The Nature of the Reverse Type I (Modified Type II) Spectral Change in Liver Microsomes†

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ABSTRACT: The reverse type I (RI) spectral change, formerly referred to as the modified type II spectral change, is due to the formation of a peak at about 420 nm and a trough at about 392 nm in the difference spectrum. Interaction of the cytochrome P-450 containing mixed function oxidase with RI compounds is at a site different from the type I site and distinct from the type II (heme iron) site. The RI spectral change, like the type I spectral change, does not affect the combination of carbon monoxide with reduced cytochrome P-450. Also like the type I spectral change, the RI spectral change occurs at the same wavelengths regardless of the compound (*e.g.*, methanol, ethanol, butanol, phenacetin, etc.) causing its appearance. The RI spectral change is suggested as being due to interaction of lipid soluble compounds with the substrate-bound form of cytochrome P-450 at a site other than the type I site; this interaction shifts the equilibrium between the 394-nm form and the 419-nm form of the enzyme toward the 419-nm form with an attendant decrease in the rate of reduction of cytochrome P-450. Both free (419-nm form) and endogenous substrate-bound forms (394-nm form) of cytochrome P-450 exist in liver microsomes. Phenacetin, which causes the RI spectral change, also causes a type I spectral change of lesser magnitude. Only the type I spectral change appears to be related to metabolism.

The first report that substrates of a cytochrome P-450-containing mixed-function oxidase of adrenal cortex microsomes cause spectral changes when added to the microsomal suspension appeared in 1965 (Narasimhulu *et al.*, 1965). It was followed shortly by other studies on another cytochrome P-450-containing mixed-function oxidase, the liver drug oxidase system (Remmer *et al.*, 1966; Imai and Sato, 1966).

Earliest studies indicated that the interaction of substrates with the mixed function oxidase caused three types of alteration of the difference spectrum; these were termed type I, type II, and modified type II spectral changes (Schenkman *et al.*, 1967a).

The type I spectral change was characterized by the formation of a peak at about 390 nm and a trough at about 420 nm in difference spectrum (Schenkman *et al.*, 1967a); it was shown to be the result of a shift in the absorption maximum of the oxidized form of cytochrome P-450, from 419 to 415 nm, and the appearance of a shoulder, or new absorption band, at 394 nm (Schenkman and Sato, 1968). This type of spectral change was reported not to be caused by interaction of substrate with the heme moiety of the hemoprotein, but to be a modification of an existing ligand of the heme to the protein (Schenkman and Sato, 1968). A wide variety of compounds have been found to cause the type I spectral change. Of these, all appear to be substrates of the enzyme; they range from drugs of many pharmacological categories (Schenkman *et al.*, 1967a) to fatty acids (Orrenius and Thor, 1970) and steroids (Ellin *et al.*, 1972). All cause a similar spectral change.

The type II spectral change was reported to be similar to the reaction between a basic amine and a ferrihemoprotein, and was shown as being a ferrihemochrome spectral change (Schenkman *et al.*, 1967a). The compounds causing this type of spectral change react with both the ferricytochrome P-450 (absorption maximum 419 nm) and the substrate-bound hemoprotein (394-nm peak) as determined in absolute spectrum (Remmer *et al.*, 1969) and in difference spectrum (Schenkman, 1970). The peak position of the complex depends upon which basic amine is added, and varies between 425 and 435 nm. At present, only a few type II compounds are known to be substrates. One of these, aniline (Schenkman *et al.*, 1967a), like the type II compound ethyl isocyanide (Omura and Sato, 1964), can displace carbon monoxide from the reduced hemoprotein, indicating interaction at the heme iron; like ethyl isocyanide, aniline also has been shown to react with the reduced cytochrome P-450 to form a spectral change containing two Soret peaks (Imai and Sato, 1968).

The most neglected of the spectral changes has been the modified type II category. Although originally assigned as a catchall for unusual spectral changes (Schenkman *et al.*, 1967a), which resembled the type II category, it soon became apparent that this spectral change was as specific with respect to wavelength as the type I spectral change; it was shown to be the reverse of the type I spectral change in both absolute and difference spectra, and was suggested as being due to displacement of some preexisting substance, bound *in vivo*, from the enzyme (Schenkman *et al.*, 1969).

Similar binding spectra have also been seen in adrenal cortex mitochondrial preparations (which contain cytochrome P-450), with steroid substrates and nonsubstrates (Whysner *et al.*, 1969), and in kidney cortex microsomes (Ellin *et al.*, 1972) with fatty acids and steroids. Because the modified type II spectral change appears spectrophotometrically similar to the type II spectral change, it has often been likened to the latter (Whysner *et al.*, 1969) and often erroneously referred to as a type II spectral change (Anders, 1971). In this paper we show that this spectral change is clearly dis-

