The Effect of Carbon Monoxide Inhalation on the Mixed-Function Oxidase Activity in the Chick Embryo and the Adult Mouse[1]

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KLINGENBERG (1958) and GARFINKEL (1958) discovered a carbon monoxide (CO) binding pigment in rat liver microsomes. This mixed-function oxidase (mfo) called P-450 by OMURA and SATO (1964) is a TPN-linked cytochrome and can incorporate atmospheric oxygen into a molecule as in the hydroxylation of steroids and drugs (GILLETTE et al. 1969). It is also involved in the hepatic detoxification processes (REMMER 1970).

Preliminary studies (BAKER and TUMASONIS 1971) in our laboratory have shown that viability and hatchability of exposed chick embryos are inversely related to the CO concentration used. The morphologic, hematologic, and enzymatic alterations occurring in chick embryos exposed to CO have established the sensitivity of this developing organism to CO. Alteration in lactic dehydrogenase (LDH) and cytochrome oxidase (COX) activity of heart tissue of CO-exposed chick embryos (TUMASONIS and BAKER 1972) indicated that the 14th day of development represented a turning point in the pattern of enzymatic response between the 10th and 18th day of development. BAKER et al. (1971) have reported an increase in mfo activity in livers of chick embryos exposed to CO. In view of these findings and the reported influence of CO upon the heme-containing enzyme COX, the present study was designed to investigate the effect of CO on two enzymes of the mfo system, hydroxylase and O-demethylase, in the liver of 12- and 17-day-old CO-exposed chick embryos. To determine whether a comparable alteration occurs in mammals, livers of NYLAR mice were analyzed for mfo activity following 1-week exposure to 100, 200, and 500 ppm of CO.

Materials and Methods

Exposure and handling of embryonated eggs: White Leghorn embryonated eggs, 55.0 g ± 0.5 g, were used in this study. Storage, handling, and experimental conditions have been described (BAKER and TUMASONIS 1971). A preliminary 24-hour exposure study of 11-, 13-, 15-, and 17-day-old chick embryos to 425 ppm CO was performed to determine the effect of CO on mfo activity. Subsequently, chick embryos of known age were exposed to 425 ppm CO from 24 to 144 hours to

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determine the effect of length of exposure upon mfo activity. To determine the influence of age, sex, and exposure upon mfo activity 12- and 17-day-old chick embryos were exposed to 425 ppm CO. Chick embryos were sexed at the time of sacrifice.

Exposure and handling of NYLAR-A mice: Rigid plastic isolators, modified by the addition of one-inch inlet and exhaust ports, served as exposure and control chambers. CO was mixed with air and a flow rate effecting a complete change of atmosphere every 5 minutes was maintained. The desired level of CO was established and monitored continuously by a nondispersive infrared-type analyzer. Standardization of the infrared analyzer was carried out as described previously (BAKER and TUMASONIS 1971). While each isolator is capable of holding 6 to 8 mouse cages, we considered 4 cages with 10-15 mice per cage as optimal to eliminate dead spaces and provide maximum access to the contaminated environment. Food and water were provided ad lib. Mice used were NYLAR-A-strain (Division of Laboratories and Research, New York State Department of Health - Albany strain inbred for 38 years at this laboratory).

Carboxyhemoglobin: Carboxyhemoglobin (COHb) determinations were made on both control and exposed chick embryo and NYLAR-A mouse blood according to the method of BUCHWALD (1969). Chick embryo blood was obtained from the allantoic artery, and mouse blood by orbital bleeding.

Enzyme preparations: Embryonated eggs (13 and 18 days old) were opened, livers removed, weighed after removal of gall bladder, and homogenized in 3 volumes of ice-cold 1.15% KCl solution with a Teflon-glass homogenizer. The homogenate was centrifuged at 12,000 x g for 20 minutes in a Sorvall RC2-B centrifuge with a SM-24 head and then filtered through glass wool. One millimeter of the supernatant was equivalent to 250 mg of liver. All enzymic assays were carried out the day suspensions were prepared.

