Adenosine Analogs: Structure–Activity Relationships in Vascular and Intestinal Smooth Muscle

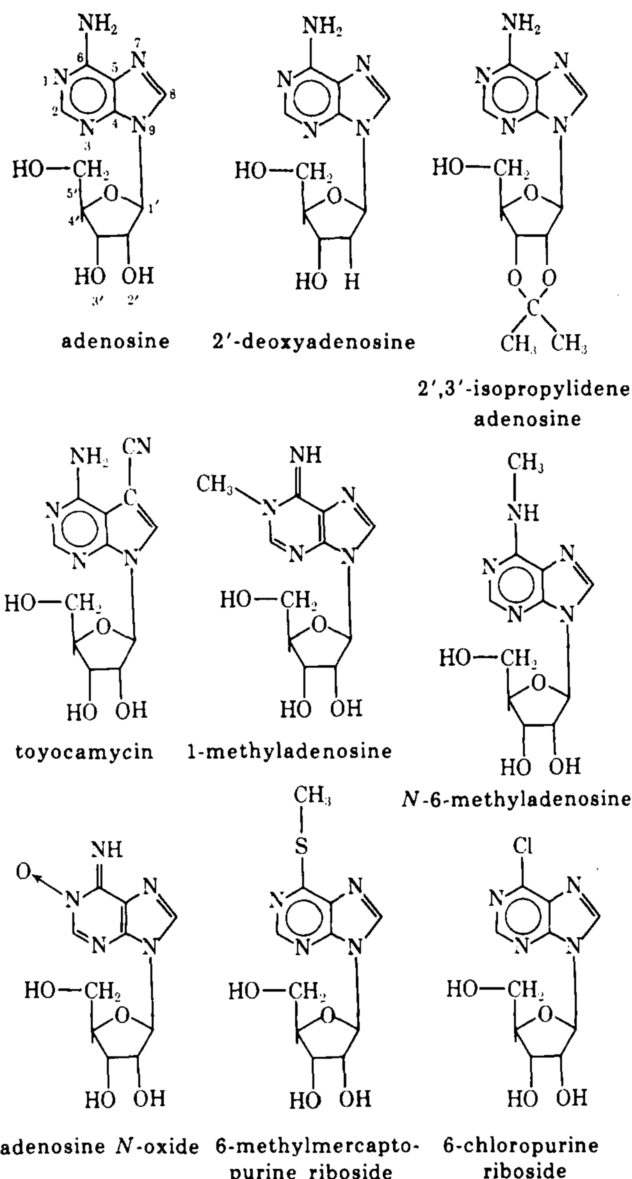
S. W. LESLIE▲, J. L. BOROWITZ, and T. S. MIYA

Abstract ☐ The relaxant effects of a series of adenosine analogs were compared on isolated intestinal and aortic smooth muscle of the guinea pig. Blood pressure-lowering effects of these compounds were also compared in the guinea pig and dog. Analogs with a modified purine ring system (1-methyladenosine and toyocamycin) were more effective in relaxing intestinal than vascular smooth muscle. Substituents that decreased the basicity of the N_1-position or interfered with hydrogen bonding in the 6-position decreased activity in both vascular and intestinal smooth muscle. Deoxyadenosine, which lacks an oxygen in the 2′-position of the ribose portion of the molecule, was ineffective in intestinal but still relaxed vascular smooth muscle. It was concluded that intestinal smooth muscle interacts significantly with the 2′-position of the ribose portion of the adenosine molecule whereas vascular smooth muscle requires an intact purine ring system in the molecule for maximal activity.

Keyphrases ☐ Adenosine analogs, structure-activity relationships—relaxant effects on isolated ileal and aortic smooth muscle, blood pressure depression compared in guinea pig and dog ☐ Smooth muscle relaxation, isolated ileal and aortic muscle—effects of adenosine analogs, structure-activity relationships ☐ Relaxation of isolated ileal and aortic smooth muscle—effects of adenosine analogs, structure-activity relationships ☐ Blood pressure depression—adenosine analogs, guinea pig and dog ☐ Structure-activity relationships—adenosine analogs, smooth muscle relaxation and blood pressure depression

Adenosine is thought to be a breakdown product of the purinergic transmitter (1) and is found circulating in the blood in a concentration of 4×10^{-8} M (2). It is known to be released from intestinal (3), cardiac (4, 5), and adrenal (6) tissue. Adenosine relaxes bronchiolar (7), intestinal (8), and certain vascular (9) smooth muscle.

