Improved Delivery through Biological Membranes. 2. Distribution, Excretion, and Metabolism of *N*-Methyl-1,6-dihydropyridine-2-carbaldoxime Hydrochloride, a Pro-Drug of *N*-Methylpyridinium-2-carbaldoxime Chloride†,[1]

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N-Methyl-1,6-dihydropyridine-2-carbaldoxime hydrochloride, the pro-drug of 2-PAM, was found to be converted in vivo to 2-PAM, rapidly and quantitatively. The significantly changed properties of the pro-2-PAM resulted in a longer biological half-life and a favorable distribution of 2-PAM formed upon its oxidation. No new metabolite was found when pro-2-PAM was administered intravenously; however, a new metabolic product was formed when the pro-drug was given by oral route.

A dihydropyridine type pro-drug (pro-2-PAM) of *N*-methylpyridinium-2-carbaldoxime chloride (2-PAM) has been developed[1] as the first example of a novel drug delivery concept based on a dihydropyridine–pyridine type redox system. This pro-drug approach provides a unique and efficient mechanism for delivery through lipoidal biological membranes of highly hydrophilic, nonlipoidal molecules. The general concept suggests the use of a dihydro form of an *N*-alkylated heteroaromatic quaternary salt type drug in order to allow its passage through various biological barriers [such as intestinal epithelium, blood–brain barrier (BBB), etc.], after which the dihydro form is quickly oxidized back to the active quaternary compound at the site(s) of action. A more general aspect of the concept would use the redox system as a carrier part in the derivatizable nonlipoidal molecule to be delivered.

In the present paper, the distribution and excretion characteristics, as well as the limited metabolic fate of pro-2-PAM as compared to 2-PAM, are described in order to verify the basic pro-drug concept.

Distribution studies[2] on ^{14}C-labeled 2-PAM administered intraperitoneally to mice have shown that after 1 hr 64% of the labeled compound was recovered as follows: 20% of the activity was contained by the liver, duodenum, jejunum, ileum, kidney, caecum, and blood, while the urine contained the remaining 44%. Only trace amounts of radioactive material were found in the brain. It was also found[3] that 10 min after the completion of an intravenous injection of 2-PAM to rabbits, the highest concentration was found in the kidney, while the least amount was in the brain. It was also noted[3] that no 2-PAM enters the erythrocytes in either human or dog subjects. The concentration of 2-PAM in whole blood was roughly 45–50% of the plasma concentration after an iv administration to human volunteers,[4] which would confirm that almost no 2-PAM penetrates the red-blood cells, although in a recent study[5] it was shown that 2-PAM enters the red-blood cells to some extent.

Because 2-PAM is a highly polar and water-soluble compound, it is rapidly eliminated from the body;[3] thus, its half-life in the human serum after iv administration was only 0.9 hr. Values of 1.35[3,6] and 1.67 hr[7] for biological half-life in man were also reported. The concentration of 2-PAM in blood usually falls below effective levels within 1–2 hr after administration.[8,9] The primary route of elimination is by tubular secretion[10] which results in a rapid renal excretion. Thus, the distribution of 2-PAM is restricted by its polar characteristics which greatly facilitate its renal excretion by the organic base excretory mechanism.[3,10,11] The fast excretion of 2-PAM leads to a significant reduction in the antidotal action of 2-PAM when the drug is injected more than 1 hr before exposure to the toxic agent[12,13] or if the poison is applied topically.[14]

Most of the excreted drug (80–90%) is in the form of the unchanged oxime,[3,4,6] although when 2-PAM is incubated aerobically with rat liver homogenate it is completely metabolized.[3,15] This indicates that 2-PAM when given iv does not reach its possible sites of metabolism in the liver, probably due to its high polarity. Although 2-PAM is slightly more extensively metabolized when given orally to rats, in which case it must pass through the portal system to the liver, it is still mainly eliminated unchanged.[16]

Besides the unchanged 2-PAM, numerous other metabolites could be identified,[17] among which the 1-methyl-2-cyanopyridinium ion, 1-methyl-2-pyridone, 1-methyl-4-pyridone-2-carboxamide, and cyanide ion are the major components.

Although the oral route is not the recommended way to administer an antidote with signs or symptoms of moderate to severe anticholinesterase poisoning, there may be circumstances in which the oral route is acceptable,[18,19] such as mild poisoning or in prophylaxis. Since pro-2-PAM was designed to enhance penetration of the drug through all kinds of lipoidal barriers, the oral route was also considered.

The reported extent of absorption of an orally administered 2-PAM in humans is low. Thus, 27% of 2-PAM was recovered in the urine after oral administration,[20] while 5–35% was found in the feces. It is possible that 2-PAM is decomposed extensively by intestinal bacteria. Large variations in blood levels among individuals receiving the same dose were also observed;[9] the mean unchanged 2-PAM found in the urine was 20–25% of the dose. Another study[21] reported 31.9% unchanged 2-PAM in the urine. The large difference between effective oral[22,23] and intravenous doses can be attributed to the incomplete absorption, characteristic for orally administered quaternary ammonium compounds.[24,25] It was shown that 2-PAM is not transferred by an active transport[26] system and its diffusion through the intestinal wall is also independent of the water transport, which eliminates the solvent–drag effect characteristic for small neutral molecules.[27] Neither did the extent of absorption correlate with the chloroform–water partition ratio. On the other hand, the movement of 2-PAM across the intestinal wall was dependent on its concentration and the transmural potential difference. It was assumed that 2-PAM crossed the

† Dedicated to the memory of Professor Edward E. Smissman.

Table I. Concentration of 2-PAM in Blood after Iv Administration of 5 mg/kg of 2-PAM and Pro-2-PAM to a Beagle Dog[a]

	Drug administered			
		Pro-2-PAM		
Time, min	2-PAM, plasma, μg/ml	Plasma,[b] μg/ml	Whole blood,[b] μg/ml	Ratio of amt of plasma/whole blood[c]
5	11.45[d]	4.40	6.60	1:3.0
10	11.50	3.70	4.80	1:2.6
15	5.68	3.03	4.70	1:3.1
30	5.55	1.46	4.50	1:6.2
45	2.45	2.08	4.27	1:4.1
60	1.25	1.65	3.90	1:4.7
120	0.80		2.75	
180		1.39	2.30	1:3.3
240		0.86	1.63	1:3.8
300			1.50	
360			0.82	

[a] The same dog was given both 2-PAM and pro-2-PAM. The dog was allowed to rest for 10 days between the two experiments. [b] Analyzed as 2-PAM. [c] Ratio was calculated assuming that the volume of plasma is exactly half of the volume of whole blood. [d] The 2-PAM concentration in whole blood at this time was determined and found to be 5.50 μg/ml.

mucosal membrane by diffusion through the aqueous "pores".[26] Neglecting the hydration, it was calculated that 2-PAM cation can be fitted into a sphere of 4.3-Å radius for the anti form and 4.8 Å for the syn form. The average pore radius is about 4 Å[28] and was thus assumed that 2-PAM cation could diffuse through pores, the radii of which were slightly larger than the mean value. In vivo study[29] supports this theory, since by changing the N-substituents of 2-PAM the percentage adsorbed was reduced significantly. Furthermore, any "abnormality" in size of "pores" or change in transmural potential among species or numbers of the same specie would influence the amount absorbed. This might explain the large variations in blood levels of human volunteers after oral administration of 2-PAM.[19]

Results and Discussion

Preliminary oral and iv biopharmaceutical studies have been carried out. Beagle dogs were injected iv with 5 mg/kg of 2-PAM and pro-2-PAM, respectively, and the concentration of 2-PAM was determined in blood samples withdrawn at various time intervals, using the analytical procedure described.[30] After administration of pro-2-PAM, the samples were analyzed again for 2-PAM, after removing a very small portion of nonconverted pro-2-PAM, by extraction. The results are shown in Table I.

It can be seen that the amount of 2-PAM in whole blood was higher than in the plasma after iv administration of pro-2-PAM, while 2-PAM is distributed mainly in the plasma[3,4] as was also confirmed by the present study. In the case of pro-2-PAM, based on its pK_a (6.32 ± 0.06) about 90% was in the lipophilic free base form at the pH of blood and thus it was expected to distribute from the plasma into or on the surface of the red blood cells. Indeed, only one part out of three of 2-PAM (when pro-2-PAM was administered) was found in the plasma. This difference in distribution between 2-PAM and its pro-drug was felt to be very important. It suggested that by administration of pro-2-PAM the distribution pattern of 2-PAM in a biological system is significantly changed and that pro-2-PAM would help the quaternary pyridinium compound to reach organs which were impermeable to the parent drug.

Additional blood clearance studies were carried out using ^{14}C-radiolabeled 2-PAM and pro-2-PAM, respectively.

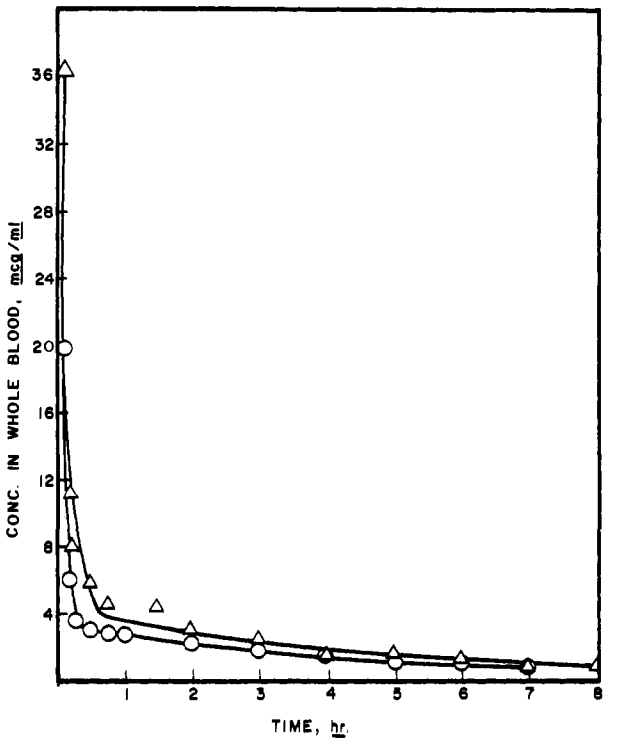


Figure 1. Blood levels of pro-2-PAM (△) calculated from total radioactivity and of 2-PAM (○) determined by uv method after iv administration of 4.5 mg/kg of [^{14}C]pro-2-PAM to the beagle dog.

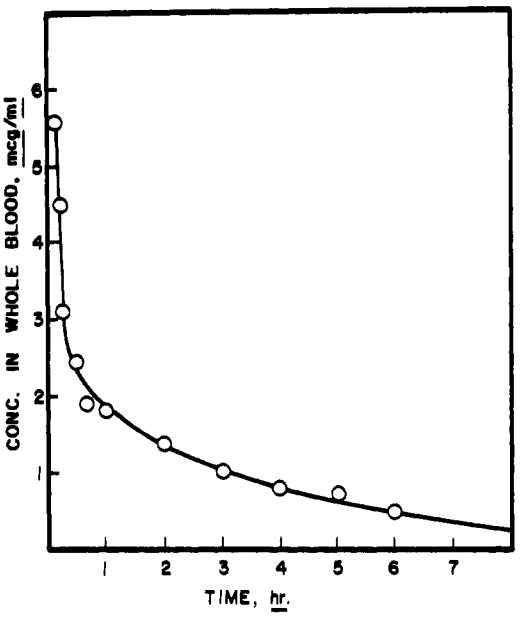


Figure 2. Blood level of 2-PAM iodide calculated from total radioactivity found in blood after iv administration of 7.0 mg/kg of [^{14}C]-2-PAM iodide to the beagle dog.

The ^{14}C was introduced as the NCH_3 group, since it has been established that demethylation is not one of the methabolic pathways of 2-PAM.[16] Both radioactive materials were administered again to the same dogs in order to avoid some of the usual variables. When pro-2-PAM was administered, the total radioactivity was measured as well as 2-PAM was determined by the classical uv method. The results are shown in Figure 1.

Figure 2 shows the blood levels of 2-PAM obtained when radiolabeled 2-PAM iodide was given iv, calculated from the total radioactivities and confirmed by the uv method.

In order to estimate the in vivo rate of transformation of the pro-drug to 2-PAM, a simple two-compartment

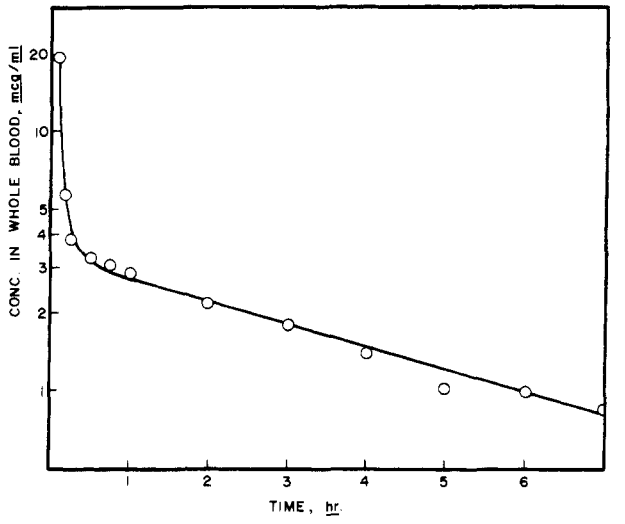
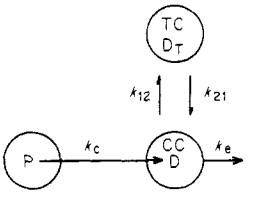


Figure 3. Semilogarithmic plot of blood levels of 2-PAM after administration of 4.5 mg/kg iv of pro-2-PAM to the beagle dog. The solid line was calculated by a Simplex optimization method.

model was constructed,[31] assuming that after iv injection of pro-2-PAM, a source (P) for 2-PAM is formed in the body, which supplies 2-PAM (D) into the central compartment (CC) by a first-order rate constant k_c. From the central compartment, 2-PAM is distributed into a tissue compartment (TC) (k_{12} and k_{21}) and eliminated by a first-order rate constant (k_e).



The concentration of 2-PAM in the central compartment can be expressed as

$$[D] = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-k_c t} \quad (1)$$

where C, A, B, α, and β are constants containing the rate constants k_{12}, k_{21}, k_c, and k_e, the dose of pro-2-PAM, and a constant for conversion of the unit of the dose to a concentration unit. Using an optimization program based on the Simplex algorithm,[32] the blood level values of 2-PAM obtained after iv administration of pro-2-PAM (Figure 1) were fitted to eq 1 as shown in Figure 3.

The following values for the rate constants were obtained: $k_c = 40.17$ hr^{-1}; $k_{12} = 16.13$ hr^{-1}; $k_{21} = 1.39$ hr^{-1}; $k_e = 2.95$ hr^{-1}. The curve described by eq 1 is

$$[D] = 106.7e^{-20.265t} + 3.3e^{-0.202t} - 110.1e^{-40.17t} \quad (2)$$

The k_c value is an approximate one, since it is based on the assumption that the first data point (5 min) was of the highest concentration. Using this simplified model, however, it has to be considered that the k_c calculated does not represent only the oxidation process of pro-2-PAM to 2-PAM but includes also terms for the distribution of the pro-drug. Since the obtained rate constant corresponds to a $t_{1/2} = 1.04$ min for the in vivo transformation of pro-2-PAM to 2-PAM, the main conclusion is that the distribution and oxidation of the pro-drug are extremely fast. It was considered that the data are quite satisfactory for supporting this conclusion, without any statistical studies on large numbers of animals, particularly because the metabolic studies, as it will be shown, provide additional support.

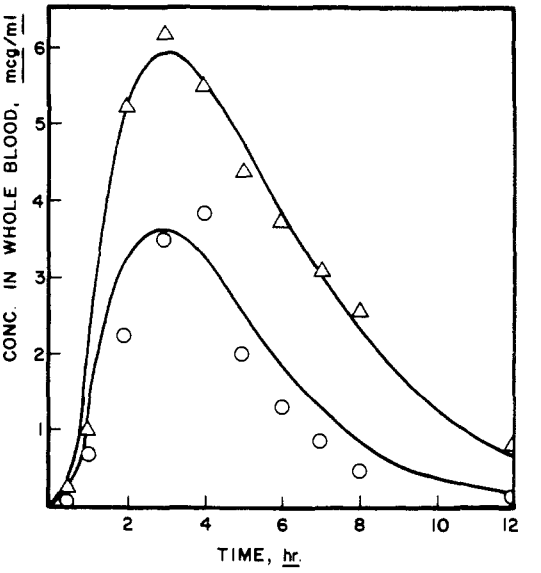


Figure 4. Blood level of pro-2-PAM (△) calculated from total radioactivity and of 2-PAM determined by a uv method (○) after oral administration of 44.6 mg/kg of [^{14}C]pro-2-PAM in gelatin capsule to the beagle dog.

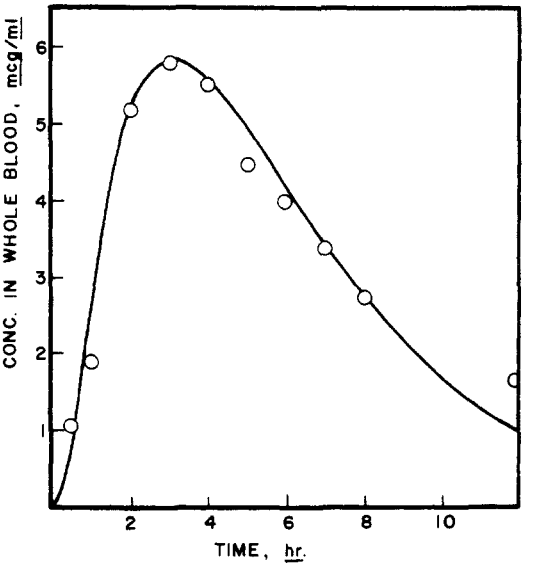


Figure 5. Blood level of 2-PAM after oral administration of 43.9 mg/kg of 2-PAM in gelatin capsule to the beagle dog.

The biological half-lives for 2-PAM administered as such and as pro-2-PAM were also calculated from the linear descending portion of semilogarithmic plots of blood levels vs. time. There was a significant difference between the observed biological half-lives, namely, when pro-2-PAM was given iv to beagle dogs, the half-life of 2-PAM was approximately 60 min longer ($t_{1/2} = 168$ min) when compared to dosing with 2-PAM ($t_{1/2} = 105$ min). The increase in the half-life cannot be attributed to a rate-limiting step of oxidation of pro-2-PAM to 2-PAM, because this step was fast. The change in the elimination rate of 2-PAM was assumed to be the result of a direct change in the distribution ability; pro-2-PAM diffuses into many more tissues, including lipoidal ones of the biological system.[33]

2-PAM blood levels were also determined after oral administration of pro-2-PAM and 2-PAM, respectively, as shown in Figures 4 and 5. Urine samples were also collected in all experiments, both after iv and oral administration.

An average of 65% unchanged 2-PAM was excreted in

Table II. Percent of Radioactivity and of 2-PAM Excreted after Iv and Oral Administration of ^{14}C-Radiotagged Pro-2-PAM and 2-PAM to a Beagle Dog

Drug	Iv urine, radioact., %	Oral			
		Urine		Feces	
		2-PAM, %	Radioact., %	2-PAM, %	Radioact., %
Pro-2-PAM[b]	98.04	67.62	43.66	19.16	50.90
2-PAM[a]	92.20	79.80	*c*	62.10	*c*

[a] Dose of 7 mg/kg given iv as the iodide salt which corresponds to 4.6 mg/kg of 2-PAM. Orally a dose of 43.9 mg/kg of 2-PAM was given. The same dog was used in all studies. [b] Dose of 4.5 mg/kg was given iv and 44.6 mg/kg orally. [c] No radiolabeled 2-PAM was given orally.

the urine of the dogs after oral administration of 2-PAM. This implies that at least this fraction of the dose was absorbed from the gastrointestinal system. This was quite a surprising result, since it has been reported[20] that in man only 27% of unchanged 2-PAM was found in the urine after oral dosing. No bioavailability data have been, however, reported on beagle dogs. In order to verify that the results obtained are indeed due to the species used, a human subject was given 1 g of 2-PAM orally. The cumulative excreted fraction of 2-PAM in the urine was only 34%, which is in good agreement with the reported values. This would suggest that beagle dogs have a higher ability to absorb 2-PAM than man.

The average amount of 2-PAM excreted in urine when the dogs were dosed orally with pro-2-PAM (45 mg/kg) was only about 20–25% of the dose given. The amount of radioactivity found in the urine after oral dosing of radiotagged pro-2-PAM was, however, 43.7%. The other half of the dose was found in the feces. The lower blood level of 2-PAM after oral dosing to beagle dogs with the pro-drug indicated a lower oral bioavailability. As it will be seen, a new metabolite which was not observed when pro-2-PAM was given iv or when 2-PAM was administered either iv or orally[16] has appeared in the urine when the dogs were given oral pro-2-PAM. It is possible that this metabolite was a fraction of pro-2-PAM which has been decomposed in the gastrointestinal system and absorbed as such. Since pro-2-PAM has a low pK_a, 6.32, it should be in the un-ionized form in most parts of the gastrointestinal tract and should be absorbed in a similar way to other weak electrolytes.[34] The lack of good oral absorption indicates some other interfering process. It was shown that pro-2-PAM was rapidly decomposed in the free base form even under anaerobic conditions.[1] Since the neutral form is also the absorbable form of the pro-drug, it can be assumed that as the process of absorption was theoretically optimized (the pro-drug was mainly in the free base form) the rate of decomposition of pro-2-PAM was also increasing. This increased decomposition of pro-2-PAM to products other than 2-PAM might account for the poor oral bioavailability of 2-PAM from pro-2-PAM. The absorbed fraction of the dose is significantly decreased if the decomposition products are less absorbed than pro-2-PAM or 2-PAM.

Another possibility of decomposition would be addition of various nucleophiles,[35] including bile acids, to the dihydro system, which would occur mainly under acidic conditions in the stomach.

The main objective of the limited metabolic studies was to determine if any new metabolite is forming after administering pro-2-PAM, as compared to the metabolism of 2-PAM. The total urine was collected for 72 hr after iv and oral administration, while in the case of oral administration of pro-2-PAM, the feces were also collected. The percentages of the total radioactivity found in urine and feces were given in Table II.

Table III. 2-PAM and Metabolite Content of the Urine of the Beagle Dog after Administration of Radiotagged 2-PAM and Pro-2-PAM, as Calculated from Bands Area of Radiochromatograms

Metabolite	Pro-2-PAM		2-PAM, iv
	Oral	Iv	
2-PAM[a]	50%	66%	87%
Known metabolites[b]	35%	34%	13%
New metabolites[c]	15%		

[a] Band corresponds to $R_f = 0.53$.[16] [b] Bands correspond to R_f = 0.33–0.36, 0.46–0.48.[16] [c] New band with $R_f = 0.08$.

Urine samples were paper chromatographed and radioactive spots were counted in order to evaluate whether the metabolism of the pro-drug was any different to that of 2-PAM. The comparison was based on the R_f values. In all cases (oral and iv administration of 2-PAM and pro-2-PAM, respectively), the main metabolite was unchanged 2-PAM identified as having $R_f = 0.53$.[16] The area under the various bands of the chromatograms was measured and the fractions were calculated and shown in Table III.

The elimination of a drug is composed of at least two physiological processes: metabolism and excretion. While the elimination of 2-PAM administered by iv route occurred primarily by excretion (around 90% as unchanged 2-PAM), only 66% of 2-PAM was excreted after an iv dose of pro-2-PAM. On the other hand, as it is shown in Table III, no new metabolites could be detected. This might suggest that mainly the oxidized form of the pro-drug, 2-PAM, was the one which underwent metabolism. This suggestion is well supported by the fast oxidation processes of pro-2-PAM, which can be considered the main metabolic pathway of pro-2-PAM itself. It is very likely that the enhancement of the metabolism of 2-PAM (34%) when administered as pro-2-PAM is the result of the change in distribution. As it has been shown, 2-PAM is very rapidly metabolized by liver homogenates. However, very little metabolism was observed in vitro. This apparently is the result of the hydrophilic character of 2-PAM.

When radiolabeled pro-2-PAM was administered orally only about 50% of the radioactivity was found to be due to 2-PAM. A new metabolite with an R_f value of 0.08 was also observed. This metabolite could be formed only in the gastrointestinal tract, prior to or during the absorption process.

Our limited biopharmaceutical and metabolism studies on pro-2-PAM have thus established that in vivo pro-2-PAM administered by iv route transforms very rapidly and exclusively into 2-PAM, and no new metabolite could be detected. These facts as well as the increased biological half-life and more favorable distribution observed when 2-PAM was administered as its pro-drug indicate that pro-2-PAM should represent an important step in improving the antidotal characteristics of 2-PAM.

Experimental Section

1. **Materials.** 2-PAM chloride was obtained from Ayerst Laboratories, Inc.; Aquasol, a cocktail for LSC, and [^{14}C]methyl iodide were supplied by New England Nuclear.

[^{14}C]Methyl-labeled 2-PAM iodide was synthesized as follows. To a mixture of 2.4 g (0.0187 mol) of 2-pyridinecarbaldoxime was added 0.617 g of [^{14}C]methyl iodide (10 mCi) and 0.8 g of methyl iodide in 35 ml of dry acetone. The mixture was stirred in a pressure bottle at 95° for 6 hr. The yellow product was separated, washed with acetone, and vacuum dried: mp 224–226°; specific activity 1 mCi/mol or 3.79 mCi/mg.

[^{14}C]Methyl-labeled pro-2-PAM chloride was synthesized from [^{14}C]methyl-labeled 2-PAM as described for the unlabeled material.[1] The product was characterized and identified to have the purity of the unlabeled material. After dilution with cold product, a material of 0.65 mCi/mmol or 3.72 mCi/mg specific activity was obtained.

2. Analytical Procedures. Determination of 2-PAM in Biological Materials. Concentrations of 2-PAM in biological materials were estimated by uv spectrophotometry using the procedures described by May et al.[30] as follows.

Blood and Plasma. To 2 ml of whole blood or plasma in a centrifuge tube, 3 ml of water and 1 ml of 0.26 *M* barium hydroxide were added and mixed by shaking. After 3 min, 1 ml of 0.3 *M* zinc sulfate and 1 ml of 4% sodium chloride were added and the tube was vigorously shaken. The precipitate was centrifuged and the supernatant solution was filtered. To the filtrate, 0.1 ml of 30% sodium hydroxide was added and the absorbance determined at 335 nm. When pro-2-PAM was administered, the whole blood samples were extracted twice with 7 ml of ether before preparing the protein-free filtrate.

Urine. It was shown[30] that uv analysis of human urine gives rather inaccurate data because of the large variations in the absorbance at 335 nm of the control samples. The same problem was encountered during the present study of the analysis of beagle dog urine samples. We have found, however, that as in the case of human urine, the ratio of the absorbance at 335 nm between an alkaline protein-free dog urine sample and an acidic one is a constant value, namely 1.73 ± 0.09.

The urine samples were diluted 1:50 with water; then a protein-free filtrate was prepared as described for blood. The filtrate was divided in two equal portions; to one portion 0.1 ml of 6 *N* hydrochloride acid was added, while 0.1 ml of 30% sodium hydroxide was introduced to the other portion. The absorbance at 335 nm of the acidic solution was multiplied by 1.73 and the value obtained was used as the control and, as such, it was subtracted from the absorbance of the basic solution determined at the same wavelength. In the case of human urine, the acid–base absorbance ratio was found to be 1.67.

3. Liquid Scintillation Counting (LSC) of Radioactivity in Biological Tissues. The procedure of Wiebe et al.[36] for preparation of biological materials for LSC (using AquASOL) was followed. Counting was performed on a liquid scintillation system LS-150 (Beckman Instruments) using ^{14}C & ^{3}H block. The efficiency of the counting was determined using standard quenched samples and an external standard.

Blood. To 1 ml of whole blood, 1 ml of 30% hydrogen peroxide was added. After the blood was discolored, the mixture was homogenized; 1 ml of water and 10 ml of Aquasol were added, mixed thoroughly, and counted.

Feces. The entire sample of feces was mixed in a blender with three times the sample weight of water to make a thin suspension. An aliquot of 10 ml of the suspension was discolored with 60 ml of 30% hydrogen peroxide. After 12 hr, 1 ml of the mixture and 10 ml of Aquasol were well mixed and counted.

4. Radiochromatography. Radiochromatography was performed using No. 1 Whatman paper strips 1 in. wide in a descending chamber over 24 hr in a system of 1-butanol–acetic acid–water (4:1:1). During this time, the solvent traveled about 50 cm.

In the case of the urine, the undiluted samples were chromatographed directly. The strips were air-dried and were counted on a radiochromatograph "Actiograph III" (Nuclear-Chicago Corp.).

5. Preliminary Oral and Iv Biopharmaceutical Studies. Beagle dogs (11–14 kg) were used. They were kept in metabolism cages for 5 days before the start of the experiment. When dosing orally, the dogs were fasted for 12 hr before and 4 hr after administration. The compounds were given in gelatin capsules followed by 150–200 ml of water administered through a stomach tube. When dosing iv the compounds were dissolved in approximately 3 ml of citrate buffer, pH 3, just before injecting them into the jugular vein.

Blood samples (5–7 ml) were drawn from one of the jugular veins. The blood was preserved with 0.15% EDTA and was analyzed on the same day. Urine samples were collected when the dogs voided and were frozen until assayed.

When 2-PAM was tested on a human volunteer, he was allowed no solid food from midnight until 4 hr after he had taken the drug. The drug was given in gelatin capsule. The volunteer was encouraged to drink and urine samples were collected at appropriate intervals.

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