An incubation mixture consisted of 1 ml of a 12,000 x g supernatant; 1 ml $MgCl_2$-nicotinamide solution ($MgCl_2$-24µmoles ; nicotinamide - 100µmoles); 1 ml 0.25 M phosphate buffer, pH 7.4, containing cofactors sodium glucose-6-phosphate hydrate, 25µmoles, and nicotinamide adenine dinucleotide phosphate ((NADP) 0.5µmoles); 0.1 ml substrate solution (p-nitroanisole - 10µmoles, aniline 0.5µmoles); and 2 ml distilled water. The mixtures were incubated aerobically in a water bath at 37° C for 30 minutes.

The amount of demethylated p-nitroanisole was determined from the concentration of p-nitrophenol present according to the method of McMAHON et al. (1963). The hydroxylation of aniline was determined by measuring the p-aminophenol by the modified indophenol method of KATO and GILLETTE (1965b).

Measurements of absorption spectra: CO-difference spectra of microsomal preparations from control and exposed male and female mice and 18-day-old chick embryos were measured in a Beckman DBG spectrophotometer. Microsomal preparations containing about 2 mg of protein per ml of 0.25 M phosphate buffer (pH 7.4) were placed in both the sample and reference cells. After the base-line was recorded, the sample containing cells was treated with dithionite and CO, and the spectral differences measured. When CO (100%) was used, it was carefully bubbled through the sample for 1 minute. Samples were reduced by the addition of a few milligrams of dithionite ($Na_2S_2O_4$). All spectrophotometric measurements were made at room temperature (20-25°C).

Student's t test was used to establish statistical significance.

Results and Discussion

Carboxyhemoglobin levels: Carboxyhemoglobin (COHb) determinations on blood of chick embryos of known age following exposure to 425 ppm CO for 1, 3, 5, 7, and 24 hours have established that within 3 to 5 hours COHb percentages reach maximum value. After 24 hours of CO-exposure, COHb levels were found stabilized slightly lower than the maximum level obtained during initial hours of exposure. COHb equilibrium levels of exposed chick embryos were incubation-age related, averaging 7.4 ± 1.4% (x ± S.D.) for the 12- to 14-day-old chick embryos; 16.4 ± 2.8% for 16-day-old embryos; and 35.7 ± 3.9% for 18-day-old embryos. The mean COHb level of 12- through 18-day-old control chick embryos was 1.5 ± 0.8%. No significant difference was found in COHb levels of exposed male or female, or control embryos. In NYLAR mice exposed to 100, 250, and 500 ppm CO, COHb levels were found stabilized at 9.7 ± 3.1%, 13.5 ± 2.0%, and 25.6 ± 3.8%, respectively, during the 7-day exposure period. The mean COHb level of control mice was 0.5 ± 0.07%. Again, no significant difference was noted in COHb levels of exposed male and female, or control mice. Statistical data were based on 20 samples for each category.

Effect of exposure to 425 ppm CO on mixed-function oxidases of chick embryo livers: A preliminary report (BAKER et al. 1971) on mixed function oxidase activity of chick embryo liver homogenates suggested that mfo activity was age- and exposure-related. Hydroxylase and O-demethylase activity increased with the incubation age of the chick embryo and changed with CO-exposure. Since these studies also indicated an alteration in mfo activity occurring about the 14th-15th day of incubation, 24-hour CO-exposure experiments were undertaken on 12- and 17-day-old chick embryos in which the influence of sex, as well as age, upon mfo activity at these two periods of development was determined.

