Experimental Methanol Toxicity in the Primate: Analysis of Metabolic Acidosis

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Experimental Methanol Toxicity in the Primate: Analysis of Metabolic Acidosis. CLAY, K. L., MURPHY, R. C., AND WATKINS, W. D. (1975). Toxicol. Appl. Pharmacol. 34, 49–61. Methanol was administered to rats, rhesus monkeys (Macaca mulatta), and pigtail monkeys (Macaca nemestrina). Of these animals, only the pigtail monkey reliably developed a severe metabolic organic acidosis resembling that observed in humans. Blood and urine specimens drawn from methanol-treated pigtail monkeys were analyzed for organic acid content by gas chromatography–mass spectroscopy and specific assays were performed for formate. The anion gap resulting during methanol acidosis was accounted for in full by increased blood concentrations of formate. Systemic formate concentrations remained low in rats and monkeys which failed to become acidotic following methanol administration.

Methanol produces toxic effects expressed primarily in the human. These include a profound metabolic acidosis and an impairment of vision, the latter being widely variable in its severity. It is generally agreed that these toxic effects of methanol occur as a result of its metabolism and are not due to the alcohol itself. Studies reported thus far have not succeeded in defining the specific acid(s) causing the decline in pH and carbon dioxide content of the blood measured following exposure to methanol.

'An important factor limiting our understanding of methanol acidosis has been that only in recent years has the marked species difference in response to methanol been appreciated fully. No acidosis resembling the human condition has been reported for a nonprimate laboratory animal (Koivusalo, 1970) and methanol toxicity studies limited to primates have produced widely varying results. Gilger and Potts (1955) administered methanol to the rhesus macacque (Macaca mulatta) and observed acidosis leading to death in all animals which received 3 g/kg or more. Cooper and Felig (1961) also employed the rhesus monkey in their studies but observed death in none of the animals which received 6 g/kg or less and did not see definite acidosis even in those animals which died from higher doses of methanol.

Metabolic differences reported to date in the primate and nonprimate are difficult to relate to the striking species differences in response to methanol administration. Recent work (Mannering et al., 1969; Tephly et al., 1964; Watkins et al., 1970) has defined different primary catalysts in the rat and the rhesus monkey for the oxidation of methanol to formaldehyde. When the rates of production of formaldehyde in the rat and monkey are considered in the overall rate of conversion of methanol to carbon

dioxide, however, it does not appear likely that the reported enzymatic differences account for the observed toxicological differences.

A second restriction in the study of methanol acidosis has been the technical difficulty of accurately measuring the many acid species which could contribute to a decrease in blood pH and carbon dioxide content. In earlier studies, formic acid, lactic acid, and hydroxybutyric acid were estimated to account for less than 25% of the total organic acids measurable in human urine obtained from a patient suffering methanol acidosis (Van Slyke and Palmer, 1920), thereby directing attention to other possible organic acids.

The purpose of this investigation was to identify the acid species responsible for the metabolic acidosis associated with methanol toxicity. The study consisted of two parts: First it was necessary to determine a suitable experimental model of human methanol acidosis, and second, by use of that model, we wanted to identify and quantitate those acids which contribute to the metabolic acidosis following methanol treatment.

METHODS

All chemicals used were reagent grade unless otherwise indicated. [^{14}C]sodium formate (3 mCi/mmol) was obtained from New England Nuclear Corporation (Boston, Mass.). 4-Methylpyrazole was obtained from Analabs (North Haven, Conn.).

Animals employed in this investigation were as follows: rhesus monkeys (*Macaca mulatta*) adult females, 4–6 kg; pigtail monkeys (*Macaca nemestrina*) adult females and 1 male, 3–6 kg; Sprague–Dawley rats, 250–450 g.

Serum electrolytes, glucose, blood urea nitrogen, and creatinine were determined by the methods given by O'Brien (1968). Blood pH, pCO_2, and pO_2 were determined with a digital blood-gas analyzer (model PHM 72, Radiometer, Copenhagen). pH, pCO_2, and pO_2 electrodes were calibrated immediately prior to each determination. Blood bicarbonate was calculated from the values for blood pH and pCO_2.

