Study of the Metabolic Conversion of Imipramine and Desipramine to *N*-Nitrosodesipramine by Bacteria Using a Nitrogen-Selective GC Analysis

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Received September 9, 1981, from the **Department of Medicinal Chemistry and the* ‡*Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677.* Accepted for publication November 12, 1981.

Abstract □ A GC method using dual nitrogen selective and flame ionization detectors was developed for the determination of *N*-nitrosodesipramine using *N*-butyryldesipramine as the internal standard. The precision of the method was found to be ±5.0% and the accuracy was ±4.9%. The method could be used to detect 10 ng/ml of *N*-nitrosodesipramine in bacterial cultures. When desipramine and sodium nitrite were incubated with aerobic or anaerobic bacteria, the nitrosamine level was found to be 10–300 times higher than the controls. When imipramine and potassium nitrate were incubated with a mixed anaerobic culture, the level of *N*-nitrosodesipramine was found to be 4.5 times higher than the control.

Keyphrases □ Imipramine—metabolic conversion to *N*-nitrosodesipramine by bacteria, nitrogen-selective GC, desipramine □ *N*-nitrosodesipramine—metabolic conversion of imipramine and desipramine by bacteria, nitrogen-selective GC □ Desipramine—metabolic conversion to *N*-nitrosodesipramine by bacteria, nitrogen-selective GC, imipramine □ Metabolic conversion—imipramine and desipramine to *N*-nitrosodesipramine by bacteria, nitrogen-selective GC

Previous studies have shown that secondary amines can be converted to carcinogenic *N*-nitroso amines when incubated with bacteria found in the intestine or bladder (1–4). It has also been demonstrated that bacteria can convert trimethylamine to dimethylamine and produce *N*-nitrosodimethylamine (3, 5). Though the conversion of these simple amines to *N*-nitroso compounds may have an impact on public health, the level of exposure to these compounds would be fairly low. The exposure to maintenance drugs such as desipramine and imipramine would be considerably higher, however, and these drugs might be converted to the *N*-nitroso derivatives in the intestine or bladder by bacteria. Recent studies in these laboratories with fungi have shown that these microorganisms *N*-demethylate imipramine in yields of 2–11% depending on the organism studied (6). Work presently in progress with aerobic and anaerobic bacteria has shown that many of the organisms give a 1–3% conversion from imipramine to desipramine. The objective of the present study was to develop an analytical method for *N*-nitrosodesipramine suitable for complex matrixes and to determine if either desipramine or imipramine would be converted to the nitrosamine in the presence of the bacteria.

EXPERIMENTAL

Synthesis of *N*-Nitrosodesipramine—Though there are references to *N*-nitrosodesipramine in the literature (7, 8), no synthetic information has been given nor have any of the spectral properties of the alleged compound been reported.

In the present study, 200 mg of the desipramine hydrochloride (0.66 mmole) was dissolved in 10 ml of water, made basic with 4 drops of concentrated ammonium hydroxide, then extracted with 15 ml of methylene chloride. The extract was dried with anhydrous sodium sulfate, then the extract was added to a solution containing 115 mg of nitrosonium tetrafluoroborate (0.66 mmole $NOBF_4$) in 5 ml of methylene chloride at room temperature. Over a 15-min period, 100 mg of pyridine in 2 ml of methylene chloride was added to the well-stirred suspension. After 1.5 hr the mixture was washed with 10 ml of 2 *N* HCl, 15 ml of 10% Na_2CO_3, then dried with anhydrous sodium sulfate. The solvent was evaporated to give 165 mg (85% yield) of the nitrosamine as a thick oil (IR: 1445 cm^{-1}, N=O). The mass spectrum of the nitrosamine obtained using the solid probe showed a fairly intense molecular ion at 295 (12%) and a peak at 265 characteristic of the loss of the nitrosyl group. The nitrosamine was also observed to produce peaks at 193 (100%), 208 (96%), 220 (50%), and 234 (32%) which were similar to those observed for the fragmentation of desipramine. The mass spectrum obtained by the GC mode (Fig. 1) was observed to be essentially the same.