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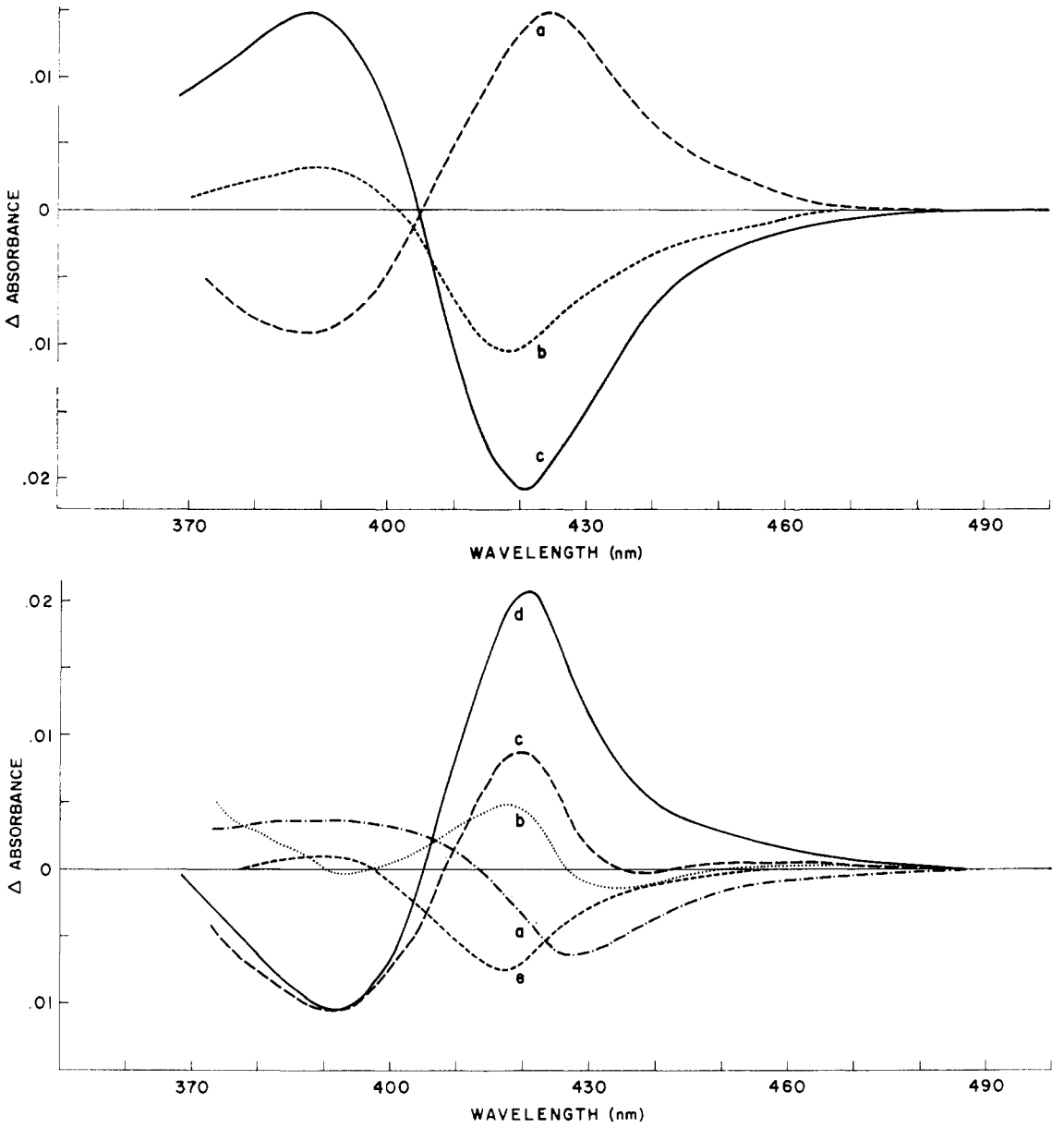

FIGURE 1: Spectral changes. (A, top) Phenacetin-induced reverse type I spectral change in untreated rat liver microsomes. Liver microsomes from adult male rats were suspended to 2.0 mg of protein/ml in 0.1 M Tris-HCl (pH 7.5) and were distributed between two cuvets; the cytochrome P-450 content was 1.43 μM. Phenacetin in acetone was added on a stirring rod after evaporation of the acetone. (a) 0.96 mM phenacetin in a sample cuvet; (b) 1.63 mM aminopyrine in a sample cuvet; (c) 3.3 mM hexobarbital in a sample cuvet. (B, bottom) Hexobarbital- and phenacetin-induced reverse type I spectral change in benzopyrene-induced rat liver microsomes. Liver microsomes from benzopyrene-(20 mg/kg) pretreated adult male rats were suspended to 1.43 mg of protein/ml in 0.1 M Tris-HCl (pH 7.5); the cytochrome P-450 content was 2.4 μM. Addition to the cuvets was (a) 1.7 mM hexobarbital in a sample cuvet, (b) 6.8 mM hexobarbital in a sample cuvet, (c) 10 mM hexobarbital in sample cuvet, (d) 0.86 mM phenacetin in a sample cuvet and (e) 3.3 mM aminopyrine in a sample cuvet.

tinct from the type II spectral change and that it involves interaction between compounds and a non-heme portion of the mixed function oxidase. For clarity of nomenclature the modified type II spectral change will be referred to in this paper as the reverse type I (RI) spectral change—a more appropriate and descriptive name.

Materials and Methods

Male albino rats of the Wistar strain (150 g) were killed by decapitation and the livers were removed and perfused with cold 0.15 M NaCl. Microsomes were prepared from 0.25 M sucrose–1 mM EDTA and were washed with 0.15 M KCl as previously described (Remmer *et al.*, 1966).

Spectra were recorded using an Amino-Chance dual-wavelength recording spectrophotometer as previously described (Schenkman, 1970; Schenkman *et al.*, 1967b, 1969). Compounds added to the microsomal suspension were contained either in aqueous solution or in acetone; if in the latter solvent, samples were rapidly evaporated on the stirring rod and were stirred into solution. The maximal volume of addition per 3 ml of suspension was 50 μl.

In vitro assays of aminopyrine oxidation relied upon formaldehyde production as previously described (Schenkman *et al.*, 1967b). Assay for phenacetin metabolism involved a modification of an assay for acetanilide metabolism (Kritsch and Staudinger, 1961), since the product of acetanilide p-hydroxylation and phenacetin O-dealkylation are the same. The assay

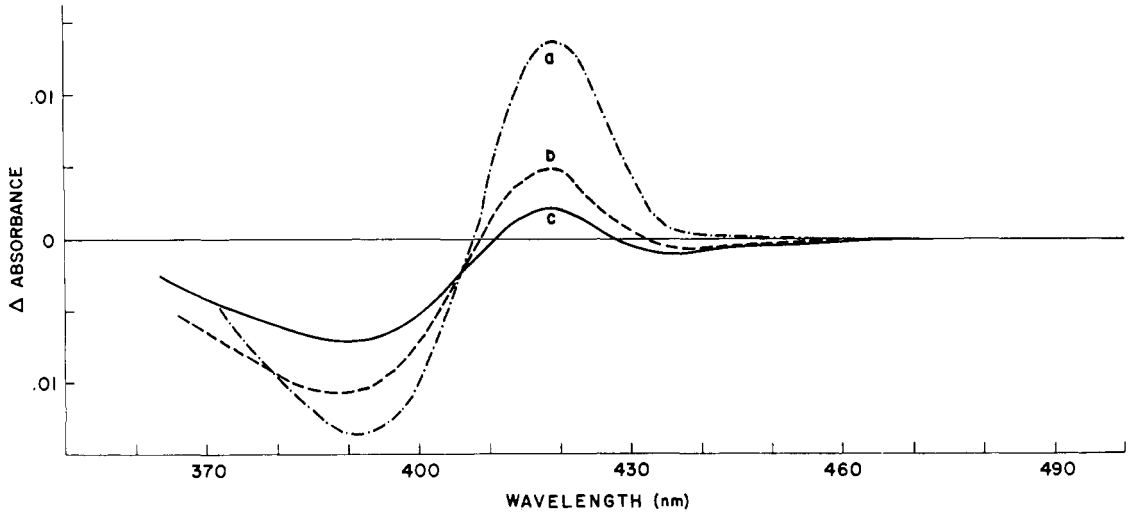

FIGURE 2: Effect of addition of alcohols to microsomal suspensions. Liver microsomes of untreated male rats were suspended in 0.1 M Tris-HCl (pH 7.5) to 2.0 mg of protein/ml (1.5 μM P-450). Alcohols were added to the sample cuvet to a concentration of 20 μl/ml: (a) 1-butanol, (b) ethanol, and (c) methanol.

consisted of stopping the reaction of 3 ml of the assay medium (Kritsch and Staudinger, 1961) with 1 ml of 15% trichloroacetic acid, centrifuging to remove the protein precipitate and extracting 2.5 ml of the supernatant with 1.5% amyl alcohol in ether (10 ml). The aqueous layer was removed, along with any material lying between the phases, and the ether phase was shaken with 2 ml of 1 M NaOH. The ether phase was removed and 1 ml of 10% Na_2CO_3 was added to the NaOH. After mixing, 1 ml of Folin reagent (Folin–Ciocalteau reagent, diluted 1 : 5) was added and after 30 min at 37°, the absorbance of the blue color was read at 690 nm.