Plasma was prepared for gas chromatography–mass spectrometry (GC–MS) by the following procedure: Internal standard (decanoic acid) was added to plasma prepared from 2 ml heparinized blood and the protein was precipitated by addition of 4 ml absolute ethanol and centrifugation at 2000g. The volume of the supernate was reduced to 0.1 ml by flash evaporation at 30°C and then taken up in water to a total volume of 3 ml. The solution was adjusted to pH 8.0 with 1 N sodium hydroxide and extracted three times with a total volume of 45 ml water-saturated diethyl ether. The organic phase was discarded. The aqueous phase was adjusted to pH 2.0 with 1 N HCl and then extracted with 45 ml ether in three aliquots. The ether extracts were evaporated under a nitrogen stream in a 60°C heating block. The extracted organic compounds were converted to their trimethylsilyl derivatives by reaction with 100 μl bis(trimethylsilyl)-trifluoroacetamide in 100 μl CH_3CN at 60°C for 10–12 hr.

Samples were injected on a gas chromatograph (Perkin-Elmer model 990, flame ionization detector) fitted with a glass column (6 ft × 2 mm i.d.), packed with 5% OV-22 on Chromosorb W (100–200 mesh). The carrier gas was nitrogen. The injection port temperature was maintained at 300°C. Temperature programming was employed after an initial 30°C for 2 min, followed by a rise from 30° to 250°C at 8°/min. Sample components were identified by their methylene unit values (Dalgleish *et al.*, 1966), by

cochromatography with standard compounds, and by mass spectral identification. Comparison with the added internal standard allowed estimation of the amount of each component in the chromatogram. Combined GC–MS was performed on an AEI MS 12 interfaced to a Beckman GC 7 with a PDP-8i computer for data acquisition (Markey, 1970; Plattner and Markey, 1971).

Blood samples (2–4 ml) were collected from monkeys either from the femoral vein with sealed, heparinized syringes, or directly from a cannula inserted in the femoral vein and advanced to the region of the right heart. Blood was collected from the rat by cardiac puncture with heparinized vacuum tubes while the animal was under light ether anesthesia or from a cannula in the carotid artery of the anesthetized animal. Monkey urine was collected on ice from an indwelling Foley bladder catheter. Rat urine was collected in plastic metabolism cages.

Following methanol administration, formate was assayed in whole blood and urine by the method of Rietbrock and Hinrichs (1964) or by steam distillation and colorimetric assay by the method of Lang and Lang (1972). Standard curves for quantitations were made with steam distillate from control blood or urine samples.

Steam distillation of whole blood samples by the procedure recommended by Rietbrock and Hinrichs (1964) recovered 87–90% of formate added to the sample. Following administration of [^{14}C]formate to rats and monkeys, [^{14}C]formate in serial blood samples was determined by the specific oxidation method of Yang (1969). Recovery of [^{14}C]formate as $^{14}CO_2$ with this method was better than 90% in all cases, with blood concentrations of [^{14}C]formate from 0.5 mg/100 ml to 100 mg/100 ml and with 100–22,000 dpm.

RESULTS

It has been generally agreed, although never directly demonstrated, that "methanol acidosis" arises from the processes of metabolism rather than from the alcohol itself. We have obtained more direct support of this concept employing 4-methylpyrazole, a potent and relatively specific inhibitor of hepatic alcohol dehydrogenase (Theorell et al., 1969) the principal catalyst in the primate for the oxidation of methanol to formaldehyde. Pigtail monkeys were treated with 4-methylpyrazole (50 mg/kg, iv) 30 min before administration of methanol in a dose which routinely produces acidosis (4 g/kg). 4-Methylpyrazole administration was repeated every 6 hr. In these experiments, no significant decrease in blood pH or other signs of toxicity were observed for the duration of the study (48 hr). Monkeys were observed further after this time and appeared normal in all respects.

