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The Metabolism of Aminopyrine in Chick Embryo Hepatic Cell Culture: Effects of Competitive Substrates and Carbon Monoxide

ALAN POLAND[1] AND ATTALLAH KAPPAS

The Rockefeller University, New York, New York 10021

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SUMMARY

Chick embryonic liver cells grown in monolayer cultures metabolize aminopyrine to 4-aminoantipyrine, which is released into the medium. This *N*-demethylation is performed by liver cell cultures but not by renal or intestinal cell cultures from chick embryos. The formation of 4-aminoantipyrine is linear with time and is inhibited by SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl), piperonyl butoxide, hexobarbital, lauric acid, and testosterone. Inhibition in all cases is at least partially reversible. The rate of 4-aminoantipyrine formation is also reversibly inhibited by carbon monoxide. From the $CO:O_2$ ratio and rate of formation of the metabolite, it is possible to estimate the partition coefficient, K_L, for cytochrome P-450 in living cells, which agrees quite well with estimates from incubations *in vitro*.

INTRODUCTION

During the past decade, extensive investigations of hepatic microsomal mixed-function oxygenase have demonstrated that this electron transport system metabolizes many xenobiotics and naturally occurring compounds (1). Cytochrome P-450 has been identified as the terminal oxygen activator of this system, which combines with molecular oxygen and the substrate. A variety of drugs and naturally occurring metabolites competitively inhibit each other's metabolism and binding spectrum with cytochrome P-450 (2–4). Carbon monoxide binds to the heme prosthetic group of this unusual hemoprotein and prevents oxygen activation and hence substrate metabolism (5, 6).

All the above observations have been derived from experiments *in vitro* using microsomes or more inclusive cellular fractions. Hepatic cell culture offers an isolated system in which one can study mixed-function oxidations more physiologically, in whole cells. The fact that the affinity of carbon monoxide for cytochrome P-450 is much greater than for cytochrome oxidase suggested the possibility of selectively inhibiting microsomal oxidation in cell culture without significantly affecting oxidative phosphorylation (6). This is not possible in the whole laboratory animal, because hemoglobin has a greater affinity for carbon monoxide than either of these cytochromes (6). The ability to inhibit microsomal oxidations selectively in whole cells may shed some light on the physiological role of

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[1] Present address, Department of Pharmacology, University of Rochester Medical Center, Rochester, N. Y. 14642; to whom requests for reprints should be addressed.

microsomal oxidation in the metabolism of endogenous substrates.

In this paper, studies are presented employing hepatic cell cultures from chick embryos, which metabolize aminopyrine. The metabolism of this drug is reversibly inhibited by a variety of substances and carbon monoxide.

METHODS

Culture of chick embryo liver cells. The livers of 14–16-day-old chick embryos were removed under sterile conditions and cultured according to the method of Granick (7), with a few modifications. The livers were pooled, washed three times in Earle's solution without calcium or magnesium (modified Earle's solution), blotted dry, and weighed. Then the livers were minced very finely with a sterile razor blade and digested in 12 ml of modified Earle's solution, containing 100 mg of trypsin (Worthington) and 30 mg of Pangestin (Difco), for 20–40 min at 37°. During this period the cells were gently aspirated into and out of a large-bore pipette to aid in cell separation. The cell suspension was then diluted with modified Earle's solution to 150–200 mg/ml. One milliliter of the diluted cell suspension was added to 20 ml of medium in a 10-cm-diameter Petri dish (Falcon Plastics or Nunc). The medium consisted of 1 liter of Eagle's basal medium without phenol red, 100 ml of fetal bovine serum, 10 ml of a 100-fold concentrate of amino acid mixture, 10 ml of 0.2 M glutamine (all purchased from Microbiological Associates), 0.5 ml of Mycostatin (200 units), 6 mg of penicillin G, 10 mg of streptomycin, and 3.0 g of $NaHCO_3$; the pH was adjusted to 7.4. The cells were placed in an air–5% CO_2 atmosphere at 37°, and after 5–8 hr the medium was removed by aspiration and replaced with fresh medium. This was done because of the heavy inoculum of cells, and to remove the unattached cells. The experiments were performed between 16 and 48 hr of culture. Under phase microscopy the growth was very heavy, with liver parenchymal cells easily distinguishable from other cell types.

