series may be dissociated. 4. The drug has a more marked uricosuric effect than phenylbutazone.

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## Phospholipid Studies of Different Serum Lipoproteins Employing $P_{32}$. (21264)

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The distribution of the lipoproteins of human serum into 2 main types, the $\alpha$ and the $\beta$ lipoproteins, is now well established as a result of chemical(1,2) and electrophoretic fractionation(3,4). Each of these contains phospholipid and the average normal serum shows approximately equal amounts of $\alpha$ and $\beta$ phospholipid. The two lipoproteins differ considerably in cholesterol and protein concentration(2,3,5). A number of studies have been directed toward an investigation of phospholipid turnover employing $P_{32}$ both in animals and humans (6-8). These have been mainly concerned with total serum phospholipid measurements. The purpose of the present report is to present data concerning phospholipid $P_{32}$ analyses in the individual types of lipoproteins separated electrophoretically.

*Material and Methods.* Six patients, 3 normal individuals and 3 patients with mild liver disease, were employed in this study. Each was given approximately 0.2 millicuries of $P_{32}$ in the form of $Na_2HPO_4$ orally. Bleedings were usually made at 6, 12, 24 and 48 hours after administration. Electrophoretic separation of the lipoproteins was carried out in a starch supporting medium as described previously(3,9). Broad starch blocks were usually employed, measuring 22 cm in width, 45 cm in length and 1.2 cm in thickness. This permitted the separation of 3 samples at the same time furnishing a direct comparison. Samples ranging from 2-10 cc were applied to the starch block and the separation carried out at 350V over a period of 18 hours in a cold room at 4°C. The starch block encased in wax paper was kept in a relatively dry state throughout the procedure. Following the separation, the starch block was cut up into ½ inch segments which were then thoroughly dried before a fan. These were then each extracted with 25 cc of alcohol-ether (Bloors reagent) and these extracts employed for phosphorus analyses and for radioactivity measurements. The alcohol-ether extracts were evaporated down to approximately 4 cc volume and transferred to planchettes for further evaporation to dryness followed by counting. Recoveries of serum lipid phosphorus in the fractions ranged from 85-102% by this procedure. An alternate procedure that was employed for purposes of verification was to obtain the aqueous protein solution directly from the undried starch segments by displacement filtration(9). The protein was then precipitated by $ZnSO_4$ and NaOH(10), washed, and extracted with alcohol-ether. Similar results were obtained by the two procedures although the latter was more time-consuming and recoveries were not as good as by direct extraction of the dry segments. Barbital buffer pH 8.6, $\Gamma/2$ 0.05 was used in these experiments. Phosphorus was converted to phospholipid by the factor 25.

*Results.* The distribution of radioactivity in the lipid extracts of the starch segments following electrophoretic separation of normal sera taken 12, 24 and 48 hours after oral ad-

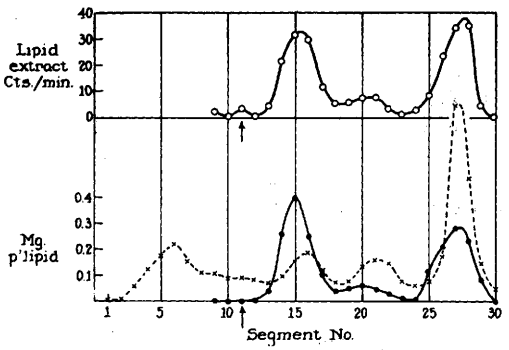


FIG. 1. Curves showing distribution of phospholipids and radioactivity in various fractions of a normal serum separated by zone electrophoresis. Upper curve illustrates radioactivity of alcohol-ether extracts; lower curve phospholipid on similar extracts. The $\beta$ lipoprotein peak, segment 15; $\alpha_2$ peak, segment 20; $\alpha_1$ peak, segment 27. Broken line represents protein curve on the same fractions. Arrows show the site of application of serum.

