Summary. 1. Liver homogenates prepared from rats given large doses of isonicotinic acid hydrazide (INAH) exhibited reduced glutaminepyruvate transaminase-deamidase activity and increased glutaminase activity. 2. Purified enzyme preparations (free of glutaminase) prepared from livers of INAH-treated rats catalyzed the glutamine-pyruvate reaction at markedly reduced rates compared to controls. Addition of pyridoxal (or pyridoxamine) phosphate produced a considerable increase in activity of the preparations obtained from INAH-treated rats, but not of that of the untreated controls. 3. The evidence supports the concept that vit. B_6 participates in the glutamine transaminase system.

1. Meister, A., Morris, H. P., and Tice, S. V., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 301.

2. Biehl, J. P., and Vilter, R. W., *ibid.*, 1954, v85, 389.

3. Meister, A., Sober, H. A., Tice, S. V., and Fraser, P. E., *J. Biol. Chem.*, 1952, v197, 319.

4. Meister, A., and Tice, S. V., *ibid.*, 1950, v187, 173.

5. Meister, A., *ibid.*, 1954, v210, 17.

6. Yoneda, M., and Osano, N., *Science*, 1953, v117, 277.

7. Yoneda, M., Kato, N., and Okajuna, M., *Nature*, 1952, v170, 803.

8. Sakai, J., *Kekkaku*, 1954, v27, 161. (Chem. Abstr., 1954, v48, 9463.)

9. Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M., *J. Biol. Chem.*, 1954, v209, 453, 467.

10. Beaton, J. R., and Ozawa, G., *ibid.*, 1955, v214, 685.

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Effect of Dihydrocholesterol and Soybean Sterols on Cholesterol Metabolism in Rabbit and Rat.* (22167)

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Because considerable evidence exists showing that such sterols as dihydrocholesterol and soybean sterols are absorbed from the gut, it was considered worthwhile to restudy the effects of these sterols on cholesterol metabolism in rabbit and rat. The data in this paper provide an explanation for the conflicting reports heretofore published and demonstrate the fallacy inherent in attempts to produce a negative cholesterol balance by inhibition of cholesterol absorption from the gut.

Experimental. Preparation of sodium acetate-1-C^{14}. Methylmagnesium bromide was carbonated with $C^{14}O_2$. Control diets consisted of Purina Rabbit Chow for rabbits and Purina Laboratory Chow for rats. Sterol diets were prepared by dissolving the requisite amount of dihydrocholesterol‡ or mixed soybean sterols§ in ether, adding the solution to control diets and evaporating the ether. Sera and tissues were prepared for sterol analysis as described previously(1). *Sterol determination.* Digitonin precipitable, Liebermann-Burchard (L.B.) positive sterols were determined by the method of Sperry and Webb(2) and total digitonin precipitable sterols by a modification of the Pollak-Wadler turbidimetric method(3). The latter method was modified as follows: Sterols were precipitated with digitonin in graduated glass-stoppered tube at 10°C. After precipitation the volume was adjusted in the tube, and one drop

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‡ Dihydrocholesterol (Lot no. 105-220 I MRS), generously supplied by Schering Corp., contained 98% Liebermann-Burchard negative sterols.

§ The mixed soybean sterols (Control no. S-7577 and S-9707), generously supplied by Distillation Products Industries, contained 79% and 94% Liebermann-Burchard positive sterols respectively.

TABLE I. Recovery of Soybean Sterols from Acetic Acid Solution by Various Methods for Cholesterol Determination.

L.B. positive sterol added, mg		Representative recoveries of L.B. positive sterols							
		Methods							
		Sperry-Webb		Abell *et al.*		Modified Pollak-Wadler with			
						Acetone-ethanol		Acetone-methanol	
Cholesterol	Soybean sterols	mg	%	mg	%	mg	%	mg	%
.160	—	.170	106	.163	102	.163	102	.169	105
—	.160	.133	83	.150	94	.134	84	.162	101

