Primary and β-Secondary Deuterium Isotope Effects in N-Deethylation Reactions

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Lidocaine (1), labeled specifically with deuterium in the α-methylene (lidocaine-d_4, 2) and β-methyl (lidocaine-d_6, 3) carbon atoms of the terminal amino group, was used to probe the mechanism of oxidative N-deethylation by rat liver microsomes. The reaction rates were determined by measuring the formation of acetaldehyde colorimetrically. This general assay for oxidative N-deethylation reactions has the advantages of being rapid, producing a relatively stable colored derivative and being linear over the range of 0.25–4 μg of acetaldehyde formed per milliliter of incubate. Deuterium substitution at the methylene carbon atoms, the presumed site of initial oxygen insertion, revealed a k_H/k_D = 1.49 ± 0.11 and a K_m^D/K_m^H = 1.23. Deuterium substitution on the terminal methyl groups showed a k_H/k_D = 1.52 ± 0.10 and a K_m^D/K_m^H = 0.92. The results are explained in terms of both primary and secondary isotope effects on a possible rate-determining step in the N-deethylation sequence.

Many biologically important amines such as nicotine,[1] morphine,[2] and others[3] undergo oxidative N-dealkylation and N-oxidation reactions which often represent primary metabolic pathways for such compounds. These metabolic processes have been the subject of an extensive amount of research in the last decade. (For reviews, see ref 4.)

The mechanism of microsomal N-dealkylation reactions probably involves oxidation of the carbon atom α to the amino group. Evidence for this comes from several studies involving isolation of various carbinolamine metabolites,[5] from a study of N-debenzylation using $^{18}O_2$ as the source of oxygen,[6] and from the effects of isotopic substitution in which deuterium replaces hydrogen on the carbon atom α to the amino group.[7-11] All of these studies show kinetic primary isotope effects on the rate of N-dealkylation.

To better elucidate the nature of the transition state in the N-dealkylation reactions we initiated a series of in vitro studies with lidocaine (1), a widely used local anesthetic and antiarrhythmic agent, and the specifically deuterated analogs 2 and 3 (Figure 1). Isotopic substitution on a carbon atom β to a reacting center, such as in 3, can provide substantial information about the geometry of the transition state.[12]

Results

Acetaldehyde Determination. To measure the acetaldehyde produced by N-deethylation of lidocaine (1), an assay was devised based on the 3-methyl-2-benzothiazolone hydrazone test of Sawicki et al.[13] The method was linear from 0.25 μg of aldehyde formed per milliliter of incubate to 4 μg/ml with absorbances ranging from 0.06 to 0.76. The usual range of absorbance for monitoring acetaldehyde production from the N-deethylation of lidocaine was from 0.15 to 0.50 at 666 nm. A standard curve obtained from 0.25 to 4 μg/ml of acetaldehyde in the concentration of buffer used in the microsomal incubations is shown in Figure 2.

Initial Velocity Determinations. A comparison of the initial velocities of the N-deethylation of lidocaine and its deuterated analogs showed a significant ($p > 0.05$) slowing of the rate with both of the deuterated compounds. Plots of the amount of acetaldehyde formed vs. time are shown in Figure 3. Each reaction was virtually linear for 0–10 min (r = 0.990) although a better fit was obtained when the reactions were run from 0 to 7.5 min (r = 0.995). The calculated relative rates of oxidative N-deethylation were k_H/k_{D_4} = 1.49 ± 0.11 and k_H/k_{D_6} = 1.52 ± 0.10. (Mean ± standard deviations of four determinations from two microsomal experiments.)

K_m and V_m Determinations. The apparent K_m and V_m values obtained for the three substrates are given in Table I. From these results we find that $K_m^{D_4}/K_m^H$ = 1.23 and $K_m^{D_6}/K_m^H$ = 0.92 while $V_m^H/V_m^{D_4}$ = 1.36 and $V_m^H/V_m^{D_6}$ = 1.40.