Pretreatments with phenobarbital, 3,4-benzopyrene, or 3-methylcholanthrene were as previously described (Schenkman *et al.*, 1969).

Results

Although phenacetin can cause a type I spectral change with rabbit liver microsomes (Imai and Sato, 1966), a difference was noted when rat liver microsomes were used; phenacetin (Figure 1A) caused the formation of the reverse type I (RI) spectral change. This was seen even when animals were pretreated with phenobarbital or benzopyrene (Figure 1B). The magnitude of the RI spectral change did not appear to be a function of the concentration of cytochrome P-450. The addition of aminopyrine or hexobarbital caused formation of a type I spectral change in liver microsomes of both untreated (Figure 1A) and phenobarbital-treated rats. However, with liver microsomes from the benzopyrene-treated rats, both aminopyrine and hexobarbital also caused the formation of a RI spectral change. This latter effect was concentration dependent, and as pointed out earlier (Schenkman *et al.*, 1969), required considerably higher drug levels than needed for the formation of the type I spectral change. As seen in Figure 1B, increasing the amount of hexobarbital in the medium from 1.7 mM up to 10.0 mM caused a conversion of a type I spectral change to a RI spectral change. This conversion was not observed in untreated or phenobarbital-treated animal liver microsomes.

Previously it was suggested that pretreatment of animals with polycyclic hydrocarbons causes a decrease in the substrate induced type I spectral change due to a retention of the polycyclic hydrocarbon in the microsomes (Schenkman *et al.*, 1969; Schenkman, 1970). This suggestion was based upon the similarity between the 394-nm peak of cytochrome P-450 (in absolute spectrum) after pretreatment of animals with polycyclic hydrocarbon (Hildebrandt *et al.*, 1968; Schenkman *et al.*, 1969) and the 394-nm peak obtained upon addition of type I substrates to P-450 of untreated animals (Schenkman and Sato, 1968). In addition, it was found that a large excess of hexobarbital shifted the 394-nm peak of P-450 (of polycyclic hydrocarbon pretreated animals) to the usual 419-nm peak (Schenkman *et al.*, 1969). In difference spectrum this shift is seen as the reverse of a type I spectral change, or the RI spectral change. The RI spectral change is also seen with microsomes of untreated animals, as, for example, when phenacetin is added. Furthermore, it has been reported that ethanol addition to liver microsomes causes this reverse of the type I spectral change (Imai and Sato, 1967; Diehl *et al.*, 1970; Rubin *et al.*, 1971). The addition of two other alcohols, methanol and butanol, to microsomal suspension also caused an RI spectral change, the magnitude of which was dependent upon the concentration of the alcohol as well as upon the chain length. The comparative effects of 20 μl/ml of methanol, ethanol, and 1-butanol are shown in Figure 2.

In order to determine whether the alcohol induced RI spectral change was caused by extraction of some previously bound substrate in the microsomes, as suggested by Diehl *et al.* (1970), microsomes from benzopyrene-treated rats were extracted with 20 μl of 1-butanol/ml; these extracted microsomes still displayed a prominent band at 394 nm in absolute spectrum and still displayed an RI spectral change on addition of phenacetin (or high concentrations of hexobarbital).

Examination of these extracted microsomes and comparison with the same preparation which was not extracted revealed only a greater removal of protein; the ratio of cytochrome P-450 to cytochrome b_5 was unchanged, but the hemoprotein content per milligram of protein was increased 35%. When the heme levels were balanced, no difference was observed spectrophotometrically. Alcohol extraction did not affect the ability of phenacetin to cause the RI spectral change (Figure 3), or the type I spectral change caused by naphthalene (Netter, 1969), but did diminish the ability of aminopyrine to cause a type I spectral change (Figure 3). However, aminopyrine

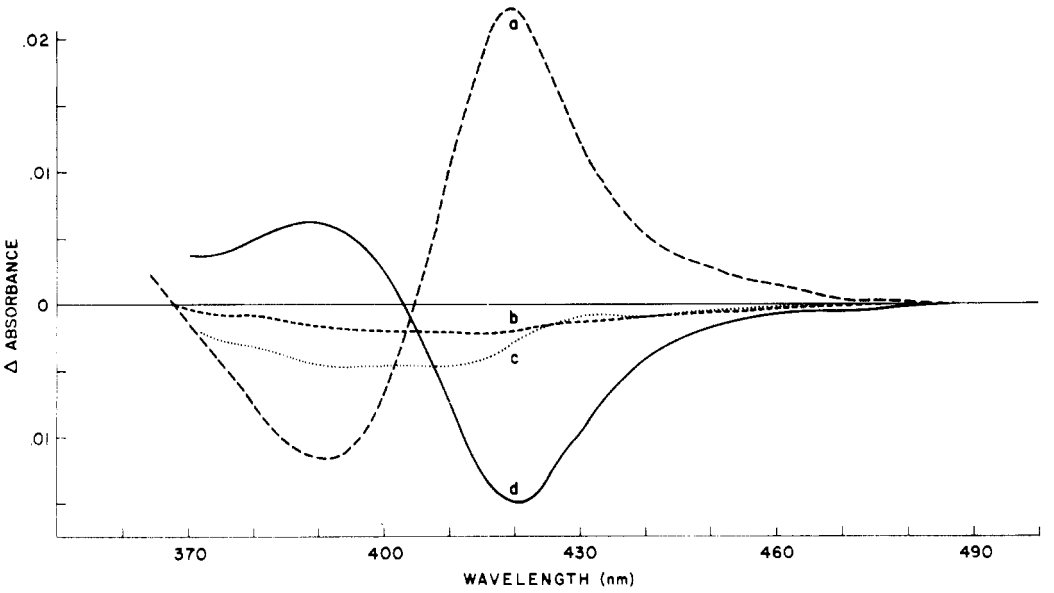

FIGURE 3: Effect of butanol extraction of liver microsomes from benzopyrene pretreated rats. Butanol (20 μl) added per ml of 10,000g supernate of liver homogenate prior to sedimentation of the microsomes at 105,000g for 1 hr. The microsomes were resuspended to an equal volume in 0.15 M KCl to wash out the butanol, and resedimented by centrifugation at 105,000g for 1 hr. The microsomes were then resuspended to 2.0 mg of protein/ml in 0.1 M Tris-HCl (pH 7.5) and distributed between two cuvets. Additions were as follows: (a) 0.86 mM phenacetin in a sample cuvet, (b) 1.67 mM aminopyrine in sample cuvet, (c) 3.3 mM aminopyrine in a sample cuvet, and (d) 0.2 mM naphthalene in a sample cuvet (evaporated on stirring rod from acetone and stirred into microsomal suspension).

