Diet as Source of Serum Cholesterol in Man.* (25664)

C. Bruce Taylor,† Dorothy Patton, Nelson Yogi and George E. Cox
Dept. of Pathology, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Serum cholesterol in animals and man may be advantageously regarded as having 3 potential sources: (1) synthesis by the liver, (2) synthesis by extrahepatic tissues, and (3) absorption of cholesterol from ingested foods. An inverse relationship between hepatic cholesterol synthesis and absorption of cholesterol from the diet has been demonstrated consistently in several species of laboratory animals. Rapid suppression of hepatic cholesterol synthesis, in response to absorption of dietary cholesterol, is believed to compensate for ingested cholesterol such that total supply of cholesterol (endogenous plus exogenous) to serum tends to remain constant (1). The existence of such a homeostatic mechanism in dogs, rats, and monkeys has been amply demonstrated; its existence in humans has been generally assumed, but never demonstrated. Cholesterol synthesis in extrahepatic tissues generally shows little change in response to dietary cholesterol. The homeostatic mechanism for serum cholesterol therefore appears to be primarily a function of the liver. It would seem, then, that the capacity of this homeostatic mechanism must be close to the maximum capacity of the liver to synthesize cholesterol; that is, the capacity of the liver to compensate for dietary cholesterol by reducing its endogenous synthesis cannot exceed its maximal capacity to synthesize cholesterol. For example, if the liver of man normally has a maximal or potential capacity to synthesize and contribute to serum about 2 g of cholesterol per day, it is reasonable to credit it with a compensation capacity of about 2 g per day. Several workers have suggested 2 g per day as a good approximation of rate of synthesis of serum cholesterol in man (2). No previous study has permitted a decisive interpretation of what tissues participate most prominently in contributing cholesterol to serum of man. Inferring from dogs and rats, it has been widely assumed that most of this 2 g per day is synthesized within the liver. Our study was not designed further to test the validity of the 2 g per day rate, but to determine what proportion of serum cholesterol normally can come from synthesis by liver tissue, and how much from synthesis by extrahepatic tissues. That is, however much cholesterol is synthesized and put into serum daily, whether it be 2 g or more or less, what percentage of this amount originates where?

Method. By feeding a cholesterol-rich diet the liver is suppressed (it is assumed that this suppression phenomenon exists in man as in lower animals) and thereby effectively eliminated as a source of serum cholesterol. If dietary cholesterol is labelled with carbon-14 (4-C-14-cholesterol) the contribution of dietary cholesterol to serum cholesterol can be easily measured by virtue of its radioactivity. Whatever percent of serum cholesterol comes directly from dietary cholesterol, the difference between this and 100% is thought to represent the contribution of extrahepatic tissue. Assuming that the cholesterol-rich diet is replacing the liver as one source of serum cholesterol, then the contribution of the diet should equal that amount that would be the contribution of the liver if the diet contained no cholesterol. In preparation of the diet, 10 g of dry egg yolk solids were taken as equal to 1 egg yolk, with a cholesterol content of 0.25 g. Appropriate amounts of dry egg yolk were suspended in absolute alcohol, radioactive and additional inert cholesterol were dissolved in hot absolute alcohol, and this was then thoroughly mixed into the egg yolk suspension. The mixture was placed in large shallow trays and the alcohol evaporated with the aid of fans. The residue was finely ground, thoroughly mixed, and packed into small souffle cups, 1 dose per cup. This pro-

* Supported by Ames Co., Elkhart, Ind., Chicago, and Am. Heart Assns.; Life Insurance Medical Research Fund; Otho S. A. Sprague Memorial Inst.; Schweppe Foundation, Chicago, Ill. and Nat. Heart Inst., NIH.

† Present address: Dept. of Pathology, Evanston Hospital, Evanston, Ill.

