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Research Article

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Serum Cholesterol Esterifying and Cholesteryl Ester Hydrolyzing Activities in Liver Diseases: Relationships to Cholesterol, Bilirubin, and Bile Salt Concentrations

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ABSTRACT Patients with acute hepatitis and chronic alcoholic liver disease had decreased net serum cholesterol esterifying activity (CEA) which correlated positively with the percentages and concentrations of cholesteryl esters in their serum. These cholesterol parameters also correlated negatively with serum bilirubin concentrations, but bilirubin added to sera in vitro failed to influence CEA. The decreased net CEA in the patients was not due to its inhibition by serum bile salts. The sera from five patients catalyzed a net hydrolysis of cholesteryl esters rather than a net esterification of free cholesterol. Since serum cholesteryl ester hydrolase activity may also have been present in the patients with decreased CEA, net CEA cannot be equated with the activity of lecithin–cholesterol acyl transferase (LCAT) in patients with liver disease. The relative contributions of LCAT and cholesteryl ester hydrolase activities to CEA in disease states remain to be evaluated by mutually independent assays. Nevertheless, the correlations found between net CEA and the concentrations and percentages of cholesteryl esters support the concepts that serum cholesterol esterifying activity is physiologically important in the formation of serum cholesteryl esters and that decreased CEA is one mechanism for the decreased level of cholesteryl esters seen in patients with liver diseases.

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INTRODUCTION

When normal serum or plasma is incubated in vitro, cholesterol is esterified by the enzymatic transfer of a fatty acid from the beta position of lecithin to cholesterol (1, 2). The responsible enzyme is a lecithin–cholesterol acyl transferase (LCAT). Glomset has discussed the hypothesis that LCAT, acting intravascularly, catalyzes the synthesis of a major portion of serum cholesteryl esters (3). In normal sera, the over-all cholesterol esterifying activity (CEA) has been equated with LCAT activity (3, 4).

In pathological sera, the CEA may not represent LCAT activity alone, since tissue cholesteryl ester hydrolases may enter the blood stream in disease states. From Glomset's hypothesis, however, the concentration of cholesteryl esters may depend upon the over-all cholesterol esterifying activity of serum whatever factors influence it. If this is so, decreased concentrations or percentages of esterified cholesterol in patients with hepatobiliary diseases might be expected to correlate with the net serum cholesterol esterifying activity (CEA). To test this, we compared the serum CEA and the concentrations of the total, the unesterified, and the esterified cholesterol in normal sera and in sera from patients with acute viral hepatitis and chronic alcoholic liver disease.

The CEA correlated with the per cent and the concentration of serum cholesteryl esters, but both the CEA and the cholesteryl ester levels also correlated negatively with serum bilirubin concentrations. We therefore studied the effects of bilirubin in vitro on CEA. In addition, since bile salts are known inhibitors of CEA in vitro, we examined the relationships of bile salt concentrations to CEA.

METHODS

Subjects. 25 control subjects, aged 22–36 (median, 29), had no evidence of disease. 28 patients, aged 15–56 (median, 28), had viral hepatitis diagnosed by history and clinical and laboratory findings. Of these, 15 were classified as having serum hepatitis, and 13 probably had infectious hepatitis. We studied 16 patients with viral hepatitis during the stage of increasing icterus and 12 during convalescence. 25 other patients, aged 30–57 (median, 48), were alcoholics and had clinical and laboratory signs of chronic alcoholic liver disease. We studied them from 2 to 87 days after admission (median, 19) when their disease activity was stable. We excluded patients from the study if the concentration of their serum total cholesterol was greater than 275 mg/100 ml, if their FBS was greater than 120 mg/100 ml, or if their BUN was greater than 21 mg/100 ml.

Blood drawing. Blood was drawn after the patients had fasted for 12 hr. The sample was iced at once, allowed to clot, and centrifuged while still cold. The serum was separated immediately into 3–5-ml aliquots and frozen at −15°C until analysis. It was stored a maximum of 1 month prior to assay. Blood samples were also sent for the routine analysis of blood sugar, blood urea nitrogen, bilirubin, alkaline phosphatase, serum glutamic-oxaloacetic transaminase (SGOT), total protein and albumin, prothrombin time, and a hemogram.

Incubation of serum to determine its cholesterol esterifying activity. A serum sample was thawed in a 37°C water bath, and 1 ml was extracted with ethanol-acetone for the determination of total and unesterified cholesterol. The remaining serum was incubated at 37°C for 5 hr, after which another 1 ml was extracted for cholesterol analysis.

