evidence is incomplete. In spite of these uncertainties, the points shown in Fig. 2 are probably as good as most of those for low sea levels about 10,000 years ago; in fact, they fall within the scatter of points based on many kinds of materials through which the curve of Fig. 2 was drawn by Shepard (*12*).

Medcof (*15*) reported several age determinations of oyster shells; one shell, aged 10,600 ± 130 years, came from Georges Bank (42°05′N; 67°15′W) and was at a depth of 53 m of water. Another, aged 6850 ± 100 years, came from Northumberland Strait in the Gulf of St. Lawrence (46°00′N; 62° 37′W), from a depth of 37 m. These results (*16*) corroborate and seem likely to extend our own findings.

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1. This species is not to be confused with *Ostrea equestris* Say, which was found alive at a depth of 110 m between Delaware Bay and Chesapeake Bay, a new northern limit (*2*).
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Photochemical Action Spectrum of the Terminal Oxidase of Mixed Function Oxidase Systems

Abstract. *The reversal of the carbon monoxide inhibition by bands of monochromatic light was determined for the oxidative demethylation of codeine and monomethyl-4-aminopyrine and the hydroxylation of acetanilide by rat liver microsomes and for the hydroxylation of 17-hydroxyprogesterone at carbon-21 by bovine adrenocortical microsomes. Maximum reversal occurred at 450 millimicrons, the light absorption maximum of the CO compound of the CO-binding pigment of microsomes. The agreement between photochemical action spectrum and spectrophotometric difference spectrum supports the conclusion that the CO-binding pigment is the terminal oxidase of mixed function oxidase systems of mammals.*

Mixed function oxidases, also termed mixed function oxygenases or aerobic hydroxylases (*1*), catalyze the incorporation of atmospheric oxygen into organic compounds (AH) according to Eq. 1:

$$AH + TPNH + H^+ + O_2 \rightarrow AOH + TPN + H_2O \quad (1),$$

where TPN and TPNH are triphosphopyridine nucleotide and its reduced form. The enzymes are strongly inhibited by sulfhydryl reagents but they are not inhibited by respiratory poisons such as cyanide and azide. However, Ryan and Engel (*2*) discovered that one of these enzyme reactions, the hydroxylation of corticosteroids at carbon-21 by the steroid 21-hydroxylase of bovine adrenocortical microsomes, was inhibited by carbon monoxide and that the inhibition was reversed by light. This potential clue to the nature of the oxygen-activating enzyme of hydroxylase systems was not further exploited because Ryan and Engel were unable to detect in their preparations a pigment that combined with carbon monoxide. Re-examination (*3*) of the difference spectrum of bovine adrenocortical microsomes with appropriate spectrophotometric methods revealed that the preparations contained the so-called CO-binding pigment previously observed in liver microsomes by Klingenberg (*4*) and Garfinkel (*5*) and designated by Omura and Sato (*6*) as an