Synthesis of *N*-Butyryldesipramine—Using 15 ml of toluene as the solvent, 200 mg of desipramine hydrochloride (0.55 mmole), 200 mg of butyric anhydride (1.3 mmoles), and 200 mg of pyridine were heated on a steam bath for 1 hr. The mixture was washed with 20 ml of aqueous 10% Na_2CO_3, the toluene layer was dried, then the solvent was evaporated under vacuum to give a 270-mg residue. The IR spectrum of this material indicated that the desired product was contaminated with a small amount of butyric anhydride. The residue was then sonicated with 10% Na_2CO_3 for 30 min, then extracted with methylene chloride, dried with anhydrous sodium sulfate, and evaporated to give 230 mg (95%) of the final product as an oil; IR (thin film): 2950, 1637, 1483, 1227, and 750 cm^{-1}; mass spectrum (GC mode): 336 (M^+, 7%), 234 (9%), 222 (13%), 208 (100%), 193 (45%), and 142 (89%).

Determination of *N*-Nitrosodesipramine—Sulfamic acid (30 mg) was added to a 10.0-ml sample of the bacterial culture to remove residual nitrite. Then, 20.0 μg of *N*-nitrosodesipramine (internal standard) in ethyl acetate was added, and the sample was extracted with 5 ml of isooctane. The isooctane layer was transferred to a conical evaporation tube[1],

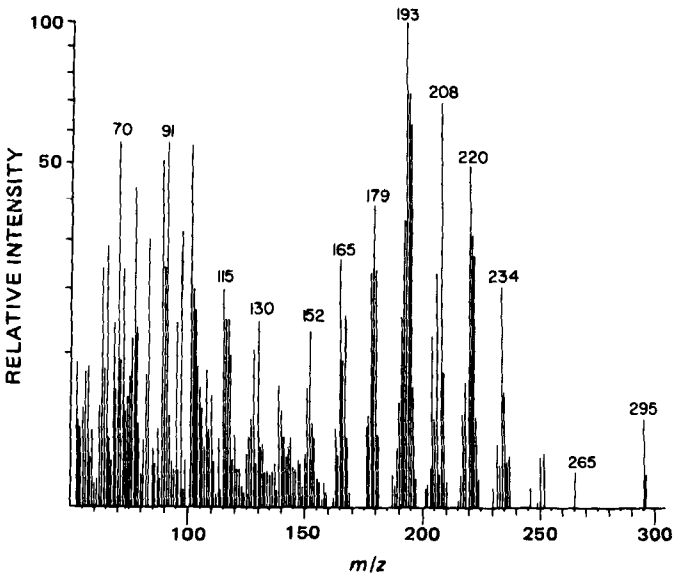

Figure 1—*Electron impact mass spectrum of N-nitrosodesipramine. The spectrum was obtained using the GC mode of operation with background correction with a sample of the nitrosamine obtained by synthesis.*

[1] Brinkmann 48 Place Concentrator, Brinkmann Instruments, Des Plaines, Ill.

0022-3549/82/0800-0901$01.00/0

Table I—Recovery of *N*-Nitrosodesipramine and *N*-Butyryldesipramine

Sample Medium	Extraction Recovery	
	N-Nitroso-desipramine, %[a]	Internal Standard, %[b]
Water	103.6	105.2
	96.6	101.4
	101.4	102.3
Mean ± *SD*	100.5 ± 3.6	103.0 ± 2.0
Sterilized Bacterial Culture	81.4	73.5
	96.3	90.9
	86.7	83.3
	93.0	94.3
Mean ± *SD*	89.4 ± 6.6	85.5 ± 9.2

[a] The sample was spiked with 1.0 μg/ml of *N*-nitrosodesipramine and the percent recovery was determined by comparison to the peak height obtained for 10 μg/50 μl of the nitrosamine in ethyl acetate. [b] The sample was spiked with 2.0 μg/ml of the internal standard and the percent recovery was determined by comparison to the peak height obtained for 20 μg/50 μl of the internal standard in ethyl acetate.

then evaporated at 80° under a stream of nitrogen. When the samples were almost dry, the walls of the evaporation tubes were washed down with 500 μl of methanol, then the samples were evaporated to dryness. The residue was taken up in 50 μl of ethyl acetate and 1.0 μl was used for the GC analysis.