Cholesterol and bile salt analyses. The concentrations of total and unesterified cholesterol in the samples were determined by the method of Sperry and Webb (5). The concentrations of esterified cholesterol were calculated as the difference between the concentrations of the total and the unesterified cholesterol. Total serum bile salts were determined by the enzymatic method of Palmer (6).

Expression of the cholesterol esterifying and cholesteryl ester hydrolyzing activity of serum. This is expressed as ΔF, which is the difference in the concentration of unesterified cholesterol (in mg/100 ml) before and after incubation. When cholesterol was esterified, the concentration of unesterified cholesterol decreased during incubation, and ΔF is expressed as a negative value. When cholesteryl esters were hydrolyzed, unesterified cholesterol increased during incubation, and ΔF is expresssed as a positive value.

Effect of bilirubin in vitro on ΔF. 0.1 ml of 0.1 N NaOH was added to a test tube containing 2.34 mg of unconjugated bilirubin.[1] Immediately after the bilirubin was in solution, 10 ml of freshly thawed test serum was added. The contents were mixed, after which 0.1 ml of 0.1 N HCl was added and mixed. An equal amount of test serum was treated with base and acid without bilirubin. These sera were mixed to form appropriate test concentrations of bilirubin. Untreated sera provided a control. The addition of bilirubin did not change the pH of the test serum from that of the control serum. ΔF was determined in test and control sera from four normal subjects, two subjects with hepatitis, and one with cirrhosis.

Effects of bile salts in vitro on ΔF. Sodium glycocholate was prepared from recrystalized pure glycocholic acid by dissolving the latter in methanol, neutralizing it with NaOH, and recrystalizing the sodium salt from the methanol solution with ethyl acetate. The bile salt was dissolved in 0.03 N NaOH, and 0.15–0.3 ml of the solution was added to 3.0–6.0 ml of test sera, followed by equivalent amounts of HCl. Base and acid were added without bile salts to another aliquot of test sera. The aliquots were mixed to give final bile salt concentrations from 25 to 800 μmoles/100 ml. The effect of these concentrations of bile salts on ΔF was determined in test and control sera from three normal subjects, two subjects with hepatitis, and one with cirrhosis.

Statistical methods. We used duplicate determinations of cholesterol on 50 serum samples to estimate the random error in ΔF due to chemical methodology. From the 50 differences between the duplicate cholesterol determinations, we estimated the variance of a single determination of cholesterol according to the formula $s^2 = (X_1 - X_2)^2/2N$, where s^2 = the estimated variance of a single determination; X_1 = the value of the first duplicate; X_2 = the value of the second duplicate; and N = the number of samples (7). Since ΔF is expressed as the difference between two concentrations of cholesterol, one before and one after incubation, we estimated the standard deviation of ΔF due to variation in chemical technique by combining the estimated error of two single determinations of cholesterol, $SD_{\Delta F} = \sqrt{2s^2}$. Using $SD_{\Delta F}$ in the relationship $t \times SD_{\Delta F} = \Delta F$ with t 0.05 (d.f. = 50), we defined a value of ΔF that included 95% of the random error due to chemical technique. We considered an observed ΔF greater than this value to be greater than methologic error at the 0.05 probability level.

We used the t test for ungrouped data (8) to compare the means of the concentrations of the total, unesterified, and esterified cholesterol, the per cent of total cholesterol present as esterified cholesterol, ΔF, and bilirubin among the three study groups (normal subjects, patients with hepatitis, and patients with chronic alcoholic liver disease).

We tested for the following correlations (8): (*a*) in all study groups, for simple correlations of ΔF with each of the four parameters of cholesterol measurements (the per cent of total cholesterol present as cholesteryl esters, the concentrations of esterified, unesterified, and total cholesterol); (*b*) in the patient groups only, for simple correlations of ΔF with the concentrations of total bilirubin and total bile salts and with the activities of alkaline phosphatase and SGOT, and for simple correlations of total bilirubin concentrations with each of the four parameters of cholesterol measurements.

RESULTS

Methodologic error. The differences between duplicate determinations of cholesterol were independent of the cholesterol concentrations of the samples. The estimated variance of a single cholesterol determination was 1.55 mg/100 ml. The estimated standard deviation of ΔF due to random error in chemical technique was 1.8 mg/100 ml. The value of ΔF which included 95% of random error due to chemical technique was 3.6 mg/100 ml. Accepting a 0.05 probability of error, we considered an observed value of ΔF greater than 3.6 mg/100 ml to be greater than methodologic error.