The interaction between adenosine and adenosine deaminase was studied at the molecular level (10), but the structure–activity relationships for adenosine's relaxant effects on intestinal and vascular smooth muscle are not fully known (11–14). Therefore, structural characteristics of a series of adenosine analogs important for relaxation of these smooth muscles were determined.



METHODS

A series of purine ribosides was tested *in vitro* on longitudinal smooth muscle of the guinea pig ileum and on circular smooth muscle of guinea pig descending thoracic aorta. *In vivo* blood pressure studies were also performed in guinea pigs and dogs to determine whether analogs that were inactive *in vitro* in the descending thoracic aorta were also inactive within the vascular bed as a whole.

Fasted (18–24 hr.) male guinea pigs (300–600 g.) were sacrificed by cervical dislocation, and a segment of ileum 20 cm. from the ileocecal junction was excised. Ileal strips 2–4 cm. in length were suspended in an oxygenated bath with Tyrode's solution maintained at 37°.

The descending thoracic aorta was also excised and prepared as described by Furchgott and Bhadrakom (15). The spirally cut segments were suspended in an oxygenated bath with the Krebs-Henseleit solution maintained at 37°.

One gram of tension was placed on both the longitudinal muscles of the ileum and the circular muscles of the aorta, and contraction was recorded[1]. The aortic strips were allowed to equilibrate in the bath for 2 hr. and the ileal strips for 1 hr.

Nicotine was used as the agonist in the ileum, and epinephrine was used as the agonist in the aorta. The concentrations of nicotine or epinephrine were the same for all experiments (ranging from 10^{-9} M to 3×10^{-6} M or from 10^{-7} M to 3×10^{-6} M, respectively). Drugs were pipeted directly into the baths and did not dilute the baths by more than 1%.

The purine ribosides used in this study were adenosine[2], 2′-deoxyadenosine, 6-methylmercaptopurine riboside, 2′,3′-isopro-

[1] Using a Grass force-displacement FTO 3C transducer on a model 5 polygraph.
[2] Sigma Chemical Co., St. Louis, Mo.

Table I—Inhibitory Effects of Adenosine and Its Analogs on Isolated Guinea Pig Ileum and Thoracic Aorta

Compound	Concentration, *M*	Tissue	Inhibition[a] of Contraction, %	*N*
Adenosine	10^{-5}	Ileum	49	6
Adenosine	10^{-5}	Aorta	30	6
N-6-Methyladenosine	10^{-5}	Ileum	35	5
N-6-Methyladenosine	10^{-5}	Aorta	39	5
Isopropylidene adenosine	5×10^{-5}	Ileum	43	6
Isopropylidene adenosine	5×10^{-5}	Aorta	0	5
Isopropylidene adenosine	10^{-4}	Aorta	53	5
Deoxyadenosine	10^{-4}	Ileum	0	5
Deoxyadenosine	10^{-4}	Aorta	59	7
1-Methyladenosine	10^{-4}	Ileum	43	8
1-Methyladenosine	10^{-4}	Aorta	0	4
Toyocamycin	10^{-5}	Ileum	34	6
Toyocamycin	10^{-6}	Aorta	0	6

[a] Percent inhibition produced by adenosine and adenosine analogs was calculated for concentrations of 1×10^{-6}, 3×10^{-6}, and 5×10^{-6} *M* nicotine in ileal tissue and for concentrations of 4×10^{-9}, 1.4×10^{-8}, 4.4×10^{-8}, and 1.4×10^{-7} *M* epinephrine in the aorta. The values obtained were then added and a mean percent depression was calculated.

pylidene adenosine, adenosine *N*-oxide[3], 1-methyladenosine, *N*-6-methyladenosine[4], and toyocamycin (a pyrrolo [2,3-*d*]pyrimidine nucleoside antibiotic)[5].

Male guinea pigs (300-600 g.) or healthy mongrel dogs (8-13 kg.) were anesthetized with pentobarbital (35 or 50 mg./kg., respectively) and carotid blood pressures were recorded. Drugs were injected *via* the external jugular vein in the guinea pig and *via* the femoral vein in the dog. A control series of five doses (25-400 mcg./kg.) of adenosine was first injected into each animal, followed by a series of doses of the adenosine analogs (25 mcg./kg.-5 mg./kg.). A test dose of adenosine (25 mcg./kg.) was injected after each series of doses of each analog to ensure that no potentiation or desensitization of the blood pressure response had occurred.