The uncertainty surrounding the suitability of the monkey as a model with which to study human methanol poisoning is based primarily on two conflicting sets of experiments utilizing rhesus monkeys (Gilger and Potts, 1955; Cooper and Felig, 1961). Our experience with the rhesus (Table 1) indicates that there is a wide variability within this strain with respect to their response to methanol. The first three rhesus macacques (methanol ip, 4 g/kg) exhibited no apparent sign of toxicity, although a relatively mild metabolic acidosis was produced. The fourth rhesus in the series became progressively weaker after treatment with methanol and developed a severe metabolic acidosis which led to coma and death. In contrast to the results obtained with the rhesus monkey, four pigtailed monkeys treated with 4 g/kg methanol displayed a sharp decrease in

TABLE 1

EFFECTS OF METHANOL ADMINISTRATION ON BLOOD ACID-BASE STATUS IN THE PIGTAIL AND RHESUS MONKEYS[a]

Experiment	Dose (g/kg)	Blood pH[b] minimum	pCO_2[b] (mmHg)	Time after methanol treatment (hr)	Other signs of toxicity
Rhesus 1	4	7.22	30	24	No apparent signs of toxicity
Rhesus 2	4	7.24	34	22	No apparent signs of toxicity
Rhesus 3	4	7.22	32	24	No apparent signs of toxicity
Rhesus 4	4	7.03	14	27	Comatose from 18 hr until death at 28 hr after methanol
Pigtail 1	2	7.29	30	24	No apparent signs of toxicity
Pigtail 2	3	7.21	32	22	No apparent signs of toxicity
Pigtail 3	4	7.03	15	21	Obvious distress after 15 hr; comatose from 18 hr until death at 22 hr after methanol
Pigtail 4	4	7.10	18	20	Comatose at 18 hr after methanol; recovered
Pigtail 5	4	7.10	20	21	Comatose 17–19 hr after methanol; recovered
Pigtail 6	4	7.04	33	33	Weak and apathetic after 12 hr; comatose 32 hr after methanol; sacrificed at 36 hr

[a] Monkeys were given methanol as a 20% solution in physiological saline, ip.
[b] Control values average: pH—7.40; pCO_2—37 (see Table 2).

blood pH from normal values of 7.40–7.45, accompanied by a decline in pCO_2 from a normal 40–44 mmHg (Table 1). Methanol doses of 2 and 3 g/kg produced metabolic acidosis in the pigtailed monkey, but the decrease in blood pH and pCO_2 was not as marked as with 4 g/kg. The four pigtailed monkeys and the single rhesus which became severely acidotic following 4 g/kg methanol exhibited signs which were remarkably similar to those reported in cases of human poisonings (Kane *et al.*, 1968). In addition, the time course of development of the acidosis corresponded well to the course of the poisoning in human cases.

Table 2 presents data from blood chemistry parameters determined in a single pigtailed monkey and subsequently confirmed in two additional animals. The only

TABLE 2

Some Blood Chemistry Parameters in the Pigtail Monkey During the Course of Acute Methanol Intoxication

Parameter	Control[a] average	Hours after methanol treatment[b]				
		7.5	11.5	14.5	19	21
pH	7.40	7.36	7.29	7.23	7.13	7.03
pCO_2[c]	37	27	20	24	20	15
Total CO_2[d]	22.0	15.7	9.8	10.5	7.1	4.4
Sodium[d]	152	—	157	155	150	148
Potassium[d]	3.4	—	3.0	3.2	3.3	4.3
Chloride[d]	106	—	102	103	111	105
Glucose[e]	73	—	89	85	105	106
Urea N[e]	20	—	20	16	—	17

[a] Average of values from three different blood samples over the 12-hr period immediately prior to methanol administration.
[b] Methanol was administered as a 20% solution in physiological saline, ip.
[c] mmHg.
[d] mEq/liter.
[e] mg/100 ml.

measured parameters which changed significantly were blood pH, carbon dioxide content, and glucose concentrations. Electrolytes and urea nitrogen remained unchanged during the course of the experiment. Blood pO_2 remained constant or slightly elevated, indicating that the animals were well-oxygenated.

The most severe signs of methanol poisoning are usually observed in humans 12–24 hr following methanol ingestion (Bennett *et al.*, 1953). It can be seen in Table 2 that this was also the case in the pigtailed monkey.

