and 2 or more bags to permit comparison in terms of a standard plasma. It has been demonstrated that under these conditions dialysis of the free cortisol through the membrane is rapid. Dissociation of the steroid-protein complex, and therefore equilibrium, is achieved. Specific activity in each compartment is equivalent. Then, by direct measuring the radioactivity in each compartment, an accurate determination of relative total binding capacity may be made.

The procedure has practical significance. Sandberg and Slaunwhite(3) reported that diluted pregnancy plasma has a higher binding capacity than normal, but Daughaday's data from undiluted plasma, dialyzed against a control plasma, revealed that number of available binding sites was approximately the same(2). The difference is readily resolved because the former authors were at least partially measuring total capacity while the latter was determining free binding sites almost exclusively. Thus, the higher concentrations of cortisol which occur in pregnancy plasma were occupying some of the available sites in the latter incubations, and the amount of free sites (and possibly free cortisol) was equivalent.

The physiologic significance of the steroid-protein complex remains undetermined. Presumably, the complex may be physiologically inactive; in this case, it might act as a buffer against excessive concentrations of steroid. On the other hand, the slow rate of dissociation *in vitro* would mitigate against its being a reservoir of readily available steroid. It is possible, however, that the organism has a more efficient method of dissociating the complex or that the complex may be effective in certain systems as an entity. Finally, if one considers synthesis of this protein as an artifact of pregnancy (and it has been demonstrated to be elicited by estrogen in both males and females), it may have no "role" whatsoever and the higher levels of corticoids found in the plasma in pregnancy may reflect nothing other than homeostatic response to lack of available unbound steroid.

*Summary.* Simultaneous dialysis of 2 diluted plasma samples in the same system at 37°C is an acceptable method for measurement of the relative amounts of corticosteroid binding protein present. Dissociation of the steroid-protein complex in undiluted samples is slow even at 37°. The kinetics of binding do not permit attainment of equilibrium at 4°, even when the plasma is diluted. The differences noted by previous investigators in comparison of pregnant and non-pregnant samples are explainable by this incomplete equilibrium.

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## Factors Affecting Extractability of Cholesterol from Lyophilized Sera by Cold Chloroform.* (26199)

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The significance of the fraction of serum cholesterol which is extracted when lyophilized serum is shaken with cold *anhydrous* chloroform for 3 hours has been studied in

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our laboratory for some years. The excellent reproducibility of replicates suggests that the technic measures a definite fraction of total cholesterol. The possible relationship between atherosclerosis and high values for this fraction, designated "readily extractable" cholesterol (REC), has been emphasized(1,2). Although concentration of this REC fraction increased in most subjects as total serum cholesterol increased, the marked individual variations suggested that other factors may be involved. We wish now to report on 4 additional factors which affected its concentration: (a) cholesterol and triglyceride content of whole serum and chylomicra ("chylomicra" as used here represents material rising to the surface when serum is centrifuged in a refrigerated centrifuge at 20,000 × g for 2 hours), (b) addition of sodium salts of fatty acids and of bile salts to the serum prior to lyophilization, (c) addition of crystalline human serum albumin to the serum prior to lyophilization, and (d) moisture in the chloroform.

*Methods.* The "readily extractable" cholesterol (REC) of whole serum was determined by the technic described by Forbes *et al.*(1). Triglycerides of serum and infranate were determined by a modification of a procedure described by Van Handel and Zilversmit(3). Two 3 ml aliquots of the chloroform extract were evaporated, and 0.5 ml of 0.4% alcoholic KOH was added to one tube and 0.5 ml of alcohol to the other, followed by heating at 60-70°C for 30 minutes. Two ml of water and 0.1 ml of 10 N $H_2SO_4$ were then added, followed by 0.5 ml of saturated $KIO_4$. After 7 minutes, 0.5 ml of M $NaAsO_2$ was added, and each tube diluted to 10 ml with water. One ml aliquots were transferred, 10 ml chromotropic acid (prepared as described by Van Handel and Zilversmit) added to each, and the solutions heated in a boiling water bath away from direct light for 30 minutes. After cooling, color was read at 570 mμ. Total cholesterol content of serum and infranate were determined by a minor modification of a previously described procedure(4).