of polyoxyethylene sorbitan mono-laurate (Tween 20) added to stabilize the suspension. Optical density of the suspension was determined photoelectrically. The digitonide precipitate was removed by centrifugation, and optical density of supernatant determined. Amount of digitonide was computed from the difference in optical densities. For a more complete precipitation of the soybean sterol digitonides it was necessary to change the alcohol in the precipitating medium from ethanol to methanol (Table I). *Feeding experiments.* Male New Zealand white rabbits weighing 2-2.5 kg were used. These were divided into 3 groups (Tables II and III). The first group received control ration. The second received 3 g dihydrocholesterol (DHC) 3 times weekly. The third received 3 g mixed soybean sterols 3 times weekly. Animals were maintained on these diets for 4 weeks at which time they were sacrificed and blood and tissue samples removed. Male rats of Sprague-Dawley strain weighing 200-300 g were divided into 3 groups. The first group received control ration, the second received 1% DHC (Table II), the third received 3% mixed soybean sterols (Fig. 1). Three animals from each group were sacrificed weekly for 5 weeks. Pooled liver samples were removed each time for determination of cholesterol synthesis and sterol content. The sterol contents of control and DHC groups were also determined after 8 weeks on the diet. Rabbits and rats were allowed to eat *ad libitum,* with weight gain determined weekly. There were no significant differences in diet consumption or weight gains between control and experimental animals. *Isotopic experiments.* Liver cell clusters(4) were prepared from livers of control and sterol-fed rabbits and rats; incubation with C^{14}-carboxyl-labeled acetate, isolation of cholesterol and analysis of its radioactivity were performed as described previously(5).

Results. The use of proper analytical procedure for sterol analysis was essential in this study. Soybean sterols, which consist largely of isomers of sitosterol and some stigmasterol, form a digitonide and give the L.B. reaction (6). We found that recovery of soybean sterols from solution by the commonly employed technics of Sperry and Webb(2) and Abell *et al.*(7) was incomplete. Table I shows the efficiency of recovery of soybean sterols by these methods as well as by the modified Pollak-Wadler procedure. The recovery of soybean sterols by the Sperry-Webb method and modified Pollak-Wadler procedure with acetone-alcohol was poor. The reason for the failure of recovery was solubility of soybean sterol-digitonin complex in 1:1 acetone-ethanol. In gravimetric experiments we have found that washing 28.4 mg of soy sterol digitonides with two 5-ml aliquots of 1:1 acetone-ethanol dissolved away 61% of the digitonides.

Recovery of soy sterols by the method of Abell *et al.* was better. However, the determinations in Table I were performed with a minimum exposure to the stream of air used in the method to evaporate petroleum ether extract to dryness. We have determined, in a manner similar to that employed by Sperry and Brand(8), that soybean sterols were 2 times as sensitive to air oxidation as cholesterol; like cholesterol, air oxidation destroyed soy sterols' ability to give the L.B. reaction. It is probable then that loss of soybean sterols during analysis by the method of Abell *et al.* resulted from air oxidation and

TABLE II. Liver and Serum Sterols of Rabbits Fed Dihydrocholesterol (DHC) and Incorporation of Sodium Acetate-1-C^{14} into Cholesterol by Excised Livers* of Rabbits and Rats Fed DHC.

Group	Animal	Diet	Duration of feeding, wk	Serum, mg % A Total digitonin precipitable sterols	Serum, mg % B Cholesterol†	Serum, mg % C DHC‡	Liver, mg/g A Total digitonin precipitable sterols	Liver, mg/g B Cholesterol†	Liver, mg/g C DHC‡	Specific activity of liver cholesterol§
I	Rabbit 1	Control	4	42	50	—	5.3	4.6	.7	—
	2	"	4	33	36	—	5.6	5.1	.5	306
II	3	9 g DHC/wk	4	100	72	28	21.3	12.7	8.6	34
	4	"	4	87	59	28	18.6	10.5	8.1	—
I	4 rats	Control	8	—	49	—	5.8‖	5.6	.2	387
II	4 "	1% DHC	8	—	30	—	8.4‖	5.2	3.2	127

* Each vessel contained 3-4 g (wet wt) of liver cell clusters, 40 ml of buffer and 5 mg of $CH_3C^{14}OONa \cdot 3H_2O$ (5.1 mc/mM).
† L.B. positive sterol as determined by method of Sperry and Webb.
‡ Digitonin precipitable, L.B. negative sterol (column A minus column B).
§ Counts/min./mg of cholesterol/g dried liver.
‖ Determined gravimetrically as the digitonide.

that this loss may be far greater than shown in Table I. It should be added, however, that substitution of N_2 for air in the petroleum ether evaporation step in the Abell *et al.* procedure obviates this difficulty. From this evidence it seems clear that neither the Sperry-Webb nor Abell *et al.* procedures for cholesterol determination is adequate for analysis of soybean sterols. One may further conclude that use of these methods to determine serum and tissue total L.B. positive sterols may result in falsely low results when soybean sterols are present. For this reason reports, based on these methods, of a lowering of cholesterol levels after feeding soybean sterols may be suspect if soybean sterols are present in the body.

As the last column of Table I demonstrates, substitution of methanol in Pollak-Wadler type procedure provided the best recovery of soy sterols from solution.