Drug incubations. The drugs, aminopyrine (Eastman Organic Chemicals), 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) (a gift of Smith Kline & French, Inc.), piperonyl butoxide (a gift of FMC Corporation, Niagara Chemical Division), sodium hexobarbital (Winthrop Laboratories), testosterone (Calbiochem), and lauric acid (Sigma Chemical Company), were dissolved in the medium prior to addition. Testosterone, lauric acid, and piperonyl butoxide were all first dissolved in ethanol so that the final solvent concentration in the medium was never more than 0.10%. After 18–30 hr, the medium was removed from the cultures and an exact volume of fresh medium (7 or 10 ml) containing aminopyrine and the test drug was added. Following various periods of incubation, 5 ml of the medium were removed for analysis.

In some experiments, after the medium had been removed and the plates washed with buffered salt solution, all the cells were removed by adding 0.1 M potassium phosphate buffer, pH 7.4, containing 200 mg of EDTA per liter. The cells were analyzed for protein by the alkaline-copper method of Sutherland *et al.* (8), with bovine serum albumin as a standard.

Gas mixture experiments. Four low-flow flowmeters (Matheson Gas Products) were connected to a common manifold, and this in turn was connected to a water flask to saturate the gas with water vapor, then run into a specially built Lucite gassing chamber. The chamber was placed in the regular culture incubator. The total flow rate in the 1-cu-ft chamber was 100–200 ml/min.

This design permitted the simultaneous investigation of aminopyrine metabolism in control plates (air–5% CO_2) and in cultures exposed to various CO–O_2 mixtures; both were exposed to the same temperature, humidity, and carbon dioxide concentrations. The CO:O_2 gas ratios were determined from the readings on the individually calibrated flow valves. To determine the partition coefficient, the CO:O_2 gas ratios were corrected for the relative solubility of these gases in water at 37° (0.77).

Methods of analysis. 4-Aminoantipyrine was analyzed by the method of Brodie and Axelrod (9), with one minor modification. Five milliliters of medium were extracted in

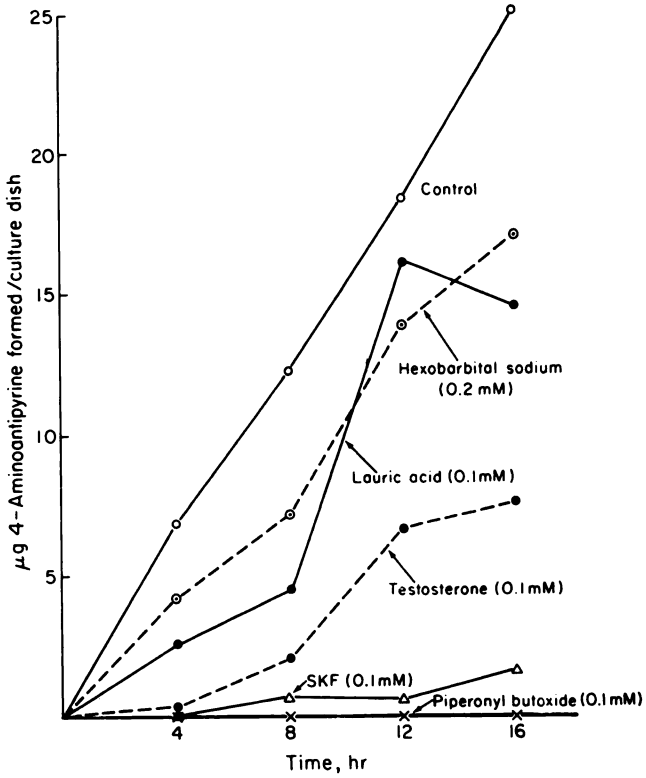

FIG. 1. *Effect of various agents on 4-aminoantipyrine formation in liver cell cultures*

Liver cell cultures were grown for 24 hr; then 10 ml of medium containing 10 μmoles of aminopyrine (1 mM) were added to each Petri dish. Appropriate inhibitory drugs were added to each Petri dish: SKF 525-A (0.1 mM), △——△; piperonyl butoxide (0.1 mM), ×——×; hexobarbital sodium (0.2 mM), ⊙- - -⊙; lauric acid (0.1 mM), ●——●; testosterone (0.1 mM), ●- - -●. At appropriate time intervals (4, 8, 12, and 16 hr), dishes were removed and the media were analyzed. The results are averages of three samples at each point for the control plates, and of duplicate samples for the drug-inhibited cultures. After 16 hours about 1.2 per cent of the substrate is converted to 4-aminoantipyrine.