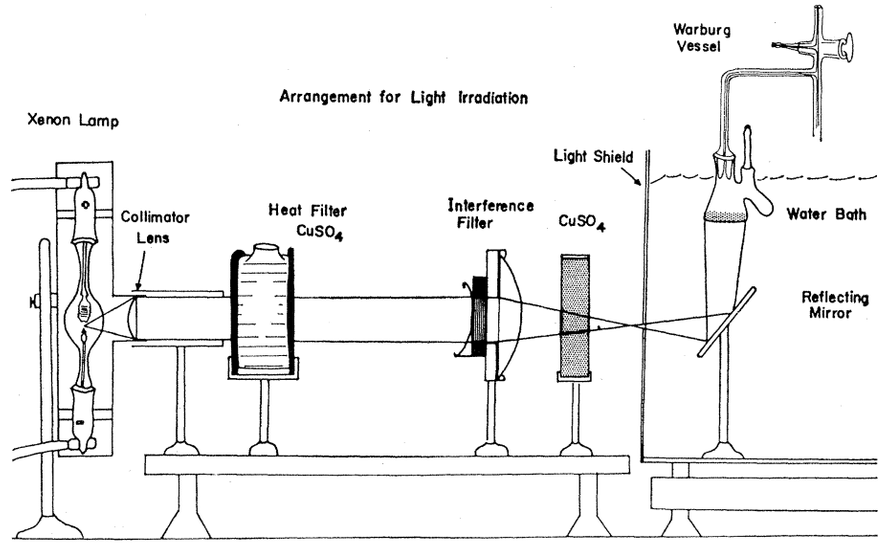

Fig. 1. Arrangement for determining the photochemical action spectrum. The collimated beam of a 1600-w high-pressure xenon lamp passes through heat filter (5.0-cm layer of 7 percent $CuSO_4$ solution), interference filter and neutral density filter if required, focusing lens, and 2.5-cm layer of $CuSO_4$ solution for absorption of the second order spectrum, enters the light-shielded glass-walled water bath through an opening in the shielding, and is reflected by the mirror onto the bottom of the Warburg vessel, which is shaken within the area of the beam at 130 oscillations per minute. Less than 1 percent of the irradiating light was absorbed by the bottom of the incubation vessel and the reaction mixture. The half band width of the interference filters was ± 10 to 12 mμ for filters with transmission maxima at 401, 465, and 502 mμ (*13*) and ± 4 mμ for the filters at 412, 419, 426, 433, 441, 450, and 475 mμ (*14*).

unusual cytochrome provisionally called P-450. The CO compound, P-450-CO, of the reduced form of this pigment has its absorption maximum at 450 mμ.

To determine whether P-450-CO was the CO derivative of the oxidase of the steroid 21-hydroxylase system the photochemical action spectrum method (*7*) was used. This method is based on the fact that the degree of reversal of the CO-inhibition of an enzyme reaction by different bands of monochromatic light of equal intensities corresponds to the light absorption spectrum of the CO compound of the enzyme. Since mixed function oxidases are characterized by the fixation of molecular oxygen into a specific substrate rather than by the overall consumption of oxygen, the rate of formation of the hydroxylation product was used as the measure of photochemical action. The photochemical action spectrum of the adrenocortical steroid 21-hydroxylase was in accord (*8*) with the spectrophotometric difference spectrum of P-450-CO. We now report that the same relation holds for several aerobic hydroxylases of liver microsomes, and that the CO-binding pigment P-450 appears to be the terminal oxidase of mixed function oxidase systems of mammalian tissues.

The reactions studied were the oxidative demethylation of codeine and monomethyl-4-aminopyrine (MAP) and the hydroxylation of acetanilide by rat liver microsomes. Enzyme activity was induced (*9*) by the intraperitoneal injection of sodium phenobarbital (80 mg/kg) into male Wistar rats (80 to 100 g). Two days after injection the animals were killed. The microsomal fraction was isolated from the liver homogenates by a procedure similar to that for adrenocortical microsomes (*8*). Portions of the microsome suspension were transferred to the main compartment of conical Warburg vessels containing buffered substrate solution while a TPNH generating system was placed in the side arm of the vessel. After attachment to the manometers, the assay system was equilibrated for 10 minutes at 25°C with appropriate gas mixtures. The stopcocks were then closed, the contents of side arm and main compartment were mixed, and the incubation was continued for an additional 10 to 15 minutes with or without illumination (Fig. 1). The reaction was then stopped and the hydroxylation product was determined.

The effect of varying the ratio of CO to O_2 on the oxidative demethylation of codeine by liver microsomes is shown in Fig. 2. The inhibition is of the competitive type as evidenced by its dependency on the ratio of CO to O_2 rather than on the CO concentration. The ratio at which half inhibitions occurred, the distribution constant K of the enzyme between CO and O_2, was close to 1 (range 0.5 to 1.5) with the three aerobic hydroxylation systems of liver microsomes as well as with the steroid 21-hydroxylase of adrenocortical microsomes (8). This value is about one order of magnitude lower than the K value for the respiration enzyme reported by Warburg (*7*).

The photochemical action of monochromatic light on the CO-inhibition has been expressed by Warburg's (*7*) light sensitivity factor, L, which is the reciprocal of the quantum energy required for doubling the distribution constant K. The 450-mμ light band effected maximal reversal of the CO inhibition of the three hydroxylase systems (Fig. 3). Light from the 441- and 462-mμ regions of the spectrum was about half as effective. Concerning the degree of reversal at a given wavelength the differences between the enzyme systems are of doubtful significance, and probably reflect the difficulty of determining the small differences in product formation between irradiated and nonirradiated samples with sufficient precision.

