

zation reaction. The methods described by Irreverre and Martin (11) were employed. Authentic 5-hydroxy-L-pipecolic acid gave a spot just to the left of proline (refer to Fig. 5A, of Irreverre and Martin, 11). The *R* value (relative to alanine) was 1.31 in *t*-amyl alcohol-2,4-lutidine. The ninhydrin color was bright purple, fading to yellow over several days. Under ultraviolet light, it fluoresced bright cherry-red, or reddish-orange at lower concentrations. A greenish-blue color was produced with isatin. The reaction mixture from the cyclization of hydroxylysine showed this same spot. Another spot, identical in every respect, appeared slightly lower on the chromatogram. The *R* value was 1.00 in the basic solvent. It seemed probable that the diastereoisomers had separated on the paper. This hypothesis was tested by chromatographing hydroxy-L-proline and allohydroxy-D-proline. These diastereoisomers separated to approximately the same degree and in the same direction. The *R* values were 1.04 and 0.83 in the basic solvent. Also, authentic 5-hydroxy-L-pipecolic acid showed only the lower spot after inversion to allohydroxy-D-pipecolic acid. The inversion was accomplished by refluxing with acetic anhydride containing acetic acid (12) and subsequent hydrolysis.

A large, immobile spot was also present on the paper chromatograms of the cyclization mixture. This probably represented linear polymerization of the monochloro acids which could have accounted for the low yield of 5-hydroxy-pipecolic acid. Studies on the stereochemistry of 5-hydroxy-L-pipecolic acid are in progress.

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### Effects of Intravenously Administered Fat on the Serum Lipoproteins

It has been shown in our laboratories that daily intravenous infusions of a fat emulsion cause a significant decrease in the elevated serum lipid values of patients with idiopathic hyperlipemia or primary hypercholesteremic xanthomatosis (1). In order to study these effects in more detail, electrophoretic analyses of serum were carried out in eight normal human beings and in eight dogs before and after an intravenous infusion of emulsified fat (2).

The human beings were given a single intravenous infusion of an emulsion containing in a total volume of 500 ml either 50 g of cottonseed oil or 50 g of synthetic triolein, with soybean phosphatide, pluronic, and dextrose added as emulsifying and stabilizing agents (3). The dogs weighed about 15 kg and were given 300 ml of this emulsion. The time for the infusion averaged 4 hours in the human beings and 2 hours in the dogs. Blood samples were taken before and at the end of the infusion as well as for several hours afterward. Samples of serum were analyzed by paper electrophoresis as described previously (4).

In the eight human beings, the paper strips stained for lipids showed, prior to the infusion, two bands: (i) an intensely stained band in the beta globulin area, corresponding to the beta lipoproteins, and (ii) a less intensely stained band intermediary between alpha-1 globulin and albumin, corresponding to the alpha lipoproteins. At the end of the intravenous infusion, the beta lipoprotein band showed a densely stained trail extending back to the starting line, representing the chylomicrons. In addition, both lipoproteins moved faster: the beta lipoproteins moved either with a speed intermediary between beta globulin and alpha-2 globulin or with a speed of alpha-2 globulin (Fig. 1). The alpha lipoproteins in some subjects were split into two components, the larger component migrating with the speed of albumin and the other smaller component migrating ahead of albumin; in other subjects, all alpha lipoproteins migrated together as a broad band ahead of albumin. On the corresponding protein-stained paper strips, small amounts of

protein were seen ahead of albumin, representing the protein moiety of the alpha lipoproteins. The increase in the electrophoretic mobility of the alpha and beta lipoproteins persisted for about 2 hours after the end of the infusion.