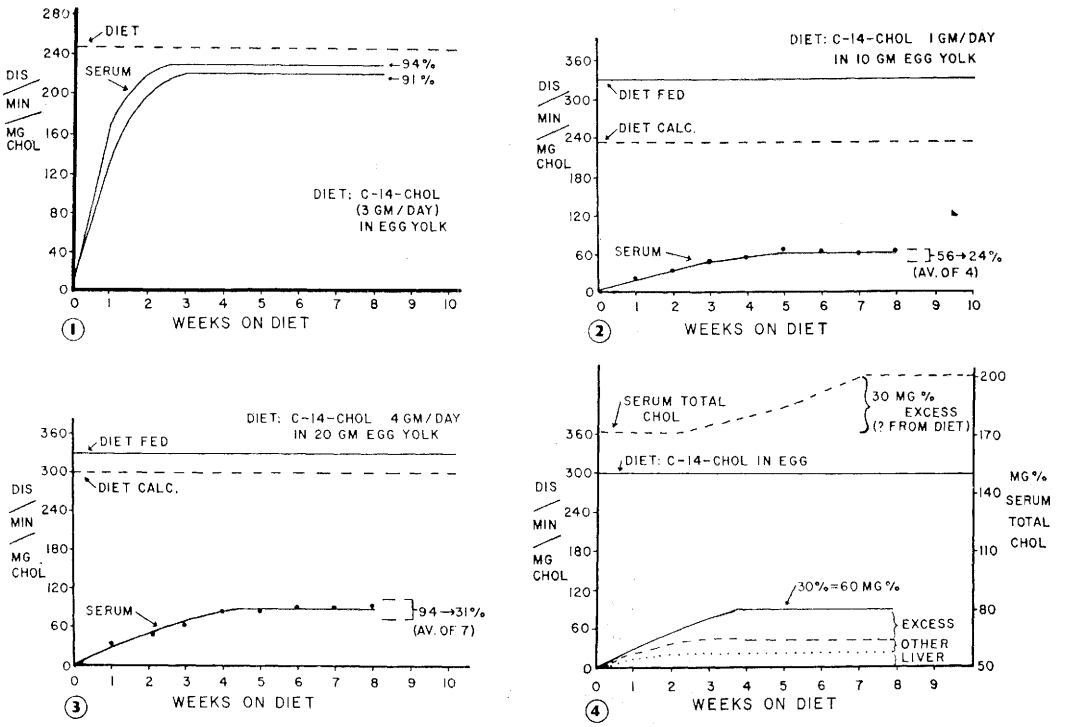

FIG. 1. Proportion of serum cholesterol derived from diet, in dogs fed a cholesterol-rich diet with C-14-cholesterol mixed in egg yolk.

FIG. 2. Proportion of serum cholesterol derived from diet, in humans receiving a daily total of about 1.4 g cholesterol with 1.0 g supplied as C-14-cholesterol mixed in 10 g egg yolk.

FIG. 3. Proportion of serum cholesterol derived from diet, in humans receiving a daily total of about 4.4 g cholesterol with 4.0 g supplied as C-14-cholesterol mixed in 20 g egg yolk.

FIG. 4. Theoretical considerations in estimating contribution of the liver to serum cholesterol, from data that specifically and directly measures contribution of dietary cholesterol to serum cholesterol.

cedure did not improve the taste quality of the egg yolk, but did facilitate a uniform mixing of the labelled cholesterol with the unlabelled cholesterol including that naturally present in the egg yolk. Volunteer healthy adults ingested this diet for 8 week periods. In Exp. 1, 4 subjects each ingested a daily dose of 10 g egg yolk containing 1 g C-14-cholesterol. In Exp. 2, 7 subjects each ingested a daily dose of 20 g egg yolk containing 4 g C-14-cholesterol. With few exceptions the daily amount was taken in split doses, to facilitate absorption. Blood samples were drawn once weekly, serum cholesterol isolated, washed by the procedure of Sperry and Webb(3), and radioassayed in a Packard Tri-Carb liquid scintillation spectrometer. Aliquots of the diet mixture were also assayed as a check on its composition. Total cholesterol determinations were done by the method of Sperry and Webb.

