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ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Division of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy, Ohio State University, Columbus, OH 43210*

The invaluable assistance of Ms. Denise M. Ragaji in the preparation of the manuscript is gratefully acknowledged.

RESEARCH ARTICLES

Metabolism of 8-(Methylthio)cyclic 3′,5′-Adenosine Monophosphate by Rats and Dogs after Oral or Intravenous Dosing and *In Vitro* by Subcellular Preparations of Dog Liver

JACQUES DREYFUSS ˣ, JAMES M. SHAW, KEITH K. WONG, and ERIC C. SCHREIBER

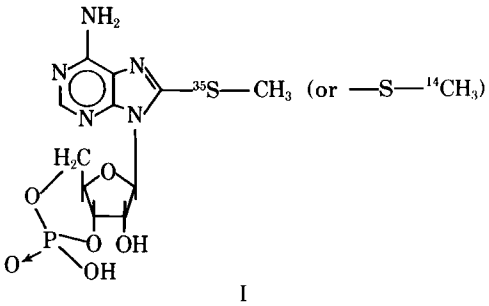
Abstract □ 8-(Methylthio-^{14}C or -^{35}S)cyclic 3′,5′-adenosine monophosphate (I) was given intravenously to rats (5 mg/kg) and orally and intravenously to dogs (0.25, 2.5, or 50 mg/kg). Oral doses were absorbed well but slowly. Plasma half-lives in dogs were about 3 hr after oral or intravenous doses of 0.25 or 2.5 mg/kg and ranged from 5 to 12 hr after oral or intravenous doses of 50 mg/kg. Plasma glucose and insulin concentrations in dogs were increased by oral or intravenous doses of the compound. Regardless of the route, excretion of radioactivity by rats and dogs at all doses was chiefly in the urine (74–87% of the dose); the remainder was excreted in the feces or bile. Compound I was rapidly distributed to most tissues of dogs but entered the brain and certain portions of the eye slowly and to a limited extent. Urine and plasma of dogs and urine of rats contained I, 8-(methylthio)adenosine, and at least two other unidentified metabolites. Compound I and cyclic 3′,5′-adenosine monophosphate were metabolized *in vitro* by the soluble fraction of dog liver to form 8-(methylthio)adenosine-5′-monophosphate and adenosine-5′-monophosphate, respectively. These compounds were further converted to 8-(methylthio)adenosine and adenosine, respectively. Compound I was metabolized *in vitro* more slowly than cyclic 3′,5′-adenosine monophosphate.

Keyphrases □ 8-(Methylthio)cyclic 3′,5′-adenosine monophosphate—metabolism, dogs and rats after oral and intravenous doses, *in vitro* metabolism by subcellular dog liver preparations □ Adenosine monophosphate—activity mimicked by 8-(methylthio)cyclic 3′,5′-adenosine monophosphate, metabolism of analog *in vivo* and *in vitro* □ Metabolism—8-(methylthio)cyclic 3′,5′-adenosine monophosphate in dogs and rats after oral and intravenous doses and *in vitro* by subcellular dog liver preparations

Ample documentation now exists that the biological effects of cyclic 3′,5′-adenosine monophosphate can be mimicked by certain of its analogs (1, 2). Such biologically active analogs might exhibit greater po-

tency, penetrability, tissue specificity, or stability than is the case with cyclic 3′,5′-adenosine monophosphate (2). 8-(Methylthio)cyclic 3′,5′-adenosine monophosphate (I) is one such analog that has exhibited activity in the stimulation of a purified cyclic 3′,5′-adenosine monophosphate-dependent protein kinase (3), the inhibition of cyclic nucleotide phosphodiesterase from cat heart and rat brain (4, 5), and the activation of tyrosine transaminase from hepatoma cells grown in culture (6).

The metabolism of I given to rats and dogs by oral and intravenous routes was studied to determine whether the 8-(methylthio) substituent altered its disposition as compared with that of cyclic 3′,5′-adenosine monophosphate. The results show that I was converted to 8-(methylthio)adenosine and other unidentified metabolites. In addition, I was metabolized *in vitro* more slowly than cyclic 3′,5′-adenosine monophosphate.



I

EXPERIMENTAL

Animals—Albino rats (Sprague–Dawley or Charles River CD) and purebred beagles were used. Animals were fasted overnight before the administration of drug and were housed in metabolism cages which permitted the separate collection of urine and feces.

Purity and Specific Activity—Compound I-^{35}S had an initial specific activity of 12.1 μCi/mg; no radiochemical impurities were detectable. Compound I-^{14}C had a specific activity of 2.76 μCi/mg; no radiochemical impurities were detectable.

Surgical Preparation of Dogs—Dogs were anesthetized with 30 mg/kg of pentobarbital sodium administered intravenously. A catheter was inserted into the bladder for the collection of urine. The radial vein was cannulated and infusion of the following solution, at the rate of 3 ml/min, was begun: mannitol, 100 g; KH_2PO_4, 200 mg; K_2HPO_4, 900 mg; pentobarbital sodium, 25.5 mg/kg; and sufficient water to make 2 liters. Mannitol was given to ensure an adequate flow of urine.

A midline incision was made, and the entrance to the gallbladder was clamped just above its point of entry into the common bile duct. A polyethylene catheter (No. 100) was then inserted about 1.3 cm (0.5 in.) into the common bile duct. The catheter was tied in place, and the midline incision was closed with wound clips.

Analysis of Urine and Bile—Samples of urine and bile were counted directly in 15 ml of Bray's scintillation fluid (7).

Analysis of Blood for ^{35}S—A 0.2-ml sample of heparinized blood was digested in 0.5 ml of 1.0 *N* NaOH by heating overnight at 80°. The sample was then bleached with 30% hydrogen peroxide, followed by neutralization with 0.2 ml of 2-ethylhexanoic acid. The sample was counted with 15 ml of Bray's scintillation fluid.

Analysis of Blood for ^{14}C—Samples of blood (0.2 ml) were combusted with a biological material oxidizer[1].

Analysis of Plasma—A 0.4-ml sample of plasma was dissolved in 2 ml of solubilizer[2]. The sample was counted with 15 ml of Bray's or toluene scintillation fluid. The toluene scintillation fluid contained, per liter, 5 g of 2,5-diphenyloxazole and 300 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

Analysis of Feces for ^{35}S—Fecal samples were homogenized in about three volumes of methanol. A 100-mg sample of the homogenate was digested in 1 ml of solubilizer for 16 hr with shaking and then bleached with 30% hydrogen peroxide. The sample was counted with 15 ml of Bray's scintillation fluid.

Analysis of Feces for ^{14}C—Samples of feces were homogenized with two to three volumes of methanol. About 400 mg of homogenate was combusted with the biological material oxidizer.

Analysis of Tissue for ^{35}S—Samples of brain were homogenized in water, using an all-glass homogenizer[3]; about 200 mg of homogenate was digested in 2 ml of solubilizer. Certain portions (about 200 mg) of the eye (lens, cornea, aqueous and vitreous humors, and combined retina, choroid, and sclera) or representative portions of the pancreas, renal and omental fat, skin (without hair), adrenal, thyroid and pituitary glands, and ovary were each placed in 2 ml of solubilizer. Samples of tissue from the heart, kidneys, liver, lungs, thigh muscle, and testes or the entire rat carcass were analyzed by first grinding the tissue in a meat grinder and then digesting about 200 mg of the well-mixed sample in 2 ml of solubilizer. All samples were counted in 15 ml of Bray's scintillation fluid.

Analysis of Tissue for ^{14}C—Samples of brain were homogenized in water, using an all-glass homogenizer; about 400 mg of homogenate was combusted with the biological material oxidizer. Certain portions of the eye (lens, cornea, aqueous and vitreous humors, and combined retina, choroid, and sclera) and representative portions of the pancreas, renal and omental fat, skin (without hair), adrenal, thyroid and pituitary glands, and ovary were combusted. Samples of tissue from the heart, kidneys, liver, lungs, thigh muscle, and testes or the entire rat carcass were analyzed by first grinding the tissue in a meat grinder and then combusting about 400 mg of the well-mixed sample.

Measurement of ^{14}C-Carbon Dioxide—To measure the $^{14}CO_2$ evolved, each rat was placed in a plastic metabolism cage[4] enclosed in a plastic bag. Air was drawn from the cages through two carbon dioxide-absorbing traps, connected in series with a vacuum line and containing monoethanolamine–2-methoxyethanol (1:3). The amount of radioactivity in the expired air was measured by adding a 0.5-ml sample of the absorbing solution to 2.0 ml of solubilizer, followed by 15 ml of toluene scintillation fluid.

Binding of Drug to Plasma Proteins—Binding measurements were made on 3-ml samples of plasma which were transferred to ultrafiltration membrane filter cones[5]; they were centrifuged at 25–30° for 30 min at 2000 rpm. After centrifugation, an aliquot of the ultrafiltrate and the plasma sample prior to centrifugation were solubilized in 2.0 ml of solubilizer and counted with 15 ml of toluene scintillation fluid.

Extraction Procedure for Feces—Samples of feces were homogenized with two to three volumes of methanol and then centrifuged; the supernatant fluid was recovered and saved. Chloroform–methanol (1:1), equal in volume to the supernatant fluid recovered, was added to the fecal residue, and the mixture was shaken for 30 min and centrifuged; the supernatant fluid was saved. This last step was repeated one more time. The supernates were combined and spotted directly for chromatography or were first concentrated to a suitable volume at 40–50° under reduced pressure. This extraction procedure removed an average of 59.8% of the radioactivity present in the fecal homogenates of dogs.

Chromatography—Samples of urine, bile, and extracts of feces and plasma were spotted on cellulose Q2-F[6] plates and developed in one or more of the following solvent systems: 1, isopropanol–28% ammonia–water (7:1:2); 2, *n*-butanol–pyridine–acetic acid–water (30:20:6:24); and 3, 95% ethanol–buffer (70:30). (The buffer was prepared by mixing 1 *M* ammonium acetate and 3×10^{-3} *M* ethylenediaminetetraacetate and adjusting the pH to 5.0 with acetic acid.)

Counting of Samples—The radioactivity in each sample was

[1] R. J. Harvey Instrument Corp., Hillsdale, N.J.
[2] NCS Solubilizer, Amersham/Searle Corp., Des Plaines, Ill.
[3] Potter-Elvehjem, Fisher Scientific Co., Springfield, N.J.
[4] Microchemical Specialties Co., Berkeley, Calif.
[5] Centriflo, Amicon Corp., Lexington, Mass.
[6] Quantum Industries, Fairfield, N.J.

Table I—Plasma Half-Lives of Radioactivity after the Oral or Intravenous Administration of I to Dogs

Dog[a]	Sex	Route[b, c]	Dose, mg/kg	Time to Maximum Plasma Concentration, hr	Plasma Half-Life, hr	Time Interval, hr
Q2-168	M	Intravenous	0.25	—	3.7	1–12
Q1-349	F	Intravenous	0.25	—	2.9	1–12
Q2-56	M	Intravenous	2.5	—	3.0	1–12
Q2-80	F	Intravenous	2.5	—	2.9	1–12
Q2-240	M	Intravenous	50	—	4.8	1–24
Q3-53	F	Intravenous	50	—	4.3	1–24
Q3-39	M	Oral	2.5	3	2.3	6–12
Q0-297	F	Oral	2.5	3	2.7	6–12
Q3-10	M	Oral	2.5	2	3.0	3–12
Q2-198	F	Oral	2.5	4	2.8	4–12
Q3-10	M	Oral	50	8	9.6	8–48
Q2-198	F	Oral	50	4	11.9	12–48

[a] The type of radioactivity given to these dogs is indicated in Table V. [b] Intravenous doses were dissolved in 0.1 *M* Na_2CO_3. [c] Oral doses were administered in capsules. Tepid water (100 ml) was given to each dog immediately after the capsule.

determined with a liquid scintillation spectrometer[7]. Counting efficiency was determined with automatic external standardization and the use of previously prepared quench curves. Chromatograms were scanned for the presence of radioactivity with a scanner[8]. Areas of the various radioactive peaks were integrated by the use of a planimeter.

Calculation of Biological Half-Lives and Absorption and Elimination Rate Constants—Biological half-lives in the plasma were estimated graphically from linear portions of the appropriate semilogarithmic plots. Absorption rate constants were calculated as described by Rescigno and Segre (8).

Determination of Glucose Concentrations in Plasma—These analyses were performed automatically[9] by the method of Kornberg and Horecker (9).

Determination of Insulin Concentrations in Plasma—These analyses were performed employing an insulin radioimmunoassay kit[10]. The results were calculated from standard curves prepared from standardized human insulin.

Preparation of Subcellular Fractions from Dog Liver—Male beagle dogs (10–12 kg) were anesthetized intravenously (thiopental sodium, 30 mg/kg) and killed by exsanguination; the livers were removed. Samples of liver from different lobes were homogenized immediately in three volumes of 1.15% KCl, using a homogenizer[3]. The whole homogenate was centrifuged at 9000×*g* for 20 min, and the supernatant fraction was decanted through a single layer of gauze to remove some fat droplets; this fraction was designated the "postmitochondrial supernate."

The postmitochondrial supernate was centrifuged at 105,000×*g* for 60 min. The supernatant material, filtered through a single layer of gauze, was designated the "soluble fraction," and the pellet was resuspended in 6 ml of 1.15% KCl. This suspension was designated the "microsomal fraction," which was centrifuged again at 105,000×*g* for 60 min. After removal of the supernatant fluid, the pellet was resuspended again in 6 ml of 1.15% KCl and was designated the "washed microsomal fraction."