The GC analysis[2] was conducted using dual nitrogen selective and flame ionization detectors with a dual pen recorder. The column (2 mm × 183 cm) was operated at 260° with helium as the carrier gas (30 ml/min) and with a packing of 3.0% methyl–phenyl silicone polymer[3] on a 110–120 mesh silanized support[4]. The carrier gas coming from the column was split equally between the flame ionization detector (H_2 = 19 ml/min) and rubidium bead nitrogen selective detector (H_2 = 2.3 ml/min), and each detector received 300 ml/min of air. Using these conditions, the retention time for *N*-nitrosodesipramine was 9.2 min and 14.9 min for *N*-butyryldesipramine. The nitrogen detector response index of the nitrosamine (response index = 0.155) was measured relative to caffeine as described previously (9).

In the analysis of the bacterial cultures, the presence of the nitrosamine was ascertained through the use of the retention times of the components

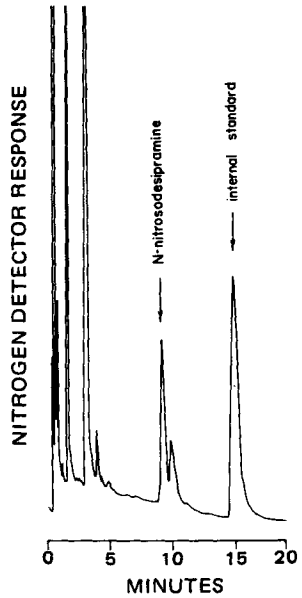

Figure 2—*Nitrogen-selective GC of bacterial cultures. The chromatogram was obtained from the extract of a culture of* Enterobacter aerogenes *containing desipramine and sodium nitrite. Though a dual flame ionization detector–nitrogen detector system was used for all samples, the flame ionization detector response has been omitted from the figure for simplicity.*

[2] Model 900, Perkin-Elmer Inc., Norwalk, Conn.
[3] OV-17, Analabs Inc., North Haven, Conn.
[4] Anachrom ABS, Analabs Inc., North Haven, Conn.

Table II—Accuracy and Precision of the Analytical Method

Bacterial Culture	*N*-Nitrosodesipramine, μg/ml[a]
1	1.108
2	1.060
3	1.042
4	0.987
Mean ± *SD*	1.049 ± 0.050

[a] A full-term Stage II culture not containing any drug or nitrite was sterilized, then spiked with 1.0 μg/ml of the nitrosamine. A 10-ml sample was then taken for analysis.

and the response index (9) of each component. Electron impact mass spectra[5] of the GC peaks were used as a secondary means of peak identification for some of the samples. Quantitations were based on the nitrogen-selective detector peak heights of the nitrosamine relative to the internal standard (*N*-butyryldesipramine). The method was calibrated using a simple solution containing a known quantity of the nitrosamine and the internal standard.

Incubation of Bacterial Cultures—Aerobic bacteria were grown according to a two-stage fermentation procedure using a defined medium (10) supplemented with either 25 m*M* $NaNO_2$ or 25 m*M* KNO_3. The Stage II cultures were incubated (37°, 250 rpm) with either 100 μg/ml of desipramine hydrochloride or 100 μg/ml of imipramine hydrochloride for 48 hr.

The anaerobic bacteria used in the study were isolated as pure cultures from the intestinal contents of a naive male Wistar rat. Mixed cultures of anaerobic bacteria from the large intestine and small intestine were also used.

Stage I cultures of the anaerobes were grown (24 hr, 37°) in 10 ml of the defined medium supplemented with either 25 m*M* $NaNO_2$ or 25 m*M* KNO_3 using an anaerobic chamber[6] for the culture tubes. Then 0.5 ml of the Stage I culture was transferred to the Stage II tubes containing 10 ml of the defined medium and 25 m*M* $NaNO_2$ or KNO_3 and 100 μg/ml of desipramine hydrochloride or imipramine hydrochloride. The Stage II cultures were incubated for 48 hr (37°) in fresh anaerobic chambers.

Control studies were conducted in which the defined sterile medium containing 25 m*M* $NaNO_2$ and 100 μg/ml of desipramine hydrochloride was adjusted to pH 5.0, 6.0, 7.0, and 8.0, then incubated with the same method for the Stage II aerobic cultures or the Stage II anaerobic cultures.

RESULTS AND DISCUSSION

Initial attempts to prepare *N*-nitrosodesipramine using desipramine, aqueous sodium nitrite, and hydrochloric acid (pH ≅ 3 maintained) were not successful. Even after 24 hr, the reaction mixture did not show significant amounts of the nitrosamine, but a large amount of another product that gave a GC–mass spectrum [*m/z* 240 (M^+, 100%), 193 (74%), 179 (15%), 167 (25%), and 83 (79%)] that would be consistent with a *C*-nitro derivative of iminodibenzyl. Due to a lack of pure reference standards of the four possible isomers of nitroiminodibenzyl, no attempts were made to establish the identity of the product.

