since this substance was absent from large quantities of it analyzed chemically, and also because normal rabbits maintained on it in our laboratory for periods of 2 years have not shown any elevation of blood cholesterol. In view of the results to be described later, it should be emphasized that cholesterol was not added to the diet of the animals in these studies. Water was available *ad libitum*.

Lipid Determinations.—Blood for lipid determinations was drawn from an ear vein, 1 to 2 cc. of blood sufficing for the determination of cholesterol, phospholipid, and total lipid. Serum cholesterol was determined by a modification of the method of Bloor, Pelkan, and Allen (12). Neither Tween 80 nor Triton A20 give the Liebermann-Burchard reaction when added to serum *in vitro*, though they may cause an interfering color that can readily be compensated for by using a red filter. In those instances in which the cholesterol esters are reported, both the total and free cholesterol were determined by the method of Schoenheimer and Sperry (13). Serum total lipids were measured by the gasometric lipid carbon method of Van Slyke and Folch (14). Lipid phosphorus was determined by a modification of the method of Fiske and SubbaRow (15). A number of trials have shown that the color reaction in this determination is not affected by the addition of either Tween 80 or Triton A20 to serum *in vitro*. Phospholipid levels are reported as milligrams of lecithin per 100 cc., and were calculated by multiplying the figure for lipid phosphorus by 25.

Toxic Effects of Intravenously Administered Tween 80 and Triton A20.—The intravenous injections of Tween 80 and Triton A20 were in general well tolerated by the animals, though certain toxic reactions were observed. The initial injection of Tween 80 caused varying degrees of intravascular hemolysis in most of the animals, as was determined by pink to red discoloration of the blood plasma and by a decrease in the packed cell volume, but no relation could be discerned between the severity of the hemolysis and the degree of lipemia (to be described later). The hemolytic effect of intravenous Tween 80 disappeared with repeated injections, indicating perhaps that the elevated blood lipids exerted a protective action against the hemolytic effect. Triton A20 caused only slight hemolysis upon intravenous injection.

The initial injection of Triton A20 was usually followed by a very dramatic series of events. A few minutes after the injection the animals became rigid, the rigidity being most marked in the posterior extremities. This was followed by convulsions, often extremely violent, lasting 5 to 10 minutes. The animals gradually recovered and about 30 minutes after the injection they appeared entirely normal. The frequency and severity of these reactions decreased significantly with repeated injections, suggesting a protective action due to the lipemia. Such convulsive reactions were not observed following injections of Tween 80. It should be emphasized that despite the apparent severity of these reactions, the effects were quite transitory, and upon recovery there were no obvious sequelae. The animals appeared healthy, ate well, and gained weight.

Twenty-four hour urine specimens were collected from four rabbits that had received multiple injections of Triton A20; these disclosed no abnormal proteinuria or glycosuria upon

test. Blood urea nitrogen was determined at the height of lipemia in several rabbits and found to be within the normal range. No tests of hepatic function were performed. The fact that animals injected with Triton were found to have a moderate decrease in per cent of cholesterol esters in the blood serum suggests that there may be some impairment of liver function in these animals; on the other hand, it is possible that the decreased cholesterol ester percentage is merely a reflection of a disproportionate increase in free cholesterol induced by the injected agent.

