Lamm, M. E., and Small, P. A., Jr. (1966), *Biochemistry 5*, 267.
Lark, C. A., Eisen, H. N., and Dray, S. (1965), *J. Immunol. 95*, 404.
Levy, H. B., and Sober, H. A. (1960), *Proc. Soc. Exptl. Biol. Med. 103*, 250.
Light, A., and Smith, E. L. (1963), *Proteins 1*, 2.
Little, J. R., and Eisen, H. N. (1966), *Biochemistry 5*, 3385.
Mayer, M. M., (1961), *in* Experimental Immunochemistry, Kabat, E. A., and Mayer, M. M., Ed., Springfield, Ill., C. C Thomas, p 476.
Nisonoff, A. (1964), *Methods Med. Res. 10*, 134.
Noelkin, M. E., and Tanford, C. (1964), *J. Biol. Chem. 239*, 1828.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Porter, R. R., and Weir, R. C. (1966), *J. Cellular Comp. Physiol. Suppl. 67*, 51.
Press, E. M., Givol, D., Piggot, P. J., Porter, R. R., and Wilkinson, J. M. (1966), *Proc. Roy. Soc. (London) B166*, 150.
Ramachandran, L. K., and Witkop, B. (1959), *J. Am. Chem. Soc. 81*, 4028.
Smith, E. L., McFadden, M. I., Stockwell, A., and Buettner-Janusch, V. (1955), *J. Biol. Chem. 214*, 197.
Snedecor, G. W. (1937), Statistical Methods, Ames, Iowa, Iowa State University, p 240.
Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.
Steiner, L. A., and Lowey, S. (1966), *J. Biol. Chem. 241*, 231.
Teale, F. W. J. (1960), *Biochem. J. 76*, 381.
Utsumi, S., and Karush, F. (1965), *Biochemistry 4*, 1766.

# The Effects of Actinomycin D on the Biosynthesis of Plasma Lipoproteins*

Gerald R. Faloona,† Bruce N. Stewart, and Melvin Fried

ABSTRACT: Intraperitoneal administration of actinomycin D, particularly when supplementary injections were given, severely inhibited orotic acid incorporation into the trichloroacetic acid precipitate of rat liver for several hours and resulted in a decrease in the principal low density lipoprotein component of rat serum, which was still apparent after 21 hr. At the same time the concentration of high density lipoproteins was found to increase slightly. The biosynthesis of lipoproteins was measured by following L-[$^3$H]lysine incorporation into the lipoprotein protein moieties, both *in vivo* and in the isolated perfused liver.

In both cases actinomycin D pretreatment caused a greater inhibition of low density lipoprotein synthesis than that of high density lipoprotein. Specific activity determinations on a number of protein fractions isolated from the perfusate and from the liver after a liver perfusion demonstrated that the decreases in synthesis observed after actinomycin D treatment vary considerably for different proteins, indicating a general heterogeneity of hepatic template stability.

Evidence has been presented that mRNA of significantly prolonged stability exists in reticulocytes, a highly differentiated mammalian tissue essentially synthesizing a single type of protein (Marks *et al.*, 1962). As an organ displaying a wide variety of enzymatic functions and synthesizing a number of different proteins for export, the liver might be expected to exhibit a heterogeneous stability of its mRNA population. Pitot *et al.* (1965) have presented evidence that the templates coding for several liver enzymes have quite different lifetimes. A number of investigators have measured hepatic protein synthesis after blocking or inhibiting RNA synthesis with actinomycin D (Singer and Leder, 1966). Recently John and Miller (1966) measured the synthesis of the specific plasma proteins, albumin and fibrinogen, in a perfused liver system and found the synthesis of the latter molecular species to be more sensitive to inhibition by actinomycin D.

With the exception of the chylomicrons, serum lipoproteins are synthesized mainly, if not exclusively, by the liver (Roheim *et al.*, 1967). Two species of

* From the Department of Biochemistry, University of Florida College of Medicine, Gainesville, Florida 32601. *Received September 20, 1967.* Supported by National Institutes of Health Research Grant AM 02457 and Florida Heart Association Grant 67-AG-5. Taken in part from a dissertation submitted by G. R. F. to the University of Florida in partial fulfillment of the requirements of the degree of Doctor of Philosophy, Dec 1966.