RESULTS

Effects of Adenosine Analogs on Isolated Ileum and Thoracic Aorta—A summary of the inhibitory effects of adenosine and adenosine analogs on isolated intestinal and arterial smooth muscle is presented in Table I. Adenosine and *N*-6-methyladenosine had inhibitory effects in both tissues (Table I). These compounds were previously reported to inhibit vascular smooth muscle contractions (14), and adenosine was reported to relax intestinal smooth muscle (8).

At a concentration of 1×10^{-4} *M*, isopropylidene adenosine significantly depressed contractions of the aorta in response to 1.4×10^{-7} *M* ($p < 0.001$) and 4.4×10^{-7} *M* ($p < 0.025$) epinephrine. At a lower concentration, 5×10^{-5} *M* isopropylidene adenosine depressed ($p < 0.05$) nicotine- (3×10^{-6} *M*) induced contractions in the ileum.

Deoxyadenosine depressed contractions in arterial tissue (Fig. 1) but not in the ileum at a concentration of 10^{-4} *M*. By contrast, 1-methyladenosine and toyocamycin (Figs. 2 and 3, respectively) depressed contractions in the ileum but not in isolated aorta at the concentrations used. 6-Chloropurine riboside, 6-methylmercaptopurine riboside, and adenosine *N*-oxide were each tested (10^{-4} *M*) in at least three ileal and three aortic preparations and were found to be ineffective in inhibiting the contractile responses.

Since toyocamycin and 1-methyladenosine appear to produce a rather selective relaxation of ileal contractions induced by an indirect-acting agonist (nicotine), further experiments were performed with these two analogs ($N = 6$ in each case) against acetylcholine, a direct-acting agonist. Results were similar to those with nicotine, although these analogs were somewhat less potent in depressing acetylcholine-induced contractions. Toyocamycin (1×10^{-4} *M*) produced a mean depression of 44%, while 1-methyladenosine (1×10^{-4} *M*) resulted in a 16% mean depression of acetylcholine-induced contractions (mean percent depression was calculated by averaging percent depressions produced by 1×10^{-7}, 3×10^{-7}, 1×10^{-6}, and 3×10^{-6} *M* acetylcholine).

Concentration-response curves of the guinea pig ileum to nicotine were consistently reproducible. However, a decrease in sensitivity of the aorta to epinephrine was seen, as previously reported (15). No further desensitization was noted after the first concentration-response curve (four experiments). Therefore, in the guinea pig ileum, the responses obtained in the presence of the inhibitors were compared statistically with the mean value of the control and recovery responses, while in the aorta the responses obtained in the presence of the inhibitors were compared statistically only with the recovery responses.

Effects of Adenosine Analogs on Guinea Pig and Dog Blood Pressure—Adenosine (Fig. 4) was found to produce a transient blood pressure depression in the guinea pig, resulting in a 27-mm. Hg decrease after the highest dose of 400 mcg./kg. Depressor responses were generally short lived, and pressure returned to control levels in about 30 sec. The effect was more prolonged (about 3 min.) when doses of 1 mg. or more of the analogs were given. *N*-6-Methyladenosine (Fig. 4) also depressed guinea pig blood pressure but was only one-fourth to one-half as potent as adenosine.

Although 1-methyladenosine was approximately 10 times less

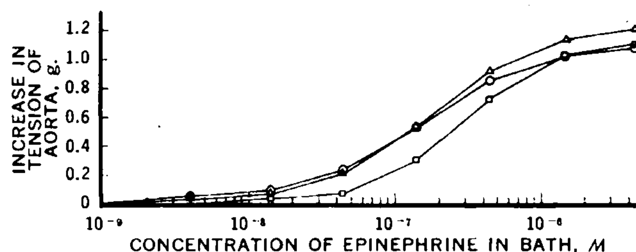


Figure 1—*Effect of deoxyadenosine (1×10^{-4} M) on epinephrine-induced contractions in the guinea pig descending thoracic aorta. Each point represents the mean response of seven aortic strips. Deoxyadenosine significantly depressed the contractile response to 1.4×10^{-7} M ($p < 0.01$) and 4.4×10^{-7} M ($p < 0.01$) epinephrine. The paired difference form of the Student t test was used to compare the recovery response with the responses obtained in the presence of the adenosine analog in all experiments involving the aorta. Key: ○, control; □, deoxyadenosine, 1×10^{-4} M; and △, recovery.*