and hexobarbital still were able to cause an RI spectral change, when added to the extracted microsomes.

Even precipitation of microsomes of benzopyrene-treated rats by addition of cold acetone (microsomes 1 : 5,v/v) did not prevent the RI spectral change by phenacetin or hexobarbital, although the magnitude of response was considerably diminished (60%).

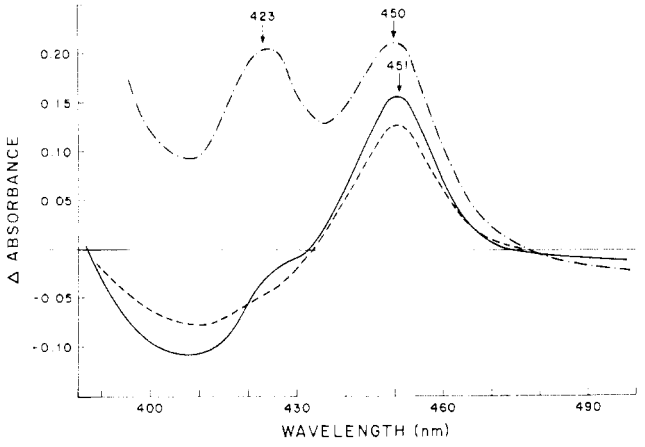

FIGURE 4: Effect of phenacetin and aniline on the carbon monoxide complex of cytochrome P-450. Rat liver microsomes were suspended in 0.1 M sodium phosphate buffer (pH 7.5) to give a concentration of 1.7 μM cytochrome P-450. The microsomal suspensions were divided into Thunberg cuvets and were degassed under vacuum before filling the air space with carbon monoxide. One Thunberg cuvet air space was filled with nitrogen as a reference. A few milligrams of solid $Na_2S_2O_4$, a chemical reductant, was present in the side arm of the cuvets, and was tipped into the suspension before running spectra. (——) CO in a sample cuvet, N_2 in a reference cuvet; (– – –) CO + 28 mM aniline in a sample cuvet, N_2 + 28 mM aniline in a reference cuvet; (– · –) CO + 28 mM aniline in a sample cuvet, N_2 in a reference cuvet. When phenacetin (3.3 mM) was present in one or both cuvets, the spectra obtained were identical with (—).

P-450 particles were formed by tryptic removal of cytochrome b_5 (Schenkman and Sato, 1968) from microsomes of untreated and 3-methylcholanthrene-treated rats. The microsomes were kept in 25% glycerol to prevent conversion of P-450 to cytochrome P-420 (Ichikawa and Yamano, 1967). Before formation of the P-450 particles, "off-balance" absolute spectra (Remmer *et al.*, 1969) obtained by comparison of the microsomes from control and methylcholanthrene-treated animals yielded a type I spectral change. In this procedure the microsomal concentrations are diluted to equal cytochrome b_5 levels. In the above experiment the ratios of cytochrome P-450 to cytochrome b_5 were identical in treated and untreated rats; in treated animals more of the cytochrome P-450 was present as the substrate-bound form, absorbing at 394 nm (Schenkman *et al.*, 1969), while in the untreated animal more was present as the substrate-free hemoprotein, absorbing at 419 nm. Formation of P-450 particles involved centrifugation to a pellet after overnight digestion by trypsin. The pellets resuspended to original volume had no P-420, and still formed an RI spectral change with phenacetin. Comparison of the microsomal particles of the treated and untreated animals diluted to equal cytochrome P-450 content again revealed a type I spectral change, but of lower magnitude. When related to cytochrome P-450 content, the magnitude of the phenacetin binding spectrum was diminished more than 50% by tryptic digestion in glycerol.

In an attempt to determine whether the RI spectral change and the type I spectral change are indications of a binding to different hemoprotein moieties (free and substrate bound) or to an allosteric site on the same moiety, simultaneous binding studies were performed. It was observed that in spectral titrations with phenacetin in the presence of 1.6 mM aminopyrine in the microsomal suspension, both the maximal phenacetin-induced RI spectral change as well as the spectral dissociation constant were increased (K_s = 0.37 mM to K_s = 1.7 mM phenacetin). Titration of aminopyrine-induced decrease in absorption at 420 nm in the presence of 0.7 mM