† Present address: Research Division, Veterans Administration Hospital, Dallas, Texas.

peptide subunits are believed to predominate in lipoprotein molecules; $\alpha$ peptide is associated with high density lipoproteins (HDL)[1] and $\beta$ peptide with low density lipoproteins (LDL). The protein moiety associated with very low density lipoproteins (VLDL) is very similar to, although apparently not identical with, the $\beta$ peptide (Granda and Scanu, 1966).

In the present paper, the synthesis of different lipoprotein fractions are compared in a perfused rat liver system and in the whole animal at various times after treatment with actinomycin D. The results indicate that the synthesis of the LDL is affected earlier and more extensively than that of HDL, and that the observed differences are part of a general heterogeneity of hepatic template stability. A preliminary report has appeared (Faloona *et al.*, 1967).

## Methods and Materials

*Rats.* Male rats (Holtzman, 200–400 g) were maintained in a fed state except when used as blood donors, in which case they were fasted overnight.

*Chemicals, Isotopes, and Antisera.* Actinomycin D was a gift of Merck Sharp and Dohme Research Laboratories. L-[$^3$H]Lysine (270 $\mu$Ci/$\mu$mole) was purchased from Nuclear-Chicago. Antiserum to rat serum proteins was obtained from Hyland Laboratories.

*Protein Isolation.* Blood was obtained from the abdominal aorta under ether anesthesia and was either allowed to clot or was defibrinated by collecting the fibrin by stirring with wooden sticks. Lipoproteins were isolated from the serum, or from perfusates, by flotation in a Spinco Model L or L-2 ultracentrifuge using a 40 or a 50 Ti rotor. The density of the serum or perfusate was sequentially adjusted to selected values by addition of a concentrated solution of KBr, $d$ = 1.37. After an ultracentrifuge run the lipoproteins were aspirated from the top 1–1.5 ml and recentrifuged at the same initial density. The density of the next milliliter was measured by pycnometry and the isolated lipoproteins defined as less than that density. The clear intermediate zone (2–5 ml), which contained only traces of protein, was discarded and the residual portion readjusted with KBr to the succeeding density. The initial background densities and minimal average forces used to isolate each fraction are shown in Table I. Another component, intermediate density lipoproteins (IDL), with hydrated density $\cong$ 1.07, could be excluded by discarding the center of the tube after centrifugation at $d$ = 1.07. In lipoprotein fractions prepared in this manner no other components were detectable by analytical ultracentrifugation, and serum albumin could not be detected immunochemically. Serum albumin was isolated by trichloroacetic acid–ethanol extraction (Debro *et al.*, 1957) and checked for homogeneity by ultracentrifugation and electrophoresis. For the isolation of total liver protein, livers were homogenized in 95% ethanol and the precipitate was washed once with 10% trichloroacetic acid and twice with 95% ethanol.

TABLE I: Conditions for Isolation of Lipoproteins.

| Fraction | Initial Density[a] | Final Density[a] | Min Av Force ($g$ min) |
|---|---|---|---|
| VLDL | 1.01 | 1.006 | $1 \times 10^8$ |
| LDL | 1.07 | 1.05 | $1.5 \times 10^8$ |
| HDL | 1.21 | 1.18 | $2 \times 10^8$ |

[a] Refers to background density independent of protein.

*Analytical Ultracentrifugation.* Flotation analyses were conducted in a Spinco Model E analytical ultracentrifuge using an An-D rotor and a 12-mm double-sector cell. Runs were made at 42,040 rpm at 23°, with schlieren photographs taken at a 65° bar angle. To prepare samples for analytical ultracentrifugation, serum was adjusted to $d$ = 1.235 with solid KBr and centrifuged for $2 \times 10^8 g$ min. Lipoproteins were isolated with the top 1 ml of solution at a background density of 1.21.

