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AZOTOMYCIN—TOXICOLOGIC, BIOCHEMICAL AND PHARMACOLOGIC STUDIES IN MICE

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Abstract—A biochemical basis was sought for the acute and cumulative toxicity of Azotomycin, (glutamine, *N*[1-[(1-carboxy-5-diazo-4-oxopentyl) carbamoyl]-5-diazo-4-oxopentyl]-, sodium salt), an antagonist of L-glutamine. Whereas mice can tolerate relatively large single doses of the drug, very small repeated doses are toxic. L-Asparaginase augments the toxicity of the latter regimen but not the former. Brain, liver, intestines and spleen are the organs principally damaged by Azotomycin. A systematic study of the synthesis and concentration of L-glutamine, L-asparagine and other metabolites in these organs has revealed that Azotomycin is also a powerful inhibitor of L-asparagine synthetase *in vivo*. A variety of murine organs have been shown to hydrolyze Azotomycin to yield 1 mole L-glutamic acid and approximately 2 moles 6-diazo-5-oxo-L-norleucine (DON). On this evidence it is suggested that DON, a classical antagonist of L-glutamine, is responsible for the toxicity of Azotomycin in these organs. When the analogue interfered more with the utilization than with the breakdown of L-glutamine, L-glutamine accumulated to a pathologic degree; this situation has been seen in liver. When both utilization and breakdown were curtailed to a similar degree, the steady state concentration of L-glutamine was maintained; this situation was seen in spleen, intestines and brain. In no case did Azotomycin inhibit the biosynthesis of L-glutamine.

UNTIL RECENTLY, interest in the tripeptide antibiotic, Azotomycin (glutamine, *N*-[1-[(1-carboxy-5-diazo-4-oxopentyl)carbamoyl]-5-diazo-4-oxopentyl]-, sodium salt), focused on its role as an antagonist of L-glutamine with significant antitumor properties.[1] It has been reported that Azotomycin, like 6-diazo-5-oxo-L-norleucine (DON), one of its constituents, exhibits an unusually broad spectrum of activity against the early and late stages of sarcoma 180 (S180), carcinoma 755 (Ca755) and leukemia 1210 (L1210);§ maximal antitumor activity is demonstrable when low doses of Azotomycin are given on a schedule of widely spaced injections.[2] Relatively high concentrations of the drug are required for the demonstration of cytotoxicity against human malignant cells in culture: 70 μg/ml is a cytotoxic level and 250 μg/ml is a lethal level of Azotomycin in the HeLa cell system.§

With the observation of Haskell and Canellos[3] that Azotomycin was a powerful inhibitor of L-asparagine synthetase extracted from human malignant cells, interest

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in Azotomycin was revived. The review by Livingston *et al.*[4] of antagonists of L-glutamine reported significant but incomplete inhibition of crude murine tumoral L-asparagine synthetase by Azotomycin at a concentration of 2×10^{-3} M. In both of the studies cited, increasing the concentration of drug to $8–10 \times 10^{-3}$ M failed to augment the degree of inhibition found at the lower concentration. This finding suggests that there might have been several synthetases present in these preparations: one sensitive and the other resistant to the inhibitory action of Azotomycin. Notable also in these investigations was the finding that Azotomycin inhibited the synthesis of L-asparagine to only a very minor degree when NH_4Cl served as the source of the amide, but strongly when L-glutamine was the amido donor. This finding is consonant with the customary designation of Azotomycin as an antagonist of L-glutamine.

The demonstration that Azotomycin could inhibit the synthesis of L-asparagine made it logical to determine whether the drug would exert therapeutic synergism with L-asparaginase. Jacobs *et al.*[5] have, in fact, achieved striking therapeutic synergism with the two drugs against leukemia 5178Y in mice. This synergism is similar to that reported for L-asparaginase plus DON (a constituent moiety of Azotomycin) versus Ehrlich ascites, melanoma B16, and the Walker carcinosarcoma.[6] L5178Y, in the native state, is exquisitely sensitive to L-asparaginase. Since L-asparagine synthetase is present only at low levels in this tumor, it is unlikely that the therapeutic synergism of Azotomycin with L-asparaginase necessarily depends on inhibition of the synthesis of L-asparagine. On the other hand, Ca755, L1210 and S180 are all endowed with high concentrations of cytoplasmic L-asparagine synthetase, a finding which may explain their resistance to L-asparaginase. The oncolytic activity of Azotomycin, viewed as an inhibitor of L-asparagine synthetase, becomes still more meaningful in the case of such tumors.