By all parameters measured, the pigtailed macacque appeared to serve as a suitable experimental animal in which to study human methanol acidosis. The next objective in this study was to identify the specific acid(s) which caused the observed systemic alterations in pH, and which might be related in some manner to the species differences in response to this alcohol. Table 2 shows that total cations measured before and after methanol treatment remain constant, and the total anion content must, therefore, also remain constant. The single blood electrolyte observed to change during the course of methanol acidosis was bicarbonate. Plasma concentration of this ion decreased from

control values of 20–24 mEq/liter to values as low as 4 mEq/liter or lower during the most severe periods of acidosis. From these measurements, it was apparent that to account for the observed acidosis, it was necessary to identify and quantitate additional anions in the range of 15–20 mEq/liter.

Gas chromatography (GC) and combined GC–MS have proven to be powerful tools in the study of metabolic disorders (Jellum et al., 1973). It was felt that the same techniques might serve to identify the acids responsible for methanol acidosis. Urine and blood specimens were collected from pigtailed monkeys during the course of the acidosis produced by administration of 4 g/kg methanol. Acidic components were extracted and derivatized as described in Methods. It should be noted that these procedures were not primarily designed for quantitation, but were intended to be screening procedures for detection of changes in organic acid composition of blood after methanol treatment. Use of an internal standard, however, allows one to estimate the amount of each component present. Decanoic acid was chosen for the internal standard because it was the only acid examined which was found to elute at a point in the chromatogram where no interfering substances occurred. These procedures result in loss of some volatile acids, but that loss should be uniform in both control and postmethanol samples so that one can use these procedures to detect concentration changes in organic acid components.

Relative to controls, significant increases were measured in the following acids: lactate, α-hydroxybutyrate, β-hydroxybutyrate, α-ketobutyrate, acetoacetate, p-hydroxyphenylacetate, and p-hydroxyphenyllactate. The concentrations of a number of other acids measured by these techniques did not increase, suggesting some specificity in the increased concentration of these organic acids. The plasma acids measured in this manner were estimated to account for a maximum increase in anion concentrations of 0.5 mEq/liter above control values. The increase in these particular acids, therefore, could account for no more than 2–3% of the observed decrease in plasma bicarbonate concentrations.

Our derivatization and gas chromatographic conditions did not permit estimation of formate concentrations. In light of the considerable quantities of anion which remained to be identified, however, we felt that the participation of this acid in methanol acidosis must be reevaluated, although the prevailing view in the literature was that formate plays a minor role (Koivusalo, 1970; Tephly et al., 1974).

Figure 1 shows the results of formate assays performed on whole blood samples drawn from the pigtail monkey and the rat at intervals following ip administration of methanol (4 and 6 g/kg, respectively). Under these conditions rats exhibited signs completely different from the monkey. Despite the higher dose, the only observable effect in the rat was a transient central nervous system depression (4–6 hr), followed by a return to normal behavioral activity. Administration of methanol to the monkey produced an initial CNS depression of less than 30 min duration, after which the monkey appeared completely normal until 15–18 hr after methanol when it began to show signs of weakness and apathy which progressed to coma and death at 28 hr after treatment.