[$^{14}$C]*Orotic Acid Incorporation.* To estimate the extent of inhibition of RNA synthesis after actinomycin D treatment, rats were injected intraperitoneally with 1 $\mu$Ci of [$^{14}$C]orotic acid (1.5 mCi/mmole) and the livers were removed 1 hr later. They were homogenized and washed extensively with ethanol and with 10% trichloroacetic acid at 4°. The precipitate was dissolved in 1 M NaOH and aliquots were taken for $^{14}$C analysis.

*In Vivo Incorporation Experiments.* L-[$^3$H]Lysine (20 $\mu$Ci/100 g body wt) was injected intraperitoneally into fasted rats at varying times following administration of actinomycin D and the animals were bled from the abdominal aorta exactly 1 hr after the lysine pulse. The lipoproteins were isolated and washed as described above. Livers were removed, weighed, and frozen, and later homogenized in ethanol, washed in trichloroacetic acid, and again with ethanol before dissolving in NaOH for specific activity determination of liver protein. The total LDL fraction was separated from serum by adjusting to an initial background density of 1.085 and centrifuging for 1.5–2 $\times 10^8 g$ min. HDL was then isolated in the usual manner and albumin was extracted from the residue (Debro *et al.*, 1957).

*Liver Perfusion.* After surgical preparation similar to that described by Heimberg *et al.* (1965), the livers were perfused *in situ* with cold saline for about 3–5 min, thus washing the sinusoids free of the liver donor's blood. The liver was then introduced into the perfusion system (Heimberg *et al.*, 1965) and preperfused for 20 min before adding the L-[$^3$H]lysine. The perfusate consisted of 40 ml of defibrinated whole blood from fasting rats,

[1] Abbreviations used: VLDL (very low density lipoproteins), $d < 1.010$; LDL (low density lipoproteins), $1.01 < d < 1.06$; HDL (high density lipoproteins), $1.075 < d < 1.20$; IDL (intermediate density lipoproteins), $d \cong 1.07$.

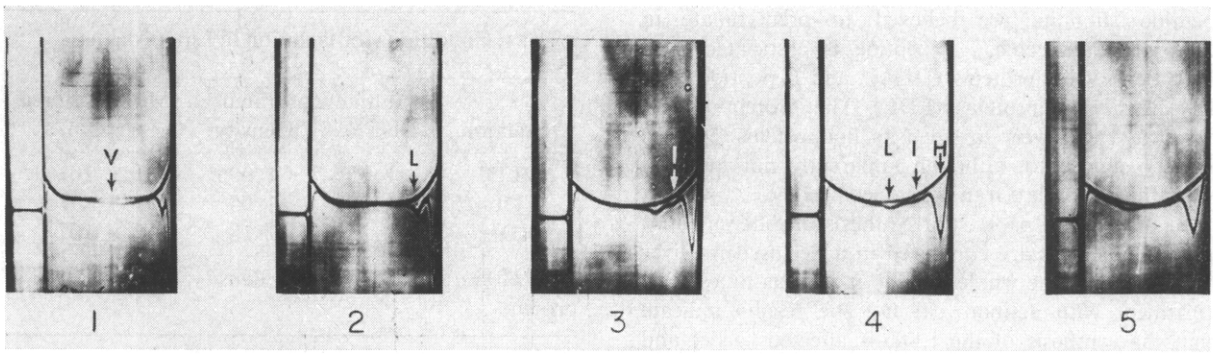


FIGURE 1: Flotation analysis of normal rat serum lipoproteins. Lipoproteins, $d$ <1.19, were concentrated 5.7-fold from sera of normal fasting rats. Photographs of schlieren patterns (1–5) were taken at 6, 9.5, 17, 23, and 30 min after reaching 42,040 rpm at $d$ < 1.19, 26°. V = VLDL, L = LDL, I = IDL, H = HDL. Flotation from right to left.

diluted to 100 ml with Krebs bicarbonate buffer (Krebs and Henseleit, 1932), to which 250 units of heparin was added. L-[$^3$H]Lysine (100 $\mu$Ci in 0.5 ml of saline) was introduced as one pulse into the portal cannula at time zero. Perfusions were terminated 2 hr after addition of isotope.