For comparison Fig. 4 shows the photochemical action spectrum of the bovine adrenocortical steroid 21-hydroxylase system. This system has the advantage of permitting the determination of small differences in reaction rates with sufficient precision. We determined (*10*) the action spectrum with our improved irradiation technique. Its close similarity to the action spectrum of the liver systems is evident, the only difference being a slightly broader maximum.

The light sensitivity values for the CO complexes of the demethylases and the acetanilide hydroxylase of rat liver were respectively lower by 60 and 30 percent than the value for the bovine adrenocortical steroid 21-hydroxylase (Table 1). The reasons for these important differences are unknown. More significant, however, is the finding that the L values of all mixed function oxidase systems are of the same order of magnitude, which is about 1/100 of the magnitude reported for the CO derivatives of the respiration enzyme and of hemoglobin (*7*). Low light sensitivity, hitherto only found of CO com-

Table 1. Light sensitivity of CO-inhibited mixed function oxidases (λ = 450 mμ; 25°C).

Substrate	V*	$\frac{CO}{O_2}$	n†	Kd‡	ΔK	10^{-6} L§
			Rat liver microsomes			
Codeine	5.7	1.05	0.48	0.96	2.17	0.97
MAP	4.0	2.16	0.34	1.14	2.86	1.17
Acetanilide	6.0	1.05	0.43	0.82	3.07	1.84
			Bovine adrenocortical microsomes			
17-Hydroxy-progesterone	2.4	2.16	0.30	0.97	3.15	2.70

* V = rate of product formation = mμmole/min per milligram of protein, without CO. † $n = V^{co}/V$, where V^{co} is the rate of product formation in the presence of CO. ‡ $K = [n/(1 - n)]$ (CO/O_2). § L = (cm² min)/mole quanta.

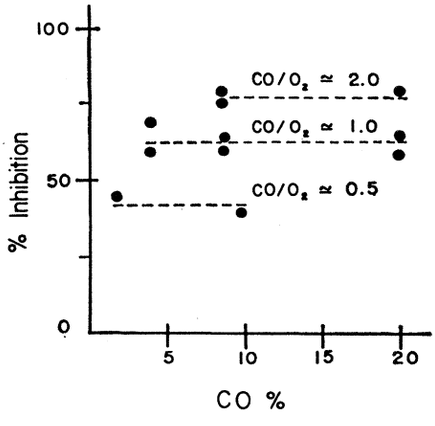

Fig. 2. Dependency of the CO inhibition of codeine demethylation on the CO/O_2 ratio. The percentage inhibition of codeine demethylation plotted as function of the CO concentration of the CO-O_2-N_2 mixtures. The ratio CO/O_2 is recorded above the horizontal lines. Inhibitions are calculated with reference to the rate of a control sample equilibrated with the same oxygen concentration as the sample exposed to CO. The main compartment of each Warburg vessel contained 0.5 ml of microsome suspension (3 mg of protein); 1.2 ml of 0.08*M* trisphosphate buffer (*p*H 7.4) containing 0.005*M* $MgCl_2$; and 0.5 ml 0.025*M* codeine phosphate solution. The side arm contained 0.3 ml of the TPNH-generating system (0.5m*M* TPN; 0.13*M* glucose-6-phosphate, 0.5 Kornberg units of glucose-6-phosphate dehydrogenase). Incubation time was 10 minutes at 25°C in darkness. The reaction was stopped by adding 0.25 ml of 6*M* trichloroacetic acid to the detached vessel. Formaldehyde, the demethylation product, was determined according to Nash (*15*).

plexes of ferrocysteine (*7*), thus appears to be a distinctive biophysical characteristic of the CO complexes of mixed function oxidases. In addition,

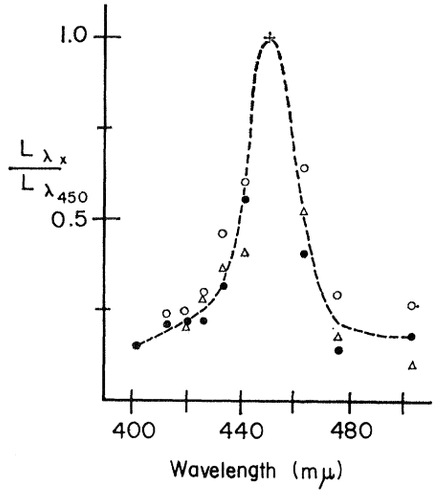

Fig. 3. Photochemical action spectrum of mixed function oxidases of rat liver microsomes. ●, Codeine; ○, MAP; △, acetanilide. Procedure as in Fig. 2. Parahydroxyacetanilide was assayed by a slight modification of the method of Krisch and Staudinger (*16*). Light sensitivity is defined by $L = (1/i)\ (\Delta K/Kd)$, where i is light intensity in terms of mole quanta/(cm² min), Kd the distribution constant in darkness, and ΔK the increase in K produced by light of a given intensity