Recently, it has been shown that nitrosonium tetrafluoroborate with pyridine can be used to produce nitrosamines of much higher purity (11). In particular, it was anticipated that the reagent would avoid the acidic conditions that are associated with *C*-nitrosation of aniline-like compounds. Indeed, it was found that this reagent gave an 85% yield of a high-purity product in 1.5 hr at room temperature. The mass spectrum of the product (Fig. 1) showed a molecular ion (295), a loss of the *N*-nitrosyl fragment (265), and the loss of the iminodibenzyl fragment (194) which would be characteristic of the *N*-nitroso compound as compared to the *C*-nitroso compound. The IR spectrum of the product was also characteristic of a nitrosamine.

In selecting an internal standard for the analysis of *N*-nitrosodesipramine, it was desirable to use a compound that was extremely lipophilic, like the nitrosamine and that had the same acid–base properties as the nitrosamine. The acetyl, propionyl, and butyryl amide derivatives of desipramine were investigated as potential internal standards and they were found to have retention times of 12.0, 13.0, and 14.9 min on the same GC system that gave a retention time of 9.3 min for the nitrosamine. Preliminary studies with bacterial cultures, to which no internal standard had been added, showed that cultures often produced small GC peaks

[5] Finnigan model 3221-F200 with INCOS data system, Sunnyvale, Calif.
[6] Gas Pak, Baltimore Biological Laboratories, Cockeysville, Md.

Table III—*N*-Nitrosodesipramine Formed in Aerobic Bacterial Cultures

Aerobe	Media Additives	*N*-Nitrosodesipramine, μg/ml	Final pH of Culture
Enterobacter aerogenes-13048[a]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	3.36	5.6
Escherichia coli-27165	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.35	7.4
Klebsiella pneumoniae-27889	100 μg/ml desipramine + 25 m*M* $NaNO_2$	2.31	6.0
Proteus mirabilus[b]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	2.46	5.8
Proteus vulgaris-27973	100 μg/ml desipramine + 25 m*M* $NaNO_2$	1.66	5.8
SI-1[c]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.13	7.8
SI-2[c]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.077	7.8
SI-3[c]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.067	7.8
Control (pH 5)[d]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.010	5.0
Control (pH 6)[d]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	ND	6.0
Control (pH 7)[d]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.019	7.0
Control (pH 8)[d]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.010	8.0
Mixed SI[e]	100 μg/ml imipramine + 25 m*M* KNO_3	ND[f]	8.3
Mixed LI[g]	100 μg/ml imipramine + 25 m*M* KNO_3	ND	5.6
Control[h]	100 μg/ml imipramine + 25 m*M* KNO_3	ND	7.8

[a] All numbers refer to the American Type Culture Collection, Rockville, Md. [b] This culture was isolated from the large intestine of a naive Wistar rat and identified using a commercial culture identification system[7]. [c] Pure culture isolate from Wistar rat small intestines. [d] Sterile defined media adjusted to the indicated pH. [e] Mixed culture obtained from Wistar rat small intestines. [f] Below detection limit of ≅0.010 μg/ml. [g] Mixed culture obtained from Wistar rat large intestine. [h] Sterile defined media.

Table IV—*N*-Nitrosodesipramine Formed in Anaerobic Bacterial Cultures

Anaerobic	Media Additives	*N*-Nitrosodesipramine, μg/ml	Final pH of Culture
SI-B1[a]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.56	7.2
SI-B5[a]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.40	7.1
SI-B6[a]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.63	6.6
LI-B2[b]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	3.37	5.3
Control (pH 5.0)[c]	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.012	5.0
Control (pH 6.0)	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.029	6.0
Control (pH 7.0)	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.014	7.0
Control (pH 8.0)	100 μg/ml desipramine + 25 m*M* $NaNO_2$	0.005	8.0
Mixed SI[d]	100 μg/ml imipramine + 25 m*M* KNO_3	0.028	5.6
Mixed LI[e]	100 μg/ml imipramine + 25 m*M* KNO_3	0.165	6.1
Control[f]	100 μg/ml imipramine + 25 m*M* KNO_3	0.037	7.9

[a] Pure culture isolated from Wistar rat small intestines. [b] Pure culture isolated from Wistar rat large intestine. [c] Sterile defined media adjusted to the indicated pH. [d] Mixed culture obtained from Wistar rat small intestines. [e] Mixed culture obtained from Wistar rat large intestine. [f] Sterile defined media at the normal pH of the buffer system.

at 10.1 and 11.0 min that would have caused some minor problems if the acetyl or propionyl amides had been used as the internal standard.