There were no deaths following the single intravenous injections of Tween 80. Four rabbits died during the course of repeated injections of Tween 80, and postmortem examination of these animals revealed degeneration and necrosis of the liver in two and extensive pneumonia in one. The remaining animal was too autolyzed for the cause of death to be determined. No animals died as a result of either the single or multiple injections of Triton A20. A number of animals were sacrificed for purposes of histological study at varying intervals following single or multiple injections of Tween 80 or Triton A20. There were no changes observed in the gross in any of these animals other than slight pallor of the liver and moderate to marked increase in size of the spleen. No abnormalities were noted on microscopic examination of sections of the heart, lungs, thyroid, adrenals, gonads, pancreas, body fat, striated muscle, and brain. The increase in size of the spleen was due at least in part to the presence of many large fat-filled macrophages in the red pulp. There was an increase in the amount of stainable fat in the liver and in the tubular cells of the kidney. It should be emphasized, in view of the changes in blood lipids to be described further on, that, with the exception of the two animals previously mentioned, in which hepatic necrosis was observed following multiple injections of Tween 80, there were no degenerative or necrotic changes seen in the parenchymal cells of the liver or the kidney in these experiments. The aortas of a few of the animals that received injections of Triton A20 for 9 weeks or longer contained small yellow atheromatous plaques that were identical in distribution and morphology with those observed in cholesterol-fed rabbits (16), though the incidence of this lesion was far less than would be expected in cholesterol-fed rabbits that had attained a comparable blood cholesterol elevation. The relation of intravenously injected surface-active agents to the development of atherosclerosis is discussed more fully in the associated paper (11).

Hyperlipemia Following the Intravenous Injection of Tween 80

The blood cholesterol and phospholipid levels of six rabbits, each of which had received a single intravenous injection of 20 per cent Tween 80 in the amount of 2.5 cc./kg., are shown in Table I. It will be seen that there was a sharp increase in the levels of blood cholesterol and phospholipid following the injections, and that these levels reached peaks within 6 to 12 hours and then returned to the normal range within 24 to 48 hours. Incidentally it was observed that the sera, which initially were clear, became opalescent to milky at the height of the lipemia, and then gradually reverted to their original clarity some 24 to 48 hours after injection. It is noteworthy that the increase in blood cholesterol above the initial levels ranged from 46 to 260 per cent, and the increase in blood phospholipid from 111 to 338 per cent. By contrast, we have found the diurnal variation of both blood cholesterol and phospholipid in many normal rabbits to be within $\pm$ 10 per cent.

In other experiments similar to that of Table I, 2.5 cc./kg. of 20 per cent Tween 80 was found to be optimal for the production of lipemia in rabbits. The effect on blood lipids de-

creased when smaller doses were injected, and was not apparent with doses below 1 cc./kg. Serious toxic reactions and death resulted when 4 cc./kg. of 20 per cent Tween 80 was given.

Boggs and Morris (9) have reported that frequent bleeding in rabbits may give rise to hyperlipemia. Their animals were bled 25 cc. at a time, whereas in the experiments reported here only 1 to 2 cc. of blood was withdrawn at each bleeding. It seemed worth while, however, to determine whether the bleedings may have been a factor in the lipemia that followed injection of the surface-active agent. Hence two rabbits were injected intravenously with 2.5 cc./kg. of 0.9 per cent NaCl solution and were bled at the same times and in the same amounts as were

TABLE I

Serum Cholesterol and Phospholipid Levels of Rabbits Given a Single Intravenous Injection of 20 Per Cent Tween 80, 2.5 Cc./Kg.

Rabbit No.		Time after injection, *hrs.*							Increase at peak
		0	3	6	9	12	24	48	
									per cent
1	Chol	86	106	126	104	104	106	94	46
	PL	83	153	175	165	135	148	117	111
2	Chol	69	144	177	166	170	124	87	156
	PL	145	288	358	343	300	208	158	146
3	Chol	51	128	154	155	142	118	80	204
	PL	120	253	265	263	278	183	110	131
4	Chol	40	88	100	123	91	88	70	207
	PL	68	120	173	190	125	115	90	180
5	Chol	29	78	100	94	78	50	47	240
	PL	63	150	170	185	175	98	80	196
6	Chol	28	68	100	101	94	60	49	260
	PL	45	155	185	198	175	75	55	338

Chol = total serum cholesterol, mg./100 cc.
PL = phospholipid, expressed as lecithin, mg./100 cc.
0 = immediately prior to injection.

the Tween 80–injected animals of Table I. No significant alterations were observed in their blood cholesterol or phospholipid levels or in the turbidity of their sera. In addition, the fact may be noted that in the experiments of Boggs and Morris hyperlipemia was observed only after 8 to 16 days of repeated bleeding when the circulating red cells were reduced to less than half their original number. Hematocrit determinations at 6 hours and 24 hours following injection of Tween 80 revealed a decrease of but 2 to 8.5 per cent in the volume of packed red blood cells, the findings indicating plainly that the reduction in number of red cells was not sufficient to account for the degree of lipemia observed.