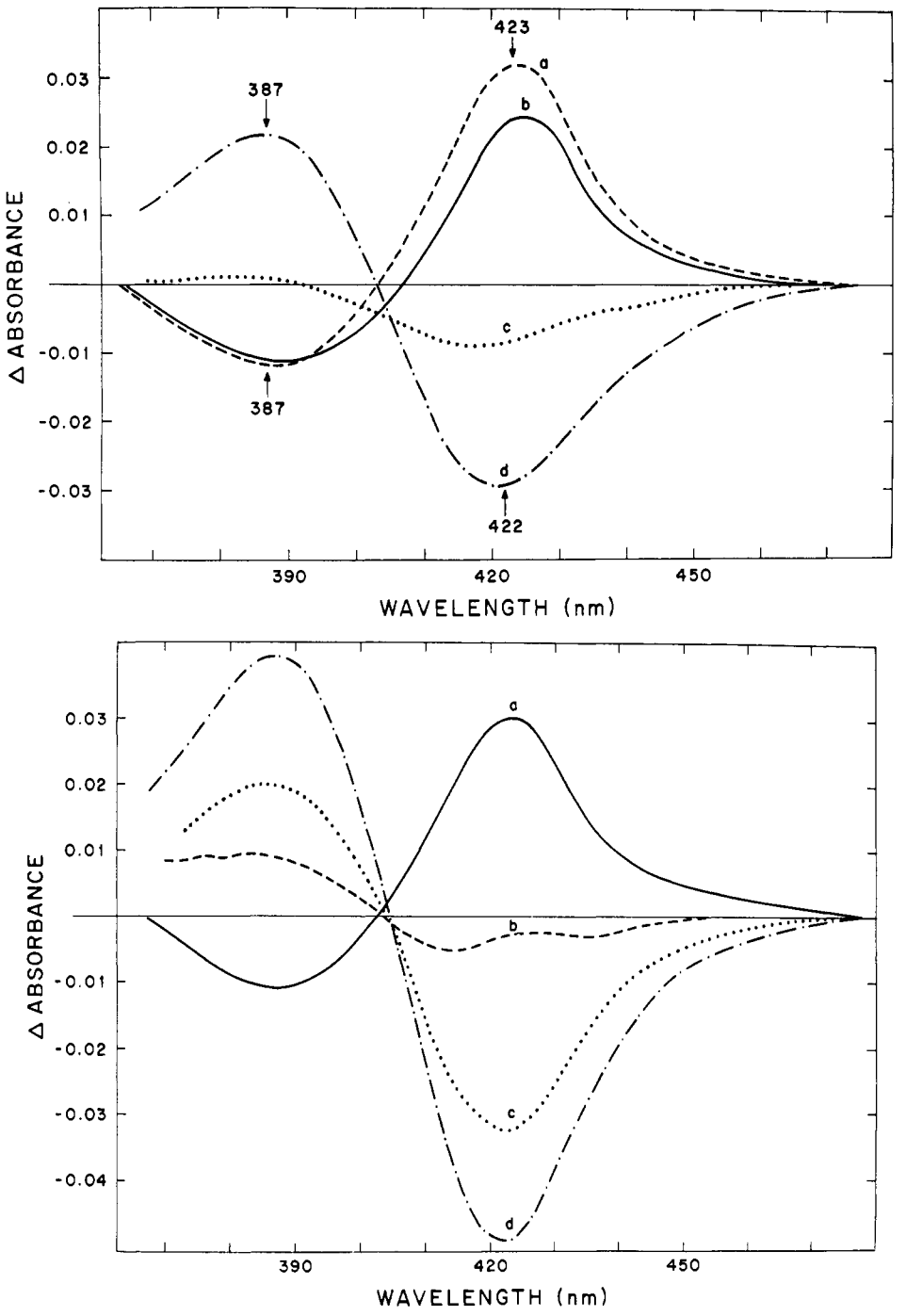

FIGURE 5: Interactions of substrate-induced RI and type I spectral changes. Liver microsomes from male adult rats were suspended in 0.1 M sodium phosphate buffer (pH 7.5) to a concentration of 3 mg of protein/ml, containing 3.4 μM cytochrome P-450 and 1.59 μM cytochrome b_5. All additions were to the sample cuvet. (A, top) (a) 3.3 mM phenacetin; (b) 3.3 mM phenacetin + 3.3 mM aminopyrine; (c) curve a subtracted from curve b; (d) 3.3 mM aminopyrine. (B, bottom) (a) 3.3 mM phenacetin; (b) 3.3 mM phenacetin + 3.3 mM hexobarbital; (c) curve a subtracted from curve b; (d) 3.3 mM hexobarbital.

phenacetin resulted in a 22% lower maximal spectral change (determined from double-reciprocal plots, Schenkman, 1970) as compared to titration in the absence of phenacetin. In addition, the presence of phenacetin increased the K_s from 0.35 to 1.1 mM aminopyrine. This strong inhibition of the aminopyrine interaction by phenacetin is not due to a binding of the phenacetin to heme iron, since the presence of 3.3 mM phenacetin in the microsomal suspension did not affect the CO complex of dithionite reduced cytochrome P-450 (Figure 4); hence the RI spectral change is not a ferrihemochrome.

Similarly, the presence of 15 μl of 1-butanol/ml in the microsomal suspension did not alter the magnitude of the P-450–CO complex. In the presence of the type II compound aniline, however (Figure 4), the resultant spectral change was considerably diminished. As pointed out by Imai and Sato (1967), aniline binding to reduced cytochrome P-450 forms two Soret peaks, one at 425 nm and one at 445 nm. When aniline was only included in the cuvet containing carbon monoxide, competitition between aniline and carbon monoxide for reduced cytochrome P450 was at once apparent; the 425-nm Soret peak was visible, along with a shift of the 450-nm peak of the CO complex to slightly lower wavelength.

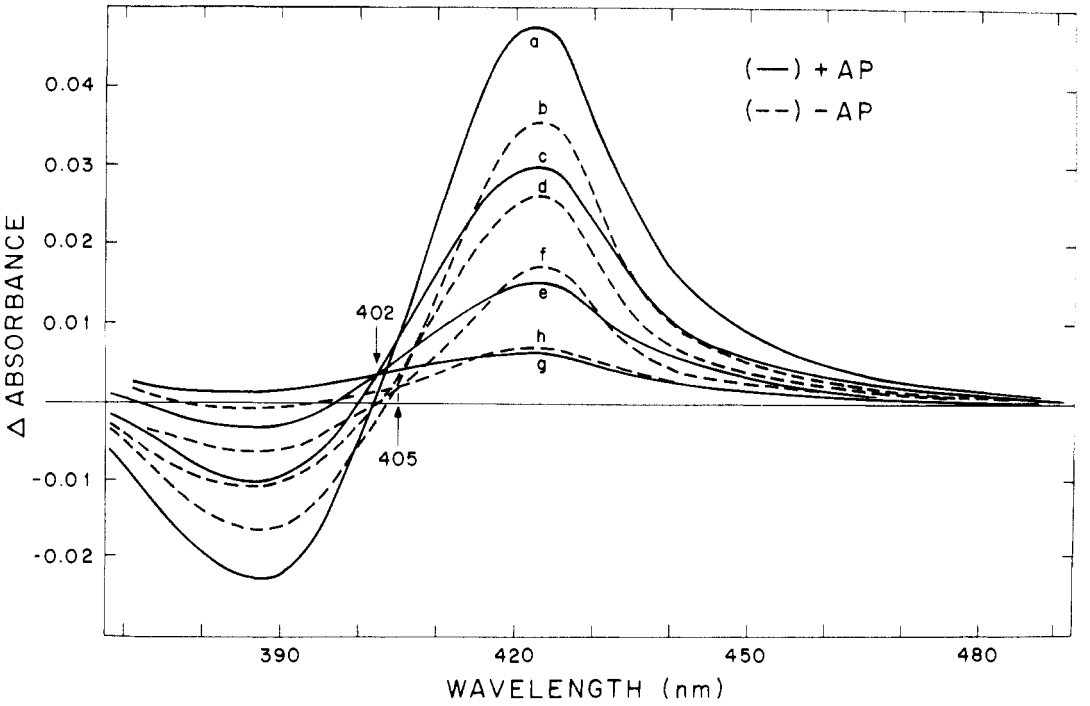

FIGURE 6: Comparison of phenacetin-induced RI spectral changes in the presence and absence of aminopyrine. Conditions were as in Figure 5, except solid curves were obtained after addition of 3.3 mM aminopyrine to the microsomal suspension. The phenacetin concentrations used were: (g,h) 0.067 mM, (e,f) 0.4 mM, (c,d) 1.1 mM, and (a,b) 2.4 mM.