Results. This study employed a principle previously used by Morris *et al.*(4). This group fed rats a 2% C-14-cholesterol diet and found that 74 to 90% of the serum cholesterol came from dietary cholesterol. The remaining 10 to 26% was believed to represent the contribution of extrahepatic tissues to serum cholesterol in the rat. Applying this principle to dogs, in preliminary studies, we obtained results typified in Fig. 1. The dogs were fed commercial dog biscuits, with daily supplements of 50 g of egg yolk solids containing 3 g cholesterol labelled with a specific activity of 242 DPM/mg (disintegrations per minute per mg of cholesterol). Specific activity of the serum cholesterol quickly rose to peak plateau values of 220 and 228 for the 2 dogs shown. By calculation (specific activity of serum cholesterol ÷ specific activity of dietary cholesterol, × 100) 91 and 94%, respectively, of serum cholesterol came from dietary

cholesterol. Presumably, then, 91 and 94% of serum cholesterol would come from the liver if the animals were eating a cholesterol-free diet. These results closely confirm previous studies on dogs by other workers using different methods, all of which demonstrated a remarkably predominant role of the liver in supplying cholesterol to serum in low-cholesterol fed dogs(5).

When the study was begun on human subjects, amount of egg yolk and cholesterol was reduced, because the diet appeared so much less palatable to humans than to the dogs that smaller doses were mandatory. The results from Exp. 1 are shown in Fig. 2. These 4 subjects included 2 females age 22, 1 female age 50 and 1 male age 24 years. Specific activity of their egg yolk ration ("diet fed") was 330 DPM/mg. Because of a calculated average ingestion of nonradioactive cholesterol (in foods) of about 0.4 g per day, overall specific activity of dietary cholesterol ("diet calculated") was estimated to be about 236 DPM/mg. Ingestion of this diet for 8 weeks effected a rise in serum cholesterol radioactivity to a peak plateau value, in one subject, of 60 DPM/mg. The curve in Fig. 2 represents this individual subject, with the points indicating the observed specific activities of serum cholesterol. The 2 horizontal bars at end of curve indicate the range of the 4 cases. Average of the 4 cases is 56 DPM/mg, which demonstrates the diet to be a source of 24% (56 $\div$ 236, $\times$ 100) of the serum cholesterol. Presumably then the remaining 76% is coming from extrahepatic tissues or residual unsuppressed hepatic cholesterol synthesis.

In Exp. 2 (Fig. 3) the 7 subjects included 5 males and 2 females, ages 20 to 30 years. The 0.4 g nonradioactive cholesterol in their diets was calculated to lower overall dietary cholesterol specific activity from 330 DPM/mg to 300 DPM/mg. A typical 8 week curve, representing actual data of 1 subject, is shown. Horizontal bars at end of curve indicate range of the cases. Average of the 7 cases is 94 DPM/mg, which demonstrates that the diet was supplying 31% of the serum cholesterol in these subjects. The remaining 69% should represent the contribution of extrahepatic tissues only, if hepatic cholesterol synthesis in the human is suppressible by dietary cholesterol.

In both experiments, serum cholesterol specific activity reached its peak value in 4 to 5 weeks, thereafter remaining quite constant to the end of the 8 week study period.

Discussion. If the human liver normally synthesizes cholesterol at a slow rate, then even its complete suppression could offer little compensation for dietary cholesterol. If the human liver synthesizes cholesterol at a rapid rate—but if this rate is not significantly reduced by dietary cholesterol—then again the "compensation capacity" of the liver could not be large. The only available data indicate a very low rate of synthesis, as estimated in liver biopsies surgically removed from patients with non-hepatic diseases(6); however, these patients generally were eating a diet of their choice prior to surgery, thereby leaving open the possibility that the observed low rate might represent a potentially high rate which was being suppressed by dietary cholesterol.

It is not known whether hepatic cholesterol synthesis in man can be markedly suppressed by dietary cholesterol. The technic of comparing uptake of C-14-acetate into serum cholesterol, in subjects on a cholesterol free *vs.* a cholesterol rich diet, shows a striking and unequivocal suppressive effect of cholesterol feeding in dogs(7); however, the same technic, with careful dietary control, showed no suppressive effect of cholesterol feeding in humans(8). This is quite consistent with the observations mentioned above, that human liver normally has a low rate of cholesterol synthesis such that its suppression would not be expected greatly to reduce overall uptake of C-14-acetate into serum cholesterol. Of course, it is also consistent with the alternate, but completely undemonstrated, hypothesis of a high rate which is not reduced by dietary cholesterol.