Mass Spectral Determination of Trapped Acetaldehyde. Because of the rather large isotope effect that was observed on the initial velocity when lidocaine-d_6 was used as substrate, and because other mechanisms involving enamine formation can be envisioned for the oxidative process, we determined if any deuterium was being lost from the β-methyl groups of the substrate or the acetaldehyde formed. The chemical ionization mass spectrum contained ions at m/e 180 (MH^+ of the unreacted reagent 3-methyl-2-benzothiazolone hydrazone), m/e 209 (MH^+ of the trideuterated azine reaction product of acetaldehyde and reagent), and m/e 241 (MH^+ of a small amount of lidocaine-d_6 substrate which carried through the extraction). These results demonstrate that deuterium was not released from the β-methyl groups of the substrate or the acetaldehyde product.

Discussion

Theoretical calculations[14] have shown that the effects of isotopic substitution on reaction rates are caused by changes in the vibrational frequencies associated with the process of activation of a molecule from its ground state to a transition state. Primary isotope effects are observed when a bond to the isotopic atom itself is broken in the rate-determining step. The magnitude of a "normal" kinetic primary isotope effect is k_H/k_D = 6–10, but theoretically and experimentally this ratio may vary considerably. In fact, an unusually low or high primary isotope effect may actually assist in a description of the transition state.[15] Secondary isotope effects on the rate of a reaction may be observed when the isotopic atom is in a position (usually near to the reacting center) from which transfer of the atom does not take place, but changes in vibrational frequencies do occur. Such secondary effects give valuable information about transition state geometry.[12,15]

Of particular interest to the work presented here are deuterium isotope effects observed in enzymatic N-dealkylation involving the microsomal oxygenases. The results obtained with lidocaine-d_4 are similar to those found in the N-demethylation of various trideuteriomethyl tertiary amines.[8-11] All cases show isotope effects on the initial velocities or V_m's, and morphine shows an increased K_m value for the deuterium-labeled substrate.[8] Our results support the contention by Henderson et al.,[9] Thompson and Holtzman,[10] and Abdel-Monem[11] that the isotope effects observed in these N-dealkylations reflect C–H bond breaking in a rate-determining step.

This interpretation requires some explanation. First of all, small isotope effects on rate-determining steps, such as those observed here, have been observed in several chemi-

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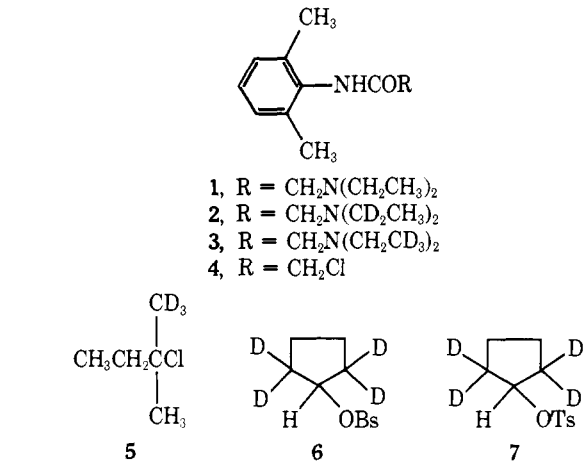


Figure 1. Structures of compounds discussed in the text.