*Specific Activity Measurements.* Isolated lipoproteins were precipitated from the solutions by addition of ten volumes of cold isopropyl alcohol. The isopropyl alcohol precipitates were washed twice with 10% trichloroacetic acid and once with ethanol and dried *in vacuo*. The dried protein was then dissolved in 1 M NaOH, aliquots were taken for protein determination (Lowry *et al.*, 1951), and the radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. In the perfusion experiments the proteins were washed more extensively because of the much greater concentration of nonprotein radioactivity. This extra washing process included dissolving in 0.2 M NaOH and reprecipitating with trichloroacetic acid. The same procedures were employed for albumin and hepatic protein except that the isopropyl alcohol precipitations and the final ethanol wash were omitted.

*Electrophoresis.* Protein fractions were separated by polyacrylamide gel electrophoresis at pH 9.1 using a 7% gel. Bands were stained with amido black or eluted with potassium phosphate buffer ($\mu$ 0.01, pH 7.0).

TABLE II: Inhibition of [$^{14}$C]Orotic Acid Incorporation into Trichloroacetic Acid Precipitate of Liver Homogenate.

| | Time after Actinomycin D (hr) | Dose | % Inhibn |
|---|---|---|---|
| *In vitro* (added to perfusate) | 1 | 0.2 mg | 90 |
| *In vivo* (intraperitoneal injections) | 3 | 0.1 mg/100 g | 82 |
| | 3 | 0.3 mg/100 g | 92 |
| | 9 | 0.1 mg/100 g | 67 |
| | 9 | 0.3 mg/100 g | 91 |
| | 9 | 0.2 and 0.1 mg/100 g every 3 hr | 95 |

## Results

*Effect of Actinomycin D on [$^{14}$C]Orotic Acid Incorporation.* Following intraperitoneal injection actinomycin D has been found to be concentrated in the liver and to inhibit hepatic RNA synthesis effectively. Such effects are dose dependent and are reversed after significant periods of time. Trakatellis *et al.* (1964) have shown essentially complete inhibition of RNA synthesis 4 hr after a dose of 0.1 mg of actinomycin/100 g of body weight. For the purposes of the present work a dose and injection schedule which would block mRNA synthesis for several hours was developed. The results are shown in Table II. Doses of actinomycin D of 0.1 mg/100 g of body weight severely inhibit (<90%) [$^{14}$C]orotic acid incorporation into total liver RNA. Tripling the dose had little additional effect. Nine hours after injection some recovery was observed. When an initial dose of 0.2 mg/100 g was followed by an additional 0.1 mg/100 g at 3–5-hr intervals, 95% inhibition was observed after 9 hr.

*Effect of Actinomycin D on the Circulating Levels of Serum Lipoproteins.* Ultracentrifugal analysis of serum lipoproteins, $d$ < 1.21, from normal fasted rats reveals at least four components (VLDL, LDL, IDL, and HDL) (Figure 1). As has been observed with proteins from the human, VLDL and LDL contain very similar, if not identical protein moieties (G. Faloona and M. Fried, manuscript in preparation). The nature of rat IDL is presently unresolved, but HDL has a protein moiety that is distinct by electrophoretic mobility ($\alpha$), immunochemical behavior (Windmueller and Levy, 1967), and amino acid analysis (G. Faloona and M. Fried, manu-