In view of the transitory hyperlipemia that resulted from a single intravenous injection of Tween 80, as shown in Table I, it was of interest to see whether

these changes could be augmented and prolonged by repeated injections at frequent intervals. Table II shows the blood cholesterol and phospholipid levels of a group of twelve rabbits on the cholesterol-free diet which received

TABLE II

*Serum Cholesterol and Phospholipid Levels of Rabbits Given Repeated Intravenous Injections of 20 Per Cent Tween 80, 2.5 Cc./Kg., at 8 Hour Intervals**

Rabbit No.	No. of injections		Time after initial injection, *days*																	
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	8	Chol	63	107	195	287‡	470§														
		PL	98	175	260	302	505														
2	11	Chol	71	65	119	156	335‡	216	150	—	—	79									
		PL	115	125	193	242	370	205	143	—	—	108									
3	11	Chol	63	91	213	250	324‡	373	199	—	—	83									
		PL	83	135	262	275	362	410	215	—	—	103									
4	15	Chol	81	106	254	451	535	697‡§													
		PL	120	225	312	473	610	722													
5	18	Chol	69	84	216	473	580	805	950‡	1030	828	—	687	—	476	—	—	—	220		
		PL	88	153	235	480	562	815	975	1050	800	—	595	—	288	—	—	—	143		
6	19	Chol	63	71	147	194	339	620	549‡	560§											
		PL	93	165	272	312	365	625	525	488											
7	25	Chol	32	120	378	385	278	396	385	363	487	482‡	385	—	343	—	—	157	—	89	
		PL	75	212	365	415	390	403	422	425	512	475	333	—	288	—	—	150	—	103	
8	25	Chol	62	145	314	416	461	482	597	650	730	747‡	875	—	712	—	—	475			
		PL	85	193	350	455	472	500	675	833	850	862	940	—	700	—	—	342			
9	25	Chol	84	148	268	400	326	300	385	403	440	445‡	360	—	320	—	—	287	—	—	137
		PL	125	213	295	385	363	375	412	425	388	412	475	—	303	—	—	250	—	—	118
10	28	Chol	89	69	168	284	367	341	406	483	544	531‡	417	—	340	—	258	—	145		
		PL	113	125	242	275	350	398	455	478	540	585	435	—	300	—	193	—	150		
11	29	Chol	31	108	238	295	403	392	440	535	547	492	545‡§								
		PL	65	153	225	250	363	385	403	425	538	562	562								
12	31	Chol	118	94	165	185	190	201	286	259	256	358	425	483‡	345	—	220	—	62	—	60
		PL	145	180	213	275	253	255	310	243	267	312	405	463	320	—	245	—	163	—	112

Chol = total serum cholesterol, mg./100 cc.
PL = phospholipid, expressed as lecithin, mg./100 cc.
0 = immediately prior to initial injection.
* Initial injection followed by rest period of 24 hours; then reinjected at 8 hour intervals around the clock.
‡ Day of last injection.
§ Died.

repeated intravenous injections of 20 per cent Tween 80 in amounts of 2.5 cc./kg. Twenty-four hours after a first injection others were given at 8 hour intervals to a total of eight to thirty-one. Blood was drawn just prior to the initial

injection and daily thereafter. It will be seen that the blood cholesterol and phospholipid levels rose progressively, with only occasional and minor deviations, during the course of the injections. The peak levels of blood cholesterol ranged from 335 to 1,030 mg./100 cc., and those of phospholipid from 370 to 1,050 mg./100 cc. In every instance there was a parallel increase in blood cholesterol and phospholipid content, with the peak phospholipid levels tending to be somewhat higher than the corresponding cholesterol levels. The blood cholesterol and phospholipid levels began to drop promptly after the injections were stopped, and in four animals (rabbits 2, 3, 7, and 12) they returned to the normal range within 5 to 8 days. Four rabbits (Nos. 1, 4, 6, and 11) died during the course of the experiment. Blood lipid levels of the remaining four animals (Nos. 5, 8, 9, and 10) had not entirely returned to normal within 6 to 10 days following the end of the injection period, though in nearly every instance the values had decreased markedly when the observations were terminated. In general, it will be noted, the phospholipid levels decreased somewhat more rapidly than did the cholesterol levels. The blood serum of most of the animals was milky during the period of hyperlipemia, and very quickly returned to normal clarity when the injections were discontinued, often within 48 hours, despite the fact that the blood cholesterol and phospholipid levels had not yet reached the normal range. In a few instances the blood serum remained clear throughout the course of the injections, despite elevations of cholesterol and phospholipid that were as great as those of other animals with milky sera.

Sustained elevations of both blood cholesterol and phospholipid were maintained in the experiment of Table II for periods ranging from 4 to 18 days; in other experiments, in which injections of Tween 80 were given twice daily, it was found possible to maintain the hyperlipemia for as long as 10 weeks. In all experiments the lipid levels continued to rise as long as the surface-active agent was administered.

Prolonged Elevation of Blood Lipids Produced by Intravenous Injection of Triton A20

The blood lipid levels of six rabbits each of which received a single intravenous injection of 12.5 per cent Triton A20, 2.5 cc./kg., are given in Table III. The blood cholesterol and phospholipid rose progressively to levels which in the case of cholesterol ranged from 244 to 545 mg./100 cc., and in the case of phospholipid, from 358 to 1,240 mg./100 cc. Peak phospholipid levels were in all cases higher than the corresponding cholesterol levels, and in some cases the discrepancy was marked. The peak levels were reached in 1 to 3 days following a single injection of Triton A20, and the blood lipids returned to the normal range only after 5 to 15 days. The blood cholesterol and phospholipid levels of a representative animal of this group are illustrated graphically in Fig. 1. The blood serum of all the animals was strikingly milky at the height

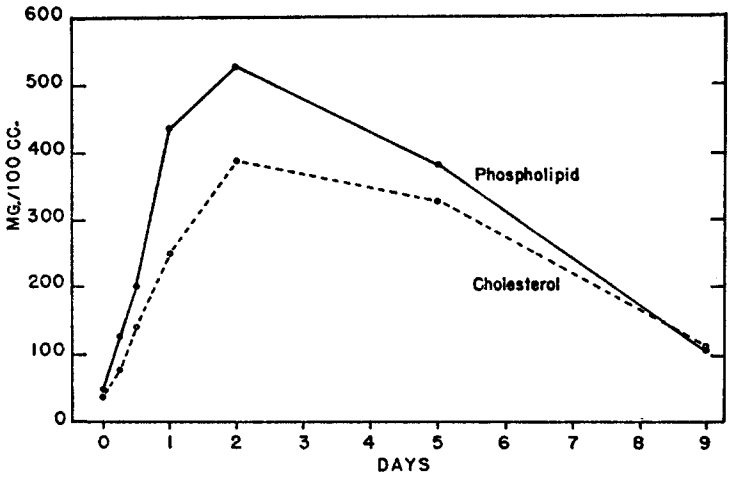

FIG. 1. Blood cholesterol and phospholipid levels of rabbit 3, Table III, given a single intravenous injection of 12.5 per cent Triton A20, 2.5 cc./kg., at day 0.

TABLE III

Serum Cholesterol and Phospholipid Levels of Rabbits Given a Single Intravenous Injection of 12.5 Per Cent Triton A20, 2.5 Cc./Kg.

Rabbit No.		Time after injection, *days*									
		0	1	2	3	5	7	9	11	13	15
1	Chol	57	443	545	520	433	—	148	—	125	104
	PL	118	873	1083	1240	595	—	185	—	155	108
2	Chol	40	260	420	—	338	—	88	—	52	
	PL	58	428	633	—	495	—	85	—	50	
3	Chol	41	252	382	—	323	—	116	—	74	
	PL	45	438	528	—	380	—	105	—	63	
4	Chol	32	238	244	160	94	42				
	PL	58	340	358	220	90	55				
5	Chol	51	360	340	223	142	101	62			
	PL	78	383	355	253	170	158	88			
6	Chol	62	312	462	350	208	142	106	79		
	PL	90	353	518	465	183	150	158	103		

Chol = total serum cholesterol, mg./100 cc.
PL = phospholipid, expressed as lecithin, mg./100 cc.
0 = immediately prior to injection.

of lipemia, and the serum gradually became clear as the blood lipids dropped toward the normal range. In another experiment the total lipid, free and esterified cholesterol, and the phospholipid content of the blood serum were determined 24 and 48 hours after a single intravenous injection of Triton A20 into each of three rabbits. The observations (Table IV) showed that there was a considerably greater increase in free cholesterol than in esterified cholesterol, resulting in a decreased percentage of cholesterol ester. Furthermore, the total lipid was markedly elevated in all three animals, even more than were the cholesterol and phospholipid fractions. This observation was regularly con-

TABLE IV

Serum Lipid Levels of Rabbits Given a Single Intravenous Injection of 12.5 Per Cent Triton A20, 2.5 Cc./Kg.

Rabbit No.	Time after injection	Cholesterol			Cholesterol ester	Phospholipid*	Total lipid
		Free	Ester	Total			
	hrs.	*mg./100 cc.*	*mg./100 cc.*	*mg./100 cc.*	*per cent*	*mg./100 cc.*	*mg./100 cc.*
1	0	22	54	76	71	82	205
	24	92	66	158	41	655	1919
2	0	26	69	95	72	130	298
	48	322	136	458	30	514	3041
3	0	25	68	93	73	125	345
	48	203	195	398	49	726	3602

0 = immediately prior to injection.
* = Phospholipid expressed as lecithin.

firmed in other experiments, and the facts considered together make it plain that the neutral fat as well as the cholesterol and phospholipid components of the serum lipids is regularly increased by the intravenous injection of Triton A20.[4]

Finally the hyperlipemia following repeated intravenous injections of Triton A20 into rabbits was studied. Rabbits maintained on a normal, cholesterol-free diet were injected intravenously with 4 cc. of 12.5 per cent Triton A20 twice weekly for 9 weeks. Marked and sustained elevations of both blood cholesterol and phospholipid were present during the entire injection period (see Fig. 2). In each animal there was a progressive increase in blood cholesterol and phospholipid content, which in some animals exceeded 2,000 mg./100 cc.

[4] In still other experiments Triton A20 was injected intravenously into mice and guinea pigs; the serum of every animal so treated became milky and manifested elevations of blood lipids quite comparable to those observed in rabbits.

The blood serum of the animals remained milky throughout the duration of the injection period. Injections were arbitrarily terminated at the end of 9 weeks. The animals, however, appeared in good health at the end of the experiment and most of them had gained weight, and it seemed likely that the injections could have been continued. More data on this point will be given in the accompanying paper.

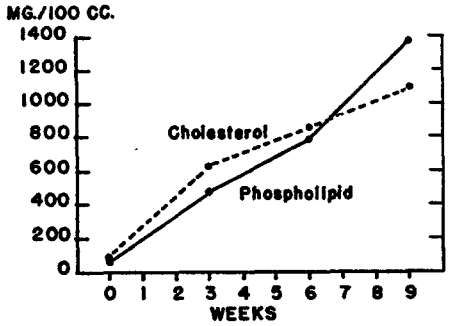

FIG. 2. Blood cholesterol and phospholipid levels of a rabbit maintained on a normal, cholesterol-free diet and injected intravenously with 4.0 cc. of 12.5 per cent Triton A20 twice weekly for 9 weeks.

DISCUSSION

The hyperlipemia that regularly followed the injection of Tween 80 and Triton A20 into rabbits, as here reported, differs notably from that produced by feeding cholesterol or that manifest in such disease states as hypothyroidism, essential xanthomatosis, or nephrosis. For in the former case the blood phospholipid was increased along with the blood cholesterol and frequently exceeded it in amount, whereas in the latter instances the elevation of blood cholesterol is almost always considerably greater than that of phospholipid (17, 18). Indeed, the surface-active agents provide the most convenient means now at hand whereby in mammals an elevation of the major lipid components of the blood serum, and particularly a parallel increase in phospholipid and cholesterol, can be produced, though it is noteworthy in this relation that a similar increase in the lipid fraction of the blood has been produced in birds by the intramuscular injection of estrogenic substances and by the subcutaneous implantation of stilbestrol pellets (19, 20). Since elevations were observed in the levels of cholesterol, phospholipid, and neutral fat in the blood of rabbits given the surface-active agents, it seemed not unreasonable to suppose that there may have been concomitant increases in other circulating lipids, for example the steroid hormones and free fatty acids. Experiments to explore this possibility are now in progress.

Precisely how the surface-active agents induce the hyperlipemia remains as yet undisclosed. In this relation it seems noteworthy, however, that the hyperlipemia that followed single intravenous injections of the maximum tolerated amounts of Tween 80 disappeared within 24 to 48 hours, whereas that following single injections of Triton A20 lasted for 5 to 15 days. This marked difference in the duration of the hyperlipemia may be due to the fact that rabbit serum and tissues contain enzymes that are capable of rapidly hydrolyzing the Tween 80 molecule but are apparently devoid of enzymes that can destroy Triton A20 (21). If this be true, it follows that the hyperlipemia depends upon the actual presence of the surface-active agent in the body. Whether the agents act directly by increasing the capacity of the plasma to hold lipids in stable emulsion, or by interfering with enzyme systems in the blood or tissue that are essential for the intermediary metabolism of fats, or by other means, cannot now be stated. The cholesterol, phospholipid, and neutral fat in the circulating blood represent only a very small fraction of the total amounts of these substances in the body, and it is quite conceivable that the hyperlipemia produced by the surface-active agents may represent, in part at least, a mobilization of lipids from the liver and other body stores. On the other hand, observations made recently in this laboratory have shown that following the intravenous injection of Triton A20 into mice the total amount of cholesterol in the body is significantly increased (22). This observation suggests that the injected surface-active agents may induce the hypercholesterolemia by augmenting the synthesis of cholesterol or by retarding its degradation, the possibilities being consistent with the observation that the animal organism is capable of synthesizing cholesterol from simple precursors such as acetate radicals and also of completely metabolizing the cholesterol molecule to carbon dioxide (23, 24). Furthermore, the fact that the hyperlipemia develops rapidly, following the injection of surface-active agents, is consistent with the observation that there is a rapid turnover of lipids in the body, as has been demonstrated by studies utilizing lipids labelled with deuterium and radioactive isotopes (25, 26).

In attempts made several years ago to influence the course of experimental atherosclerosis, Hueper (27) gave several synthetic detergents both orally and intravenously to rabbits. The agents he used, however, were highly toxic and his experiments failed to disclose any constant effect of the agents on blood lipids or on experimental atherosclerosis. Observations previously reported from this laboratory have shown that Tween 80, fed to rabbits on a normal diet, had no effect on blood cholesterol content; when fed to rabbits on a high cholesterol diet, however, the Tween 80 augmented the hypercholesterolemia usually produced by cholesterol feeding, presumably by enhancing the absorption of cholesterol from the intestinal tract, and it accelerated the development of atherosclerosis (28). The fact now disclosed—that the hyperlipemia which follows the injection of Tween 80 and Triton A20 intravenously into rabbits differs

from the hypercholesterolemia of cholesterol-fed animals—provides a new means for investigating the pathogenesis of experimental atherosclerosis, as is described in the associated paper (11).

SUMMARY

The intravenous injection of the surface-active agents Tween 80 and Triton A20 into rabbits fed a normal diet resulted in marked and sustained elevations of the cholesterol, phospholipid, and total lipid content of their blood. The increase in phospholipid in general paralleled that of the blood cholesterol.

The implications of the findings are briefly discussed.

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