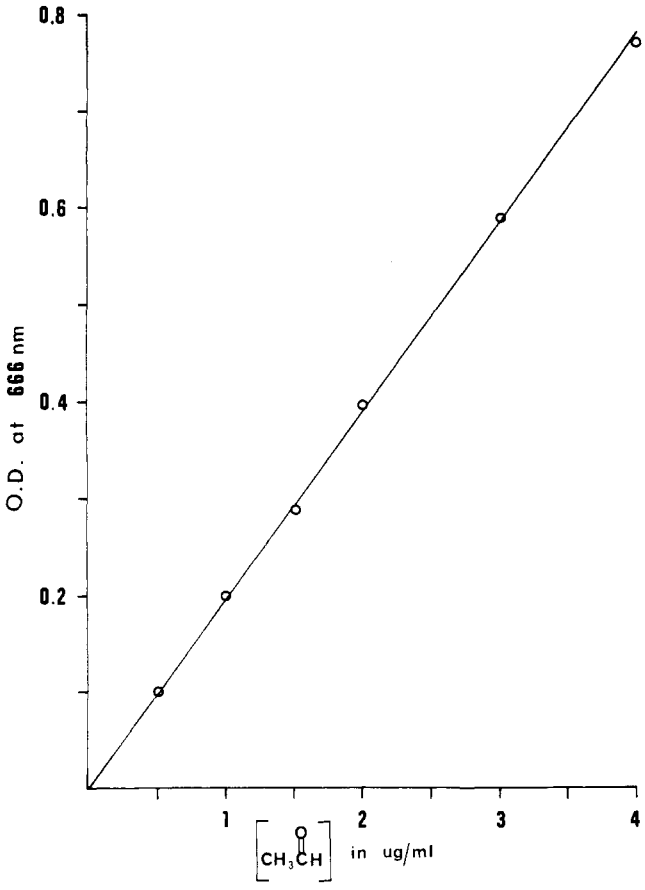


Figure 2. A standard curve of optical density (OD) at 666 nm vs. acetaldehyde concentration for the 3-methyl-2-benzothiazolone hydrazone test. Blank values of acetaldehyde were negligible in this assay carried out in buffer.

cal reactions such as the Canizarro reaction (k_H/k_D = 1.8).[16] For a normal isotope effect the assumption is made that the zero-point energy of a C–H and C–D bond is equal in the transition-state complex. This may not be true if (1) the transition state is nonlinear, (2) the transition state is asymmetric, or (3) the hydrogen atom involved is not undergoing translation at the saddle point.[15,17]

Secondly, the isotope effect on the Michaelis constants suggests that binding forces are used in the formation of the enzyme–substrate complex forcing the substrate to resemble the transition state. This results in changes in vibrational frequencies of the C–H bond to be broken in the transition state. Such an analysis has been invoked by Belleau and Moran[18] to explain the results they obtained on the interactions of deuterated tyramine with monoamine oxidase. Thus, in the N-deethylation of lidocaine, the bond-breaking process is already quite advanced in the enzyme–substrate complex since $K_m^{D_4}/K_m^{H}$ = 1.23 and V_m^{H}/V_m^{D} = 1.36. Another possibility suggested by Jencks[15] is that the Michaelis constant may contain significant kinetic terms as well as the dissociation constant.

For lidocaine-d_6 the observed kinetic β-secondary effect is similar to values obtained in solvolysis reactions involving the formation of carbonium ions. For example, Shin-

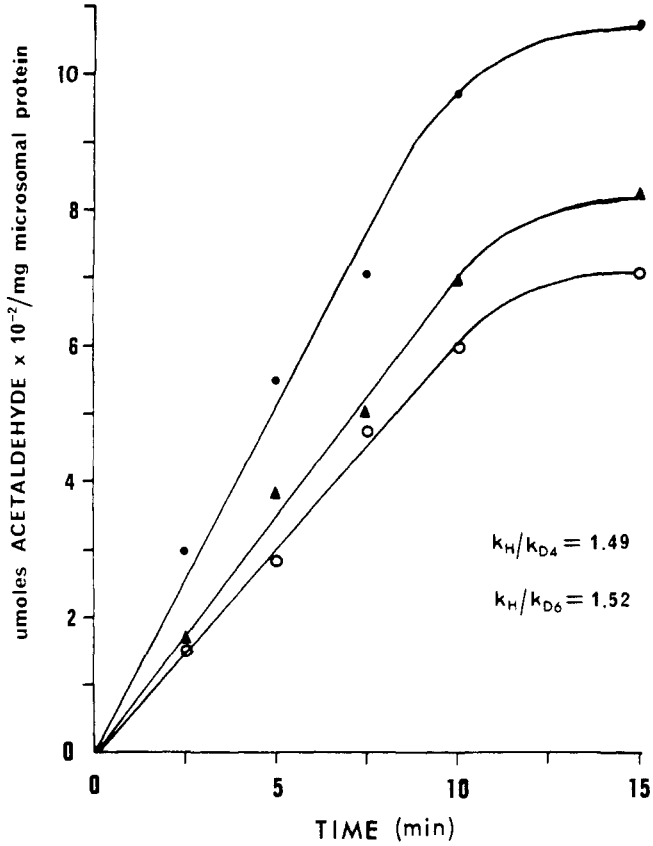


Figure 3. Curves showing the rates of N-deethylation of lidocaine (●—●), lidocaine-d_4 (▲—▲), and lidocaine-d_6 (○—○). Each point represents the mean of four determinations from two separate microsomal experiments. Blank values determined at each time point from microsomes ranged from 0.1 to 0.15 μg/ml (0.02–0.03 ODU) from 0- to 10-min incubation periods. With longer incubation periods (15–30 min) the aldehyde blank values rose to 0.60 μg/ml (approximately 0.12 ODU).