As controls, the postmitochondrial supernate or the soluble fraction was inactivated in a boiling water bath for 10 min. The coagulated protein was removed by filtration through a thin plug of glass wool, and the clear filtrate was designated the "boiled postmitochondrial supernate" or the "boiled soluble fraction."

Incubation of I and Cyclic 3′,5′-Adenosine Monophosphate *In Vitro*—Each incubation contained 0.2 ml of I, 5 m*M*; 0.2 ml of tromethamine buffer, 0.35 *M*, pH 7.4; 0.02 ml of $MgCl_2$, 250 m*M*; 0.02 ml of glucose-6-phosphate, 500 m*M*; 0.02 ml of NADP, 15 m*M*; and 0.02 ml of glucose-6-phosphate dehydrogenase[11] (two units). To each incubation was added 0.2 ml of the whole homogenate, postmitochondrial supernate, microsomal, washed microsomal, soluble, or boiled postmitochondrial supernatant fraction. Distilled water (0.32 ml) was added to provide a final volume of 1.0 ml. The incubation was carried out, with shaking, at 37° for 60 min; then the tubes were frozen immediately in an acetone–dry ice bath and stored in the freezer.

For the study of the metabolism of I-^{14}C (1 m*M*) or cyclic 3′,5′-adenosine-8-^{14}C monophosphate[12] (1 m*M*) as a function of time, the incubation mixtures were scaled up by increasing the amount of each ingredient by 3.5. Only the soluble fraction was used as the enzyme preparation, and the boiled soluble fraction served as the control. Aliquots (0.5 ml) were removed at different time intervals, as indicated in each experiment. Each aliquot was frozen immediately in an acetone–dry ice bath and then stored in the freezer. Each sample was chromatographed, as described under *Chromatography*. For the studies with cyclic 3′,5′-adenosine monophosphate, nonradioactive cyclic 3′,5′-adenosine monophosphate, adenosine-5′-monophosphate, and adenosine were used as reference compounds.

RESULTS

Absorption—In separate experiments, two dogs were given the ^{35}S-labeled compound, either 0.25 or 2.5 mg/kg iv, or the ^{14}C-labeled compound, 50 mg/kg iv. The concentrations of radioactivity found in the plasma of dogs after each dose during the first 12 hr are shown in Fig. 1. Shortly after dosing, concentrations of radioactivity in plasma reflected the amount of compound administered. After the distribution of radioactivity into tissues, the plasma concentrations of radioactivity decreased according to first-order kinetics.

Four dogs were given 2.5-mg/kg doses of I as a finely divided powder in capsules. Figure 2 shows the average concentrations of radioactivity in the plasma of these dogs during the first 12 hr after dosing. The compound was absorbed slowly, attaining an average maximum plasma concentration 3 hr after dosing, which was similar (about 1–2 μg/ml) to that found after a comparable intravenous dose. An average of 16.9 ± 6.4% (±*SE*) of the radioactivity was bound to the plasma proteins of these dogs 2 hr after they had been dosed.

Figure 3 shows similar data for two dogs that received an oral dose of 50 mg/kg. Plasma concentrations found at this higher dose reflect: *(a)* the greater dose administered, *(b)* the greater time required to absorb this larger dose, and *(c)* the greater persistence of radioactivity in the plasma, since the time scale for these two dogs extends to 48 hr.

Table I summarizes plasma half-lives for each dog after the oral and intravenous administration of I. The plasma half-lives were determined by a linear regression analysis for as long as the curves were linear and still significantly above background. Plasma half-lives after intravenous doses were about 3 hr with either the small-

[7] Packard Tri-Carb, model 3380, Packard Instrument Co., Downers Grove, Ill.
[8] Nuclear-Chicago Actigraph III, Amersham/Searle Corp., Des Plaines, Ill.
[9] Autoanalyzer, model DSA-560, Beckman Instruments, Fullerton, Calif.
[10] Amersham/Searle Corp., Des Plaines, Ill.
[11] Sigma Chemical Co., St. Louis, Mo.
[12] New England Nuclear, Boston, Mass.

Table II—Concentrations of Glucose in the Plasma of Dogs as a Function of Time after the Intravenous Administration of I

Time	0.25 mg/kg[a]		2.5 mg/kg[b]		50 mg/kg[c]	
	Dog Q2-168	Dog Q1-349	Dog Q2-56	Dog Q2-80	Dog Q2-240	Dog Q3-53
	Percent Change					
0 min	(0)	(0)	(0)	(0)	(0)	(0)
5 min	25	73	48	41	103	135
15 min	33	58	49	20	211	294
30 min	−4	13	1	5	231	564
1 hr	0	8	14	−7	188	496
1.5 hr	−1	5	4	15	−13	197
2 hr	−8	−20	−9	2	−55	96
3 hr	−2	−6	−18	1	−4	−47
4 hr	−7	2	−10	−14	26	−51
6 hr	—	—	—	—	8	−38

[a] Average glucose concentration in the plasma of these dogs at zero time was 84 ± 1 mg %. [b] Average glucose concentration in the plasma of these dogs at zero time was 83 ± 4 mg %. [c] Average glucose concentration in the plasma of these dogs at zero time was 76 ± 4 mg %.

est or the intermediate dose and about 5 hr with the largest dose. After oral doses of 2.5 mg/kg, it took 2–4 hr to attain maximum plasma concentrations of radioactivity; after oral doses of 50 mg/kg, 4–8 hr was required.

Plasma half-lives after oral doses of 2.5 mg/kg ranged from 2.3–3.0 hr for the indicated time intervals, which were essentially the same as those found after a comparable intravenous dose. After oral doses of 50 mg/kg, plasma half-lives ranged from 9.6 to 11.9 hr and were about twice as long as those cited previously for the same intravenous doses. Absorption half-times, which were 1.1 hr with the smaller dose and 3.2 hr with the larger dose, support the slower absorption of the compound at the larger dose.

Studies of Glucose and Insulin—Compound I, like cyclic 3′,5′-adenosine monophosphate, is capable of producing a hyperglycemic effect. The data of Table II show plasma glucose concentrations as a function of time after intravenous doses of 0.25 or 2.5 mg/kg; they are expressed as the percent change from control values for each individual dog. Within 5 min, intravenous doses of 0.25 or 2.5 mg/kg of I produced significant elevations of plasma glucose; these levels returned to control values within 30–60 min after dosing. In these particular dogs, the larger intravenous dose did not produce a greater elevation of glucose concentration than did the smaller dose.

After an intravenous dose of 50 mg/kg, a marked elevation in glucose concentrations was seen within 5 min; these levels persisted for 1–2 hr and then returned toward control values. The elevations in plasma glucose seen after an intravenous dose of 50 mg/kg were considerably greater than after either of the two smaller doses. In fact, the response was so marked that both dogs seemed to exhibit an overshoot phenomenon in which plasma glucose concentrations were transiently depressed to approximately one-half the control values.

Changes in plasma glucose concentrations as a function of time after oral doses of 2.5 or 50 mg/kg are shown in Table III. At the smaller dose, average elevations of glucose were slight because one dog showed a good response, one dog showed a mild response, and two dogs showed no response. However, with the larger dose, both dogs showed hyperglycemic responses, beginning between 30 and 60 min in one case and between 1 and 1.5 hr in the other. This response was more delayed than that seen after intravenous dosing and correlated with the slow absorption of the compound. The extent of the response after oral dosing was also much less than that seen after comparable intravenous doses.

Plasma insulin concentrations were measured as a function of time in dogs that had received intravenous doses of 0.25 or 2.5 mg/kg of I (Table IV). Insulin concentrations in plasma generally followed a time course similar to that observed for glucose concentrations; they were steeply elevated within 5 min after dosing and returned to control values within 30–60 min. However, the elevations found for insulin concentrations, rising more than an order of magnitude for two dogs, were generally much greater than those found for glucose.

Excretion Studies—Excretion of radioactivity by rats after in-

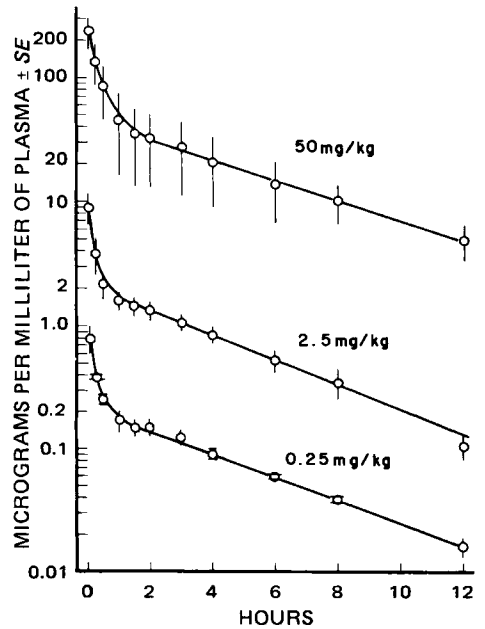


Figure 1—*Concentrations of radioactivity in the plasma of dogs after the intravenous administration of I. A male and a female dog were given the compound at each dose.*

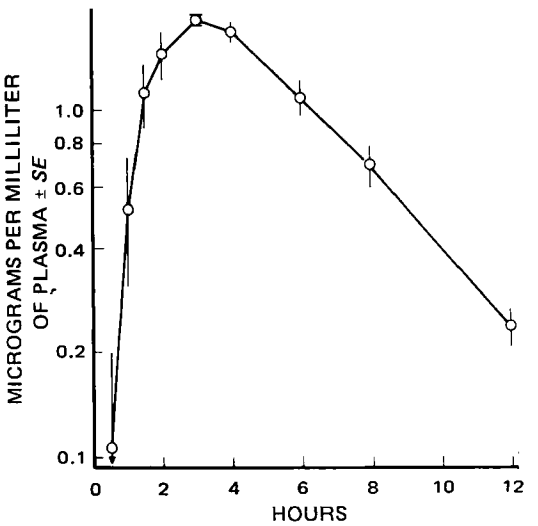


Figure 2—*Concentrations of radioactivity in the plasma of dogs after the oral administration of 2.5 mg/kg of I. Two male and two female dogs were given the compound in capsules.*

Table III—Concentrations of Glucose in the Plasma of Dogs as a Function of Time after the Oral Administration of I

Hours	2.5 mg/kg[a]	50 mg/kg[b] Dog Q3-10	50 mg/kg[b] Dog Q2-198
	Percent Change		
0	(0)	(0)	(0)
0.5	2 ± 6[c]	−1	15
1	5 ± 3	4	51
1.5	12 ± 14	40	80
2	21 ± 15	61	61
3	5 ± 10	4	27
4	4 ± 6	1	28
6	5 ± 6	22	30

[a] Four dogs, two of each sex, were used. Average glucose concentration in the plasma of these dogs at zero time was 110 ± 6 mg %. [b] Average glucose concentration in the plasma of these dogs at zero time was 80 ± 6 mg %. [c] Standard error.

travenous doses of 5 mg/kg of I bearing either the ^{35}S or ^{14}C label is shown in Table V. After administration of either radioactive compound, 74–87% of the dose was excreted in the urine and 8–14% in the feces. Dogs given intravenous doses of the ^{35}S-labeled compound at 0.25 and 2.5 mg/kg excreted radioactivity in a manner quantitatively similar to that seen for rats.

A single dog, prepared surgically for the collection of bile and given a 2.5-mg/kg dose of I intravenously, excreted 64% of the dose in the urine and 19% in the bile in 7 hr. One hour after it had been dosed, this dog had 8.8% of the radioactivity in the plasma bound to the plasma proteins. After an intravenous dose of 50 mg/kg, excretion of the ^{14}C-labeled compound was quantitatively similar to that of the ^{35}S-labeled compound when the latter had been given at 0.25 and 2.5 mg/kg.

After oral doses of 2.5 mg/kg, the excretion of radioactivity by dogs over a 5-day period was similar to that found after 50 mg/kg (Table V). From 76 to 82% of the dose was excreted in the urine and the remainder was excreted in the feces. Most of this radioactivity was excreted during the first 24 hr. These data are very similar to those found after comparable intravenous doses and indicate that at least four-fifths of an oral dose of I was absorbed by dogs.

Table VI shows the rate of excretion by rats of a 5-mg/kg iv dose of I-^{14}C. Of the 89% of the dose recovered in 4 days, 81% was excreted during the 1st day. Only 0.22% of the dose was excreted by these rats as $^{14}CO_2$, indicating that the methylthio side chain is not removed from the molecule to any appreciable extent.

Tissue Distribution—The distribution of radioactivity was studied in dogs 5 and 60 min after receiving 2.5 mg/kg iv of I (Table VII). One dog of each sex was studied at each time. Five minutes after dosing, when compared with the values found in the blood, radioactivity was highly localized in the excretory organs and widely distributed to most of the other tissues including the skin, pancreas, and other endocrine structures. Radioactivity was present in much smaller concentrations in the portions of the brain

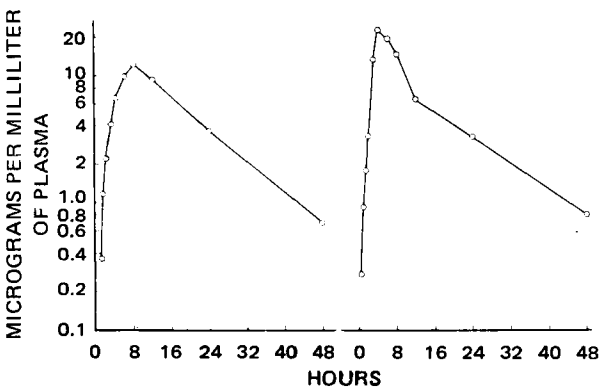


Figure 3—*Concentrations of radioactivity in the plasma of dogs after the oral administration of 50 mg/kg of I. A male (left) and a female (right) dog were given the compound in capsules.*

Table IV—Concentrations (Microunits per Milliliter) of Insulin in the Plasma of Dogs as a Function of Time after the Intravenous Administration of I

Minutes	0.25 mg/kg Dog Q2-168	0.25 mg/kg Dog Q1-349	2.5 mg/kg Dog Q2-56	2.5 mg/kg Dog Q2-80
0	Lost	17	15	22
5	58	145	135	214
15	96	90	278	235
30	17	18	30	95
60	27	14	13	18

examined and in the various portions of the eye. Sixty minutes after dosing, the distribution of radioactivity in the various organs was much more uniform, but the amounts present in the brain were still only about one-tenth those present in the blood.