Further evidence that the phenacetin RI spectral change is not a ferrihemochrome was provided by the lack of effect of this compound on the aniline-induced type II spectral change when added to the sample cuvet with aniline and a microsomal suspension. The reason for this lack of effect is that aniline forms a ferrihemochrome with *both* the free (419-nm peak) and substrate-bound (394-nm peak) form of cytochrome P-450 (Schenkman, 1970). Phenacetin also appears to bind at the type I site. As seen in Figure 5, the addition of a type I substrate to the cuvet containing phenacetin markedly diminished the magnitude of the RI spectral change. The extent of diminution was greater with hexobarbital (Figure 5B) than with aminopyrine (Figure 5A), presumably because the former has a greater affinity for the type I site (K_s = 0.09 mM *vs.* 0.33 mM, Schenkman, 1970). Subtracting curves a from curves b in each figure gives the extent of decrease due to formation of the type I spectral change (curves c) by aminopyrine or hexobarbital. Curves d in Figure 5A,B are the magnitudes of the type I spectral changes that aminopyrine and hexobarbital cause in the absence of phenacetin. Note that there is a greater loss of type I spectra with aminopyrine (69%) in the presence of phenacetin (Figure 5A) than with hexobarbital (34%) (Figure 5B).

In order to test the possibility that phenacetin interacts at another site on cytochrome P-450, reversing an equilibrium between the 394-nm-absorbing form and the 419-nm-absorbing form, the effect of the presence of both aminopyrine and phenacetin in various concentrations was studied (Figure 6). At high concentrations of phenacetin (curves a and c) the magnitude of the RI spectral change was greater when aminopyrine was in the medium than when it was absent (curves b and d), as would be expected, since more 394-nm form (aminopyrine-bound form) was present. However, at low phenacetin concentrations, the magnitude of the RI spectral change was lower in the presence of aminopyrine (curves e and g) than in its absence (curves f and h). A plot of the phenacetin-induced spectral peak at about 420 nm in the presence of aminopyrine exhibited a sigmoidal shape relative to the plot in the absence of aminopyrine, suggesting an allosteric binding.

Since compounds forming the type I spectral change cause an acceleration of the rate of reduction of cytochrome P-450 (Schenkman, 1968), compounds causing the RI spectral change should decrease this positive modifier effect if they reverse the type I structural orientation of the enzyme. That this is indeed the case is shown in Figure 7. Both phenacetin and 1-butanol, which cause the RI spectral change with rat liver microsomes, decreased the fast phase of P-450 reduction in anaerobic medium.

Metabolic studies were also performed to assess the effect of aminopyrine on phenacetin oxidation and the effect of phenacetin on aminopyrine oxidation. The results agreed with the spectral observations; the K_m for phenacetin oxidation was found to be about 0.045 mM, or about one-tenth the magnitude of the K_s for the phenacetin RI spectral change (0.37 mM). In the presence of 0.3 mM aminopyrine the oxidation of phenacetin was strongly inhibited, with a suggestion of mixed-type inhibition; the K_m was doubled and the V_{max} was markedly lowered (about 50%). Higher concentrations of aminopyrine (3 mM) effectively stopped phenacetin metabolism. The addition of 1 mM phenacetin to the medium caused inhibition of aminopyrine oxidation. The inhibition also appeared to be of a mixed type at the high phenacetin concentration.

Spectral Changes in Rabbit Liver Microsomes. In agreement with the report of Imai and Sato (1966) phenacetin does cause a type I spectral change in rabbit liver microsomes (Figure 8, curve b). In agreement with our previous

report (Schenkman *et al.*, 1969), hexobarbital causes a RI spectral change with these microsomes. In this study, rabbits pretreated with polycyclic hydrocarbon were used to enhance the magnitude of the 394-nm band. The reason for the opposite spectral changes in rabbit from that obtained with rats is not known. However, interaction does occur between substrate-induced spectral changes, as in rat liver microsomes, as shown in Figure 8. The addition of phenacetin to the cuvet containing hexobarbital decreased the RI spectral change markedly (curve c). The magnitude of the phenacetin-induced type I spectral change is lower in the presence of hexobarbital (curve d) than would be obtained in the absence of the barbiturate (curve b).

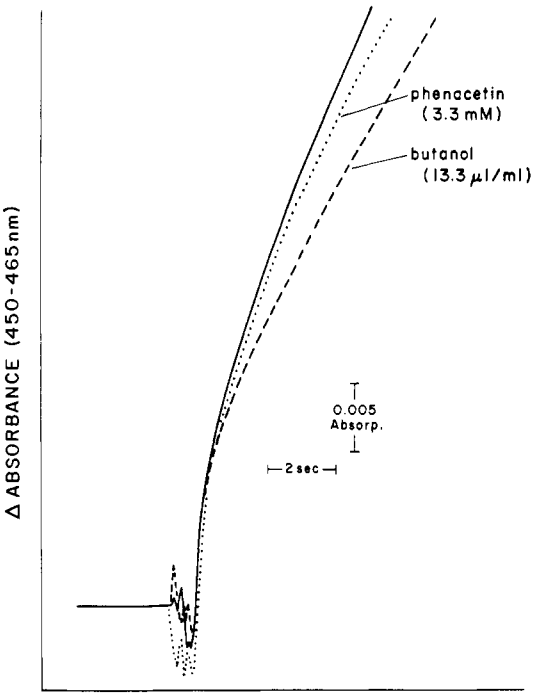

FIGURE 7: P-450 reductase activity in the presence and absence of RI compounds. Liver microsomes were suspended in 0.1 M sodium phosphate buffer to a concentration of 2.6 μM cytochrome P-450. Each anaerobic cuvet contained 6.7 mM glucose, 70 μg of glucose oxidase (Sigma)/ml and 5 μg of catalase (Sigma)/ml. The reaction mixture was gassed for 3 min with carbon monoxide and temperature equilibrated at 30° for 3 min in the spectrophotometer, and the reaction was started by plunging 20 μl of 5% NADPH into the 3-ml suspension. Aminco cuvets were used.