Table 1 summarizes O-demethylase and hydroxylase activity of 13- and 18-day-old male and female chick embryos following a 24-hour exposure to 425 ppm CO in air. The presumably normal increase in O-demethylase activity in livers of both male and female control chick embryos occurred from the 13th to 18th day of incubation. Hydroxylase activity in male control chick embryo livers increased from day 13 to 18 of incubation; however, the level of hydroxylase activity in control female chick embryos remained unchanged between day 13 and 18. Following CO-exposure two patterns of response were noted: one in which the activity of the mfo's appeared unaffected by CO-exposure in the 13-day-old chick embryos; the other in which both hydroxylase and demethylase activity showed a significant change ($P<0.05$) in the CO-exposed 18-day-old chick embryos.

TABLE 1

Para-nitroanisole O-demethylase and aniline hydroxylase activity of livers of 13- and 18-day-old male and female chick embryos following 24-hour exposure to 425 ppm CO in air.[a]

		P-nitroanisole O-demethylase (mμ ± S.D.)		Aniline hydroxylase (mμ ± S.D.)	
I.A.[b]	Sex	Control	Exposed	Control	Exposed
13	M	112±16	123±11	18± 5	15± 6
13	F	105±29	105±21	22±19	18± 6
18	M	173±36	244±34[c]	35±15	46±14[c]
18	F	186±34	266±92[c]	22±17	50±20[c]

[a]Mean ± S.D. of the values (mμmoles p-nitrophenol and p-aminophenol per gram of liver per 30 minutes' incubation at 37°C) obtained from 5 experiments. A pool of 64 livers from 13-day-old chick embryos, and a pool of 20 livers from 18-day-old chick embryos, were used for each experiment, two determinations per experiment, (N = 10).

[b]Incubation Age (I.A.) following 24-hour exposure to 425 ppm CO in air.

[c]Significantly different from controls $P<0.05$.

The lack of any significant difference in hydroxylase and O-demethylase activity in livers of 13-day-old control and CO-exposed chick embryos indicated that the COHb level (7.4 ± 1.4%) of those exposed had no measurable influence. However, in the CO-exposed 18-day-old chick embryo, concomitant with the elevated COHb level (35.7 ± 319%), the significant increase in hydroxylase and O-demethylase levels indicated the influence of the elevated COHb. An almost equivalent correlation was noted between COHb levels and enzymatic alterations in NYLAR mice. These results suggest that increases in O-demethylase and hydroxylase activity are influenced by rather high COHb levels, and may represent one possible means of adaptation to tissue hypoxia. According to MARVER (1969), the induction of mfo activity and cytochrome P-450 activity is a distinct phenomenon involving porphyrin and heme synthesis, and most probably represents the mechanism of adaptation and/or adjustment to tissue hypoxia at the elevated COHb levels. We have noted increased hematocrits in the older chick embryos and in the NYLAR mice exposed to 500 ppm CO. The polycythemia occurring on CO-exposure reflects an attempt at adaptation and inferentially indicates an activation of the production of hemoglobin precursors. While studies on the effects of hypoxia upon mfo activity of the liver of chick embryos remain to be done, the results presented in Table 1 establish that certain of the enzymes of the hepatic mfo system can undergo CO-induced "induction."

Mixed function oxidase activity in livers of NYLAR mice after uninterrupted exposure to 100, 250, and 500 ppm CO in air for 1 week: KATO et al. (1971) have reported that no sex difference was evident in aniline hydroxylation in mice liver microsomes following adrenalectomy or morphine treatment. KATO and GILLETTE (1965a) have shown that hypoxia depresses the activity of aniline hydroxylase in male rats but not in female rats. RONDIA (1970) has reported a decrease in benzpyrene hydroxylase activity of rat liver microsomes with a concomitant increase in microsomal P-450 concentration following inhalation of 60-100 ppm CO for 120 hours. MONTGOMERY and RUBIN (1971) have shown a significant effect of CO inhalation on P-450 dependent drug metabolism and that increase in sleeping time, paralysis, and clearance of drugs from the blood could be explained by an inhibition of the hepatic microsomal system by prior exposure to CO for 90 minutes. Yet in our studies 18-day-old chick embryos showed a significant increase in O-demethylase and hydroxylase activity following 24-hour exposure to 425 ppm CO; these data suggest an effect opposite to that associated with CO-induced hypoxia. To determine whether a comparable response occurs in another organism, NYLAR mice were exposed to increasing levels of CO.