Cholesteryl ester hydrolysis. The sera from four patients with hepatitis and from one patient with cirrhosis

[1] Fisher Certified Reagent; Fisher Scientific Company, Pittsburgh, Pa.

hydrolyzed cholesteryl esters rather than esterified cholesterol during incubation. These five were among the seven patients with the seven lowest percentages and concentrations of cholesteryl esters in the study (Table I and Figs. 1 and 2). Since cholesterol esterifying activity could not be measured in these samples, we excluded these patients from the correlations of ΔF with cholesterol.

Mean values in the study groups. Table II compares the means of the total, the unesterified, and the esterified cholesterol, the per cent of esterified cholesterol, ΔF, and the bilirubin concentrations in the three study groups. Compared with the normal, the patient groups had decreased concentrations of total cholesterol. The patients with chronic liver disease had lower concentrations of total cholesterol than did those with hepatitis. The concentrations of cholesteryl esters were reduced similarly in both patient groups. The percentages of cholesterol as cholesteryl esters were also decreased in the patients and were less in patients with hepatitis than they were in those with chronic liver disease. The mean value of unesterified cholesterol in the hepatitis patients was twice that of the normals and was greater than the mean value in the patients with chronic disease. The patients with hepatitis had a higher mean serum bilirubin concentration than did the patients with chronic liver disease. The mean of ΔF was less than normal in both patient groups. It was lowest in the patients with chronic liver disease.

The concentrations of total bile salts ranged from 0.3 to 30.2 (median, 8.3) μmoles/100 ml in 12 patients with hepatitis and from 2.4 to 21.7 (median, 9.1) μmoles/100 ml in six patients with chronic liver disease.

TABLE I

Cholesterol Values in Four Patients with Hepatitis and One with Cirrhosis Whose Sera Hydrolyzed Cholesteryl Esters

Patient	ΔF*	Esters	Esterified	Total
	mg/100 ml	*%*	*mg/100 ml*	*mg/100 ml*
1	+2.4	9.5	8.0	84.4
2	+9.2	14.4	16.4	113.2
3	+5.5	10.2	9.1	89.2
4	+8.8	6.0	10.0	167.0
5 (cirrhosis)	+11.2	15.0	24.0	160.0

* A positive Δ*F* indicates an increase in the concentration of free cholesterol after incubation, due to hydrolysis of cholesteryl esters.

Correlations. No correlations were found between alkaline phosphatase or SGOT activities and ΔF. In the normals, none of the parameters of cholesterol measurements correlated with ΔF.

Table III presents all the simple correlation coefficients in the patients. In both patient groups, the per cent of esterified cholesterol and the concentration of esterified cholesterol correlated with ΔF. In both groups, both of these parameters also correlated negatively with the concentration of serum bilirubin, and in both groups, the bilirubin correlated with ΔF. In addition, in the patients with hepatitis, the unesterified cholesterol correlated with bilirubin. No other correlations were significant. The per cent of cholesterol present as cholesteryl esters correlated

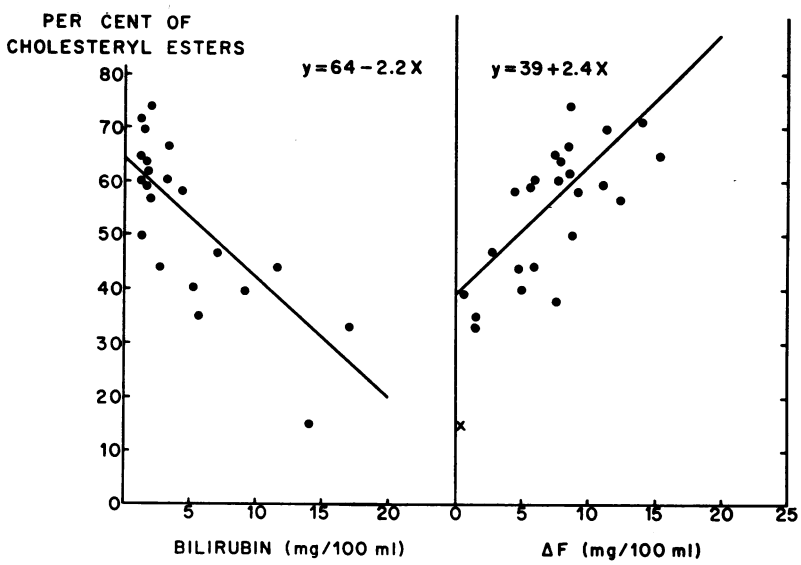

FIGURE 1 Regressions of the per cent of cholesteryl esters on ΔF and on bilirubin in patients with chronic alcoholic liver disease. (X = case with cholesteryl ester hydrolase activity, not included in the correlation.)