*Results.* Part (a). Effect of cholesterol and triglyceride content of serum and chylomicra: Experimental results are shown in Table I. Considering average serum cholesterol and triglycerides, there was a general tendency for REC concentration to increase as concentration of these 2 fractions in-

TABLE I. Ranges of Triglyceride and Cholesterol Concentrations of Whole Serum and Chylomicra and Whole Serum "Readily Extractable" Cholesterol (REC).

| No. of subjects | No. of sera | Whole serum ranges | | Chylomicron ranges | | Whole serum REC ranges |
|---|---|---|---|---|---|---|
| | | Cholesterol | Triglyceride | Cholesterol | Triglyceride | |
| | | mg % | | | | |
| Heterogeneous groups* (arranged in order of increasing triglyceridemia) | | | | | | |
| 51 | 54 | 164–362 (275) | 51–172 (113) | 0–37 (7) | 0–65 (21) | 14–44 (29) |
| 47 | 52 | 169–584 (325) | 184–300 (234) | 0–68 (15) | 0–198 (50) | 16–71 (38) |
| 28 | 31 | 184–465 (330) | 309–492 (373) | 0–48 (23) | 12–203 (87) | 24–336 (72) |
| 9 | 12 | 230–765 (531) | 539–960 (674) | 33–429 (190) | 164–495 (274) | 59–556 (244) |
| 3 | 7 | 385–600 (517) | 1100–1460 (1334) | 121–385 (196) | 492–792 (691) | 323–502 (430) |
| Familial hyperlipemic subjects | | | | | | |
| 3 | 14 | 263–913 (433) | 1280–9630 (3239) | 195–865 (384) | 1145–9280 (3060) | 112–840 (347) |
| Nephrotic syndrome subjects | | | | | | |
| 9 | 13 | 312–1019 (606) | 100–620 (337) | 0–104 (31) | 0–105 (31) | 165–959 (544) |

* Includes normals, subjects with arteriosclerotic heart disease with and without myocardial infarction, as well as a variety of other conditions.

( ), mean values.

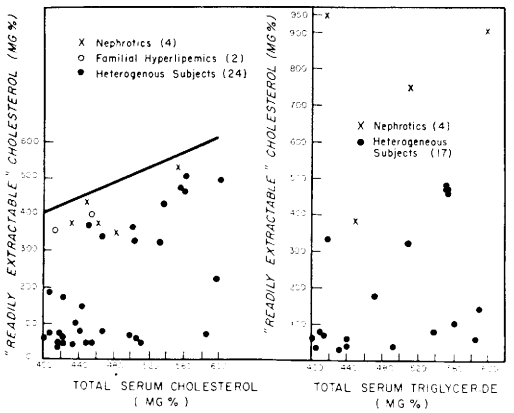


FIG. 1 FIG. 2

"Readily extractable" cholesterol of serum plotted against total cholesterol (Fig. 1) and triglycerides (Fig. 2).

creased. However, there was such marked individual variability from this generalization that it appeared that factors other than total cholesterol and triglyceride probably affected REC concentration. To emphasize this variability, REC values on sera with total serum cholesterols between 400 and 600 mg% (chosen arbitrarily for illustration) are plotted against total serum cholesterol in Fig. 1. Familial hyperlipemics and nephrotics consistently showed a high percentage of total serum cholesterol in the REC fraction. The heterogeneous group showed wide variability and differences could not be correlated with the disease condition. Similar widespread distribution is shown when REC values are plotted against total serum triglycerides in Fig. 2. Turning attention to chylomicron values, REC concentrations rose in general as the amounts of triglycerides and cholesterol in chylomicra increased. However, it was particularly interesting that, in the singular instance of the nephrotic subjects, REC concentration was consistently very high even though amounts of cholesterol and triglycerides in chylomicra were small. A possible explanation for this finding is presented in the discussion.