Bicarbonate and formate were measured in blood specimens drawn at intervals after the administration of methanol to the pigtail macacque (Fig. 2). With the rise in blood formate concentration there was a corresponding decrease in bicarbonate. It can be
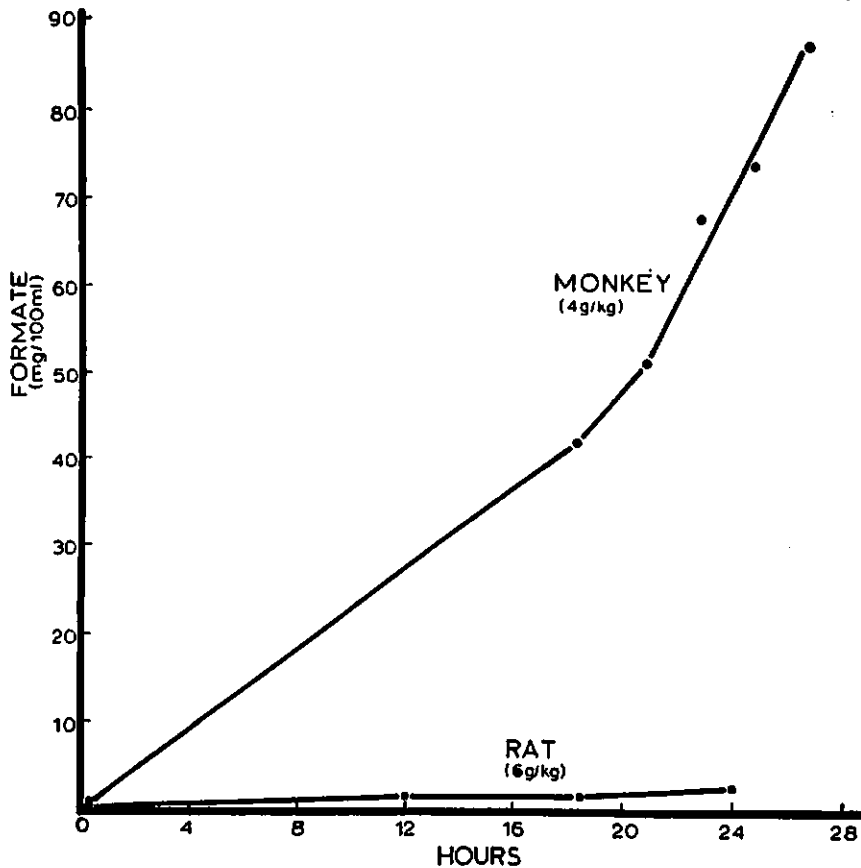
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FIG. 1. Blood formate concentrations in the monkey and rat after administration of methanol. Methanol was administered as a 25% solution in saline to the monkey (4 g/kg, ip) and the rat (6 g/kg, ip). Blood specimens were drawn at the indicated times after methanol administration.
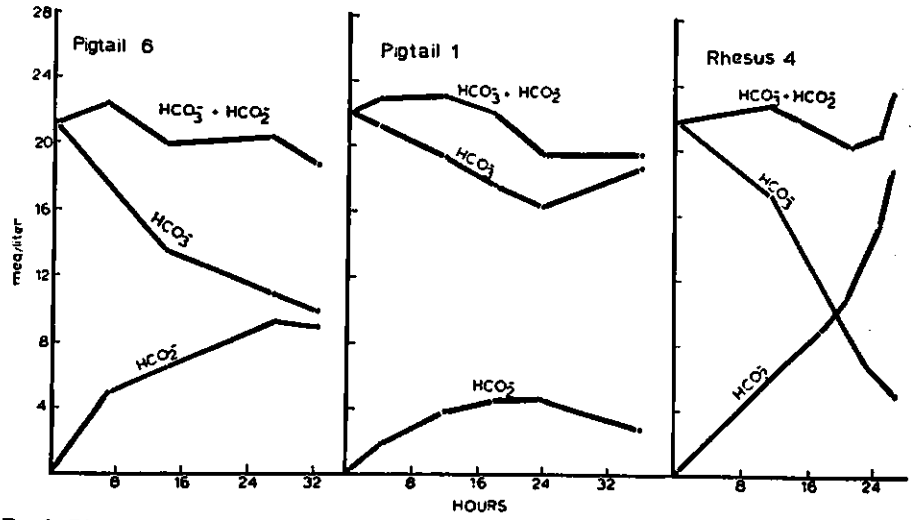

FIG. 2. Blood formate and bicarbonate concentrations in the monkey after methanol administration. Methanol (25% in saline) was administered ip in doses of 4 g/kg to Pigtail no. 6 and Rhesus no. 4, and 2 g/kg to Pigtail no. 1. Formate and bicarbonate were analyzed in blood samples drawn at the indicated times after methanol administration.