Discussion

From the data shown in this paper it is at once apparent that the reverse type I spectral change (RI) is completely different from the type II spectral change it so closely resembles. The latter is a ferrihemochrome, *i.e.*, reacts with ferricytochrome P-450 to bind to the heme iron, and, when the hemoprotein is reduced, can displace carbon monoxide. The RI spectral change does not compete with aniline for binding to the hemoprotein, nor does it compete with carbon monoxide for the reduced cytochrome P-450. The RI spectral change is, therefore, the spectral manifestation of interaction of a class of compounds (not always substrates) with the apoenzyme component of the mixed-function oxidase.

The substrate-bound enzyme has been shown in rat liver P-450 particles (Schenkman and Sato, 1968), in off-balance P-450 absolute spectrum (Hildebrandt *et al.*, 1968; Schenkman *et al.*, 1969) and in bacterial (*Pseudomonas*) P-450$_{cam}$ (Peterson, 1971) to have a peak at about 394 nm. The formation of the RI spectral change has been suggested (Schenkman *et al.*, 1969; Schenkman, 1970) as being due to the displacement of some *in vivo* bound substrate (or product) from the enzyme. The data shown in this paper indicate that this may indeed be the case; the magnitude of the RI spectral change can be markedly increased by the prior addition of a type I compound to the microsomal suspension, since it provides more substrate-bound (394-nm form) enzyme. Furthermore, formation of an RI spectral change slows the rate of P-450 reduction, as would be expected, since substrate binding (formation of a type I spectral change) enhances the rate of P-450 reduction. However, some formation of the RI spectral change in rat liver microsomes still occurs after the microsomes are treated with butanol or precipitated with acetone. If indeed some endogenous compound is bound, it is not readily extractable by these procedures. The RI spectral change does not appear to be related to substrate metabolism like the type I spectral change (Jansson *et al.*, 1972).

Observed interaction between substrate evoked RI and type I spectral changes may be explained in one of three ways: (1) RI compounds act at the same site on the enzyme as type I compounds; (2) compounds causing the RI spectral change interact at a different site on the enzyme than type I compounds; and (3) RI compounds interact at both the type I site and some other site on the enzyme.

The presence of phenacetin in the microsomal suspension decreases the maximal magnitude of the aminopyrine or hexobarbital-induced type I spectral change (from double-reciprocal plots), even at relatively low phenacetin concentrations, while markedly increasing the dissociation constant (amount of type I compound required for half-maximal spectral change). From this it must be concluded that phenacetin, a substrate of the mixed-function oxidase (Conney *et al.*, 1966), also causes a type I spectral change, the magnitude of which is less than its RI spectral change.

However, the increase in the phenacetin-induced spectral change by type 1 compounds like aminopyrine is not solely due to removal of the type I spectral overlap (as was shown with aniline; Schenkman, 1970). At low phenacetin levels, the presence of aminopyrine (Figure 6) actually decreased the magnitude of the RI spectral change. This indicates that aminopyrine and phenacetin are acting as opposing forces on the direction of equilibrium between the two forms (394 and 419 nm) of the enzyme. In fact, double-reciprocal plots of titrations of the phenacetin-induced spectral change in the presence and absence of aminopyrine show the dissociation constant (K_s) for phenacetin to be increased in the presence of the type I compound. These effects are highly suggestive of the mixed-function oxidase being a homotropic regulatory enzyme; plots of the phenacetin-induced spectral change in the presence of aminopyrine exhibit the suggestive sigmoidal curve (see Figure 9-9 in Lehninger, 1970).

Possibility 3 then appears to be the most probable explanation for the observed phenomena. The RI spectral change reflects a reversal of equilibrium between two forms of cytochrome P-450, from one with substrate at its active, or type I, site (absorbing at about 394 nm), to a form resembling the nonsubstrate-bound enzyme. From these studies it is not clear whether phenacetin interaction actually displaces the type I substrate from the enzyme active site, because there is a difference in the isosbestic point (but not spectral peak and trough) in the presence of aminopyrine (Figure 6). Although binding at different sites, there is a competition be-

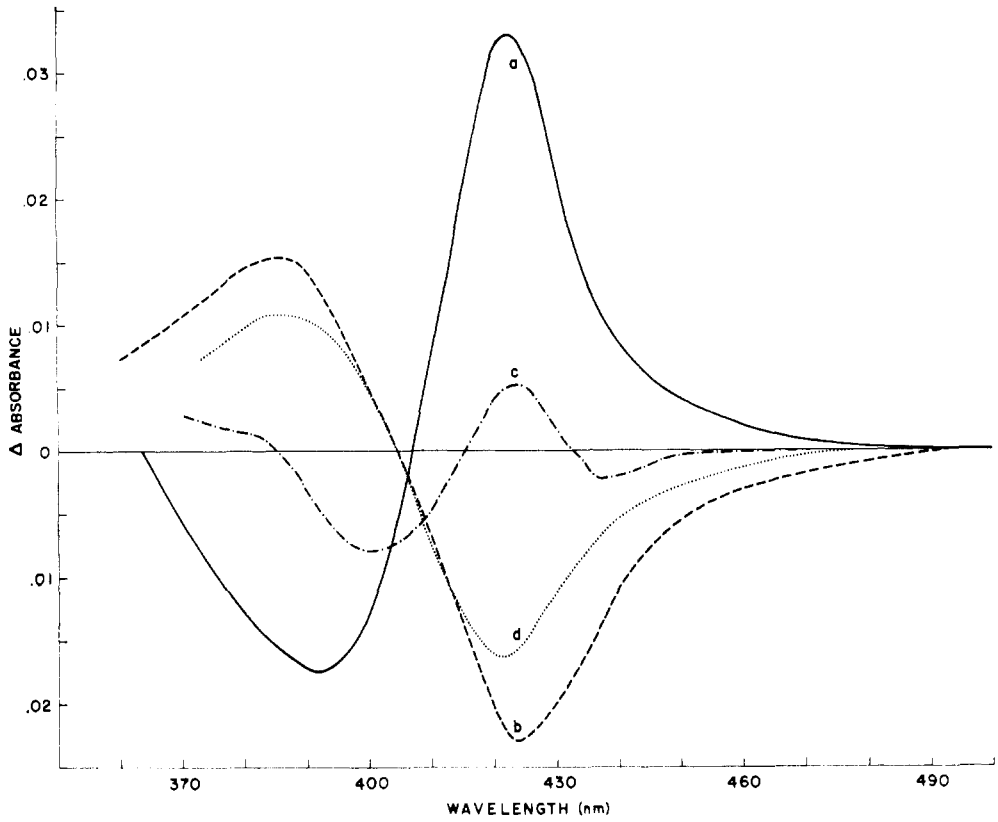

FIGURE 8: Binding of phenacetin and hexobarbital to liver microsomes of 3-methylcholanthrene-treated rabbits. Microsomal concentration 2 mg of protein/ml. Additions were: (a) 3.3 mM hexobarbital in a sample cuvet; (b) 1.5 mM phenacetin in the sample cuvet; (c) 1.5 mM phenacetin + 3.3 mM hexobarbital in a sample cuvet; (d) 1.5 mM phenacetin + 3.3 mM hexobarbital in a sample cuvet and 3.3 mM hexobarbital in a reference cuvet. Note curves a and b are the opposite of effects obtained with rat liver microsomes (Figure 1A,B).