chloroform; a portion of this organic phase was re-extracted into dilute HCl, and the diazotization reaction was performed. The coupling reagent, α-naphthol, was freshly resublimed and used at a concentration of 15 mg/ml instead of 50 mg/ml (9). Five milliliters of water or medium (without drugs added) carried through the procedure gave an optical density of 0.020–0.035 at 540 nm. A 6-μg standard of 4-aminoantipyrine in medium gave an optical density of about 0.600. The appropriate blank, consisting of medium and aminopyrine with or without competing drug, was taken through the reaction, and the amount of 4-aminoantipyrine formed was calculated from the difference in absorbance between the sample and the blank.

Statistics. Results are expressed as means ± standard deviation. The level of significance was determined by Student's *t*-test.

RESULTS

The culture of chick embryo liver cells gives a monolayer of hepatic cells which can metabolize aminopyrine to its product, 4-aminoantipyrine. As shown in Fig. 1, the formation of 4-AAP[2] was roughly linear over a 16-hr period. Although not measured in this

[2] The abbreviation used is: 4-AAP, 4-aminoantipyrine.

experiment, the average protein concentration per culture dish was 3–6 mg, with little variation between plates in any given culture. Modest cellular division and growth took place during the first 24 hr of culture.

The oxidative *N*-demethylation of aminopyrine is enzymatic rather than chemical because (a) incubation of medium and aminopyrine at 37° for 8 hr produced no 4-AAP and (b) cells fixed with 5% trichloracetic acid for 10 min, and then washed and exposed to aminopyrine in medium, formed no 4-AAP.

The metabolism of aminopyrine does not appear to be limited by O_2 under normal culture conditions, as shown in Table 1. Adding NADPH to the medium or using 95% O_2–CO_2 instead of air–5% CO_2 had no apparent effect on the rate of formation of 4-AAP. (The NADPH probably does not cross the cell membrane.)

Cultures of embryonic chick intestines and kidney incubated with aminopyrine for 6 hr formed no measurable 4-AAP. The metabolism of aminopyrine to 4-AAP (Fig. 2) has been demonstrated to involve two oxidative *N*-demethylations requiring NADPH, molecular oxygen, and the microsomal mixed-function oxidase system (10, 11).

TABLE 1

Effect of NADPH and O_2 on formation of 4-aminoantipyrine

In experiment 1, cells grown for 20 hr were incubated for 8 hr with 10 ml of fresh medium containing 10 μmoles of aminopyrine. Results are expressed as micrograms of 4-AAP formed per plate and are averages of duplicate Petri dishes. In experiment 2, dishes were incubated for 6 hr with 7 ml of medium containing 5.6 μmoles of aminopyrine. Results are expressed as micrograms of 4-AAP per milligram of cellular protein. In this experiment the amount of cellular protein per plate was between 3.7 and 4.3 mg.

Conditions	4-AAP formed
Experiment 1	
Control	10.5
0.125 mM NADPH	11.8
0.50 mM NADPH	11.7
Experiment 2	
Air (20% O_2)	1.27 ± 0.30[a] (n = 4)
95% O_2	1.23 ± 0.13 (n = 3)

[a] Mean ± standard deviation.

A number of drugs, mostly with a type I binding spectrum,[3] have been shown to be competitive inhibitors of aminopyrine metabolism *in vitro* (2, 3, 12). Representative drugs were chosen among the competitive inhibitors: 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) and piperonyl butoxide, as classic inhibitors; hexobarbital, as a well-studied competitive drug with the same relative magnitude of binding affinity as aminopyrine; testosterone, as a representative steroid; and lauric acid, which is ω-hydroxylated.