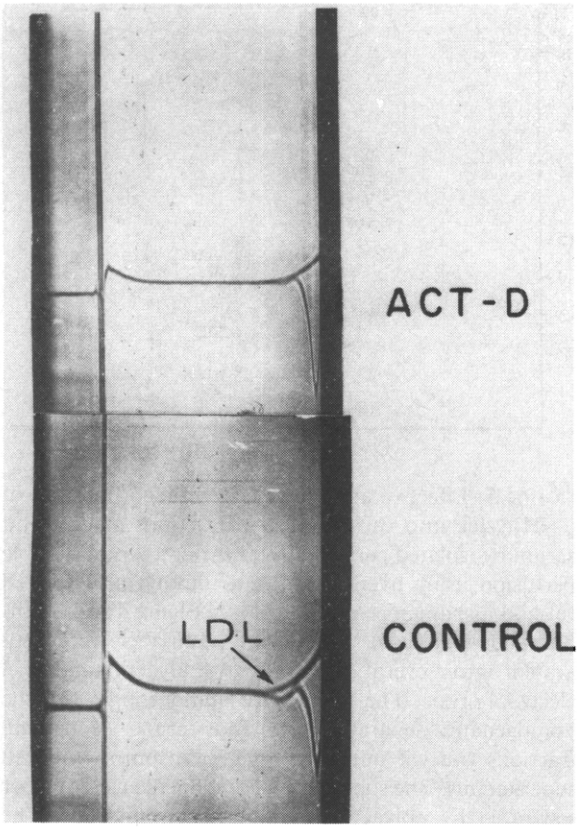


FIGURE 2: Effect of actinomycin D on the concentration of circulating rat serum lipoproteins. Rats were injected intraperitoneally with 0.2 mg/100 g at zero time and 0.1 mg/100 g at 9 and 17 hr and bled at 21 hr. Controls received sham injections. Lipoproteins $d < 1.21$ were isolated from 5 ml of serum. Photographs of schlieren patterns were taken 6 min after reaching 42,040 rpm at $d = 1.21$. Flotation is from right to left.

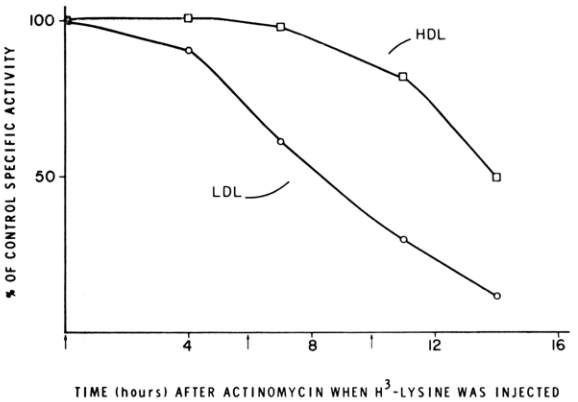


FIGURE 3: Incorporation of L-[$^3$H]lysine into LDL and HDL *in vivo*. Rats were injected intraperitoneally with actinomycin D in saline (1 mg/ml) at an initial dose of 0.2 mg/100 g body wt. Arrows ( ↑ ) indicate additional injections of 0.1 mg/100 g. Control rats received sham injections of saline. At the indicated times L-[$^3$H]lysine (20 $\mu$Ci/100 g body wt) was injected intraperitoneally and the animals were sacrificed by exsanguination 1 hr later. HDL and LDL were isolated and analyzed as described in Methods and Materials. Specific activities are plotted as per cent of control values. Each point represents the average of data from at least five rats.

script in preparation). The average normal fasting serum concentrations of the protein moiety of each fraction in the rat are 2, 10, 4, and 48 mg per 100 ml for VLDL, LDL, IDL, and HDL, respectively.

After actinomycin D treatment, the LDL concentration decreases until by 20 hr it is nearly absent from the ultracentrifugal pattern of the serum. Concomitantly, the concentration of HDL has increased (Figure 2). Such a change in the relative concentrations of LDL and HDL indicates that their relative rates of either synthesis or removal from plasma have been altered in this period.