ministration of $P_{32}$ was found to be very similar to that for total lipid phosphorus. Fig. 1 illustrates a typical experiment with a non-fasting 12 hour serum. The lower curves show the protein and phospholipid distribution obtained from analyses of the same starch segments. The protein curve shows no $\alpha_1$ peak and a wide gap between the $\beta$ and $\gamma$-globulin peaks. This is characteristic of the pattern obtained in this buffer of 0.05 ionic strength. Three phospholipid peaks are visible, a high $\beta$ lipoprotein (segment 15), a small $\alpha_2$ lipoprotein and a large $\alpha_1$ lipoprotein peak. The $\alpha_2$ lipoprotein is well separated from the $\beta$ in this weak buffer. The separation of these 2 lipoproteins is much more difficult in $\Gamma/2$ 0.1 barbital buffer. The upper curve shows the distribution of radioactivity in the alcohol-ether extracts obtained from another aliquot of the same serum separated on the same starch block and cut into identical ½ inch segments. Because of the low radioactivity of the serum, it was not possible to obtain this broad distribution of fractions when protein, phosphorus and radioactivity measurements were carried out on identical fractions. The curve for radioactivity closely parallels that for phosphorus suggesting that the different lipoproteins have very similar specific activities. This was also found to be the case at 24 and 48 hours. Table I shows the specific activities for the 3 lipoprotein fractions at various time intervals. Here the counts and the phosphorus analyses were carried out on the same pooled fractions. A second sample on another portion of the starch block was used for phosphorus analyses to indicate where to cut the main portion of the block into the three lipoprotein fractions. The specific activities obtained were similar and the variations fell within the experimental error.

The $\alpha_2$ lipoprotein illustrated in Fig. 1 and Table I has not been well characterized. In a previous report(3), this fraction was described as being evident in certain normal sera. Further observations have indicated that it is very low in fasting normal sera but rises after meals, particularly after fatty meals, and is associated with a large part of the turbidity of non fasting sera. In 5 normal individuals where this fraction was specifically studied, it was found to be very low in 4 of the 5 individuals after fasting for 15 hours. In all cases it rose after a meal and disappeared rapidly after heparin injections. This fraction usually contained slightly more phospholipid than cholesterol but the cholesterol-phospholipid ratio was not found as low as in the $\alpha_1$ lipoprotein. In the radioactivity experiments where blood was drawn at variable times after the patient had eaten, the $\alpha_2$ lipoprotein phospholipid varied considerably in concentration. In Table I the levels at 7½ hours and 10½ hours in non-fasting sera were considerably higher than the level in the fasting 24 hour serum. The specific activity of this fraction, consisting at least in part of post prandial phospholipid was not very different from the other lipoprotein fractions, although

TABLE I.

| Hr | | Phospho-lipid, mg | Ct/min. | Sp. act., ct/mg | |
|---|---|---|---|---|---|
| 7½ | $\beta$ | .69 | 36 | 52 | |
| | $\alpha_2$ | .27 | 16 | 59 | |
| | $\alpha_1$ | .42 | 24 | 57 | |
| 10½ | $\beta$ | .62 | 74 | 120 | 123* |
| | $\alpha_2$ | .31 | 46 | 150 | 152* |
| | $\alpha_1$ | .51 | 64 | 126 | 145* |
| 24 (fasting) | $\beta$ | .53 | 75 | 140 | |
| | $\alpha_2$ | .08 | 9 | 110 | |
| | $\alpha_1$ | .47 | 63 | 135 | |

* Separate experiment at 10½ hr.

the relatively low concentration of this component made this assay less accurate than for the other lipoproteins.

In certain experiments where sera at early time intervals were separated immediately after removal from the patients some difference in specific activity was noted. The $\alpha_1$ lipoprotein appeared to have a slightly higher specific activity than the $\beta$. This difference seemed to decrease in the same serum with time even when it was stored at 4°C. Special care was employed in these experiments to avoid contamination by other phosphorus containing compounds that show similar mobility to these lipoproteins. The phospholipids were subjected to repeated extractions from the dry state with different solvents. Similar results were obtained and the $\alpha_1$ lipoprotein contained higher counts relative to phosphorus than the $\beta$ although the difference was not great. This difference was noted in the sera of certain individuals; others showed identical specific activities for the two lipoproteins at each time interval. In no experiments was a higher $\beta$ lipoprotein specific activity encountered; the specific activities were either equal or slightly higher in the $\alpha_1$ fraction.

A number of phosphorus compounds other than phospholipid that separated in the electrophoretic experiments were encountered. The starch medium proved less suitable for the localization and identification of these substances than for the separation of the lipoproteins. Better resolution of the former was obtained in a paper pulp system(9) and the results of these experiments will be described separately.