In the eight dogs, fasting serum samples showed on the lipid-stained paper strips an intensely stained band in the area between albumin and alpha-1 globulin, representing the alpha lipoproteins. In five dogs, small amounts of beta lipoproteins were present in the beta globulin region; in three dogs, no visible amounts were present. At the end of the infusion, the lipid-stained paper strips showed a densely staining trail extending to the starting line, representing the chylomicrons. In those dogs in which beta lipoproteins had been present in visible amounts prior to the infusion, they then migrated with increased speed in the region of alpha-2 globulin. The alpha lipoproteins were found in all instances ahead of albumin as a broad band on both the lipid- and the protein-stained paper strips.

So far, we have not been able to demonstrate electrophoretic changes in the serum after oral feeding of the fat emulsion, possibly because the concentration of lipids in the serum of the test persons did not rise as high as it did after the intravenous infusion.

The importance of the observed electrophoretic changes following the infusion of the fat emulsions lies in the fact that the changes are identical with those induced by the intravenous injections of heparin in normal persons during alimentary hyperlipemia (4, 5) and in patients with idiopathic hyperlipemia even when fasting (4, 6). In addition, in patients with idiopathic hyperlipemia, an injection of heparin had caused a de-

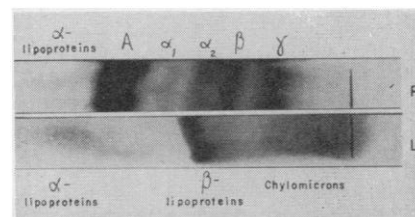


Fig. 1. Paper electrophoretic pattern of human serum obtained at the end of an intravenous infusion of fat. Upper half of the strip is stained for proteins (P), lower half is stained for lipids (L). On the lipid-stained paper strip, the beta lipoproteins, normally present in the beta globulin area, move now with the speed of alpha-2 globulins. The alpha lipoproteins, normally present in the area between albumin and alpha-1 globulins, move now ahead of albumin. On the protein-stained paper strip (P), small amounts of protein migrate ahead of albumin, representing the protein moiety of the alpha lipoproteins.

crease in the serum lipids (7). In the case of heparin, the electrophoretic changes and the reduction in the serum lipids are produced by the action of a "clearing factor" that is a heparin-activated lipoprotein lipase (8). It remains to be determined whether the infusion of a fat emulsion also activates this lipoprotein lipase or has a different mode of action.

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### Pyridine Nucleotide Analogs and the Sulfhydryl Nature of Some FAD Enzymes

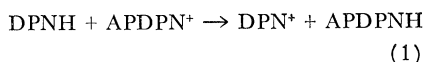
It has recently been demonstrated by Kaplan and Ciotti (1) that pig brain DPNase will catalyze an exchange between the nicotinamide moiety of DPN (2) or TPN and 3-AP, resulting in the formation of APDPN or APTPN. These analogs have been shown to be active in a number of dehydrogenase systems (3). The average potential of APDPN/APDPNH has been found to be approximately 0.08 v more positive than the DPN/DPNH system. It was therefore of interest to determine whether flavin enzymes could catalyze a transfer of hydrogen or electrons from DPNH or TPNH to the respective 3-AP analogs (4).

All measurements were performed using a Beckman DU spectrophotometer with 3.0-ml cuvettes having a 1.0-cm light path, and all reactions were run at room temperature. APDPNH and

APTPNH formation was determined by an increase in optical density at their maximum extinction at 365 mμ and at 400 mμ, where they have significant absorption in contrast to DPNH and TPNH.

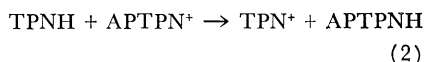
Reaction mixtures contained 50 μmoles of phosphate buffer, pH 7.5, and 50 μmoles of trisodium citrate dihydrate. In addition, the diaphorase reaction mixtures contained 0.25 μmoles of DPNH, 0.6 μmoles of APDPN, and 0.09 mg of enzyme protein in a total volume of 3.0 ml. The cytochrome-*c* reductase reaction mixtures contained 0.22 μmoles of TPNH, 0.53 μmoles of APTPN, and 0.2 mg of enzyme protein in a total volume of 3.0 ml. Additions of *p*CMB and GSH are noted in Table 1 as final molar concentrations. When *p*CMB was used it was incubated with the enzyme in the phosphate buffer for 5 minutes at 0°C. In measuring GSH reversal of *p*CMB inhibition, GSH was incubated for another 5-minute period before testing. All reactions were started with APDPN for the diaphorase and APTPN for the cytochrome-*c* reductase.