Table I. Apparent K_m and V_m for the N-Deethylation of Lidocaine and Its D_4 and D_6 Analogs by Microsomes Isolated from Rat Liver[a]

Compound	K_m, mM[b]	V_{max}[c] (nmol/mg of protein/min)
Lidocaine	0.338 ± 0.090	12.7 ± 1.84
Lidocaine-d_4	0.415 ± 0.077	9.3 ± 0.66
Lidocaine-d_6	0.312 ± 0.031	9.1 ± 0.27

[a] The results are expressed as means and standard deviations of four determinations from two separate microsomal experiments. [b] The K_m for lidocaine-d_4 differed statistically from the K_m of lidocaine and lidocaine-d_6 ($p < 0.10$ and $p < 0.05$, Student's t test). The difference in the K_m's between lidocaine and lidocaine-d_6 was not statistically significant ($p > 0.35$, Student's t test). [c] The V_{max} for lidocaine differed statistically from the V_{max} of lidocaine-d_4 and lidocaine-d_6 ($p < 0.01$).

er[19,20] found a β-secondary effect k_H/k_D = 1.40 for the rate of solvolysis of the trideuterio compound **5** and 1.52 for the solvolysis of **6**. Streitwieser et al.[21] found k_H/k_D = 2.06 for cyclopentyl tosylate-d_4 (**7**).

The kinetic β-secondary deuterium isotope effect observed in these reactions is best explained by the lowering of force constants to the C–H and C–D bonds adjacent to a carbonium-like center in the transition state through hyperconjugation.[14] Thus, in going from an sp^3 configuration to an sp^2 configuration, substitution of deuterium for hydrogen at a contiguous center will lead to a $k_H/k_D > 1$.

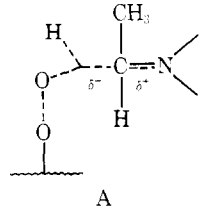
The inference to be made from the small primary isotope effect and relatively large secondary isotope effect in the oxidative N-deethylation of lidocaine is that an assymetric and/or nonlinear transition state is involved in the removal of an α-methylene hydrogen and that the carbon atom from which the hydrogen is removed has considerable sp^2-like character in this transition state. This hypothesis is supported by several lines of reasoning, assuming for the moment that insertion of oxygen between the carbon hydrogen bond is involved in the rate-determining step.

(1) The transition state for hydrogen removal is very likely asymmetric considering that the hydrogen atom is probably being transferred between two very different atoms, carbon and oxygen. That is, the hydrogen will probably not be equidistant between these two atoms in the transition state. This will, therefore, decrease the primary isotope effect.

(2) McMahon and Craig[22] found a kinetic isotope effect of 1.8 for the microsomal oxidation of α-deuterioethylbenzene and also showed that the hydroxylation was stereospecific with retention of configuration, i.e., the oxygen was inserted on the same side of the carbon as the hydrogen was removed. For such an event to occur, the reaction probably involves an electrophilic reaction of an oxenoid species on the C–H bond. Analogies to this reaction have been studied in detail by Olah[23] in his prolific work on carbenium ion insertions into single bonds via two-electron three-center bonds. Of particular importance to the discussion is that such electrophilic reactions involve nonlinear transition states which will considerably decrease the magnitude of the isotope effect.[17]

(3) In the special case of oxidative N-dealkylation, the nitrogen can lower the activation energy for the breaking of the α-C–H bond by stabilization of an electron-deficient intermediate through inductive and resonance participation.[24] The consequences of such stabilization will be to lower the magnitude of the primary isotope effect and increase the amount of sp^2-like character in the transition state. This might account for the large β-secondary isotope effect observed.

A transition state which is consistent with the isotope effects in the N-deethylation process of lidocaine is depicted in structure A. The complex shows the formation of a two-



A

electron three-center bond with oxene-type oxygen insertion[25,26] into the α-C–H bond wherein the transition state for insertion is nonlinear and asymmetric, and the α-carbon atom has substantial sp^2 character. Thus, for the N-deethylation of lidocaine, we favor direct carbon–hydrogen insertion of oxygen. The intermediate carbinolamine formed then decomposes spontaneously to acetaldehyde and secondary amine.