In the experiment presented in Fig. 1, cells were cultured for 20 hr; then 10 ml of medium containing aminopyrine (0.5 mM) and the appropriate competitive drug—SKF 525-A (0.1 mM), piperonyl butoxide (0.1 mM), hexobarbital (0.2 mM), testosterone (0.1 mM), or lauric acid (0.1 mM)—were added, and the plates were incubated for 4, 8, 12, or 16 hr. The medium was then removed for analysis. On a molar basis the relative order of potency as a competitive antagonist was: piperonyl butoxide > SKF 525-A > testosterone > lauric acid > hexobarbital. Inhibition measurements made after 8 hr are probably less reliable because of (a) utilization of the competitive substrate or (b) enzyme induction, which is perhaps unlikely in this short time period.

Despite the fact that all the above drugs act as competitive inhibitors of aminopyrine metabolism *in vitro* in rat and chick embryo liver microsomes (or 9000 × *g* supernatant fraction), they do not necessarily exert their effect in tissue culture in this manner. Conceivably, the drugs could alter membrane permeability to aminopyrine or exert a cytotoxic effect (13). In fact, at the concentration of SKF 525-A employed in Fig. 1, some cells lost adhesiveness, indicating a membrane effect or cell death.

[3] The binding of substrates to cytochrome P-450 produces characteristic difference spectra. The type I spectrum, typified by aminopyrine, has a peak at approximately 390 nm and a trough around 420 nm; the type II spectrum, typified by aniline, has a trough at approximately 395 nm and a peak around 425 nm.

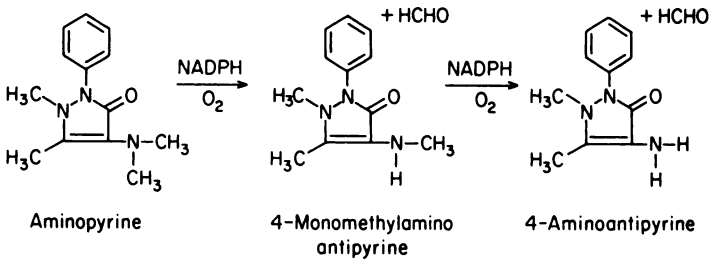

FIG. 2. *Metabolism of aminopyrine*

TABLE 2

Reversible inhibition of 4-aminoantipyrine formation by competitive substrates

Chick embryonic hepatic cells were cultured in 10-cm Petri dishes for 20 hr. Each dish was then incubated with 7 ml of medium containing 5.6 μmoles of aminopyrine (0.8 mM) and the appropriate competitive drug. After 6 hr, the medium was removed and analyzed, and the Petri dishes were gently washed twice with phosphate-buffered 0.9% NaCl and twice with regular medium. Another 7 ml of medium containing the same amount of aminopyrine without any other drugs were added to each dish and again incubated. After 6 hr, medium was removed for analysis, and the cells were washed, detached with EDTA, centrifuged, and analyzed for protein.

Comparative substrate	Concentration	n	4-AAP formed		p^b
			1st incubation period	2nd incubation period	
	mM		*μg/6 hr/mg protein*		
None (control)		4	1.27 ± 0.30[a]	1.39 ± 0.07	NS[c]
SKF 525-A	0.04	3	0.34 ± 0.13	0.55 ± 0.04	<0.05
Piperonyl butoxide	0.04	3	0.20 ± 0.12	0.90 ± 0.20	<0.01
Hexobarbital	0.20	3	1.02 ± 0.07	1.60 ± 0.06	<0.001
Testosterone	0.10	3	0.44 ± 0.04	1.55 ± 0.19	<0.001
Lauric acid	0.10	3	1.04 ± 0.06	1.60 ± 0.26	<0.05

[a] Mean ± standard deviation.

[b] p is the level of significance using Student's t-test, comparing the first and second incubation periods for each treatment group.

[c] Not significant.

A washout experiment was performed to examine the reversibility of drug inhibition of aminopyrine metabolism (Table 2). In the first period the cultures were incubated with aminopyrine and the competing substrate. The medium was then collected, and the plates were washed and incubated with aminopyrine alone. As shown in Table 2, more 4-AAP was always formed during the second incubation period, after washout of the competing substrate, than during the first period. In the control cultures there was no significant difference between the first and second incubation periods. The level of significance is given in Table 2.