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seen that the sum of the formate and bicarbonate concentrations measured over the course of the entire experiment lies within the normal range for bicarbonate. This inverse relation between formate and bicarbonate concentrations also holds for doses of methanol which produce only moderate acidoses (2 and 3 g/kg).
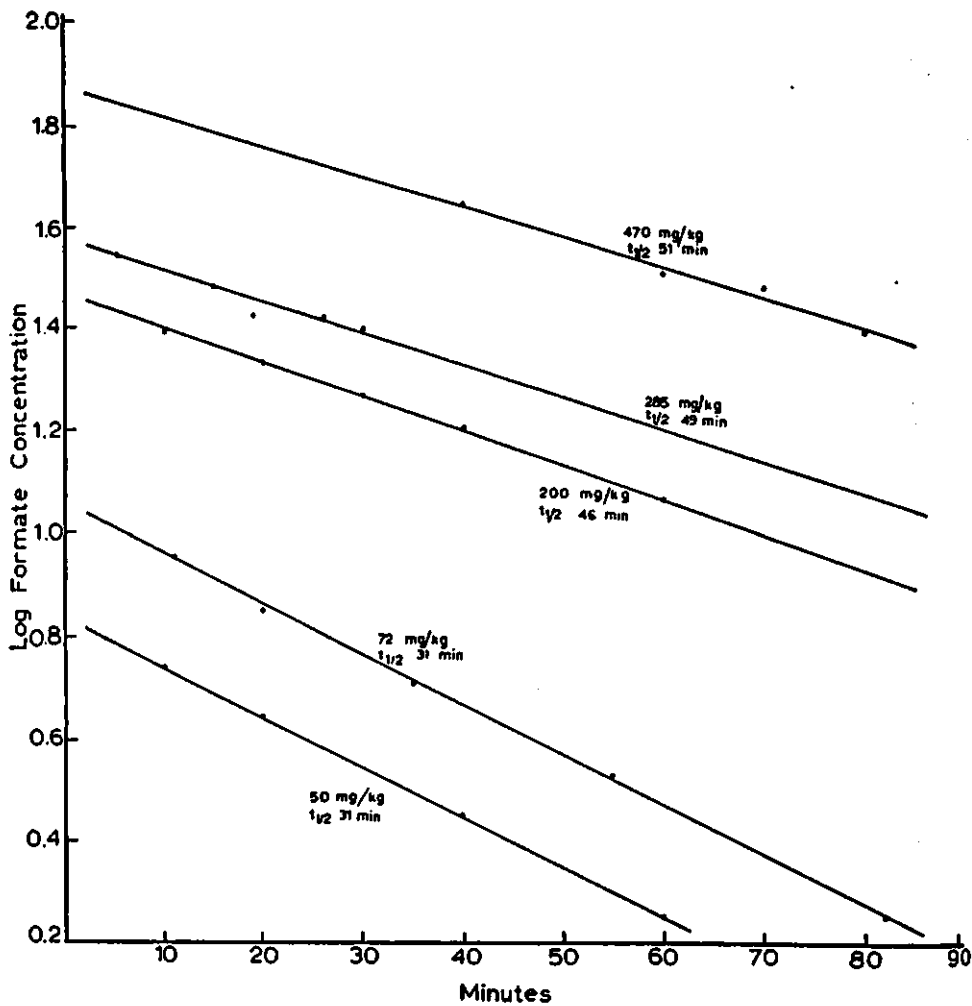

Fig. 3. Disappearance of formate from the monkey. Sodium formate (100 mg/ml) was administered intravenously in the indicated doses. Blood formate concentrations were determined on serial samples drawn from a femoral vein catheter. Curves were fit to data points by a least squares linear regression analysis and half-time ($t_{1/2}$) values were calculated from the resulting slope and intercept data.

Previous measurements in the rat and monkey have shown that there is little significant difference in the rates of methanol oxidation to carbon dioxide *in vivo* (Watkins *et al.*, 1970; Bartlett, 1950), an observation which we have confirmed in this laboratory. It appeared, therefore, that the species difference in blood formate accumulation observed in this study was not explainable by different rates of production of formate. Renal

disposition, however, could differ in the two species, so we carried out studies to assess the role of the kidney in the elimination of formate. Urine collected from rats for 24 hr after administration of methanol (6 g/kg) contained only 0.2% of the administered dose as formate, whereas urine collected from the pigtail for 23 hr following adminis-tration of methanol (4 g/kg) contained more than 2.0% of the administered dose as formate. These observations suggest that the urinary excretion of formate is directly related to systemic formate concentrations and that differences in the renal excretion of formate do not account for the observed species differences in the systemic concen-trations of this anion following methanol treatment.