*Incorporation of [$^3$H]Lysine in Vivo.* In the *in vivo* experiments in which [$^3$H]lysine was injected intraperitoneally, the lipoproteins were separated into only two fractions, LDL ($d < 1.065$) and HDL ($1.08 < d < 1.20$), in order to ensure recovery of sufficient LDL protein for accurate specific activity measurements. The assumption that these two fractions are metabolically distinct is based on the finding that the protein moiety of VLDL synthesized and released by the liver may be converted to LDL, but not to HDL. This is based on data from experiments in humans with $^{131}$I-labeled lipoproteins by Gitlin *et al.* (1958). A pulse of labeled amino acid appears in plasma protein in about 10–15 min and these plasma proteins have usually reached maximum specific activity at 1 hr (Peters, 1962; Majumbar *et al.*, 1967). Therefore, by sacrificing the animal 1 hr after the pulse, the specific activity is measured at a time when labeled amino acid incorporation is essentially complete; turnover of the plasma proteins which have been synthesized and released in that period is minimal.

After an initial lag the specific activities of LDL decreased more rapidly than those of HDL after actinomycin D treatment (Figure 3). Since the LDL/HDL concentration ratio is also decreasing with time, the net incorporation difference is even greater. These data are useful in demonstrating the time course of the relative incorporation into the two fractions after actinomycin D treatment. However, the use of a perfused liver system permitted testing the possibility that the results of the *in vivo* experiments might reflect selective inhibition of the synthesis of LDL of extrahepatic origin. The use of identical perfusate composition for actinomycin D treated and control livers allowed more precise measurement of the extent of inhibition.

*Incorporation of [$^3$H]Lysine by Perfused Livers.* The specific activity and net incorporation data from Table III demonstrate that VLDL and HDL are the two principal forms of lipoprotein synthesized and released by the normal liver. Presumably LDL is produced *in vivo* by the action of lipoprotein lipase on VLDL. This enzyme

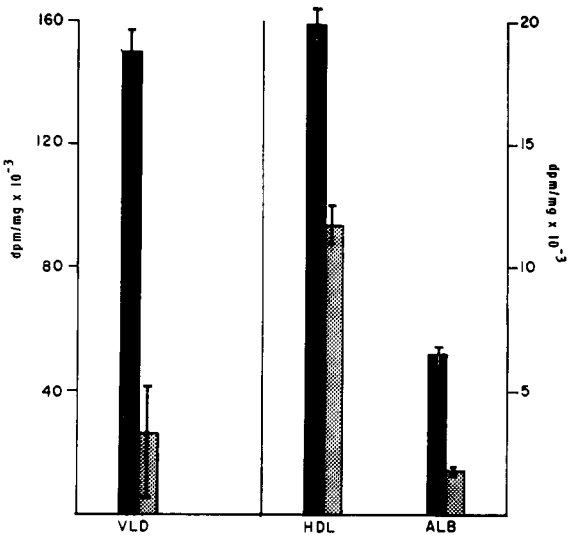


FIGURE 4: L-[$^3$H]Lysine incorporation into VLDL, HDL, and albumin by isolated perfused rat livers. Liver donors were pretreated with actinomycin D, 0.2 mg/100 g at zero time and 0.1 mg/100 g at 6 and 9 hr. Controls received sham injections. At 10 hr livers were isolated and perfused as described in Methods and Materials. Data presented are from three actinomycin D treated livers (shaded bars) and three controls (solid bars) with the range of values indicated.