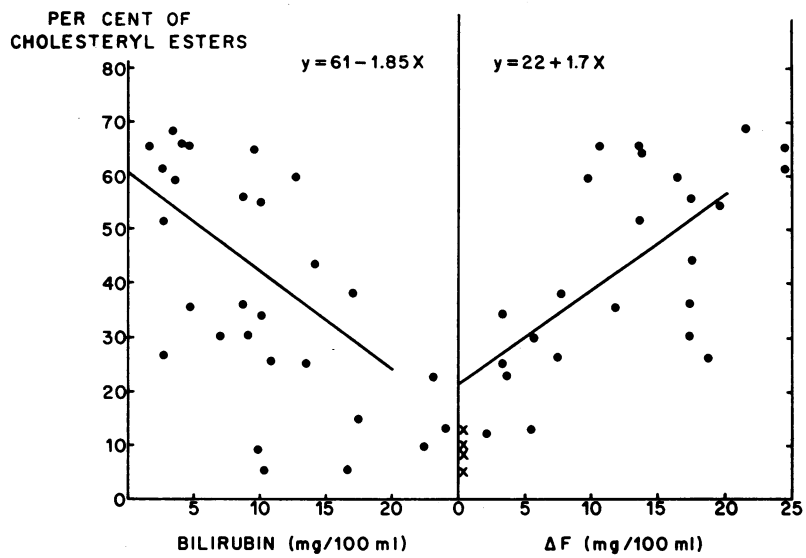

FIGURE 2 Regressions of the per cent of cholesteryl esters on ΔF and on bilirubin in patients with viral hepatitis. (X = cases with cholesteryl ester hydrolase activity, not included in the correlation.)

more closely with ΔF and with bilirubin than did the concentrations of cholesteryl esters.

ΔF did not correlate with the concentrations of total serum bile salts in either patient group.

Regression equations. Scattergrams of the per cent of cholesteryl esters plotted against bilirubin and ΔF, together with their regression equations, show these relationships in Fig. 1 for the patients with chronic liver disease, and in Fig. 2 for the patients with hepatitis.

The regression equations of the per cent of cholesterol present as cholesteryl esters on bilirubin were similar in the two patient groups (Figs. 1 and 2). If the groups were combined, the correlation coefficient of these parameters was greater than it was in either group alone ($r = -0.722$).

The regression coefficients of the per cent of cholesteryl esters on ΔF were also similar in the two patient groups, but the Y intercept of the regression equation was less in the patients with hepatitis than it was in those with chronic liver disease. Thus, although a given increment in ΔF resulted in a similar increment in the per cent of cholesterol present as cholesteryl esters in both patient groups, for any absolute value of ΔF, the absolute value for the per cent of esters was less in the patients with hepatitis than it was in the patients with chronic liver disease. Due to this difference in the Y intercepts of the regression equations between the patient groups, the correlation coefficient of the per cent of cholesteryl esters and ΔF was less when the groups were combined ($r = 0.420$) than it was in either group alone, yet it was still significant.

Patients in the icteric phase as opposed to the convalescent phase of hepatitis had no differences in the concentrations of cholesterol or bilirubin, in ΔF or in the correlations of these parameters.

TABLE II

Mean Concentrations (±SEM) of Total, Unesterified, and Esterified Cholesterol, Per Cent of Cholesteryl Esters, ΔF, and Bilirubin in Each Study Group

n	Total	Unesterified	Esterified	Per cent of esters	ΔF	Bilirubin
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	%	*mg/100 ml*	*mg/100 ml*
25 Normal	*209* (7)*	*56* (2)	*153* (5)	*73* (0.3)	*−15.6* (0.8)	*<1.2*
28 Hepatitis	*187* (8)	*112* (7)	75 (9)	*39* (4.0)	*−10.0* (1.4)	*10.1* (1.1)
25 Chronic liver disease	*160* (10)	*69* (5)	92 (8)	*55* (2.5)	*−7.0* (0.7)	*4.0* (0.9)‡

* Values in italics are significantly different ($P < 0.05$) from each of the other two means in the same column.
‡ n = 22 (see Table III).