tween type I and RI substances for the direction of the equilibrium; the presence of type I substrate in the medium is responsible for the increase in the K_s for phenacetin. Competition causes an increase in the K_s for aminopyrine and for hexobarbital-induced spectral changes in the presence of phenacetin too. But in these titrations the maximum spectral change from ordinate intercepts in double-reciprocal plots is never as large as in the absence of phenacetin, even though hexobarbital has a much greater affinity for the enzyme (K_s = 0.09 mM *vs.* 0.35 mM for phenacetin), because of the phenacetin type I component.

Specific substrates cause opposite spectral changes in rabbit liver microsomes from those obtained with rat liver microsomes, although spectral interactions are similar. The reason for this is not known, but may reflect differences in the two receptor sites responsible for recognizing substrates.

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Isolation and Identification of 24,25-Dihydroxycholecalciferol, a Metabolite of Vitamin D_3 Made in the Kidney†

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ABSTRACT: A metabolite of vitamin D_3 found in normocalcemic and hypercalcemic animals has been isolated in pure form from chicken kidney homogenates. It has been identified as 24,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions.

It is now clear that vitamin D_3 must be converted first in the liver to 25-hydroxycholecalciferol (25-OHD_3)[1] (Blunt *et al.*, 1968; Ponchon *et al.*, 1969; Horsting and DeLuca, 1969) and then in the kidney to 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) (Fraser and Kodicek, 1970; Gray *et al.*, 1971; Holick *et al.*, 1971a,b; Lawson *et al.*, 1971) before it can stimulate either intestinal calcium transport (Boyle *et al.*, 1972a) or bone calcium mobilization (Holick *et al.*, 1972). Of great importance is the observation that the 25-OHD_3–1-hydroxylase in the kidney is tightly regulated either directly or indirectly by blood serum calcium levels. Boyle *et al.* (1971) have demonstrated in young rats that the synthesis of 1,25-$(OH)_2D_3$ is maximal in hypocalcemic rats and is almost completely repressed in normocalcemic animals. Furthermore, they showed that the normocalcemic animals produced a metabolite proposed to be 21,25-dihydroxycholecalciferol (21,25-$(OH)_2D_3$) (Suda *et al.*, 1970a). Similarly Omdahl and DeLuca (1971) reported that the inhibition of intestinal calcium transport due to dietary strontium is the result of a block of the kidney hydroxylase which produces 1,25-$(OH)_2D_3$ from 25-OHD_3. They also found that instead of synthesizing 1,25-$(OH)_2D_3$ from 25-OHD_3 these animals make a new metabolite designated as peak Va. This metabolite is made exclusively by the kidney (Boyle *et al.*, 1972b; Omdahl *et al.*, 1972) and can be generated *in vitro* from 25-OHD_3 with kidney mitochondria from chickens that are fed a high calcium diet or a strontium diet.

It is the purpose of this report to establish firmly the structure of the chicken *in vitro* and porcine *in vivo* peak Va metabolite as 24,25-dihydroxycholecalciferol (24,25-$(OH)_2D_3$) based on ultraviolet absorption spectrophotometry, mass spectrometry, and its sensitivity to periodate treatment.

General Procedures

Radioactive determinations were carried out with a Packard Tri-Carb Model 3375 liquid scintillation counter equipped with an automatic external standard system. Samples were dried in 15 × 45 mm glass vial inserts with a stream of air and dissolved in 4 ml of toluene counting solution (2 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene] per 10 of toluene).

Ultraviolet absorption spectra were recorded with a Beckman DB-G recording spectrophotometer while mass spectrometric determinations were carried out with an AEI MS-9 mass spectrometer using a direct probe inlet at temperatures of 120–150° above ambient. Gas–liquid chromatography was carried out with an F & M Model 402 high-efficiency gas chromatograph equipped with a 0.25 in. × 4 ft glass column packed with 3% SE-30 on gas chrom Z, 100–120 mesh (Applied Science Labs, Inc., State College, Pa.). The column temperature was 250° and an outlet flow rate of 80 ml/min was maintained. All solvents used were redistilled before use.

In Vitro Preparation of Peak Va. Chickens (50 one-day old white Leghorn cockerel chicks obtained from Northern Hatcheries, Beaver Dam, Wis.) were kept in cages at 38° and fed *ad libitum* for 2 weeks as previously described (Omdahl *et al.*, 1971). At the end of the second week the chicks were switched to a high calcium diet (3% calcium) and given 0.25 μg of vitamin D_3 orally each day for an additional 12 days. The animals were sacrificed, and the kidneys were removed and homogenized in three volumes of buffer solution (pH 7.4) containing 14 mM Tris-OAc, 0.19 M sucrose, 1.87 mM MgOAc, 5 mM succinate, and 0.4 mM NADP. A total of 450 ml of homogenate was obtained and this was incubated as 6-ml aliquots in 75 erlenmeyer (250 ml) flasks at 37° for 90 min. Each flask contained 4.2 μg of [26,27-^{3}H]25-OHD_3 (specific activity 57,000 dpm/μg) (Suda *et al.*, 1971) in 25 μl of 95% ethanol. The homogenate was extracted with $CHCl_3$ and MeOH as previously described (Lund and DeLuca, 1966).

The resulting yellow lipid residue (1 g) was dissolved in 1.5 ml of 65:35 $CHCl_3$–Skellysolve B (petroleum ether fraction redistilled at 67–69°) and applied to a 2 × 60 cm glass column packed with 60 g of Sephadex LH-20 (a hydroxypropyl ether

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[1] Abbreviations used are: 25-OHD_3,-25-hydroxycholecalciferol; 21,25-$(OH)_2D_3$, 21,25-dihydroxycholecalciferol; 25,26-$(OH)_2D_3$, 25,26-dihydroxycholecalciferol; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 24,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol.