COMMUNICATIONS

Cytochrome P-450 as a Microsomal Peroxidase in Steroid Hydroxylations

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Cholesterol 20 α -hydroperoxide was found to undergo an enzymic rearrangement to 20 α ,22R-dihydroxycholesterol and 20 α ,21-dihydroxycholesterol by a cytochrome P-450 preparation and has been proposed as an initial intermediate in pregnenolone biosynthesis (van Lier & Smith, 1970a,b,c). Hydroperoxides have also been identified as intermediates in the NADPH-dependent hydroxylation of fluorene and tetralin by a 10 000g liver supernatant fraction (Chen & Lin, 1968, 1969). In the following, cytochrome P-450 seems to be responsible for the decomposition of steroid and other organic hydroperoxides to hydroxy derivatives by microsomal fractions.

The peroxidase activity of rat liver and adrenocortical microsomal fractions was measured with NNN'-N'-tetramethyl-p-phenylenediamine as hydrogen donor (O'Brien & Hrycay, 1971). The hydroperoxides that could act as substrates were, in order of effectiveness, progesterone 17 α -hydroperoxide, allopregnanolone 17 α -hydroperoxide, linoleic acid hydroperoxide, cumene hydroperoxide, cholesterol 7 β -hydroperoxide and cholesterol 25-hydroperoxide. Purified cytochrome b₅ had low peroxidase activity, whereas a cytochrome P-450 preparation was very active. Liver microsomal fraction from phenobarbital-injected rats with a threefold higher specific cytochrome P-450 content was much more active. Further evidence for cytochrome P-450 being a microsomal peroxidase was the inhibition by type I and type II ligands. Detergents, alcohols and ketones, p-hydroxymercuribenzoate and trypsin also inhibited peroxidase activity, probably as a result of cytochrome P-420 formation from cytochrome P-450.

The rate of NADPH or NADH oxidation by microsomal fractions was markedly enhanced by these hydroperoxides. The reaction rate was first-order with respect to protein concentration with K_m values for NADPH and NADH less than 10 μ M and an apparent K_m 0.4 mM for cumene hydroperoxide. Evidence for the involvement of the flavoenzyme NAD(P)H oxidoreductases was the complete inactivation by 0.05 mM p-chloromercuribenzoate and protection by NADH or NADPH respectively. Removal of cytochrome b₅ by incubation of microsomal fraction with trypsin did not affect the NADH-hydroperoxide activity. Cytochrome P-450 seemed to be involved in both activities, since type I and type II ligands or agents

converting cytochrome P-450 into cytochrome P-420 produced inactivation whereas phenobarbital administration *in vivo* caused a marked stimulation. Although liver and adrenocortical microsomal fractions had similar activity, intact or sonicated adrenocortical mitochondria, which have similar cytochrome P-450 contents, were only slightly active. Liver mitochondria had no activity.

The hydroxy products formed in these microsomal reactions with progesterone 17 α -hydroperoxide included intermediates probably involved in testosterone, oestrone or cortisol biosynthesis. The above results suggest that cytochrome P-450 can act as a peroxidase for steroid hydroperoxides and that these hydroperoxides, like oxygen, can also oxidize reduced cytochrome P-450 to cytochrome P-450.

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Plasma 11-Hydroxy Corticosteroid Concentrations in Stressed Adult Rats after Under-nutrition in Early Life

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Elevation of plasma 11-hydroxy corticosteroid concentrations in response to stress in adult rats differs between animals handled or left undisturbed in infancy (Levine, 1962). It has been proposed (Levine & Mullins, 1966) that this is a result of experimentally produced steroid variations in infancy that permanently modify the regulatory mechanisms of the hypothalamic-pituitary-adrenal system. It is possible that nutritional status during infancy may also have lasting effects on this system, since plasma 11-hydroxy corticosteroid concentrations are elevated in malnourished children (Alleyne & Young, 1967).

Rats were undernourished by restricting their mothers to an approx.-50%-*ad-libitum* diet from the seventh day of gestation until weaning. All animals were fed *ad libitum* from 25 days, when undernourished young were 34% of the weight of normal rats. At killing (15–17 weeks) previously undernourished males were 69% and females 81% of the weights of normal animals. Finally there were eight groups of animals, separated according to (a) sex, (b) nutritional history (normal or previously undernourished) and (c) treatment (not stressed or stressed). Stressed females were killed 17 min after receiving an electric shock, and stressed males 15 min after being placed in a situation in which they had been shocked the day before. Plasma 11-hydroxy corticosteroids were determined (Mattingly, 1962) with corticosterone as standard.