Experimental Section

Chemicals. Lidocaine was supplied as the free base by Astra Pharmaceutical Products, Inc., Worcester, Mass. Lidocaine-d_4 (**2**), 89% overall deuterium incorporation determined by electron ionization (EI) and chemical ionization mass spectrometry (CIMS), and lidocaine-d_6 (**3**), 94% overall deuterium incorporation, were synthesized as previously described.[27,28] Briefly, ω-chloro-2,6-dimethylacetanilide (**4**) was prepared by acylation of 2,6-dimethylaniline with chloroacetyl chloride followed by displacement of the ω-chloro group with α,α-diethylamine-d_4 and β,β-diethylamine-d_6, respectively. The diethylamine-d_4 was prepared by AlD_3 reduction of *N*-ethylacetamide-d_2 formed previously by hydrogenation of a mixture of acetonitrile and acetic anhydride with D_2 gas; diethylamine-d_6 was synthesized in a similar manner by H_2 reduction of a mixture of acetonitrile-d_3 (Stohler Isotope) and acetic anhydride-d_6 followed by AlH_3 reduction of the *N*-ethylacetamide-d_6 product. Purity of the products was ensured by the mass spectral investigations of deuterium content as described above, as well as GLC analysis on two different columns as previously described.[27]

The 3-methyl-2-benzothiazolone hydrazone hydrochloride reagent was obtained from Aldrich Chemical Co., Inc. (Cedar Knolls, N.J.) and recrystallized from methanol prior to use. NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Tissue Preparation. Hepatic microsomes were obtained for each experiment from three male albino Sprague–Dawley rats (140–190 g). The animals were decapitated and exsanguinated. All further preparations and transfers were carried out in a cold room (0 ± 4°). The livers were removed, weighed, pooled and minced, and homogenized in 3 vol of cold 1.15% KCl–0.01 *M* sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem homogenizer. After centrifugation (Beckman L2-65B) at 10,000g for 15 min at 0° to remove cell debris and nuclei, the supernatant was recentrifuged (ultracentrifugation at 0°) at 105,000g for 60 min. The microsomal pellet was resuspended manually with the homogenizer in a volume of cold 1.15% KCl buffer equal to that of the supernatant discarded and centrifuged for 60 min at 105,000g. The final pellet was again suspended manually in a volume of cold 1.15% KCl buffer equal to that of the supernatant discarded and the protein content determined by a modified Lowry procedure[29] on a 1:100 solution of the microsomal suspension in water. The final microsomal suspension was diluted with the 1.15% KCl buffer to a concentration of 5.0 mg of protein per milliliter of suspension.

Incubation Mixture. The reaction mixture contained phosphate buffer, pH 7.4 (200 μmol); magnesium chloride (10 μmol); NADPH-generating system consisting of glucose 6-phosphate (20 μmol), glucose 6-phosphate dehydrogenase (5 units), and NADP (2 μmol); lidocaine, lidocaine-d_4, or lidocaine-d_6 (0–7.5 μmol); microsomal suspension (0.5 ml); 1.15% KCl–0.01 *M* phosphate buffer (to give a final volume, 5.0 ml).

Incubations were conducted in open 25-ml erlenmeyer flasks at 37° and 120 oscillations/min and times of 0, 2.5, 5.0, 7.5, 10, and 15 min were employed for initial velocity determinations. Since reaction rates were linear for 10 min (linear regression correlation coefficient $r \geq 0.990$) incubation times of 7.5 min were utilized for K_m and V_{max} determination ($r \geq 0.995$ from 0 to 7.5). A solution containing all components but the microsomal suspension was preincubated for 5 min and then 0.5 ml of the preincubated (5 min) microsomal suspension was added to each flask at 10-sec intervals. Reactions were terminated in the same sequence at 10-sec intervals by adding 2 ml of 15% zinc sulfate solution, followed approximately 5 min later by pouring the suspension into 20-ml Pyrex culture tubes, each containing 2 ml of saturated barium hydroxide solution. Each flask was rinsed with 1 ml of distilled water.

Acetaldehyde Determination. The analysis was performed in the following manner. Each denatured microsomal sample was centrifuged at 2500 rpm for 15 min to impact the white precipitate of barium and zinc salts, and 5 ml (one-half) of the supernatant was then pipetted into an appropriately labeled 50-ml Pyrex culture tube. To each sample was added, at 30-sec intervals, 2 ml of 0.5% 3-methyl-2-benzothiazolone hydrazone hydrochloride reagent with brisk shaking after each addition. After a 30-min reaction period, 2.5 ml of a 1% ferric chloride solution was added at 30-sec intervals in the same order as the first addition. This resulted in the formation of a pale green to dark blue color. After another 30-min reaction period, 30 ml of a 2:1 mixture of reagent grade acetone–

water was added in the same sequence at 30-sec intervals to stop the reaction with subsequent formation of a fine precipitate. This precipitate was separated by centrifugation at 2500 rpm for 5 min or was allowed to settle overnight in a refrigerator (~5°) with retention of 90–95% of the original absorbance. The optical density of each sample was then read (666 nm) against reaction mixtures from each time period containing all components except microsomes (i.e., substrate blanks). A Perkin-Elmer Coleman 101 spectrophotometer was used for the readings.

Calculations. Aldehydes present in the microsomes (0.1–0.6 μg/ml) were determined simultaneously at each time interval using incubation mixtures containing all components but substrate. The quantity of this native aldehyde was subtracted from that value obtained for the reaction mixtures at the same time period. Apparent K_m and V_{max} values were determined from the amount of acetaldehyde formed at several substrate concentrations (0.125, 0.250, 0.500, 1.00, and 1.50 m*M*) during a 7.5-min incubation period. The data were fitted to the equation $S/v = S/V_m + K_m/V_m$ by linear regression analysis of S/v on S. Initial velocity determinations were made using the amount of acetaldehyde evolved at 0, 2.5, 5, and 7.5 min from reactions 1.00 m*M* in substrate. A least-squares fit of the data to a straight line was used to obtain the slopes and thus the k_H/k_D values. All velocities of deuterated substrates were corrected for less than 100% deuterium incorporation (lidocaine-d_4, 89%, and lidocaine-d_6, 94%). The Student's t test was used for statistical analysis.

Mass Spectral Determination of Trapped Acetaldehyde. A microsomal experiment was performed with lidocaine-d_6 (**3**) as previously described, and the acetaldehyde released during a 7.5-min incubation was allowed to react with 100 μl of the 3-methyl-2-benzothiazolone hydrazone reagent (0.5%) utilizing the same procedure described for the colorimetric determinations except the ferric chloride step was omitted to avoid further oxidation of the intermediate azine. The intermediate azine was extracted into purified ether at pH 6.5–7.0, the extract was filtered through anhydrous magnesium sulfate, and the filtrate was rotary evaporated at ambient temperature to yield a yellow residue which was subjected to chemical ionization mass spectrometry with isobutane as reagent gas at 140°.

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Hypo-β-lipoproteinemic Agents. 1. Bicyclo[2.2.2]octyloxyaniline and Its Derivatives

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A new assay for agents which normalize β-lipoproteins in cholesterol–cholic acid fed rats is described. Both lowering of serum cholesterol and of serum heparin precipitable lipoproteins (HPL) were measured at the end of the treatment period. Compounds which shifted the ratio of the decrease in favor of HPL are considered hypo-β-lipoproteinemic. *p*-(1-Bicyclo[2.2.2]octyloxy)aniline and several of its derivatives proved active in this assay. The synthesis of these compounds is described.

All serum lipids are bound to proteins. These lipoproteins are broadly divided into the four classes: chylomicrons, very low density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). During electrophoresis VLDL, LDL, and HDL migrate with pre-β, β, and α mobilities, respectively.[1] In the normal adult human approximately two-thirds of the total serum cholesterol is associated with the cholesterol rich LDL. Total serum cholesterol level is usually a reflection of serum LDL concentration. Since both chylomicrons and VLDL are triglyceride rich, elevated serum triglyceride levels simply reflect an increase in either one or both of these entities.[1]

Elevations of serum lipid levels have been grouped into five basic types as classified by lipoprotein patterns. The most common hyperlipoproteinemias are types II and IV, an abnormal increase in LDL and VLDL, respectively.[1] In both these hyperlipoproteinemias the incidence of prema-