Subsequent experiments were performed in order to assess more directly the elimin-ation of formate from the systemic circulation of rats and pigtail monkeys. Sodium formate was administered as a single intravenous injection to the monkey in five different doses ranging from 50 to 470 mg/kg formate. The results of these experiments are presented in Fig. 3. The calculated half-time ($t\frac{1}{2}$) of formate elimination under these conditions ranged from 31 min (50 mg formate/kg) to 51 min (470 mg formate/kg). When formate elimination was measured in the rat, doses up to 100 mg/kg yielded a $t\frac{1}{2}$ of 12 min and 670 mg/kg gave a $t\frac{1}{2}$ of 23 min. These values for the rat agree with those previously reported (Malorny, 1969) and further confirm the dose-dependency of $t\frac{1}{2}$ value reported previously (Rietbrock, 1969).

DISCUSSION

The results of these studies suggest that pigtail macacque (*Macaca nemestrina*) may be the experimental animal of choice in the investigation of human methanol poisoning. We have observed signs of severe toxicity and a severe metabolic acidosis in all pigtail monkeys treated with methanol at a dose of 4 g/kg, which appears to be the approxi-mate LD50 in this series of experiments. It should be pointed out that the single rhesus monkey of this series which exhibited behavioral signs of methanol toxicity appeared to respond exactly as did the pigtail monkeys. Lower doses of methanol in the pigtail monkey failed to produce any overt signs of toxicity, although moderate decreases in blood pH and bicarbonate concentration were observed.

Parameters examined thus far in the pigtail macacque treated with methanol (4 g/kg) are remarkably comparable to biochemical and clinical parameters reported in cases of human methanol toxicities (Kane *et al.*, 1968; Bennett *et al.*, 1953). After adminis-tration of methanol to the pigtail monkey, there is a latent period of 15–18 hr prior to the onset of any signs of toxicity. The latent period is followed by a sequence of signs beginning with behavioral distress which progresses to coma within 24–30 hr after methanol treatment, leading to death or slow recovery. This time course parallels that reported for humans suffering from methanol intoxication (Roe, 1955). Other factors found to be similar under these conditions in the pigtail monkey and human include constant serum electrolyte concentrations (Kane *et al.*, 1968) significant elevations in blood glucose concentration (Bennett *et al.*, 1953), and extensor rigidity and tremor (Guggenheim *et al.*, 1971). Autopsy has revealed no gross abnormalities in the organs of methanol poisoned pigtail monkeys or humans (Potts *et al.*, 1955), with the exception of the basal ganglia, a tissue which we did not examine.

Recent progress in GC–MS has made the analysis of complex mixtures of organic acids practicable (Markey, 1970). Our application of this technology to the identification and quantitation of the organic acids contributing to the metabolic acidosis of methanol poisoning was prompted by the prevailing opinion that some acid species other than formic, lactic, or hydroxybutyric acids was responsible for the acidosis, an impression founded on interpretations of the early work of Van Slyke and Palmer (1920), Egg (1927), and Roe (1946). The GC–MS approach employed in this study was successful in demonstrating increases in a number of acids subsequent to methanol administration. Although GC–MS was successful in identifying only a relatively small percentage of the total anions contributing to methanol acidosis in the pigtail monkey, the importance of the technique to the study should be emphasized. Only by eliminating the wide range of compounds suitably analyzed by the GC–MS technique was our attention directed to other compounds which were not considered to play a significant role in the production of methanol acidosis. It was felt that formate represented the most logical compound for reevaluation.