*Results in vitro.* In view of the similarity of specific activities of the lipoprotein fractions at various time intervals following oral administration of $P_{32}$ and in view of the suggestive evidence for further equilibration on standing, a series of *in vitro* experiments were carried out. The $\alpha_1$ and $\beta$ lipoproteins were isolated from sera 24 hours after $P_{32}$ administration. Great care was taken not to include any of the other lipoprotein in these isolations and the $\alpha_2$ and overlapping fractions were discarded. The purity of the $\alpha_1$ fractions and the $\beta$ fractions was tested by electrophoretic separation and in each case no $\beta$ was contaminated with $\alpha_1$ or the reverse. The mobility of the isolated $\beta$ lipoprotein fractions were noted to be slightly more rapid than in the original serum but good preparations still migrated considerably slower than the $\alpha_1$ lipoproteins and could be distinguished.

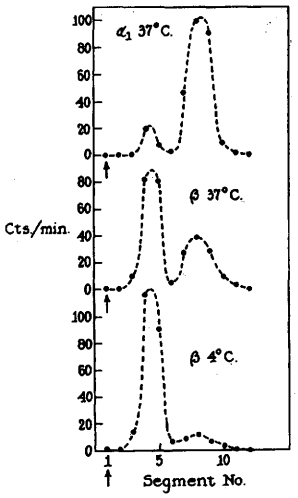


FIG. 2. *In vitro* shift in radioactivity from one lipoprotein to another. Upper curve: distribution of radioactivity of lipid extract of electrophoretic segments following 2 hr incubation of labelled $\alpha_1$ lipoprotein (segments 7, 8, 9) and unlabelled $\beta$ lipoprotein (segments 4, 5). Lower curves: 2 hr incubation of labelled $\beta$ lipoprotein with unlabelled $\alpha_1$ lipoprotein.

Following incubation of these isolated labelled lipoprotein fractions either with whole serum or with non labelled isolated fractions, a transfer of radioactivity was noted. Fig. 2 illustrates a typical experiment showing the results obtained on mixing equal amounts, in terms of phospholipid, of the labelled and unlabelled lipoprotein fractions. The incubation period in this experiment was 2 hours duration and the 3 samples were separated electrophoretically on the same starch block. The lower two curves show the shift of radioactivity from the $\beta$ lipoprotein to the $\alpha_1$ lipoprotein at 4°C. and at 37°C. A considerably greater transfer occurred at the higher temperature. The upper curve illustrates the shift of radioactivity from the $\alpha_1$ lipoprotein to the $\beta$ lipoprotein at 37°C. A definite shift occurred but this was not quite as great as in the reverse direction. Determinations of lipid phosphorus as well as

radioactivity demonstrated that the transfer of radioactivity was an exchange reaction. There was no detectable shift in phosphorus after incubation. The specific activities of the two lipoproteins approached equality in some experiments in which the incubation period was 4 hours or more; the fraction that was labelled to begin with, however, usually showed a slightly higher specific activity than the receptor fraction.

Following heating of the labelled and unlabelled fractions to 60°C. for 15 minutes prior to mixing and incubation, the transfer of radioactivity was markedly reduced. The lipoproteins did not become turbid in these experiments and their mobility was close to that observed with the unheated fractions. Potassium cyanide and potassium fluoride, when added prior to incubation did not inhibit the exchange. Addition of alcohol extracts containing labelled phospholipid did not result in significant transfer of radioactivity to the lipoproteins after incubation. In these experiments the radioactivity remained at the site of application and did not migrate with the lipoproteins. The latter experiments were not completely satisfactory technically because of the difficulty of adding the phospholipid to the aqueous lipoprotein solution. Studies of the *in vitro* exchange of $P_{32}$ phospholipid in the $\alpha_2$ lipoprotein fraction were not entirely satisfactory because of the relatively small amount of material and greater difficulty in isolation without contamination. However, the results suggested an exchange similar to that for the other two types of lipoprotein.