$$L_x/L_{450} = (i_{450}/i_x)\ ([\Delta K]_x/[\Delta K]_{450})$$

The numerical values of Kd and of the quantities from which it is derived are recorded in Table 1 which also gives the values of ΔK and L at 450 mμ.

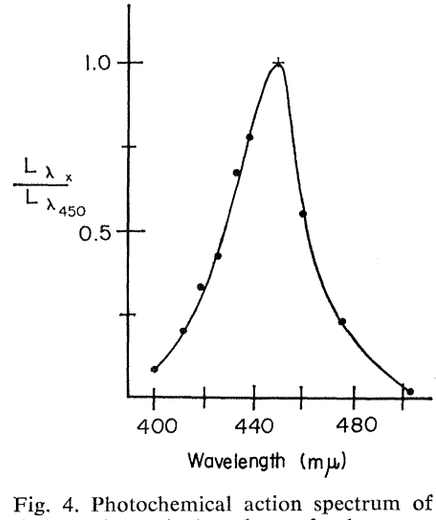

Fig. 4. Photochemical action spectrum of the steroid 21-hydroxylase of adrenocortical microsomes. The procedure has been described (*8*). The microsomal protein was 6.4 mg per vessel; incubation time 15 minutes at 25°C. Numerical data in Table 1.

this property together with the absence of detectable absorption bands outside the Soret region offers an explanation of the failure of Klingenberg (*4*) and Omura and Sato (*6*) to demonstrate spectrophotometrically any dissociation of P-450-CO by irradiating with red light of an intensity sufficient to produce partial dissociation of the CO complexes of either cytochrome a_3 or myoglobin.

Besides the accordance between photochemical action spectrum and light absorption difference spectrum of the CO derivatives, the identity of P-450 with the oxygen-activating component of aerobic hydroxylase systems is supported by reports (*11*) that treatment of animals with inducers of aerobic hydroxylase activity of liver microsomes results in a marked increase in microsomal P-450-CO. Conversely we have shown (*12*) that the development of irreversible inhibition after the addition of sulfhydryl reagents to the steroid 21-hydroxylase system is associated with the conversion of the pigment to the irreversibly inactivated derivative defined by Omura and Sato (*6*).

These observations together with our experimental evidence strongly suggest that the so-called carbon monoxide-binding pigment of microsomes is the terminal oxidase of mixed function oxidase systems of mammalian tissues.

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Cleft Palate in the Mouse: A Teratogenic Index of Glucocorticoid Potency

Abstract. Clinically equivalent doses of hydrocortisone, prednisolone, and dexamethasone have progressively increasing teratogenic activity as judged by their ability to induce cleft palate in the offspring of pregnant mice treated with these drugs during the middle period of gestation. Mole for mole, dexamethasone is at least 300 times more teratogenic than hydrocortisone. The enhanced teratogenicity of dexamethasone probably does not result from its relatively decreased mineralocorticoid activity.

A dose of 2.5 mg of cortisone acetate in suspension given intramuscularly to inbred A/Jax mice on each of days 11, 12, 13, and 14 of gestation will produce cleft palate (without cleft lip) in 100 percent of the offspring (*1*). The purposes of our study were: first, to observe the relative teratogenic activity of certain glucocorticoid drugs with cleft palate as the indicator; and second, to compare teratogenicity with other known laboratory parameters of glucocorticoid effect.

Water-soluble preparations of hydrocortisone, prednisolone, and dexamethasone (*2*) were used to avoid variations in preserving and suspending agents. The stability of these solutions was tested by comparing their teratogenic activity when injected within ¼ hour to 4 days of preparation and after 2 weeks of storage at 5°C; no differences were observed. Comparable doses of each steroid were derived from the schedule of therapeutically equivalent dosages used clinically in man (*3*). Since maternal weight is known to affect the frequency of cleft palate after a