Part (b)—Effect of addition of sodium salts of fatty acids and of bile salts: Macheboeuf and Tayeau(5) showed that addition of soaps to serum increased ether extractability of lipids. We consequently decided to determine what effect, if any, addition of sodium salts of various fatty acids to the serum prior to lyophilization would have on REC values. The effects of sodium cholate and sodium glycocholate were also studied. Experimental results are shown in Table II. Sodium butyrate exerted no definite effect, but with lengthening of the carbon chain, activity became quite evident. Sodium stearolate, even at a concentration of only 0.4%, caused approximately 70% of total serum cholesterol to appear in the REC fraction. Sodium cholate was practically inactive but sodium glycocholate showed some effect. Since commercial sodium glycocholate was used, it was possible that impurities in the preparation may have accounted for the effect noted.

Part (c)—Effect of addition of crystalline human serum albumin: Since addition of sodium salts of long chain fatty acids markedly increased cholesterol extractability, a study was carried out on the effect on REC values of addition of crystalline human serum albumin to the serum prior to lyophilization. If the presence of non-albumin bound free fatty acids in the serum were a factor in producing elevated REC values, then their removal by combination with added albumin would be expected to lower REC concentration. Experimental results are shown in Table III. Albumin caused a very marked drop in REC values of all sera with high REC concentrations. A definite drop was also noted in some sera with low REC con-

TABLE II. Effect of Various Surface Active Agents on "Readily Extractable" Cholesterol.

| Total cholesterol | Cholesterol extracted from lyophilized sera in presence of various additives | | | | Substance added |
|---|---|---|---|---|---|
| | 0 mg | 2 mg* | 5 mg* | 10 mg* | |
| | mg % | | | | |
| 255 | 29 | 29 | 27 | 25 | Na butyrate |
| 265 | 24 | 42 | 46 | 235 | Na caprylate |
| 265 | 24 | 50 | 196 | 243 | Na laurate |
| 247 | 33 | 167 | 246 | — | Na stearolate |
| 266 | 32 | 94 | 111 | 186 | Stearolic acid |
| 272 | 24 | 43 | 41 | 57 | Na glycocholate |
| 272 | 24 | 39 | 36 | 32 | Na cholate |

* mg added/.5 ml of serum.

All sera were incubated for 3 hr at 37°C after addition of the respective agent and before lyophilization. Control tubes were similarly incubated.

TABLE III. Effect of Addition of Albumin to Serum on REC Concentration.

| Cholesterol | | REC values following addition of different amounts of albumin to serum | | | | | |
|---|---|---|---|---|---|---|---|
| Serum | Chylomicron | 0 mg | 20 mg* | 40 mg* | 80 mg* | 100 mg* | |
| mg % | | mg % | | | | | |
| 279 (247–306) | 0 | 30 (22–38) | 26 (20–32) | 22 (20–26) | 19 (17–20) | 16 (13–20) | Avg of 6 sera with normal REC values |
| 357 | 27 | 66 | 37 | 33 | — | — | Sera from various subjects with elevated REC values but excluding those with nephrotic syndrome |
| 465 | 85 | 392 | — | 123 | — | — | |
| 475 | 50 | 123 | — | 52 | 39 | — | |
| 467 | 187 | 264 | — | 60 | 55 | 55 | |
| 391 | 98 | 188 | 97 | 66 | — | — | |
| 858 | 136 | 860 | — | 676 | 230 | 117 | Nephrotic syndrome |
| 810 | 0 | 752 | — | 76 | 45 | 39 | " " |

* mg albumin/.5 ml of serum. ( ), range.

centrations but in no case did REC value drop to zero.

Part (d)—Effect of traces of moisture in the chloroform used for REC extraction: The results (Table IV), revealed that moisture (.02% $H_2O$) exerted no definite effect on REC values of most sera with low REC concentrations, but markedly increased it in sera with high REC concentrations, emphasizing the importance of using anhydrous chloroform. The dried zeolite used for cholesterol and triglyceride determinations is an excellent drying agent for this purpose.