TABLE III

Simple Correlation Coefficients—Chronic Liver Disease and Hepatitis

	Per cent of esters	Esterified cholesterol	Unesterified cholesterol	Total cholesterol	ΔF
Chronic liver disease (n = 21)*					
ΔF	*0.772*‡	*0.612*	*−0.365*	0.316	—
Bilirubin	*−0.740*	*−0.437*	*0.542*	−0.089	*−0.700*
Hepatitis (n = 25)*§					
ΔF	*0.662*	*0.537*	−0.498	0.050	—
Bilirubin	*−0.645*	*−0.596*	*0.430*	−0.187	*−0.603*

* Patients whose sera hydrolyzed cholesteryl esters are omitted. Bilirubin concentrations were not available in three patients with chronic liver disease. If these are included, the correlation coefficients of ΔF with the per cent esters and the esterified cholesterol are, respectively, 0.720 and 0.594. Neither is significantly different from the corresponding coefficients in the table.

‡ Italics indicate significant correlations:

When n = 21, if $r > 0.433$, $P < 0.05$; if $r > 0.549$, $P < 0.01$.

When n = 25, if $r > 0.404$, $P < 0.05$; if $r > 0.515$, $P < 0.01$.

§ If the patients with hydrolase activity are included, assuming ΔF = zero in these patients, the correlations of ΔF with the per cent and concentration of cholesteryl esters is strengthened (r = 0.780 and r = 0.698, respectively), while those of bilirubin with the same parameters are not affected (r = − 0.638 and r = −0.608, respectively).

Possible direct effects of bilirubin on ΔF were examined in experiments summarized in Table IV. Bilirubin added to sera in vitro in concentrations up to 400 μM did not influence ΔF in either normal or pathological sera.

The effect of sodium glycocholate added to sera in vitro was tested in experiments summarized in Table V. ΔF was not significantly inhibited at glycocholate concentrations of 100 μmoles/100 ml or less, whereas higher concentrations progressively inhibited ΔF.

DISCUSSION

The esterification of serum cholesterol in vitro is catalyzed by LCAT, but the reaction is influenced by its lipoprotein substrates (3). The reaction is nearly complete after 24–48 hr. Assays of cholesterol esterification at these times are influenced more by the lipoprotein substrates than are assays of initial reaction rates, which more closely reflect LCAT activity. Standard assays for unesterified cholesterol are not sensitive enough to measure true initial reaction rates, which requires assays based on the esterification of radioactive cholesterol incorporated artificially into standard lipoprotein substrates or into serum lipoproteins.

We wished to measure the rate of cholesterol esterification early in the course of the reaction, but in as natural a state as possible. Therefore, we chose the sub-

TABLE IV

Effect of Bilirubin In Vitro on ΔF

Subject	Pretest bilirubin concentration	ΔF*					
		Control	Base plus acid (without bilirubin)	Concentration of added bilirubin			
				400 μM	300 μM	200 μM	100 μM
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*			
Normal	<1.2	18.0	16.0	18.5	17.2	14.1	18.3
Normal	<1.2	21.0	19.7	21.1	20.8	19.6	22.0
Normal	<1.2	20.8	20.0	22.2	21.5	19.4	20.5
Normal	<1.2	19.6	20.2	21.6	17.4	19.6	18.5
Hepatitis	14.0	1.5	0.4	2.2	1.8	+0.5‡	1.0
Hepatitis	7.2	17.5	16.2	17.2	18.5	17.6	17.5
Chronic	3.2	7.8	7.2	7.8	8.6	7.0	7.4

* Values are negative, except as noted.

‡ The concentration of free cholesterol increased during incubation.

TABLE V

Effect of Sodium Glycocholate In Vitro on ΔF

Subject	Pretest bile salt concentration	ΔF*						
		Control	Base plus acid (without bile salt)	Concentration of added bile salt in μmoles/100 ml				
				25	100	200	400	800
	μmoles/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*				
Normal	0.2	16.2	17.8	15.8	15.2	12.0	6.2	1.8
Normal	0.5	17.3	17.0	16.8	15.0	12.1	7.2	+0.2‡
Normal	0.8	19.1	22.0	17.3	18.3	13.6	6.3	1.6
Hepatitis	5.0	8.8	7.9	9.5	7.0	5.9	3.5	2.2
Hepatitis	22.0	10.2	11.1	11.3	9.8	7.5	4.1	+1.6‡
Chronic	3.2	6.2	5.9	7.3	6.0	4.5	2.7	0.8

* Values are negative, except as noted.