Table II gives results of feeding experiments with DHC. It can be seen that DHC, which forms a digitonide but is L.B. negative (9), was found in sera and livers of rabbits and in livers of rats, thus indicating its absorption. Under these feeding conditions, that portion of the digitonin precipitable sterol which was L.B. negative was considered to be DHC. This assumption was strengthened by the fact that increased saturated sterol fractions(10) from the livers of rabbits 3 and 4 had infrared absorption spectra characteristic for DHC. It is of interest to note that the liver cholesterol content of rabbits 3 and 4 was elevated as well as the DHC content. This may have resulted from ability of liver to convert DHC to cholesterol(11). The last column of Table II demonstrates that livers of DHC-fed rabbits and rats have a decreased ability to incorporate labeled acetate into cholesterol. These results confirm the findings of Siperstein(12), Gould *et al.*(13), and Ivy *et al.*(14) that DHC is absorbed by the rat and further demonstrate absorption in the rabbit. In agreement with the report of Gould *et al.*(13) an inhibition of hepatic cholesterol synthesis in rats fed DHC has been demonstrated and a similar inhibition shown in the rabbit. The greater degree of inhibi-

TABLE III. Liver and Serum Sterols of Rabbits Fed Soybean Sterols for 4 Weeks and Incorporation of Sodium Acetate-l-C^{14} into Cholesterol by Their Excised Livers.*

Group	Animal	Diet	Serum, mg %			Liver, mg/g			Specific activity of liver cholesterol§
			Total digitonin precipitable sterols (methanol)	Cholesterol†	Soybean sterols‡	Total digitonin precipitable sterols (methanol)	Cholesterol†	Soybean sterols‡	
I	1	Control	26	25	—	5.7	5.6	—	607
	2		25	25	—	6.8	6.0	—	457
III	1	9 g soybean	40	45	0	10.2	5.1	4.6	155
	2	sterols/wk	47	49	0	9.3	5.5	3.3	—
	3		32	35	0	10.0	6.3	3.2	242
	4		26	26	0	7.1	6.2	0.4	545

* Each vessel contained 3-4 g (wet wt) of liver cell clusters, 40 ml of buffer and 5 mg of $CH_3C^{14}OONa \cdot 3H_2O$ (5.1 mc/mM).

† L.B. positive sterol as determined by method of Sperry and Webb.

‡ Digitonin precipitable (in methanol), L.B. positive sterol, corrected for cholesterol and for L.B. negative sterol content.

§ Counts/min./mg of cholesterol/g dried liver.

tion of cholesterol synthesis demonstrated in our animals undoubtedly reflects the longer feeding period for the sterol. Although atherosclerotic lesions of the aorta were not noted in our rabbits after a 4-week feeding period, it has recently been reported that a 0.5% DHC diet, fed to cockerels for 6 months, produces aortic atherosclerosis(15).

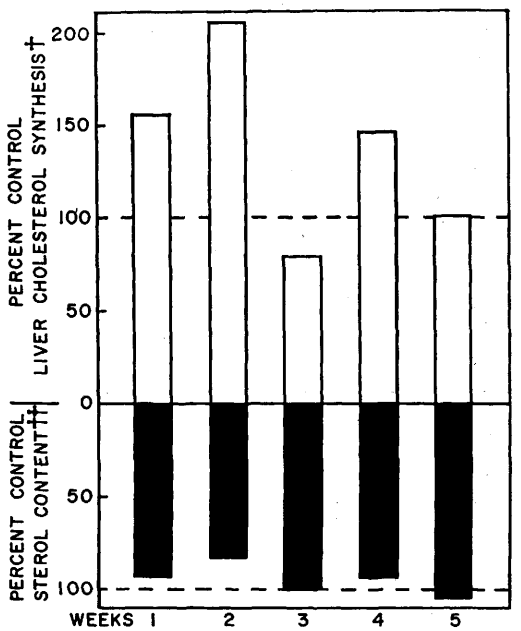

FIG. 1. Hepatic cholesterol synthesis and liver sterol content of rats fed a 3% soy sterols diet.

† $\dfrac{\text{C.P.M./mg cholesterol/g soy liver}}{\text{C.P.M./mg cholesterol/g control liver}} \times 100$

†† $\dfrac{\text{Total digitonin precipitable sterols/g soy liver}}{\text{Total digitonin precipitable sterols/g control liver}} \times 100$

Table III gives results of rabbit feeding experiments with soy sterols. It can be seen that the total liver sterols of rabbits 1, 2, and 3 of Group III are increased. As determined here, that fraction of total digitonin precipitable sterol (in methanol) which was neither cholesterol nor saturated sterol was considered to be soybean sterols. The validity of this assumption was demonstrated by the fact that infrared absorption spectra of the 1:1 acetone-ethanol soluble fraction of digitonides from livers of Group III rabbits 1, 2, and 3 were identical with infrared absorption spectrum of original mixed soybean sterols. It is of interest that rabbits fed soy sterols did not have measurable levels of these sterols in their serum. The last column of Table III shows that in livers of soy sterol diet rabbits 1 and 3 there was inhibition of cholesterol synthesis from C^{14}-labeled acetate. On the other hand Group III rabbit 4, which absorbed little soy sterol, showed a normal rate of cholesterol synthesis.