Though isooctane is too nonpolar to be ideal for the extraction of most drugs, this property proved to be critical in obtaining extracts that gave high-quality chromatograms. Solvents such as methylene chloride were found to give good recoveries of the nitrosamine, but the chromatograms gave an off-scale response for the nitrogen-selective detector for the first 3–4 min and several large peaks that eluted after the nitrosamine. When isooctane was used for the extraction, high-quality chromatograms were obtained for the bacterial cultures (Fig. 2) and quantitative recoveries of the nitrosamine and the internal standard were obtained from aqueous model systems (Table I). A bacterial culture not containing any drug or *N*-nitrosodesipramine was harvested at the end of the Stage II fermentation, then the culture was sterilized and spiked with the nitrosamine and internal standard. Though the recoveries from this matrix were not quantitative (Table I), the recoveries were very high, and the recoveries of the nitrosamine and the internal standard were the same.

When a set of four sterilized bacterial cultures was spiked with a known quantity of the nitrosamine and then analyzed with the internal standard method, it was found that the average value for the determination was 4.9% higher than the true value (Table II), however, this error was within the ±5.0% variance for the method. Using the sample sizes as described in the experimental section, the detection limit was ~0.010 μg/ml. This detection limit was not due to signal–noise limitations, but because of the presence of a peak on the shoulder of the nitrosamine that was detectable at only these very low levels.

The analysis of the aerobic bacterial cultures (Table III) showed that the amount of desipramine converted to *N*-nitrosodesipramine was 10–300 times higher in the cultures than in the controls. The identity of the nitrosamine in each of the cultures was verified by a comparison of the retention time and nitrogen detector response index (9) of the unknown and the reference standard. The GC–mass spectra of some of the extracts were examined and found to be identical to the reference standard (Fig. 1).

The chemical *N*-nitrosation of simple secondary amines increases as the pH decreases, and the rate reaches a maximum at pH 3 for most aliphatic amines. Though a buffer system was used with the bacterial cultures, the pH of several of the cultures dropped during the fermentation. However, when desipramine and sodium nitrite were incubated under identical conditions in sterile media, only trace quantities of the nitrosamine was formed even at the lower pH range (Table III). After a consideration of all of these factors, the production of the nitrosamine in the cultures of *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Proteus mirabilus*, and *Proteus vulgaris* was considerably above the control values and above that which would be expected of any simple chemical model.

Preliminary studies with imipramine, potassium nitrate, and mixed aerobic cultures (Table III) indicated that *N*-nitrosodesipramine was not formed. Though a wide variety of aerobes have been previously demonstrated to convert nitrate to nitrite (4, 12), this conversion was not monitored in the present study. Thus, the absence of the nitrosamine could be the result of a low conversion of nitrate to nitrite or a result of a slow rate of *N*-demethylation.

Of the anaerobic cultures (Table IV), isolate LI-B2 was found to produce considerably higher levels of *N*-nitrosodesipramine than the control incubations. The isolates obtained from the small intestines produced nitrosamine levels above the control values, but the concentration was nearly an order of magnitude lower than for the large intestine isolate.

A mixed culture of large intestine anaerobes (Table IV) was found to produce a concentration of *N*-nitrosodesipramine 4.5 times higher than the control incubation of imipramine and potassium nitrate alone. Though the concentration of the nitrosamine formed in the mixed culture seemed low, it was 500–1000 times greater than the simple nitrosamines commonly found in food samples (13) or human feces (14).

CONCLUSIONS

The analytical method developed for the determination of *N*-nitrosodesipramine in bacterial cultures was found to give accurate and precise (±5.0%) results with good sensitivity (0.01 μg/ml). Using the nitrogen detector response index as an additional identification aid, the nitrosamine was easily quantitated in the complex bacterial culture matrix.