Table 2 summarizes the results of one week's exposure to 100, 250, and 500 ppm CO in air upon hydroxylase and O-demethylase activity in livers of NYLAR mice. No significant difference in the activity levels of these two enzymes was noted between male and female control mice, or between male and female mice following exposure to 100 and 250 ppm CO in air for a week, or between the males and females of these three groups. However, after one week's exposure to 500 ppm CO, a significant increase ($P<0.005$) in the activity levels of these two enzymes occurred in both males and females.

TABLE 2

Para-nitroanisole O-demethylase and aniline hydroxylase activity of livers of male and female NYLAR mice following exposure to 100, 250, and 500 ppm CO in air for one week.[a]

Substrate	Sex	ppm CO Control	100	250	500
p-Nitroanisole (mμ±S.D.)	M	1658±447	1374±169	1618±96	2482±360[b]
	F	1449±265	1407±142	1448±146	2050±381[b]
Aniline (mμ±S.D.)	M	587±97	564±94	645±29	1068±125[b]
	F	543±114	565±58	523±41	788±132[b]

[a]Mean ± S.D. of the values (mμmoles p-nitrophenol and p-aminophenol per gram of liver per 30-minute incubation at 37°C) obtained from 5 experiments (6 mice/experiment), two determinations per experiment, (N = 10).

[b]Significantly different from controls $P<0.005$.

The elevated levels of hydroxylase and O-demethylase activity noted in male and female mice following exposure to 500 ppm CO for one week suggest that this effect is not associated with CO-induced hypoxia since as established in the literature (KATO and GILLETTE 1965a; MONTGOMERY and RUBIN 1971) one would expect a decrease in mfo activity. In addition little or no significant alteration in these microsomal enzymes was noted following one week's exposure to 100 and 250 ppm CO, COHb 9.7%, 13.5% respectively, sufficient to create a hypoxic condition in mice. However, the COHb level (25.6%) following exposure to 500 ppm CO may function as the inductive stimulus on the inducible enzymes of the mixed function oxidase system. Increased hematocrits of exposed animals were significantly higher than controls and there appeared to be a direct relationship between increased hematocrit and CO-exposure. To what degree this may correlate with heme synthesis (MARVER 1969) remains presently unanswered since corroborative data are required on δ-aminolevulinic acid (δALA) synthetase activity and total blood volume in both the CO-exposed chick embryos and NYLAR mice.