‡ Concentration of free cholesterol increased during incubation.

ject's own serum as substrate and measured the change in native unesterified cholesterol over the minimum period necessary to obtain accuracy by standard methods. The reaction is not strictly linear for 5 hr, thus if reaction rates per unit time are calculated from our data, they will be somewhat less than the true initial reaction rates. We attempted to avoid subjects with abnormal serum lipoproteins by excluding subjects with hyperlipemia, diabetes, or azotemia from the study.

Five patients had gross evidence of cholesteryl ester hydrolase activity in their serum. This activity is not present in normal human serum (3) but is present in liver tissue (9), and it may have entered the blood stream from the damaged liver. The lack of cholesterol esterification in these five patients does not necessarily imply that LCAT activity was absent from their serum, nor does the decreased esterification in the other patients necessarily represent only a decrease in LCAT activity, but it may represent a balance between cholesteryl ester hydrolase and LCAT activities. Direct proof of this concept will require the use of independent assays for both LCAT and cholesteryl ester hydrolase activities. Until such studies are carried out, we cannot equate CEA with LCAT activity in disease states.

Although CEA may not reflect only LCAT activity, the concentration of the serum cholesteryl esters may still depend on the over-all net CEA, whatever factors influence it, and correlations between CEA and the cholesteryl esters in liver diseases can still bear on the hypothesis that CEA is important physiologically in the formation of serum cholesteryl esters.

Turner and Pratt (10), Turner, McCormack, and Richards (11), Castro Mendosa and Jimenez Diaz (12), and Bertolini, Guardamagna, and Massari (13) found decreased cholesterol esterifying capacity in the sera of some patients with hepatobiliary diseases, but they found no correlations between the esterifying capacity and the concentrations or percentages of cholesteryl esters. These authors expressed the serum esterifying capacity as the change in the *per cent* of cholesteryl esters, which we now know fails to reflect accurately the magnitude of the esterification reaction (3). In seeking correlations between the cholesteryl esters and ΔF in our patients, we expressed the CEA as the *absolute difference* in the concentration of unesterified cholesterol before and after incubation.

The percentages and the concentrations of cholesteryl esters correlated with ΔF in both patient groups. The Y intercept of the regression equation of the per cent esters on ΔF was greater in the patients with chronic liver disease than it was in the hepatitis patients. The correlation and regression coefficients were similar in both patient groups, but the mean ΔF in patients with hepatitis was greater than the mean ΔF in the patients with chronic disease, and for a given ΔF, the per cent esters was less in hepatitis patients than in those with chronic disease. Due to this difference between the groups, if they were combined, the correlation between the per cent esters and ΔF was weakened, but it was still significant. We do not know why the Y intercepts were different between the patient groups; the difference may be related to the greater concentrations of unesterified cholesterol in the hepatitis patients. We deliberately studied the correlations in dissimilar patient populations, and considering the differences in etiology, in pathogenesis, in age incidence, chronicity, and prognosis between viral hepatitis and chronic alcoholic liver disease, we feel the similarities of the correlation and regression coefficients of the per cent esters on ΔF in both patient groups attest their significance.

The concentrations of serum bilirubin correlated with the concentrations and the percentages of cholesteryl

esters. Zieve has previously reported this association (14). Serum bilirubin concentrations also correlated with ΔF. Due to the mutual correlations of the cholesterol parameters with ΔF and with bilirubin, we speculated that bilirubin might influence ΔF directly. However, bilirubin had no effect on ΔF in vitro, and we cannot implicate bilirubin, directly at least, in the relationships between the cholesteryl esters and ΔF. Compared with the patients with chronic liver disease, hepatitis patients who had higher bilirubin concentrations also had greater mean ΔF values, which is also inconsistent with any direct effect of bilirubin on ΔF.

Since bile salts are known inhibitors of LCAT in vitro, we measured the effects of sodium glycocholate on CEA in vitro and related the results to the concentrations of bile salts in the patients. In vitro, sodium glycocholate failed to inhibit CEA at concentrations of 100 μmole/100 ml or less. Since the highest concentration of total bile salts in the patients was 30.2 μmole/100 ml, unless other bile salts affect CEA markedly differently than does glycocholate, it is evident that the decreased CEA in the patients was not due to inhibition by serum bile salts.

The correlations between CEA and the concentrations and percentages of cholesteryl esters in patients with liver disease support the concepts that CEA is important physiologically in the formation of serum cholesteryl esters, and that decreased CEA is one mechanism responsible for the decreased cholesteryl esters seen in liver diseases.

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