Biotransformation *In Vivo*—Urine that had been collected from rats and dogs during the first 24 hr after dosing with either 5 or 2.5 mg/kg, respectively, was chromatographed on thin-layer plates coated with cellulose and developed in Solvent System 1. The chromatographic reference compounds used were the parent compound, I, 8-(methylthio)adenosine-5′-monophosphate, 8-(methylthio)adenine, and 8-(methylthio)adenosine.

Four radioactive components were present in the urine of both rats and dogs (not shown), although the amounts varied between the species, particularly for the slowest moving component. Chromatography in System 1, as well as in the other two systems, indicated that the fastest moving component corresponded to 8-(methylthio)adenosine. No 8-(methylthio)adenosine-5′-monophosphate appeared to be present in these samples. Solvent System 2 clearly separates 8-(methylthio)adenosine from its adenine analog, and none of the latter was found in these samples of urine.

Figure 4 illustrates clearly the time-dependent transformation of I. These chromatograms are of urine from a dog in which the bile duct had been externalized; the urine was collected at hourly intervals. The sample collected during the 1st hr showed the presence primarily of unchanged I and of some 8-(methylthio)-

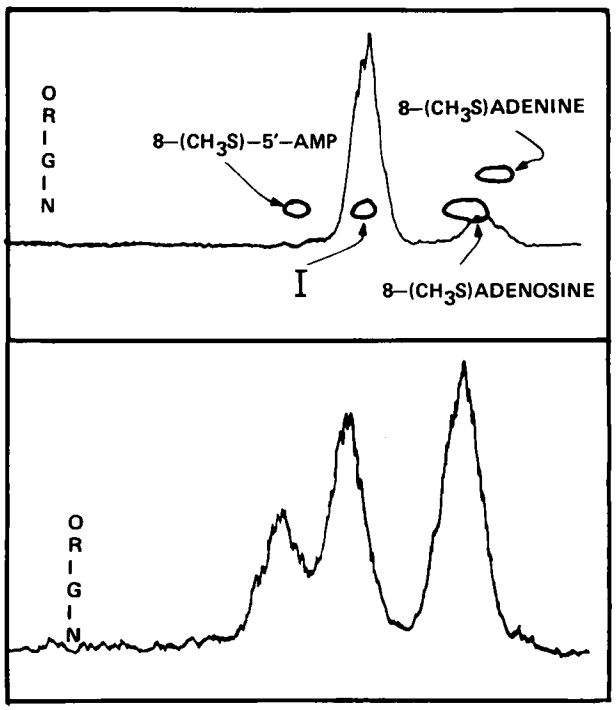


Figure 4—*Chromatograms of urine collected during the 1st (upper) and 3rd (lower) hr after the intravenous administration to a dog of 2.5 mg/kg of I. Urine was collected continuously from this dog for 7 hr. Solvent System 3 was employed. 8-(CH$_3$S)-5′-AMP is 8-(methylthio)adenosine-5′-monophosphate.*

Table V—Excretion of Radioactivity by Rats and Dogs after the Oral or Intravenous Administration of I

Species	Radio-activity	Route[a, b]	Dose, mg/kg	Average Percent of Dose ± SE: Urine	Feces or (Bile)	Total
4 Rats	^{35}S	Intravenous	5	86.66 ± 5.89	8.31 ± 2.00	94.97 ± 4.27[c]
4 Rats	^{14}C	Intravenous	5	74.15 ± 8.82	14.29 ± 5.75	88.44 ± 7.40[c]
2 Dogs	^{35}S	Intravenous	0.25	82.39 ± 1.10	22.77 ± 1.95	105.16 ± 3.05[d]
2 Dogs	^{35}S	Intravenous	2.5	80.00 ± 1.31	25.62 ± 3.04	105.62 ± 1.73[d]
1 Dog	^{14}C	Intravenous	2.5	63.87	(19.30)	83.17[e]
2 Dogs	^{14}C	Intravenous	50	77.53 ± 8.20	22.24 ± 6.10	99.77 ± 2.10[d]
4 Dogs	^{14}C	Oral	2.5	76.35 ± 11.84	16.25 ± 2.94	92.60 ± 10.98[d]
2 Dogs	^{14}C	Oral	50	82.30 ± 2.27	17.03 ± 0.46	99.33 ± 1.81[d]

[a] Intravenous doses were dissolved in 0.1 *M* Na_2CO_3. [b] Oral doses were administered in capsules. Tepid water (100 ml) was given to each dog immediately after the capsule. [c] Collections were made for 4 days from two rats of each sex. [d] Collections were made for 5 days from one or two dogs of each sex. [e] Seven-hour experiment.

Table VI—Excretion of Radioactivity by Rats after the Intravenous Administration of I

Days	Percent of Dose[a] ± SE: Urine	Feces	$^{14}CO_2$	Total[b]
1	70.16 ± 9.30	10.93 ± 4.81	0.22 ± 0.02	81.31 ± 7.41
2	3.12 ± 0.84	2.22 ± 0.95	0.00	5.34 ± 1.77
3	0.61 ± 0.24	0.84 ± 0.32	—	1.44 ± 0.56
4	0.20 ± 0.09	0.30 ± 0.22	—	0.50 ± 0.29
Cage rinse	0.07 ± 0.03	—	—	0.07 ± 0.03
	74.16 ± 8.82	14.29 ± 5.75	0.22 ± 0.02	88.66 ± 7.41

[a] Using 5 mg/kg. [b] After 4 days, the carcasses contained an average of 0.083 ± 0.044% (±*SE*) of the dose.

adenosine, based on chromatography in the three solvent systems. A sample of urine similarly analyzed during the 3rd hr after dosing showed the formation of an additional metabolite that traveled more slowly than I but was not 8-(methylthio)adenosine-5′-monophosphate and the increasing prominence of 8-(methylthio)adenosine. Studies with bile from this same animal (not shown) showed a similar time-dependent metabolic transformation.

Figure 5 shows chromatograms of urine collected from dogs during the first 24 hr after oral or intravenous doses of 50 mg/kg. Based on chromatography in the three solvent systems, these samples contained some I[13] and a good deal of 8-(methylthio)adenosine but no 8-(methylthio)adenosine-5′-monophosphate or 8-(methylthio)adenine. 8-(Methylthio)adenosine was also the principal metabolite in the feces of dogs that had been dosed orally with 2.5 mg/kg of I, as well as in the plasma of dogs that had been dosed intravenously with 2.5 or 50 mg/kg.