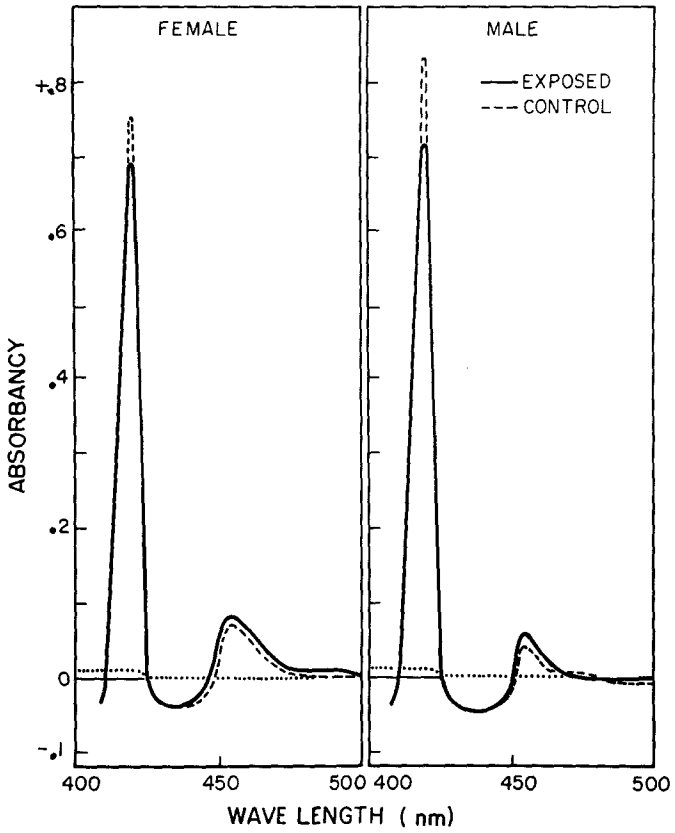

Figure 1. Carbon monoxide difference spectra of liver microsomes of control and CO-exposed male and female NYLAR-A mice (100 ppm CO). Dotted line, aerobic microsomes establishing base line. Exposed and control, dithionite reduced microsomes saturated with CO: peak at 420 indicates hemoglobin contamination; peak at 450, the microsomal CO-binding pigment P-450. Sample and reference cells contained the microsomal suspension (2 mg protein per ml., 0.25 M phosphate, pH 7.4) treated with dithionite and then saturated with CO.

Carbon monoxide difference spectra of microsomes from chick embryo and NYLAR mice: In Figure 1, the CO difference spectra of liver microsomes of control and CO-exposed (100 ppm CO) male and female NYLAR mice establish the presence of P-450. While control female mice show a higher P-450 level than males, the increase in the CO-binding pigment following exposure to 100 ppm CO is not significant. This finding correlates substantially with data in Table 2 where the difference in hydroxylase and O-demethylase activity between controls and mice exposed to 100 ppm CO is nominal. Carbon monoxide difference spectra of liver microsomes remain to be done on mice exposed to 250 and 500 ppm CO to establish whether alterations in P-450 levels correlate with those noted for hydroxylase and O-demethylase. No difference in the P-450 level from the livers of control and exposed 18-day-old chick embryos was found presumably due to the low level of the CO-binding pigment present.

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REFERENCES

BAKER, F. D., M. J. FASCO and J. W. FENTON, II: Bact. Proc. p. 67 (1971).
BAKER, F. D. and C. TUMASONIS: Arch. Environ. Hlth. 24, 53 (1971).
BUCHWALD, H.: Amer. Indust. Hyg. Assoc. J. 30, 564 (1969).
GARFINKEL, D.: Arch. Biochem. Biophys. 77, 493 (1958).
GILLETTE, J. R., A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS and G. F. MANNERING (ed.): Microsomes and Drug Oxidations. New York: Academic Press 1969.
KATO, R. and J. R. GILLETTE: J. Pharmacol. Exp. Therapeut. 150, 279 (1965a).
KATO, R. and J. R. GILLETTE: J. Pharmacol. Exp. Therapeut. 150, 285 (1965b).
KATO, R., K. ONODA and A. TAKANAKA: Biochem. Pharmacol. 20, 1093 (1971).
KLINGENBERG, M.: Arch. Biochem. Biophys. 75, 376 (1958).
MARVER, H. S.: The role of heme in the synthesis and repression of microsomal protein. In: Microsomes and Drug Oxidations (GILLETTE, J., A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS, and G. J. MANNERING, ed.). New York:Academic Press 1969, p. 495.
McMAHON, R. E., H. W. CULP, J. MILLS and F. J. MARSHALL: J. Med. Chem. 6, 343 (1963).
MONTGOMERY, M. R. and R. J. RUBIN: J. Pharmacol. Exp. Therapeut. 179, 465 (1971).
OMURA, T. and R. SATO: J. Biol. Chem. 239, 2370 (1964).
REMMER, H.: Amer. J. Med. 49, 617 (1970).
RONDIA, D. R.: C. R. Acad. Sci. Ser. D (Paris) 271, 617 (1970).
TUMASONIS, C. F. and F. D. BAKER: Bull. Environ. Contam. Toxicol. in press (1973).