Several early publications have served as the basis for the current opinion that formic acid production in the human was not sufficient to account for the observed changes in systemic pH. The calculations described by Egg (1927) and Roe (1946) assumed a lethal dose of methanol which was unrealistically low according to the current literature, and therefore the maximum formate production in the human was seriously under-estimated. The titrimetric analysis by Van Slyke and Palmer (1920) of urine obtained from a methanol-poisoned patient is frequently cited as evidence that formate concentrations are insufficient to account for the metabolic acidosis. Our results point out the difficulty of making inferences about systemic concentrations based on urinary measurements.

Following methanol administration to pigtail monkeys, we have measured increases in blood formate which parallel the development of toxicity. These formate concentrations approach those reported to inhibit certain selected enzyme activities (Watts, 1951; Kini and Cooper, 1962; Potts and Johnson, 1952). The intracellular formate concentrations may be even higher in certain tissues, as implied by the report that the formed elements of blood can concentrate formate to a level threefold greater than the plasma fraction (Annison, 1954). A preliminary study in this laboratory suggests that, following methanol administration to pigtail monkeys, formate concentrations may be significantly greater in the vitreous humor than in the blood, an observation which may have particular significance in methanol blindness. It appears likely, therefore, that intracellular concentrations of formate following methanol administration reach levels known to be strongly inhibitory for some enzymes critical to intermediary metabolism.

The correlation between the decreases in bicarbonate concentration and increases in formate concentration (Fig. 2) serves to support our belief that formic acid is the acid primarily responsible for the decrease in blood pH observed in methanol intoxication. It appears that formate, a fixed base, replaces bicarbonate, a volatile base, in the blood and that the resultant decrease in blood buffer capacity renders the organism incapable of maintaining blood pH within normal limits. Bicarbonate therapy of the acidosis would appear to constitute specific replacement therapy, but it would probably not have an effect on other possible toxic properties of the formate ion. This may explain the

observation by others (Potts, 1955) that monkeys treated with bicarbonate and not allowed to become acidotic, died from large doses of methanol at approximately the same time they would have died had the acidosis not been treated.

The basis for the marked species difference in susceptibility to methanol remains unresolved, but the comparison of the half-time of elimination of formate in the rat and monkey (Fig. 3) points to possible differences in the ability to dispose of formate. The observation that the urinary excretion of formate was higher in the monkey than in the rat would appear to eliminate differences in renal function. Little is known about the metabolic disposition of formate in the monkey, but the rat is believed to oxidize formate primarily through catalase and a folic acid-dependent reaction catalyzed by formyltetrahydrofolate synthetase (Plaut et al., 1950; Oro and Rapoport, 1959).

The differences in elimination kinetics of formate in the rat and monkey (Fig. 3) suggest the possibility of different catalysts for the metabolism of formate, analogous to the case in the first step in the oxidation of methanol (Mannering et al., 1969; Tephly et al., 1964). This suggestion is supported by the work of Aebi et al. (1964) which demonstrates that acatalasemic humans are not impaired in their production of carbon dioxide from administered formate. Further, it has been shown that 3-amino-1,2,4-triazole, a potent inhibitor of hepatic catalase, had no measurable effect on carbon dioxide production from methanol administered to the monkey (Makar et al., 1968) which supports the view that catalase does not represent the major route of metabolism of formate to carbon dioxide in the monkey.

It remains unclear which product of methanol is responsible for the toxicity associated with exposure to this alcohol. Methanol is generally believed to require metabolism to more toxic compounds for the production of intoxication, and this belief has been supported by our demonstration of lack of toxicity subsequent to inhibition of oxidation of methanol with 4-methylpyrazole. Formaldehyde continues to be a likely candidate producing much of the clinical manifestations of methanol toxicity (Potts and Johnson, 1952; Kini and Cooper, 1962; Tephly et al., 1974). However, the present study suggests that formate plays a major role in this process as well. In contrast to the rat, a species which does not experience methanol acidosis, sufficient systemic formate is measurable in the methanol intoxicated monkey to fully account for the measured decrease in systemic pH. These studies on the toxicity of methanol emphasize the importance of appreciating interspecies and intraspecies differences in metabolism and disposition of drugs and chemicals.

ACKNOWLEDGMENTS

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