Table VIII shows some of these aspects quantitatively for the individual dogs. Chromatograms of urine of a dog shown in Fig. 5 indicate that only about 27% of the parent compound was present 5 hr after dosing, whereas 43% was then accounted for as 8-(methylthio)adenosine. In the urine of other dogs that had been given 50 mg/kg, from 33 to 59% was present as I in a 24-hr collection, and a large portion of the balance was accounted for as 8-(methylthio)-adenosine. After oral doses of 2.5 mg/kg, about 30% of the radioactivity in urine was I, with a good deal of the remainder being represented by the adenosine metabolite. After 50 mg/kg po, somewhat less of the radioactivity on the average was the parent compound, with a correspondingly greater amount as the adenosine metabolite.

Biotransformation *In Vitro*—The metabolism of I *in vitro* was studied with subcellular fractions of dog liver for two reasons: *(a)* to determine whether the cleavage of the 3′-bond occurred, since no 8-(methylthio)adenosine-5′-monophosphate was detectable *in vivo;* and *(b)* to determine the comparative rates of metabolism of I and cyclic 3′,5′-adenosine monophosphate *in vitro* under identical conditions. Incubation of I and appropriate cofactors with the whole liver homogenate, postmitochondrial supernate, and the soluble or microsomal fractions indicated that the soluble fraction contained all of the enzymatic activities involved in converting I to its metabolites.

[13] Presence of I was confirmed by mass spectrometry; unpublished data.

Figure 6 shows the results obtained by incubating I with the soluble fraction of dog liver. When the samples were developed in the three solvent systems, three components were consistently obtained. The slowest one corresponded in each case to 8-(methylthio)adenosine-5′-monophosphate, the component consistently found in the middle represented the unchanged parent compound, and the fastest moving component consistently corresponded to 8-(methylthio)adenosine.

Table VII—Concentrations of Radioactivity in Selected Tissues of Dogs after the Intravenous Administration (2.5 mg/kg) of I[a]

Tissue	After 5 min, μg/g ± SE	After 60 min, μg/g ± SE
Adrenals	3.00 ± 0.02	1.43 ± 0.08
Brain: cerebellum	0.11 ± 0.02	0.14 ± 0.00
Brain: cortex	0.16 ± 0.02	0.14 ± 0.01
Brain: dorsal and hypothalamus	0.099 ± 0.032	0.10 ± 0.02
Brain: stem	0.094 ± 0.007	0.11 ± 0.00
Brain: subcortex	0.052 ± 0.004	0.096 ± 0.025
Brain: sagittal section	0.086 ± 0.008	0.11 ± 0.00
Cornea	0.22 ± 0.01	0.57 ± 0.07
Combined retina, choroid, and sclera	0.69 ± 0.07	1.35 ± 0.09
Aqueous humor	0.14 ± 0.03	0.34 ± 0.13
Vitreous humor	0.30 ± 0.02	0.69 ± 0.06
Heart	1.97 ± 0.15	1.44 ± 0.39
Kidneys	56.18 ± 25.84	4.45 ± 0.80
Lens	0.034 ± 0.013	0.22 ± 0.01
Liver	30.23 ± 0.43	6.75 ± 0.90
Lungs	3.88 ± 0.15	1.36 ± 0.05
Omental fat	0.38 ± 0.08	0.69 ± 0.02
Pancreas	2.50 ± 0.13	2.09 ± 0.72
Pituitary	3.10 ± 0.41	1.55 ± 0.21
Portal vein	3.05 ± 2.05	1.35 ± 0.35
Skeletal muscle	1.64 ± 0.02	1.34 ± 0.01
Skin	1.90 ± 0.33	1.48 ± 0.31
Testes	1.41[b]	1.36[b]
Ovaries	5.28[b]	1.75[b]
Thyroid gland	2.94 ± 0.11	1.27 ± 0.13
Blood (μg/ml)	3.19 ± 0.27	1.26 ± 0.07

[a] One dog of each sex was sacrificed at each time. [b] Single value.

Table VIII—Amounts of I and 8-(Methylthio)adenosine in the Urine of Dogs after the Oral and Intravenous Administration of I

Dog	Route	Dose, mg/kg	Collection Interval, hr	I, %	8-(Methylthio)-adenosine, %
Q2-56[a]	Intravenous	2.5	0–1	81.2	18.8
Q2-56[a]	Intravenous	2.5	2–3	36.0	43.6
Q2-56[a]	Intravenous	2.5	4–5	26.8	43.4
Q2-240	Intravenous	50	0–24	58.5	29.6
Q3-53	Intravenous	50	0–24	32.8	40.9
Q3-10	Oral	2.5	0–24	33.1	29.2
Q2-198	Oral	2.5	0–24	30.1	41.8
Q3-10	Oral	50	0–24	27.5	38.4
Q2-198	Oral	50	0–24	19.9	50.1

[a] Urine was collected continuously.

Table IX shows the conversion of I to the adenosine-5′-monophosphate and adenosine metabolites as a function of time. 8-(Methylthio)adenosine-5′-monophosphate appeared to be formed first from I, since little of the adenosine metabolite was present after 20 min. After 3 hr, there were approximately equal amounts of I and 8-(methylthio)adenosine-5′-monophosphate and about 14% of the adenosine metabolite.

Table X shows the results of having incubated I-^{14}C and cyclic 3′,5′-adenosine monophosphate with the soluble fraction of dog liver as a function of time. Cyclic 3′,5′-adenosine monophosphate was converted very rapidly to adenosine-5′-monophosphate. After 30 min, only 20% of the original amount of cyclic 3′,5′-adenosine monophosphate remained, whereas 80% of the original amount of I was still present. Two hours after incubation with the soluble fraction of dog liver, 67% of the I still remained, but *none* of the cyclic 3′,5′-adenosine monophosphate was found at this time.

DISCUSSION

The metabolism of cyclic 3′,5′-adenosine monophosphate has been studied in a number of laboratories. Verbert and Cacan (10) showed the formation of cyclic 3′,5′-adenosine monophosphate, adenosine-5′-monophosphate, adenosine, and a trace of adenine after adenosine-5′-triphosphate-^{3}H had been incubated with rat liver plasma membranes containing adenyl cyclase. Pull and McIlwain (11) studied the metabolism of adenine-^{14}C by superfused and electrically stimulated cerebral tissues of guinea pigs. The compounds released from brain tissue to superfusion fluids included adenine, adenosine, inosine, and hypoxanthine and small amounts of cyclic 3′,5′-adenosine monophosphate, adenosine-5′-monophosphate, adenosine-5′-diphosphate, and adenosine-5′-triphosphate. The output of all these compounds, except adenine, increased upon electrical stimulation. When adenosine-5′-triphosphate-^{14}C was added at low concentrations to fluids superfusing the cerebral tissue, adenosine-^{14}C was the main product.