The data reported here clearly demonstrate a striking difference between cholesterol metabolism of man, and the dog and rat. In dogs and rats on a cholesterol-rich diet, dietary cholesterol can serve as a *major* source of serum cholesterol, supplying between 74 and 94% of the serum cholesterol. In man, dietary cholesterol apparently can supply only

about ¼ to ⅓ of the serum cholesterol. Larger daily feedings of egg yolk and cholesterol might increase the percentage of serum cholesterol coming from dietary cholesterol; however the similarity of results of feeding 1 g *vs.* 4 g cholesterol per day, indicates that the picture would not be changed very much by feeding larger amounts of cholesterol. Anyway, if the liver in man is not suppressed by 4 g of cholesterol per day it is unlikely that any reasonable human diet would ever suppress it, and thus its compensation capacity must be quite inefficient at the usual levels of intake of cholesterol.

The actual contribution of liver to serum cholesterol in subjects on a cholesterol-free diet may be much less than the 24 to 31% indicated in Exp. 1 and 2. Two theoretical—unproven but reasonable and likely—considerations are outlined in Fig. 4; note that a scale for mg% serum total cholesterol is superimposed on the right side of this figure. Most of the subjects in Exp. 1 and 2 showed a serum cholesterol elevation of 10 to 40 mg%. Consider a subject whose level increased by 30 mg%, as indicated by the rise from 170 to 200 mg%: it may be reasonably assumed that much or possibly all of this excess came from dietary cholesterol. To facilitate the explanation, assume that all of it came directly from the diet. The diet is then doing more than simply replacing liver as a source of serum cholesterol—it is also supplying an excess of 30 mg%. Therefore, to calculate the potential liver contribution this excess must be subtracted from total dietary contribution. The diet contributed 60 mg% of the serum cholesterol (30% × 200 mg%). Subtracting the 30 mg% excess from the 60 mg% total contribution, leaves 30 mg% (or 15%) as the potential liver contribution. Consider further, that there likely is a little replacement of extrahepatic cholesterol synthesis by dietary cholesterol, such that of this 15%, part very probably represents suppressed extrahepatic cholesterol synthesis. (This part is labelled "other" in Fig. 4.) Subtracting this part from the 15%, leaves only 10 or 5% that may be truly regarded as the contribution of the liver.

The authors wish to emphasize, however, that they do *not* interpret these data to mean that dietary cholesterol is of little importance in hypercholesterolemia in man. The data are interpreted to emphasize the importance of extrahepatic cholesterol synthesis as a normal source of serum cholesterol in man, but not necessarily the source of excess cholesterol in cases of hypercholesterolemia. It is interesting that rats and dogs are both generally regarded as rather resistant to dietary induction of hypercholesterolemia and atherosclerosis—yet the serum in these animals readily obtains its cholesterol from dietary cholesterol, even almost to exclusion of endogenous synthesis as a source of serum cholesterol. The extension of this paradox suggests that in man, dietary cholesterol cannot be ruled out as an important factor in genesis and maintenance of hypercholesterolemia simply because it is not quantitatively the most important source of serum cholesterol.

Summary. Normally active healthy adult volunteers ingested cholesterol-rich diets labelled with 4-C-14-cholesterol. The diets supplied enough cholesterol to suppress hepatic cholesterol synthesis, if the liver is as responsive to dietary cholesterol in man as in lower animals. Radioactivity of serum cholesterol permitted direct calculation of amount of serum cholesterol coming from the diet, which should equal the amount that would come from the liver if the diet supplied none. This amount was found to be 24 to 31%. Presumably, then, at least ¾ of serum cholesterol of these human subjects was derived from cholesterol synthesis in extrahepatic tissues.

The authors are greatly indebted to Joanne Woods, Juana Alvarez, and Hellen Ellis for expert technical assistance.