*Discussion.* In view of the increase in REC obtained when soaps were added to sera with low REC concentrations and the decrease in REC when serum albumin was added to sera with high REC concentrations, it appeared that presence of non-albumin bound free fatty acids markedly affected cholesterol extractability from lyophilized sera. Albumin bound fatty acids apparently played no role.

TABLE IV. Effect of Moist Chloroform on REC Value.

| No. of sera | Avg total cholesterol | Avg REC value using | |
|---|---|---|---|
| | | Anhydrous chloroform | Chloroform containing .02% water |
| | mg % | | |
| 9 | 290 (237–394) | 28 (21–36) | 47* (22–167) |
| 4 | 444 (359–508) | 70 (50–122) | 350 (317–378) |

* Avg excluding 167 value was only 32.
( ), range.

The fact that most of the cholesterol of the nephrotic sera was "readily extractable" while little of it could be accounted for by chylomicron cholesterol seemed particularly interesting. Shafrir(6) found that concentration of free fatty acids (FFA) in nephrotic sera did not differ significantly from that of normal sera, but because of the low albumin content, only 35.7% (12 sera) was albumin bound as compared with 77.6% in 10 normal sera. In both cases, the remaining FFA was presumably bound to lipoproteins. If one assumes that lipoprotein bound free fatty acids would affect REC concentrations in a manner comparable to addition of long chain fatty acids to normal sera, an elevated REC concentration in nephrotic sera would be expected.

In a few sera from the familial hyperlipemic group the amount of cholesterol present in chylomicra was higher than the REC fraction. We do not believe this to be due to experimental error, although such cannot be excluded. The presence of high density lipoproteins in chylomicra, as suggested by Lindgren *et al.*(7), may be a partial explanation since previous work showed that $\alpha_1$-lipoproteins contribute little, if any, cholesterol to the REC fraction(8).

The tendency for REC concentration to increase with an increase in triglyceride content of the sera can probably be accounted for in 2 ways: first, an increase in chylomicron cholesterol and second, an effect of lipoprotein bound FFA. Shafrir(6) observed a definite tendency for the percentage of total

FFA bound with albumin to be reduced with an increase in total serum lipids. A concomitant increase in non-albumin bound free fatty acids along with an increase in serum triglycerides in accordance with the results herein presented would be expected to lead to an increase in REC values. The observations that traces of moisture in the chloroform used for extraction increased REC values in sera with high REC concentrations, but seldom in sera with low REC values suggests that the moisture effect may also be related to the amount of non-albumin bound free fatty acids, although we have no direct evidence that such is the case.

*Summary.* A study has been conducted on factors which affect the "readily extractable" cholesterol concentrations in serum. The results showed that (a) in general, REC values increased as triglyceride and cholesterol contents of the serum and chylomicra increased, but a notable exception was high REC values in nephrotic subjects in whom chylomicron contents of cholesterol and triglyceride were low, (b) addition of the sodium salts of long chain fatty acids to serum prior to lyophilization markedly increased cholesterol extractability, (c) addition of crystalline human serum albumin to sera with high REC concentrations markedly reduced REC values; the albumin also reduced REC concentration in most normal sera but in no case did it completely disappear, (d) traces of moisture in the chloroform used for the extraction had little or no effect on most sera with low REC concentration but markedly increased it in sera with high REC concentration. It is suggested that the concentration of non-albumin bound free fatty acids may play a major role in regulating REC concentrations in various sera.

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## Detection of Anti-HeLa Antibodies in Rabbit Antiserum by Indirect Hemagglutination* (26200)

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Production of species-specific antibodies in animals injected with cells of various mammalian cultures has been described(1-4). These antiserums agglutinated erythrocytes of the same animal species(1,2), or were effectively cytotoxic *in vitro* for cells of homologous lines(3,4). These and other findings(5) substantiate the validity of immunologic identification of the species of mammalian cells in continuous culture.

Extreme sensitivity of the antigen-antibody reaction in indirect hemagglutination suggested application of the technic to detection and measurement of antibodies to cultured mammalian cells. This preliminary report describes methodology of an indirect hemagglutination test used for detection of anti-HeLa antibodies in serum of immunized

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