Rutten *et al.* (12), employing homogenates of rat pancreas to study the assay of cyclic 3′,5′-adenosine monophosphate phospho-

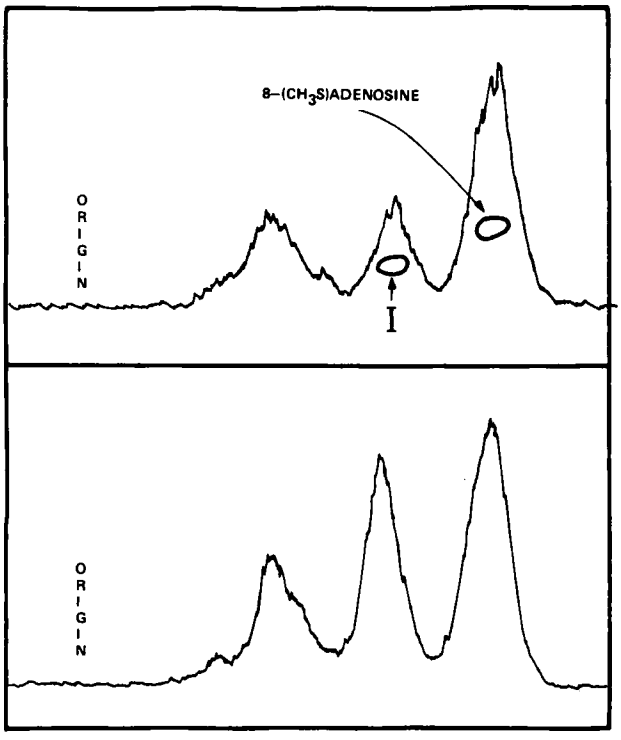


Figure 5—*Chromatograms of urine collected during the first 24 hr after the oral (upper) and intravenous (lower) administration to dogs of 50 mg/kg of I. Solvent System 2 was employed.*

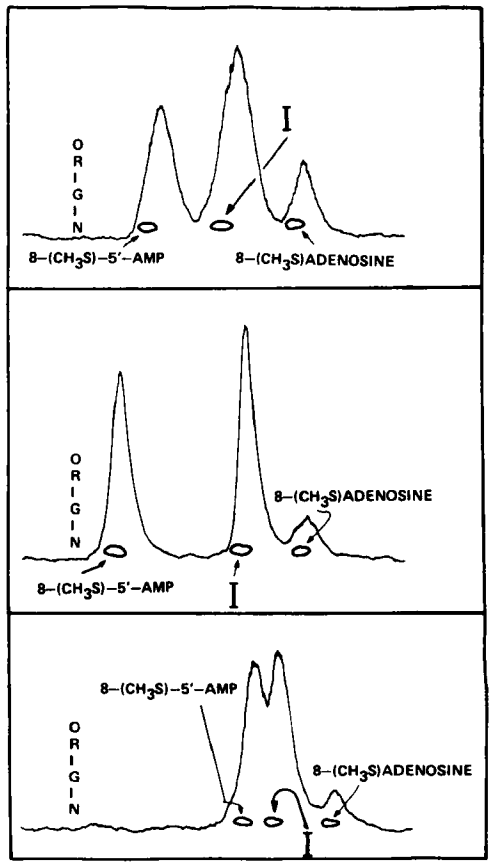


Figure 6—*Chromatograms of the incubation of I with the soluble fraction of dog liver. The samples were incubated at 37° for 150 min and developed in Solvent Systems 3 (upper), 1 (middle), and 2 (lower). 8-(CH_3S)-5′-AMP is 8-(methylthio)adenosine-5′-monophosphate.*

Table IX—Metabolism of I *In Vitro* by the Soluble Fraction of Dog Liver as a Function of Time

Incubation Time, min	I[a], %	8-(Methylthio)adenosine-5′-monophosphate[a], %	8-(Methylthio)adenosine[a], %
20	76	22	2
45	64	31	5
90	55	37	8
120	54	36	10
150	50	34	15
180	45	41	14

[a] The amount of each component was determined based on chromatography in Solvent System 3.

Table X—Metabolism of I and Cyclic 3′,5′-Adenosine Monophosphate by the Soluble Fraction of Dog Liver

Incubation Time, min	I[a], %	8-(Methylthio)adenosine-5′-monophosphate[a], %	8-(Methylthio)adenosine[a], %
30	80	17	3
120	67	16	17
Incubation Time, min	Cyclic 3′,5′-Adenosine Monophosphate[a], %	Adenosine-5′-monophosphate[a], %	Adenosine[a], %
5	33	67	0
30	20	79	1
120	0	91	9

[a] The amount of each component was determined based on chromatography in Solvent System 3.

diesterase, concluded that the hydrolysis of cyclic 3′,5′-adenosine monophosphate to adenosine *via* adenosine-5′-monophosphate was the predominant pathway but that the conversion of cyclic 3′,5′-adenosine monophosphate to cyclic 3′,5′-inosine monophosphate also occurred to some extent. In another study, Lee and Dubos (13) examined the metabolism of cyclic 3′,5′-adenosine monophosphate in mice. Nine hours after the injection of cyclic 3′,5′-adenosine-8-^{14}C monophosphate into mice, the plasma contained some adenosine-5′-monophosphate, large amounts of adenosine, small amounts of adenine, and a small amount of an unidentified metabolite.

Compound I appears to be metabolized in a manner similar to that of cyclic 3′,5′-adenosine monophosphate. After incubation with the soluble fraction of dog liver *in vitro*, both cyclic 3′,5′-adenosine monophosphate and I are metabolized by the action of a phosphodiesterase to adenosine-5′-monophosphate or its 8-(methylthio) derivative and, subsequently, by the action of a nucleotidase to adenosine or its 8-(methylthio) derivative. *In vivo*, I appears to be excreted primarily as 8-(methylthio)adenosine, but other metabolites, which have not yet been identified, are also excreted.

The hyperglycemic response found after oral or intravenous doses of I would seem to be mediated by I, rather than by some of its potential metabolites, since neither 8-(methylthio)adenosine nor 8-(methylthio)adenine produced any increases in serum glucose after intravenous administration to dogs. Comparable studies with 8-(methylthio)adenosine-5′-monophosphate resulted in only marginal increases in serum glucose[14]. Compound I appears to be absorbed at least partly as such after oral dosing, since increases in glucose concentrations in the plasma were found in each of two dogs after 50 mg/kg and in one of four dogs after 2.5 mg/kg.

In these studies, 8-(methylthio)adenosine was found to be the major metabolite of I. Adenosine, the corresponding metabolite of cyclic 3′,5′-adenosine monophosphate, has been reported to have biological activity *in vitro* in a variety of tests. McKenzie and Bar (14) studied the mechanism of the inhibition of adenylate cyclase by adenosine. They found that adenylate cyclase activities from various sources were inhibited by adenosine but not by inosine. The inhibition of cyclic 3′,5′-adenosine monophosphate-dependent protein kinases by adenosine also has been reported (15, 16). Melnykovych and Bishop (17) studied glycogen concentrations in cultured HeLa cells. In this system, the presence of adenosine decreased the degradation of glycogen, thereby resulting in an increase of the glycogen concentrations.