1. Gould, R. G., *Am. J. Med.,* 1951, v11, 209.

2. Kritchevsky, D., *Cholesterol,* New York, John Wiley & Sons, 1958, pp88-92.

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

4. Morris, M. D., Chaikoff, I. L., Felts, J. M., Abraham, S., Fansah, N. O., *ibid.,* 1957, v224, 1039.

5. Tannent, D. M., Zanetti, M. E., Atkinson, D. I., Kuron, G. W., Opdyke, D. E., *ibid.,* 1957, v228, 241.

6. Davis, C. B., Jr., Cox, G. E., Taylor, C. B., Cross, S. L., *Surg. Forum,* 1959, v9, 486.

7. Eckles, N. E., Taylor, C. B., Campbell, D. J., Gould, R. G., *J. Lab. and Clin. Med.*, 1955, v46, 359.

8. Cox, G. E., Counts, M., Wolski, J., Alvarez, J., Taylor, C. B., *Circulation*, 1958, v18, 493.

Received January 22, 1960. P.S.E.B.M., 1960, v103.

Phosphomannose Isomerase Activity in a Spectrum of Normal and Malignant Rat Tissues.* (25665)

DONALD E. KIZER AND THOMAS A. MCCOY

Biomedical Division, Samuel Roberts Noble Foundation, Ardmore, Okla.

Mannose catabolism has been observed with slices of Flexner-Jobling carcinoma(1), with Ehrlich ascites cells(2) and with Krebs-2 ascites cells(3). Yushok(3) considered anaerobic mannose catabolism to be mediated by phosphomannose isomerase (PMI) and postulated this enzyme to be rate limiting in mannose catabolism. Recently, hexosamine synthesis from mannose-6-phosphate (M-6-P) and glutamine by Walker carcinosarcoma 256 homogenates was reported(4). It was concluded that the Walker tumor contained PMI or that mannosamine was formed(4). Since these observations indicated entry of mannose into glucose metabolic pathways of malignant tissues, it was important to (a) determine whether tumors possessed PMI, (b) compare PMI activity of malignant tissue to that of normal tissue and (c) determine whether PMI activity was altered during carcinogenesis.

Materials and methods. Transplanted tumors were carried in female Holtzman rats as previously described(5), while primary hepatomas were induced by feeding 0.06% 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) in a semi-synthetic diet described by Medes, *et al.*(6). Since PMI converts M-6-P to fructose-6-phosphate (F-6-P)(7), formation of F-6-P, determined by method of Roe (8), was used to follow extent of reaction. Tissues were homogenized in 0.1 M acetate buffer pH 5.5(9) and centrifuged at 18,000 g for 60 min at 0°C. The supernatant, containing 1-2 mg/ml of protein, was added to a reaction mixture containing 0.03 M-6-P and 0.1 M acetate buffer. Protein concentration was determined by the method of Lowry(10). Aliquots for analyses were removed at appropriate intervals and total incubation period was 10 min at 38°C. A linear relationship between enzymatic activity and time of incubation was obtained. Since an activity maximum was observed at pH 5.4 to 5.5 with both 0.1 M acetate buffer and 0.1 M tris-maleate buffer, the reaction mixture was adjusted to pH 5.5. A unit of enzyme activity is defined as that which produced 0.1 μmole F-6-P mg protein/10 min. The barium salt of M-6-P† was freed of barium by treatment with excess sulfuric acid and, within sensitivity of the Roe method(8), was free of F-6-P.

Results. All normal and malignant tissues studied had PMI activity and the data are summarized in Table I. PMI activity of the Jensen sarcoma and Walker tumor was the same as that of kidney or brain but was significantly higher than the activity of muscle and spleen. Since both of these tumors were grown as intramuscular transplants and moderate contamination with muscle tissue was inevitable, PMI activities reported may be conservative.

Although characteristics of PMI from rabbit muscle(11), brewers yeast(7) and pig erythrocytes(12) were reported, this enzyme from malignant tissues has received limited attention. Thus, purification and inhibition studies were conducted with the PMI from the Walker tumor. Successive fractionations with ammonium sulfate resulted in an approximate 20-fold increase in activity; however, extensive contamination with phosphoglucose isomerase (PGI) persisted. Methanol fractiona-

* The authors express their appreciation to Boyd Howell for technical assistance.

† Nutritional Biochemicals Corporation, Cleveland, O.