The cultures treated with SKF 525-A and piperonyl butoxide did not completely recover during the second period (4-AAP levels were substantially lower than in the control culture). This may be attributable to a cytotoxic effect or to the inability completely to wash out these drugs, which have a very high affinity for cytochrome P-450. In the first incubation period the cultures treated with hexobarbital and lauric acid were not significantly inhibited in comparison with control cultures. (However, comparing periods 1 and 2, 4-AAP formation was greater after removal of hexobarbital and lauric acid.) In the second incubation period hexobarbital-treated plates formed significantly more 4-AAP than control cul-

tures ($p \leqq 0.01$). A possible explanation for this result is that the addition of hexobarbital during the first period induced drug metabolism, resulting in a faster rate of 4-AAP production in period 2 compared to control cultures.

Carbon monoxide gassing. The following experiments were performed in the same manner as those reported above, except that after the addition of aminopyrine half the cultures were placed in a Lucite gassing chamber and exposed to various $CO:O_2$–5% CO_2 gas mixtures and the other cultures were placed in a regular atmosphere of air–5% CO_2.

As shown in Table 3, the rate of formation of 4-AAP in a CO atmosphere (A) was retarded when compared to the rate of formation in a normal atmosphere (A_0). Correcting the $CO:O_2$ gas ratio for the relative solubilities of the gases in solution at 37°, and using 4-AAP formation as a measure of functional activity of cytochrome P-450, one can calculate the partition coefficient, $K_L = n/(1 - n)$ $(CO:O_2)$, where $n = A/A_0$ (14). The estimates of K_L, the relative affinity of cytochrome P-450 for CO and O_2, ranged from 0.81 to 3.85, an almost 5-fold variability. This is attributable in large part to the imprecision in estimating A and A_0, the unavoidable slight variations in the $CO:O_2$ gassing ratio over the 6-hr time course, and the amplification of the errors by the ratios of A/A_0 and $CO:O_2$ in the equation for calculating K_L.

It should be noted that the inhibition of 4-AAP formation produced by gassing with carbon monoxide was reversible. Table 4 gives the results of an experiment in which the $CO:O_2$ gas ratio was 5:1. Cultures formed less 4-AAP in the presence of CO (group 2), but cultures simultaneously exposed to CO for 6 hr and then incubated with aminopyrine in air–5% CO_2 did not significantly differ from the control cultures (air–5% CO_2) in either incubation period. If there is a more general cytotoxic effect of carbon monoxide at the concentration used (e.g., inhibition of cytochrome oxidase), apparently it is reversible, since the cells recovered their ability to form 4-AAP when placed in an air–5% CO_2 atmosphere.

It was impractical to vary the $CO:O_2$ ratio over a wider range. At $CO:O_2$ ratios much higher than 5:1 significant hypoxia would occur (a fall in the partial pressure of O_2 below that which occurs in air, approximately 20%), and at a $CO:O_2$ ratio less than 2:1 the degree of inhibition of 4-AAP formation was small and imprecise, and gave wide variations in the estimate of K_L.

DISCUSSION

The investigations presented above describe the ability of a culture of embryonic chick hepatic cells to convert aminopyrine

TABLE 3

Inhibition of aminopyrine metabolism by carbon monoxide, and estimation of partition coefficient for cytochrome P-450

Cells cultures for 20 hr were incubated for 6 hr with 7 ml of medium containing 7 μmoles of aminopyrine. A_0 is the amount of 4-AAP formed per milligram of cellular protein in control cultures in 95% air–5% CO_2, and A is the amount of 4-AAP formed in various $CO:O_2$ atmospheres with 5% CO_2. The solubility of CO with respect to O_2 at 37° in water is 0.77, and the partition coefficient for cytochrome P-450, based on Warburg's formula, $K = n/(1 - n)$ $(CO:O_2)$, is for a liquid, i.e., K_L (14).

Gas ratio, $CO:O_2$	Aqueous ratio, $CO:O_2$	4-AAP formed		$n = A/A_0$	$n/(1 - n)$	K_L
		A_0	A			
		μg/mg protein				
2:1	1.54:1	1.55 ± 0.31[a]	0.94 ± 0.25	0.61	1.54	2.37
3:1	2.31:1	1.92 ± 0.47	0.50 ± 0.11	0.26	0.35	0.81
3:1	3.08:1	1.06 ± 0.37	0.36 ± 0.11	0.34	0.51	1.58
5:1	3.85:1	1.79 ± 0.20	0.94 ± 0.10	0.53	1.11	3.85

[a] Mean ± standard deviation (three to five simultaneously gassed Petri dishes).