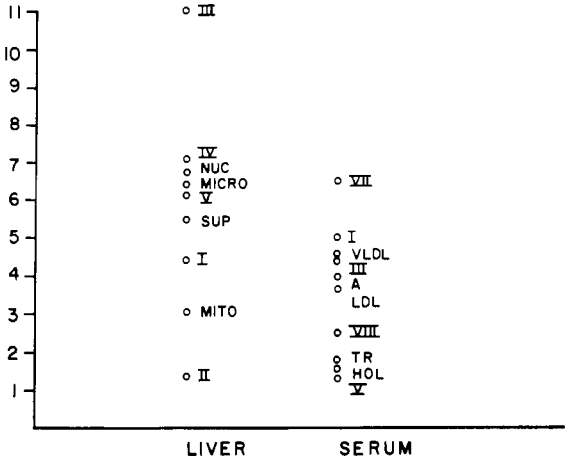


FIGURE 5: Effect of actinomycin D on incorporation of L-[$^3$H]lysine into various protein fractions of liver and serum by isolated perfused livers. After a typical double perfusion using livers from an actinomycin D treated rat and a sham injected control (see Figure 4 and Methods and Materials), the serum perfusates were separated into several fractions by polyacrylamide gel electrophoresis. The livers were homogenized and the homogenates separated into four crude centrifugal fractions (nuclei, mitochondria, microsomes, and cell supernatant). The supernatant components were further separated by polyacrylamide gel electrophoresis. The specific activity of each protein fraction was determined and the ratios of the specific activities of normal/actinomycin D treated animals are indicated on the ordinate.

activity is either absent or substantially reduced in the perfusion system. Absolute rates of synthesis cannot be calculated from the incorporation data, but the relative synthesis of the different fractions was obtained by multiplying the specific activities by the concentration of each protein in the perfusate. The validity of this calculation is based on the assumptions that no appreciable interconversion of protein moieties occurs during this period and that concentration changes are negligible. Although this may not be strictly true, it does not change the interpretation that VLDL and HDL are the main lipoprotein proteins released from the liver.

When livers were preperfused for 1 hr with actinomycin D (2 $\mu$g/ml), no apparent effect was observed on the extent of incorporation of [$^3$H]lysine into the different proteins. When livers were pretreated *in vivo* with actinomycin D and perfused between 8 and 14 hr later, decreased incorporation was observed into all fractions; but the effect was a differential one, and in each experiment the decrease in incorporation into VLDL was greater than that observed with albumin which was in turn greater than that of HDL. The data from three experiments at 10 hr after the initial actinomycin D injection are presented in Figure 4. Having received a lethal dose of actinomycin D, the rats were lethargic; however, their livers performed satisfactorily in the perfusion system. Gross appearance and perfusion flow rate were normal although bile output was reduced somewhat (0.6 *vs.* 1.0 $\mu$l/min per g of liver).

In order to ascertain whether this differential effect was occurring with other plasma proteins of hepatic origin, a sample of those serum proteins remaining after HDL was removed from a perfusate by flotation was separated into several fractions by electrophoresis in polyacrylamide gel at pH 9.1. The outer portion of each side of the gel was stained and the gel was cut crosswise into segments containing the visible bands. The proteins in each segment were extracted and their specific activities were de-

TABLE III: Incorporation of L-[$^3$H]lysine into Lipoprotein Fractions and Albumin by Normal Perfused Livers.

| | Sp Act. (dpm/mg) at 2 hr | Net Incorp (dpm) |
|---|---|---|
| VLDL | 140,000 ± 36,000[a] | 155,000 |
| LDL | 31,000 ± 9,000 | 47,000 |
| HDL | 22,000 ± 4,000 | 218,000 |
| Albumin | 6,300 ± 1,500 | 3,780,000 |

[a] Average of data from three perfusions plus and minus standard deviation.

termined. Since the original protein concentrations were identical in the actinomycin D and control perfusates and the gels were cut in an identical manner, the ratio of specific activities for each extracted protein fraction is a fair measure of the relative incorporation into that fraction during the experiment. The results indicate an extensive range of sensitivities to actinomycin D (Figure 5). Similar treatment of the supernatant fractions of homogenates prepared from the perfused livers showed even greater specific activity differences as did the cell particulates isolated ultracentrifugally from such homogenates.

## Discussion

The observation that the ratio of LDL to HDL is reduced in rat serum following the inhibition of RNA synthesis with actinomycin D can be accounted for by the results of [$^3$H]lysine incorporation studies *in vivo* and in the isolated perfused liver. In both of these systems the data indicate a considerably reduced synthesis of LDL and/or VLDL. Since the LDL protein moiety is apparently identical with the principal protein component of VLDL, and since VLDL is converted to LDL *in vivo*, the reduction of VLDL synthesis would cause a decrease in the LDL concentration of serum. The failure of the HDL level to decrease *in vivo* even 24 hr after the injection of actinomycin D is consistent with the observed persistence of its synthesis. The slight increase observed in HDL concentration (Figure 2) may be ascribed to the decreased blood volume of rats treated with actinomycin D. The total circulating pool of HDL is probably decreased. The normal half-life of circulating LDL and HDL in the rat has been determined (G. Faloona and M. Fried, manuscript in preparation) and the rate of LDL turnover is sufficiently rapid to account for its almost total absence from the circulation within 24 hr of a major block in its synthesis. While the half-lives of LDL and HDL may be altered in the presence of actinomycin D, the observed changes in their concentrations are consistent with the observed changes in their relative rate of synthesis.