[7] API 20E System, Analytab Products, Plainview, N.Y.

Several of the aerobic and anaerobic cultures were found to give high levels of *N*-nitrosodesipramine when incubated with desipramine and sodium nitrite. Though it cannot be stated that the transformation was an entirely enzymatic reaction, the yield of the nitrosamine was far higher than could be obtained by the acid catalyzed reaction between nitrite and desipramine.

Preliminary studies with mixed anaerobic cultures with imipramine and nitrate showed that the nitrosamine was formed more rapidly than the control, but the yield was very low. Additional studies with pure cultures will be needed to determine if the low yield was due to a low rate of *N*-demethylation.

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ACKNOWLEDGMENTS

Supported in part by the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi.

The authors are grateful for the sample of desipramine hydrochloride provided by Merrell-National Laboratories. They also thank Mrs. Karen Varner for technical assistance.

Blood Level Studies of All-*trans*- and 13-*cis*-Retinoic Acids in Rats Using Different Formulations

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Received July 31, 1981, from the *Department of Pharmaceutics, School of Pharmacy, University of Georgia, Athens, GA 30602.* Accepted for publication November 12, 1981.

Abstract □ Studies to determine the bioavailability of all-*trans*-retinoic acid from a microencapsulated product were carried out using rats as test animals. The microcapsules were tableted in rat food and individual rats given a tablet containing the equivalent of 10 mg of all-*trans*-retinoic acid. Comparisons were made with bioavailability data obtained after intravenous and oral administrations of a solution and a suspension. The elimination of all-*trans*-retinoic acid following intravenous administration of 1- to 5-mg doses occurred by dose-dependent kinetics. The half-lives for the terminal linear portion of the elimination phase after the plateau level were 0.78, 0.74, and 0.93 hr for the 1-, 2.5-, and 5-mg doses, respectively. Based on the doses administered and the relative area under the serum level curves, the all-*trans*-retinoic acid microcapsules were found to be ~34% as bioavailable as the solution dosage form and the microfine suspension 93% as bioavailable. The bioavailability of all-*trans*-retinoic acid in oral solution was ~40% of the intravenous dose. For comparison, rats were also dosed intravenously with 13-*cis*-retinoic acid, and this compound was found not to follow dose-dependent kinetics at similar dosage levels used for all-*trans*-retinoic acid.

Keyphrases □ Bioavailability—Blood level studies of all-*trans*- and 13-*cis*-retinoic acids using different formulations, rats □ Microencapsulation—blood level studies of all-*trans*- and 13-*cis*-retinoic acids using different microencapsulated formulations, rats □ Blood level studies—all-*trans*- and 13-*cis*-retinoic acids using different formulations, rats □ Retinoic acids, all-*trans*- and 13-*cis*- —blood level studies using different formulations, rats

A number of retinoids have been shown to prevent or inhibit the growth of epithelial tumors. The use of these compounds for chemoprevention of tumors has been reviewed previously (1, 2). Although a long-term study with a synthetic retinoid was reported (3), most studies have been relatively short term with manual dosing of the retinoids. For long-term efficacy studies, a more economical and less troublesome mode of drug administration is through the diet of the test animals. However, simple mixing of retinoids with feed is precluded because of the unstable nature of the compounds toward air, light, and moisture. Earlier studies with vitamin A compounds have shown that these chemicals can be protected from environmental hazards by microencapsulation (4, 5).

Requirements of a microcapsule product are that first it must be readily miscible with the feed of the test animals, and second, it must be soluble or digestible in the GI tracts of the animals so that the retinoid is biologically available. In the present study, all-*trans*-retinoic acid was microencapsulated, and the bioavailability of the compound from the finished product was determined and compared with intravenously and orally administered retinoid using rats as test animals. Also, for comparison, rats were dosed intravenously with 13-*cis*-retinoic acid.

EXPERIMENTAL

Chemicals—All-*trans*-retinoic acid[1], 13-*cis*-retinoic acid[2], and all-*trans*-retinol acetate[3] were used as received. All other chemicals and reagents were the highest grade commercially available.

Microcapsules—All-*trans*-retinoic acid was encapsulated in gelatin–dextrose microcapsules by a process similar to that reported for the encapsulation of vitamin A derivatives (6) using a three-phase suspension

[1] Eastman-Kodak, Rochester, N.Y.
[2] Hoffmann-LaRoche, Nutley, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.

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