In an unrelated study, Ortiz *et al.* (18) found that adenosine produced morphological alterations of iris epithelial cells *in vitro*. Adenosine has also been proposed as a regulator of lipolysis in isolated fat cells. Thus, Ebert and Schwabe (19) reported that lipolysis was increased by adenosine in a dose-dependent manner, and Fain (20) reported that the accumulation of cyclic 3′,5′-adenosine monophosphate in fat cells was inhibited by adenosine.

Adenine is toxic when administered intravenously to dogs for 3 weeks because of the formation and deposition of 2,8-dihydroxyadenine in the kidneys (21). Earlier studies (22, 23) with rats and mice that had been given adenine orally and intraperitoneally demonstrated its oxidation *in vivo* to 2,8-dihydroxyadenine and the deposition of the latter in the renal tubules. In the present studies on the metabolism of I in rats and dogs, the formation of 8-(methylthio)adenine did not appear to be a significant pathway, if it occurred at all. Moreover, the 8-position of the purine ring of I could not have been hydroxylated, since it already had a methylthio substituent.

Recently, Merits and Anderson (24) reported on the metabolic fate of the noncyclic nucleoside derivative, adenosine-5′-carboxylic acid ethyl ester-8-^{14}C, in rats, mice, and dogs. Their studies indicated that at least 94% of an oral dose of the compound was absorbed by dogs and that the compound did not traverse the blood–brain barrier to any great degree. Adenosine-5′-carboxylic acid ethyl ester was metabolized by rats, mice, and dogs, predominantly to adenosine-5′-carboxylic acid and, to a much smaller extent, inosine-5′-carboxylic acid. However, the dog eliminated only 71–82% of an oral dose of the radioactive compound within 3 days; most of the remainder was excreted slowly in the urine over the next 21 days. More detailed studies by Merits (25) of this phenomenon of prolonged elimination revealed that radioactivity from a dose of adenosine-5′-carboxylic acid ethyl ester was incorporated into the nucleic acids of mice and dogs. Most of the radioactivity seemed to be associated with ribosomal ribonucleic acid rather than with soluble ribonucleic acid or deoxyribonucleic acid. Merits (25) attributed the greater incorporation of radioactivity into the nucleic acids of dogs than those of mice to the more efficient mechanism of adenine "salvage" in dogs. The present studies of the metabolism of I in dogs indicate that there was no significant incorporation, if any, of radioactivity into nucleic acids, since the type of delayed excretion reported by Merits and Anderson (24) for adenosine-5′-carboxylic acid ethyl ester was not observed.

[14] Unpublished data, Department of Toxicology, Squibb Institute.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 7, 1974, from the *Department of Drug Metabolism, Squibb Institute for Medical Research, New Brunswick, NJ 08903*

Accepted for publication February 19, 1975.

The authors thank Mr. Peter Egli for synthesizing the ^{35}S- and ^{14}C-labeled I; Mr. Carmen Mondi, Mr. Michael Palfey, and Mr. John J. Ross, Jr., for technical assistance; Dr. Chandra Parekh for the determination of glucose concentrations in plasma; Dr. Don Harris for the determination of insulin concentrations in plasma; and Mr. Sal Meloni for preparing the illustrations. Supplies of nonradioactive I were provided by the International Chemical and Nuclear Corp.

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Microbial Kinetics of Drug Action against Gram-Positive and Gram-Negative Organisms II: Effect of Clindamycin on *Staphylococcus aureus* and *Escherichia coli*

SAMUEL M. HEMAN-ACKAH

Abstract □ Clindamycin-affected *Staphylococcus aureus* cultures show biphasic steady-state generation curves. An initial (phase I) generation of the clindamycin-affected *Staph. aureus* is followed by an ultimate (phase II) generation at the same dose level. The phase I apparent generation rate constant is greater than the phase II apparent generation rate constant and suggests the development of resistant *Staph. aureus* mutants to clindamycin action after a finite period of drug–bacteria contact at any subcompletely inhibitory concentration level. It is rationalized that the increased resistance to drug action in mutant strains is due to a comparatively reduced ribosomal binding affinity for clindamycin. In contrast, clindamycin-affected *Escherichia coli* cultures show monophasic steady-state generation curves at all concentration levels; *E. coli* cultures do not develop resistance to clindamycin action. The dependence of the apparent generation rate constant on drug concentration yields a sigmoidal curve, which is coincident by a potency factor for the phase I and phase II generations of clindamycin-affected *Staph. aureus* and suggests a common mechanism of action for both generation phases. That of clindamycin-affected *E. coli* yields an asymptote curve, which indicates a different mechanism of action. Clindamycin possesses both a bacteriostatic and a bactericidal action on initial and mutant resistant strains of *Staph. aureus*, whereas its action on *E. coli* is only bacteriostatic. Consequently, clindamycin has a minimum inhibitory concentration (MIC) against *E. coli* that is about 1000 times the MIC value against *Staph. aureus* at 37.5°. The effect of pH changes in broth media on generation inhibition of both *Staph. aureus* and *E. coli* by clindamycin action indicates that the unprotonated fraction of drug concentration contributes to the activity, possibly because of its ready penetration through cell membranes.

Keyphrases □ Microbial kinetics—drug action on Gram-positive and Gram-negative organisms, comparison of clindamycin action on *Staphylococcus aureus* and *Escherichia coli* □ Clindamycin—comparison of effects on *Staphylococcus aureus* and *Escherichia coli*, microbial kinetics □ *Staphylococcus aureus*—effects of clindamycin, compared to *Escherichia coli* □ *Escherichia coli*—effects of clindamycin, compared to *Staphylococcus aureus* □ Antimicrobial activity—comparison of clindamycin on *Staphylococcus aureus* and *Escherichia coli*

Clindamycin is one of the 7*(S)*-chloro analogs of lincomycin (1, 2) that is claimed to be more than four times as active as the parent antibiotic against a variety of Gram-positive and Gram-negative organisms (3). Like lincomycin, it interferes with ribosomal functioning in the synthesis of cell proteins (4, 5) to inhibit microbial growth and generation.

Comparative studies on the action of lincomycin and its 7*(S)*-chloro analogs against *Escherichia coli* by microbial kinetics (6) confirmed the enhanced potency of clindamycin action relative to that of lincomycin. These studies also revealed that clindamycin has only one of two mechanisms of lincomycin action against *E. coli*. Recently, it was shown (7) that lincomycin also possesses one mechanism of action against *Staphylococcus aureus*. However, *Staph. aureus* cultures, unlike *E. coli*. cultures, develop resistence to lincomycin after a finite period of organism contact with subcompletely inhibitory concentrations of the drug.

It was of interest to study the kinetics and dependencies related to modes of clindamycin action