TABLE 4

Reversibility of inhibition of aminopyrine metabolism by CO

The conditions were the same as in Table 3. Groups 1 and 3 were incubated with aminopyrine in an air–5% CO_2 atmosphere in the first and second time periods, respectively. Group 2 cultures incubated with aminopyrine in the presence of a $CO:O_2$ atmosphere (5:1 ratio) formed significantly less 4-AAP ($p < 0.001$). However, group 3 cultures, which were first exposed to a $CO:O_2$ atmosphere and then incubated with aminopyrine in air–5% CO_2, showed no significant difference in 4-AAP formation when compared with either of the control groups.

Atmosphere	4-AAP formed
	μg/mg protein
First incubation period	
1. Control (A_0)	1.79 ± 0.20[a] (n = 5)
2. CO-exposed (A)	0.94 ± 0.10 (n = 4)
Second incubation period	
3. Control (A_0)	1.95 ± 0.10 (n = 5)
4. CO-exposed, then exposed to air–5% CO_2	1.67 ± 0.28 (n = 4)

[a] Mean ± standard deviation.

enzymatically to its metabolite, 4-aminoantipyrine. This *N*-demethylase activity was present in hepatic cell cultures and was not measurable in intestinal or renal cell cultures from chick embryos. The formation of 4-AAP was inhibited by SKF 525-A, piperonyl butoxide, hexobarbital, lauric acid, and testosterone, and to a variable degree inhibition was reversible in all cases.

The inability of the washout experiment to completely reverse the inhibition produced by piperonyl butoxide or SKF 525-A might be attributable to their known strong affinity for cytochrome P-450 (2, 4), and hence tight intracellular binding, or possibly to a more general cytotoxic effect.

Carbon monoxide, in contrast to drugs, appears to be a more specific inhibitor of microsomal oxygenations. The discovery, characterization, and biochemical role of cytochrome P-450 as the terminal oxygen activator in microsomal mixed-function oxygenation were all based on the ability of this hemoprotein to bind to carbon monoxide (3, 5, 15, 16).

The experiments reported above demonstrate the ability of carbon monoxide to inhibit reversibly a cytochrome P-450-mediated reaction in living hepatocytes in tissue culture. Although the estimates of the partition coefficient, K_L, vary over a fairly wide range (almost 5-fold), they are of the same order as estimates of various P-450-mediated reactions obtained with microsomes in incubations *in vitro* (including the *N*-demethylation of monomethylaminoantipyrine) (16).

Carbon monoxide cannot inhibit drug metabolism *in vivo* in higher organisms, despite claims that it does so (17), because the relative binding affinity of hemoglobin for carbon monoxide is 200 times that of cytochrome P-450. However, Lewis (18) utilized the fact that insects do not rely on hemoglobin for oxygen transport and hence would be likely to survive CO exposure. Flies painted with aldrin showed less conversion of aldrin to dieldrin in a 90% CO–10% O_2 atmosphere than flies in a 90% N_2–10% O_2 atmosphere.

The reversible inhibition of aminopyrine metabolism in hepatic cell culture by carbon monoxide might arise from a cytotoxic effect of CO unrelated to binding to cytochrome P-450. Carbon monoxide binds to a number of other intracellular hemoproteins: myoglobin, tryptophan pyrrolase, and cytochrome oxidase. Myoglobin is not pertinent to our study, because this hemoprotein is confined to muscle cells. Inhibition of tryptophan pyrrolase ($K \cong 1$) results in diminished nicotinic acid formation; however, nicotinic acid is present in the medium. Carbon monoxide does bind to cytochrome oxidase and inhibits oxidative phosphorylation. Inhibition of oxidative phosphorylation might deprive the cell of sufficient energy to accomplish drug metabolism (19). Below are outlined the reasons we feel this is not the case in the experiments presented.