It was found that Straub's DPNH diaphorase (5), a FAD enzyme from pig heart that catalyzes the reduction of dye and inorganic iron (6) also catalyzes a transfer of hydrogen or electrons from DPNH to APDPN, as is indicated by Eq. 1.



This transfer occurred only in the presence of DPNH and APDPN. TPNH and APTPN would not serve as electron donor or acceptor, respectively, with the diaphorase. Investigation of the involvement of sulfhydryl groups in this reaction, as shown in Table 1, revealed that *p*CMB inhibited the transfer reaction. This inhibition could be reversed with GSH. The catalysis of dye reduction by this enzyme was also inhibited by *p*CMB. GSH reversal was not attempted, since the dye is reduced nonenzymatically by GSH.

TPNH cytochrome-*c* reductase (7), a FAD enzyme from pig liver, also catalyzes this transfer reaction, as is given by Eq. 2.



However, with this enzyme, DPNH and APDPN would not serve as electron donor and acceptor, respectively, but required TPNH and the corresponding analog APTPN for transfer. As can be seen in Table 1, *p*CMB inhibited the transfer reaction, and the inhibition was reversed by GSH. Since this enzyme catalyzes the transfer reaction at a much

Table 1. Inhibition by *p*CMB of diaphorase and TPNH cytochrome-*c* reductase catalyzing the reduction of APDPN by DPNH and APTPN by TPNH, respectively, and the reversal by GSH.

Reaction mixtures	Diaphorase (μmoles APDPNH formed in 3 min)	TPNH cytochrome- <i>c</i> reductase (μmoles APTPNH formed in 60 min)
Control	0.229	0.172
Plus <i>p</i> CMB ( $2 \times 10^{-4}M$ )	0	0
Plus <i>p</i> CMB ( $2 \times 10^{-4}M$ ) plus GSH ( $10^{-3}M$ )	0.234	0.172
Plus GSH ( $10^{-3}M$ )	0.233	0.172
Minus enzyme plus GSH ( $10^{-3}M$ )	0	0
Minus DPNH or TPNH plus GSH ( $10^{-3}M$ )	0	0

slower rate than the diaphorase, a 60-minute period was used to determine the formation of APTPNH rather than the 3 minutes employed for the diaphorase. The reduction of cytochrome *c* was also inhibited by *p*CMB.

These results demonstrate that, in all probability, sulfhydryl groups are involved in the catalytic properties of DPNH diaphorase and TPNH cytochrome-*c* reductase.

Other FAD enzymes such as pig heart DPNH cytochrome-*c* reductase (8), *Neurospora* TPNH nitrate reductase (9), milk xanthine oxidase, and the DPNH oxidase from *Clostridium kluyveri* (10) catalyze these transfer reactions, and demonstrate specificity toward both the reduced pyridine nucleotide and acceptor analog. Nonflavin dehydrogenases and FMN enzymes tested did not appear to catalyze this reaction.

Preliminary experiments indicate that, although only the FAD enzymes tested were active, FAD does not appear necessary for this transfer reaction. In view of this, it is of interest to speculate that FAD enzymes are in such configuration that the reduced pyridine nucleotides can transfer hydrogen or electrons directly to the protein. This reduced protein can then transfer hydrogen or electrons to the specific pyridine nucleotide analog without the involvement of flavin as an intermediate. In reactions involving reduction of dye or cytochrome *c*, the reduced protein would transfer hydrogen or electrons to FAD and then on to dye or cytochrome.