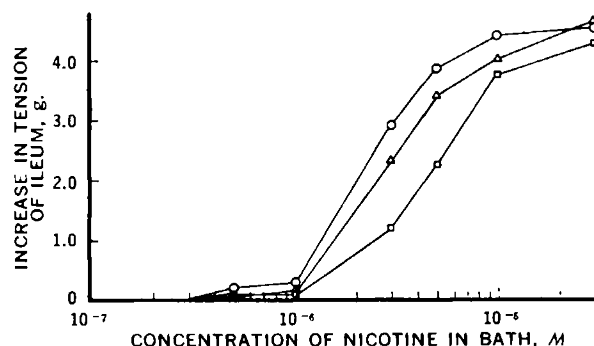


Figure 2—*Effect of 1-methyladenosine (1×10^{-4} M) on nicotine-induced contractions in the guinea pig ileum. Each point represents the mean response of eight ilea. 1-Methyladenosine significantly depressed the contractile response to 3×10^{-6} M ($p < 0.025$) and 5×10^{-6} M ($p < 0.05$) nicotine. The paired difference form of the Student t test was used to compare the mean of control and recovery responses with the responses obtained in the presence of the adenosine analog in all experiments involving the ileum. Key: ○, control; □, 1-methyladenosine, 1×10^{-4} M; and △, recovery.*

[3] Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] Terra-Marine Bioresearch, La Jolla, Calif.
[5] Obtained from Dr. Suhadolnik, Albert Einstein Medical Center, iladelphia, Pa.

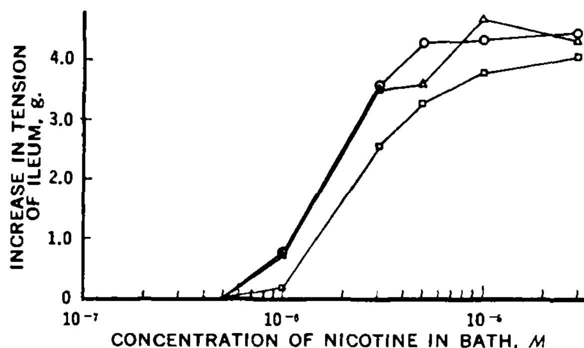


Figure 3—*Effect of toyocamycin* (1×10^{-5} M) *on nicotine-induced contractions in the guinea pig ileum. Each point represents the mean response of six ilea. Toyocamycin significantly depressed the contractile response to* 1×10^{-6} M ($p < 0.01$) *and* 3×10^{-6} M ($p < 0.005$) *nicotine. Key:* ○, *control;* □ *toyocamycin,* 1×10^{-5} M; *and* △, *recovery.*

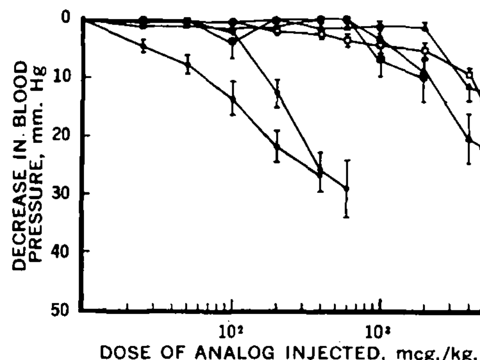


Figure 4—*Effect of adenosine and adenosine analogs on the diastolic blood pressure in the guinea pig. Each point represents the mean response* ± SE *in from five to seven male guinea pigs. Mean control blood pressure was 42.3 mm. Hg, which is comparable to reported values for the guinea pig (16). Key:* ●, *adenosine;* ○, N-6-*methyladenosine;* □, *deoxyadenosine;* △, *isopropylidene adenosine;* ■, *toyocamycin; and* ▲, *1-methyladenosine.*

potent than adenosine in relaxing ileal smooth muscle, it was 25–50 times less potent in depressing blood pressure. Thus, 1-methyladenosine was about four times more potent in ileal smooth muscle than in vascular smooth muscle.

Toyocamycin, at a dose of 1×10^{-5} *M*, was shown to be approximately equal to adenosine in inhibiting contractions of smooth muscle of the guinea pig ileum but was not effective in relaxing the descending thoracic aorta nor in depressing guinea pig blood pressure unless a large dose (1 mg./kg.) was used. The effect of toyocamycin in ileal smooth muscle was about 50 times that in vascular smooth muscle. This analog was peculiar in that, at doses of 500 mcg./kg. or greater, it markedly potentiated the vasodepressor effects of adenosine in the guinea pig. No other analog produced this effect.