Basal concentrations (means $\pm$ s.d.) (in rats that had not been stressed) did not differ significantly between normal and previously undernourished groups for either males (normal, $20.0 \pm 7.4 \mu\text{g}/100\text{ml}$, $n=19$; undernourished, $16.8 \pm 5.7 \mu\text{g}/100\text{ml}$, $n=14$) or females (normal, $33.2 \pm 18.9 \mu\text{g}/100\text{ml}$, $n=11$; undernourished, $30.5 \pm 15.7 \mu\text{g}/100\text{ml}$, $n=11$). Stressed normal males had significantly higher 11-hydroxy corticosteroid concentrations than did their previously undernourished counterparts (normal, $40.1 \pm 13.3 \mu\text{g}/100\text{ml}$, $n=21$; undernourished, $30.7 \pm 9.4 \mu\text{g}/100\text{ml}$, $n=17$; $P<0.02$). Although early undernutrition did not significantly affect concentrations in stressed females (normal, $92.8 \pm 17.6 \mu\text{g}/100\text{ml}$, $n=15$; undernourished, $81.7 \pm 16.4 \mu\text{g}/100\text{ml}$, $n=12$; $0.1 > P > 0.05$) there was among these animals a significant correlation ($r = +0.625$, $n=26$, $P<0.001$) between plasma 11-hydroxy corticosteroid concentration and body weight.

It is suggested that early undernutrition, in male rats at least, decreases their responsiveness to stressful stimulation as measured by plasma 11-hydroxy corticosteroid concentrations.

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An Assay Method for Cholesterol 7 α -Hydroxylase in Rat Liver Microsomal Fraction

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The first step in the conversion of cholesterol into cholic acid is thought to be the introduction of a 7 α -hydroxyl group into the ring system of cholesterol (Bergström, 1959). 7 α -Hydroxylase, the enzyme catalysing this reaction, is confined to the liver microsomal fraction and requires NADPH and molecular oxygen (Danielsson & Einarsson, 1966). Cytochrome *P*-450 is the terminal oxidase for this hydroxylation (Boyd, Scholan & Mitton, 1969). There is evidence to suggest that the 7 α -hydroxylation of cholesterol is rate-limiting in cholic acid formation (Danielsson, Einarsson & Johansson, 1967). Hence, by studying the effect of various physiological factors on the activity of 7 α -hydroxylase, it may be possible to obtain information about the regulation of bile acid formation in the liver.

The cholesterol molecule is susceptible to attack by molecular oxygen, with the formation of 7-oxo-cholesterol, 7 β -hydroxycholesterol, 7 α -hydroxycholesterol and other compounds. This oxidative process is enhanced in the presence of microsomal fraction fortified with NADPH. However, incubation of cholesterol with microsomal fraction from rat liver homogenized in the presence of EDTA (Shefer, Hauser & Mosbach, 1968) gives 7 α -hydroxycholesterol free from autooxidation products.

Current methods for assaying 7 α -hydroxylase activity are based on measurement of the incorporation into 7 α -hydroxycholesterol of radioactive cholesterol added to a suspension of microsomal fraction in the presence of a suitable medium. Such methods may give erroneous results because liver microsomal fraction contains appreciable amounts of cholesterol. It is possible that only a portion of this endogenous cholesterol acts as substrate for the enzyme, and that the proportion of the total endogenous cholesterol that is metabolically active varies under different conditions. It is also possible that added radioactive cholesterol mixes with the metabolically active pool to different extents depending on the way in which it is added. These considerations have led us to develop a more direct method for measuring 7 α -hydroxylase activity in microsomal suspensions.

[4-¹⁴C]Cholesterol was incubated with liver microsomal fraction and the 7 α -hydroxycholesterol synthesized was extracted and separated from other compounds by t.l.c. The steroid was located by radioautography, eluted and acetylated with [³H]acetic anhydride (Peterson & Eilers, 1965). The amount of 7 α -hydroxycholesterol was then