All rabbits receiving 9 g soy sterols per week for 4 weeks had atheromatous lesions of the aorta at autopsy. Infrared absorption spectra of the acetone-ethanol soluble fraction of the digitonides obtained from aortas of the rabbits in Group III also were identical with the spectrum of the dietary soy sterols. It is

thus apparent that soy sterols are both absorbed and atherogenic.

Fig. 1 shows the effect of a 3% soy sterols diet on the liver sterol content and rate of hepatic cholesterol synthesis in the rat. It can be seen that moderate reduction in liver cholesterol levels produced in the first 2 weeks of diet was associated with a compensatory increase in hepatic cholesterol synthesis. This increased synthesis was sufficient to restore and maintain at normal the liver cholesterol levels for the next 3 weeks of the experiment. These findings are identical with results obtained when rats were fed large amounts of a cholesterol metabolizing *Escherichia coli* which destroyed biliary cholesterol passed into the gut(16). Negative cholesterol balance, produced by either steroid or non-steroid interference with cholesterol reabsorption, is counteracted by an increase in endogenous cholesterol synthesis.

None of the livers from rats fed a 3% soy sterol diet contained a measurable amount of soy sterols. There would thus seem to have been little absorption of these sterols. The transitory reduction in liver cholesterol seen in the first 2 weeks of the experiment undoubtedly resulted from competitive inhibition of cholesterol absorption by the non-absorbable soy sterols(17).

These data demonstrate absorption of soy sterols in rabbits. It has been shown that in rabbits soybean sterols produce a depression of cholesterol synthesis resembling that seen after feeding cholesterol(18), dihydrocholesterol, and other related sterols(12).

Discussion. It is possible to conclude from our data that there is no reason to expect a reduction of tissue cholesterol levels in man by the use of dihydrocholesterol and soybean sterols. The rabbit experiments further suggest that ingestion of such substances may be hazardous.

Summary. 1. Dietary dihydrocholesterol and soybean sterols were absorbed by rabbits, and soy sterols produced atherosclerosis in these animals. 2. Livers of rabbits and rats fed dihydrocholesterol and of rabbits fed soybean sterols had an impaired ability to incorporate C^{14}-labeled-acetate into cholesterol. 3. Rats fed a 3% soybean sterols diet did not absorb significant amounts of these sterols. Soy sterols failed to produce a lowering of total liver sterol levels in rats.

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1. Curran, G. L., and Costello, R. L., *J. Exp. Med.*, 1956, v103, 49.

2. Sperry, W. M., and Webb, M., *J. Biol. Chem.*, 1950, v187, 97.

3. Pollak, O. J., and Wadler, B., *J. Lab. and Clin. Med.*, 1952, v39, 791.

4. Curran, G. L., *J. Biol. Chem.*, 1953, v200, 17.

5. Curran, G. L., and Clute, O. L., *ibid.*, 1953, v204, 215.

6. Fieser, L. F., and Fieser, M., *Natural Products Related to Phenanthrene*, 3rd Ed., New York, Reinhold Pub. Corp., 1949, pp. 285-288.

7. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.*, 1951, v195, 357.

8. Sperry, W. M., and Brand, F. C., *ibid.*, 1955, v213, 69.

9. Fieser, *op. cit.*, pp100-103.

10. Schoenheimer, R., *Z. physiol. Chem.*, 1930, v192, 77.

11. Gould, R. G., *Circ.*, 1954, v10, 589 (abstract).

12. Tomkins, G. M., Sheppard, H., and Chaikoff, I. L., *J. Biol. Chem.*, 1953, v203, 781.

13. Gould, R. G., Lotz, L. V., and Lilly, E. M., *Fed. Proc.*, 1955, v14, 487 (abstract).

14. Ivy, A. C., Tsung-Min, L., and Karvinen, E., *Am. J. Phys.*, 1954, v179, 646 (abstract).

15. Nichols, C. W. Jr., Lindsay, S., and Chaikoff, I. L., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 609.

16. Curran, G. L., and Brewster, K. C., *Bull. Johns Hopkins Hosp.*, 1952, v91, 68; Curran, G. L., unpublished experiments.

17. Hernandez, H. H., and Chaikoff, I. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 541.

18. Gould, R. G., Taylor, C. B., Hagerman, J. S., Warner, I., and Campbell, D. J., *J. Biol. Chem.*, 1953, v201, 519.

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