Adenosine and each adenosine analog were also tested on the blood pressure of two to five mongrel dogs. The vasodepressor action of these compounds was similar relative to one another in this species compared to the activity in the guinea pig. However, toyocamycin did not potentiate the vasodepressor response to adenosine in the dog.

DISCUSSION

Molecular changes that increase the electron density and thus the basicity of the N_1-position of adenosine are known to increase its affinity for adenosine deaminase (10). This study shows that a certain degree of basicity of the N_1-position is also important for adenosine's interaction with smooth muscle. Addition of an oxygen to the N_1-position of adenosine to form the *N*-oxide decreases the basicity of this area and results in loss of activity in both vascular and ileal smooth muscle.

The aromaticity of the purine ring system appears to influence the selectivity of action of adenosine analogs on smooth muscle. Replacing the nitrogen in the 7-position of the purine riboside with a cyano-carbon group, as in toyocamycin, alters electronic and steric factors which affect the electron distribution of the whole purine ring. Toyocamycin is approximately 50 times more active in intestinal muscle relative to arterial smooth muscle. Addition of a methyl group to the N_1-position of adenosine also tends to disrupt the aromaticity of the ring system and again results in a more selective activity on intestinal musculature. This methyl group also increases the basicity of the N_1-position, which may explain the increased activity remaining in vascular smooth muscle as compared to toyocamycin and reinforces the assumption that basicity of this area of the purine ring is important for activity in both tissues. Apparently, the usual purine ring system is not rigidly required for relaxation of intestinal smooth muscle by adenosine analogs, but basicity of the N_1-position is required in both tissues.

It was suggested (10) that hydrogen bonding occurs between adenosine and adenosine deaminase before removal of the amino group in the 6-position is completed. If a similar hydrogen bonding is required at the adenosine receptor to produce smooth muscle relaxation, replacement of the 6-amino group with moieties that modify hydrogen bonding should reduce or eliminate activity. Addition of a methyl group to the 6-amino group decreases the hydrogen-bonding capabilities and/or increases steric hindrance and, as shown in this study, results in a general reduction of activity. Replacement of the 6-amino group with a methylmercapto or chloride group results in elimination of hydrogen-bonding capabilities and, as shown in this study, also eliminates the effect on smooth muscle. Thus, hydrogen bonding through the *N*-6-position of adenosine may be important for the adenosine effect.

This study also showed that an intact ribose moiety is essential for complete activity of adenosine. The 2′-hydroxyl group seems to play an important role in the activity of the compound. The 2′-hydroxyl and 3′-hydroxyl groups contain electrons which are available for hydrogen bonding. If hydrogen bonding from the 2′-hydroxyl group to a site on the receptor is required for activity, it would be expected that removal of the 2′-hydroxyl group would result in loss of effectiveness. Studies with deoxyadenosine showed that a decrease in activity did in fact occur. It appears, however, that this hydrogen bonding may be more necessary in ileal smooth muscle than in vascular smooth muscle since some activity remained in the latter tissue after removal of the 2′-hydroxyl group.

Attachment of the 2′,3′-isopropylidene radical would be expected to decrease the proton-accepting power of the oxygen atoms involved. Thus, hydrogen bonding with the ribose part of the molecule is reduced but not eliminated in the isopropylidene derivative of adenosine. In this case, a relative reduction of the relaxant effect was obtained in both tissues, which suggests that some hydrogen-bonding capability of the 2′,3′-positions of the ribose moiety of adenosine is required for activity.

This study showed that the basicity of the N_1-position and hydrogen-bonding capabilities of the N_6-position of adenosine are important for activity in both intestinal and vascular smooth muscle. An intact purine ring system favors activity in vascular smooth muscle but is not required for maximal activity in intestinal smooth muscle. Hydrogen-bonding capabilities of the 2′-hydroxyl group are more important for relaxation of vascular than intestinal smooth muscle.

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 12, 1973, from the *Department of Pharmacology and Toxicology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907*

Accepted for publication April 6, 1973.

Supported by National Institutes of Health Grant GM 1500J and by a General Research Support Grant.

The authors thank Dr. Heinz Floss and Dr. R. Suhadolnik for supplying the toyocamycin. They also thank Dr. J. Wells for his advice and helpful suggestions.