It is unlikely that the results observed are due to a preferential recovery of the synthesis of specific RNA types, since the doses of actinomycin D employed were sufficiently larger to maintain nearly complete inhibition of RNA synthesis on the pretreated liver donors. Because of these large doses the influence of secondary changes in substrate or hormonal concentrations unrelated to mRNA availability in the liver cannot be excluded.

The apparent insensitivity of HDL synthesis to actinomycin D supports the finding of John and Miller (1966) that some plasma proteins of hepatic origin have a considerably greater template stability than albumin. It should be emphasized that the synthesis of each of the specific plasma proteins measured in the present work is initially insensitive to the presence of actinomycin D, as is shown by the fact that 4 hr after addition of the inhibitor L-[$^3$H]lysine incorporation into all of the proteins is still significant. This indicates a general stability of the messengers utilized in the synthesis of these plasma proteins. Trakatellis *et al.* (1964) have shown that actinomycin D, in addition to inhibiting the synthesis of RNA, increases the stability of hepatic mRNA in general. Assuming that this phenomenon includes those mRNA molecules which code for albumin and the plasma lipoproteins, the period of template stability observed in the presence of actinomycin D would be longer than the stable life of mRNA in the absence of the inhibitor.

Regardless of the actual lifetimes of the templates, the results presented in this paper suggest independent control of the synthesis of the two major classes of lipoproteins released from the rat liver. The inferred differential lipoprotein template stability appears to be only one example of a general heterogeneity of hepatic template stability. Since VLDL is responsible for glyceride transport from the liver to peripheral tissues (Heimberg *et al.*, 1965), its relatively short template stability might allow its synthesis or release to be more sensitive to metabolic regulation than that of HDL.

## References


Debro, J. R., Tarver, H., and Korner, A. (1957), *J. Lab. Clin. Med. 50*, 728.

Faloona, G. R., Stewart, B. N., and Fried, M. (1967), *Fed. Proc. 26*, 816.

Gitlin, D., Cornwell, D. G., Nakasato, D., Oncley, J. L., Hughes, W. L., and Janeway, C. A. (1958), *J. Clin. Invest. 37*, 172.

Granda, J. L., and Scanu, A. (1966), *Biochemistry 5*, 3301.

Heimberg, M., Weinstein, I., Dishmon, G., and Fried, M. (1965), *Amer. J. Physiol. 209*, 1053.

John, D. W., and Miller, L. L. (1966), *J. Biol. Chem. 241*, 4817.

Krebs, H. A., and Henseleit, K. (1932), *Physiol. Chem. 210*, 33.

Lowry, O. H., Rosebrough, R. C., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*.

Majumbar, C., Tsukada, K., and Lieberman, I. (1967), *J. Biol. Chem. 242*, 700.

Marks, P. A., Burka, E. R., and Schlessinger, D. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 2163.

Peters, T. (1962), *J. Biol. Chem. 237*, 1186.

Pitot, H. C., Peraino, C., Lamar, C., Jr., and Kennan, A. L. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 845.

Roheim, P. S., Gidez, L. I., and Eder, H. A. (1967), *J. Clin. Invest. 45*, 297.

Singer, M. F., and Leder, P. (1966), *Ann. Rev. Biochem. 35*, 195.

Trakatellis, A. C., Axelrod, A. E., and Montjar, M. (1964), *Nature 203*, 1134.

Windmueller, H. G., and Levy, R. I. (1967), *J. Biol. Chem. 242*, 2246.