1. The $CO:O_2$ partition coefficient, K_L, for cytochrome oxidase in most mammalian tissues studied is about 10 (6, 20). Without specific data on cytochrome oxidase from chick embryo liver cells, we employ $K_L = 10$ for the following argument. This means that when the $CO:O_2$ aqueous ratio is 10:1 there

is 50% inhibition of oxidative phosphorylation. At the highest $CO:O_2$ gaseous ratio employed in our experiments, i.e., 5:1 (aqueous ratio, 3.85:1), there should theoretically be only 28% inhibition of oxidative phosphorylation.

Paul (21, 22) has noted that most cultured cells exhibit a marked Pasteur effect, and can sustain themselves completely on anaerobic glycolysis for at least brief periods of time. Harris (23) found that rat fibroblasts were able to multiply at a normal rate under completely anaerobic conditions for 4 days. At gaseous $CO:O_2$ concentrations in the range of 20:1 or higher, mitosis appeared to be inhibited in chick fibroblasts (24) and sea urchin eggs (25) in culture, but survival was unimpaired.

2. If inhibition of 4-AAP formation by carbon monoxide is in part attributable to inhibition of oxidative phosphorylation and hence to a diminished level of available energy, then, for a given $CO:O_2$ atmosphere, one should find greater inhibition of aminopyrine metabolism in tissue culture than in microsomal incubations *in vitro*. Hence exposing culture cells to increasing $CO:O_2$ ratios should result in increasing deviations of observed 4-AAP formation from that theoretically expected. Another way of stating this is that, as the $CO:O_2$ ratio increases, the calculated K_L should decrease. As shown in Table 4, this is clearly not the case. Cooper *et al.* (12) observed the K values for two N-demethylations *in vitro*, using rat liver microsomes, to be 0.96 for codeine and 1.14 for monomethylaminopyrine. Despite considerable variability, our values agree quite well.

3. Finally, significant binding of carbon monoxide to cytochrome oxidase and inhibition of oxidative phosphorylation might be observable by a depression of some other energy-dependent function. We have studied ^{14}C-leucine incorporation into cellular protein in culture (ATP-dependent). Incorporation at ½ and 2 hr was not significantly different for cells in air–5% CO_2 and in 4:1 $CO:O_2$ atmospheres.[4]

The above observations do not support significant inhibition of oxidative phosphorylation by carbon monoxide.

The inhibition of microsomal mixed-function oxidation by carbon monoxide potentially has much broader uses in cell culture. In many types of experiments, the chemical under study is introduced into the culture and its effect on some cellular parameter is measured (e.g., enzyme induction, cell transformation). It is not always possible to determine whether the observed response was elicited by the parent compound or by a metabolite(s) formed in the cell culture. If the metabolite(s) is known and available, one can test this hypothesis experimentally. However, often the metabolite(s) of a drug, carcinogen, or steroid tested is unknown, unavailable; the metabolites so numerous as to preclude testing; or information is unavailable as to whether a known metabolite is formed by the cells being cultured.

If the compound under study is metabolized primarily by microsomal oxidation, it is possible to determine whether the parent drug or its metabolite(s) is responsible for the observed response by testing the drug in a regular atmosphere and a $CO:O_2$ atmosphere. The carbon monoxide should retard the oxidation of the parent compound. Hence a response that is equal or greater in the $CO:O_2$ atmosphere compared to the normal atmosphere suggests that the parent drug is responsible. Conversely, a diminished response is compatible with the idea that a metabolite(s) is responsible for the observed change. A potential application of the above hypothesis is chemical carcinogenesis. Di Paolo, Nelson, and Donovan (26) have shown that treatment of hamster embryo mixed cell cultures with benzo[*a*]pyrine produces transformed clones. Some of these transformed clones produce sarcomas when injected into hamsters. There is considerable controversy whether benzo[*a*]pyrene or its metabolite(s) produces cell transformation. One might attack the problem by comparing the number of benzo[*a*]pyrene-transformed cells in a regular and a CO atmosphere.

Investigations of microsomal mixed-function oxygenase systems in tissue cultures of liver cells offer several distinct advantages over experiments in whole animals. One can

[4] A. Poland and A. Rifkind, unpublished observations.

observe drug disappearance or product formation in the living cells in culture, analogous to serum disappearance rates in whole animals. Also, one can harvest the cells and assay the enzyme activity *in vitro*, comparable to liver homogenates.[5] The culture is an isolated system ideal for investigations of endocrine influences and metabolic requirements.

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