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Nalidixic Acid and Hydroxynalidixic Acid Analysis in Human Plasma and Urine by Liquid Chromatography

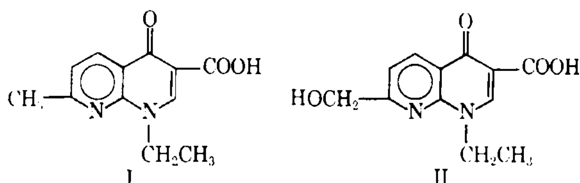
L. SHARGEL▲, R. F. KOSS, A. V. R. CRAIN, and V. J. BOYLE

Abstract □ A high pressure liquid chromatographic method was developed for the assay of nalidixic acid and hydroxynalidixic acid in human plasma and urine. This procedure measures both compounds in the same sample. The lower limits of detection for nalidixic acid and hydroxynalidixic acid were 0.25 mcg./ml. of each in plasma and 2.5 mcg./ml. of each in urine. Correlation coefficients for the analysis of nalidixic and hydroxynalidixic acids in urine of humans medicated with two different tablet formulations were 0.83 and 0.94 in comparison with a microbiological assay.

Keyphrases □ Nalidixic and hydroxynalidixic acids—analysis in human plasma and urine, high pressure liquid chromatography □ Hydroxynalidixic and nalidixic acids—analysis in human plasma and urine, high pressure liquid chromatography □ High pressure liquid chromatography—analysis, nalidixic and hydroxynalidixic acids in human plasma and urine

Nalidixic acid[1] (I) is currently used as an antibacterial agent in urinary tract infections (1–3). A major metabolite is hydroxynalidixic acid (II). This metabolite has an *in vitro* antibacterial spectrum similar to that of nalidixic acid (4). Both compounds have similar chemical structures and fluorescent spectra.

The original fluorescent method for the assay of nalidixic acid and hydroxynalidixic acid (4) involved differential extraction, which provided only partial separation of nalidixic acid and hydroxynalidixic acid. Thus, simultaneous equations were employed for the determination of the two drugs. Furthermore, blank values were occasionally high and erratic, giving unsatisfactory values for nalidixic acid.



The use of liquid chromatography for the separation and quantitation of many compounds has increased in recent years. The availability of a modern, high speed, liquid chromatograph allows the measurement of both nalidixic acid and hydroxynalidixic acid simultaneously. This report describes a new liquid chromatographic assay procedure for nalidixic acid and hydroxynalidixic acid which is more rapid and precise than previously published methods.

EXPERIMENTAL

Materials—Nalidixic acid[2] and hydroxynalidixic acid[2] were routinely prepared in the same standard solution of 0.03 *N* NaOH. Chloroform, analytical reagent grade[3], was used for all extractions. All other reagents were purchased from commercial sources.

Plasma Assay—To a 15-ml. glass-stoppered centrifuge tube were added 1.0 ml. of plasma[4] and 1.0 ml. of a standard solution containing both nalidixic acid and hydroxynalidixic acid in concentrations ranging from 0 to 40 mcg./ml. For unknown plasma samples, 1.0 ml. of sample was added to 1.0 ml. of 0.03 *N* NaOH. To this mixture was added 0.5 ml. of 0.6 *N* HCl (final pH approximately 1.0) and 5 ml. of chloroform. The tubes were stoppered, shaken 5 min., and centrifuged. The upper aqueous phase was then aspirated. A 4.0-ml. aliquot of the chloroform phase was transferred to a clean 15-ml. glass-stoppered centrifuge tube, and 1.0 ml. of 0.03 *N* NaOH was added. The tubes were shaken for 5 min. and centrifuged. The aqueous phase containing nalidixic acid and hydroxynalidixic acid was then subjected to high pressure liquid chromatography.

Urine Assay—To a 50-ml. glass-stoppered extraction tube were added 1 ml. of urine and 1 ml. of a standard solution containing 0–250 mcg./ml. each of nalidixic acid and hydroxynalidixic acid. For unknown urine samples, a 1.0-ml. aliquot was added to 1.0 ml. of 0.03 *N* NaOH. The glucuronides of nalidixic acid and hydrox-

[1] NegGram, Winthrop Laboratories, New York, N. Y.

[2] Supplied by Sterling-Winthrop Research Institute, Rensselaer, NY 12144

[3] Mallinckrodt Chemical Works, St. Louis, Mo.

[4] Since fresh human plasma caused less emulsion formation during extraction than outdated Red Cross plasma, the former was more desirable to use for processed standards.