*Discussion.* Several efforts have been made previously to study the serum lipoproteins with $P_{32}$ labelled phospholipids. Goldwater and associates(11) analyzed different levels of the ultracentrifuge cell for phospholipid radioactivity employing human sera at various times after feeding $P_{32}$. Differences in specific activity were found. Maurer(12) working primarily with rat serum separated the $P_{32}$ labelled serum fractions by filter paper electrophoresis. Considerable difficulty was encountered due to adsorption on the filter paper but labelling of the $\alpha$ type lipoproteins which predominate in the rat was noted. In the present study very little difference in specific activity of the different serum lipoprotein fractions was observed. It was only in certain sera at early times after oral $P_{32}$ that slightly higher specific activities for the $\alpha_1$ lipoproteins were encountered. These results are not surprising in view of the ready equilibration of tagged and untagged lipoproteins mixed *in vitro*. The small differences in specific activities may have resulted from variations in the ratios of lecithin, cephalin and sphingomyelin in the different lipoproteins. These various phospholipids are known to be differentially labelled in certain tissues (13). The high percentage of the total phospholipids of serum that is present as lecithin, approximately 75%(14,15), signifies that this substance is a constituent of both the $\alpha$ and $\beta$ lipoproteins.

The exact mechanism of the *in vitro* exchange remains obscure. It appears to be similar to the striking interchange of labelled cholesterol between serum and red blood cells reported by Hagerman and Gould(16), and London and Schwarz(17) and seems to involve the phospholipid molecule. No definite conclusions were drawn in the latter reports as to the mechanism of this dynamic state. The possibility of an enzymatic mechanism is perhaps raised in the present experiments by the greater exchange on incubation at 37°C. than at 4°C. and by the decreased exchange with brief exposure to 60°C. However, no definitive evidence on this point was obtained and present knowledge of phospholipid synthesis(18) does not permit visualization of such a mechanism. The enzyme inhibitors tested had no effect on the reaction. The possibility that the exchange simply involved the phosphorus must be considered although there is little evidence in the literature suggesting such lability for phosphorus in the diester linkage of lecithin and cephalin. Experiments by Weinman and associates(19) have indicated a similar turnover rate for phospholipids injected as whole serum lipoproteins when either the $P_{32}$ or $C_{14}$ label was used. This would appear unlikely if the phosphorus showed special lability. In addition, Biggs(20) has recently observed interchange of tritium labelled cholesterol between rabbit lipoproteins *in vitro*. Eder(21) has also noted the exchange of $P_{32}$

*in vitro* in rabbit sera.

*Summary.* 1. Administration of $P_{32}$ to humans by mouth resulted in labelling of the $\alpha_1$, $\alpha_2$ and $\beta$ lipoproteins separated by zone electrophoresis in a starch supporting medium. 2. Both phospholipid phosphorus and radioactivity of $\alpha_2$ lipoprotein fraction increased after ordinary meals. 3. Specific activities of the different lipoproteins were very similar even at various time intervals following the oral radioactive phosphate. The only exception was a slightly higher specific activity for the $\alpha_1$ lipoprotein in certain sera taken at early times. 4. *In vitro* experiments demonstrated transfer of phospholipid radioactivity from labelled $\beta$ lipoproteins to unlabelled $\alpha_1$ lipoproteins; also from labelled $\alpha_1$ lipoproteins to unlabelled $\beta$ lipoproteins.

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## Leukemia VI. Effect of Amicetin on Transplanted Mouse Leukemia.*† (21265)

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A new antibiotic, amicetin, isolated from *Streptomyces vinaceusdrappus* has been shown by DeBoer, Caron, and Hinman(1) to be active against *Mycobacterium tuberculosis, Staphylococcus aureus* and *Bacillus subtilis.* From the data of Hinman *et al.*(2) and Flynn *et al.*(3) describing the isolation and purification of this compound and its chemical

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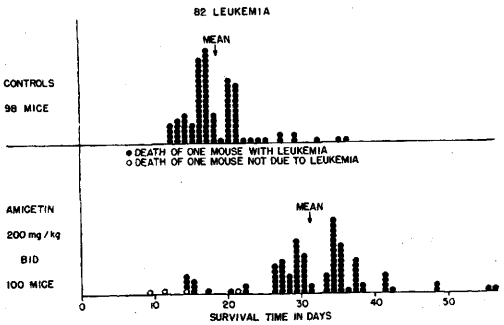


FIG. 1.