Pursuant to these experimental findings, clinical oncologists became interested in using Azotomycin in combination with L-asparaginase.[7] Toxicologic studies of the two agents were therefore undertaken as a foundation for their use in man, and for the purpose of ruling out unexpected or lethal interactions between the drugs. Jacobs *et al.*[5] have presented independent evidence that synergism of toxicity can be observed between Azotomycin and L-asparaginase under certain conditions in tumor-bearing mice. In addition, when toxicity to the central nervous system, intestines, spleen and liver was seen in mice receiving Azotomycin alone, a biochemical basis for the lesions was sought and attempts were made to uncover appropriate antidotes.

MATERIALS AND METHODS

Materials

Drugs and chemicals. The clinical formulation of Azotomycin (NSC-56654; lyophilized with mannitol) was provided by Pfizer, Inc., Maywood, N.J., and 6-diazo-5-oxo-L-norleucine (DON; NSC-7365) by Harry Wood of the National Cancer Institute, NIH, U.S.A.; L-asparagine and L-glutamine were purchased from Mann Research Laboratories, New York, N.Y., U.S.A. UL-[^{14}C]-L-valine (sp. act., 160 mCi/m-mole) and [^{14}C]-Na formate (sp. act., 56 mCi/m-mole) were obtained from Amersham Searle Corp., Arlington Heights, Ill., U.S.A. 1-[^{14}C]-α-ketoglutarate (sp. act., 14 mCi/m-mole) was purchased from New England Nuclear, Boston, Mass., U.S.A. NCS tissue solubilizer was the product of Amersham Searle. All other reagents and chemicals were of the highest quality obtainable.

Enzymes. Purified (45 ×) mammalian L-asparagine synthetase from the Novikoff hepatoma (sp. act., ~ 1084 nmoles/mg protein/hr) was a gift of Dr. M. K. Patterson, The Samuel Roberts Noble Foundation, Inc., P.O. Box 878, Ardmore, Oklahoma, U.S.A. L-Glutamate decarboxylase was partially purified by the method of Shukuya and Schwert,[8] and stored at −20° in 50% glycerine. L-Aspartic acid-β-decarboxylase was generously donated by Dr. Suresh Tate of The School of Medicine, Cornell University, New York, N.Y., U.S.A. L-Glutamate oxaloacetate transaminase, malate dehydrogenase and L-glutamate dehydrogenase were the products of Boehringer, New York, N.Y. U.S.A. L-Asparaginase (EC-2) was a generous gift of Merck.

Animals. General purpose Swiss mice, on an *ad lib.* diet of Purina chow, were used for the majority of the experiments described herein. Where indicated, BDF_1 mice were used for special toxicologic and pharmacologic experiments.

Methods

Preparation of extracts. Animals were killed by cervical dislocation and the appropriate organs removed as quickly as possible, frozen on dry ice and stored at −20°. At the time of assay, tissues were weighed and homogenized in 9 vol. (w/v) of 0·1 M Tris–hydrochloride, pH 7·6, containing 0·5 mM EDTA and 1 mM dithiothreitol.

The homogenate was divided into two parts: the first aliquot (for enzyme measurements) was refrozen at −20°. At the time of assay, it was defrosted and centrifuged at 12,000 *g* for 6 min. The second aliquot (for the measurement of L-asparagine and L-glutamine) was heated at 95° for 10 min and centrifuged at 12,000 *g* for 3 min. It is recognized that ~5 per cent of the L-glutamine present in these samples is destroyed under such conditions. No correction for this loss has been made. L-Asparagine is quantitatively recoverable after the heating step.

Measurement of L-*asparagine.* The relatively low concentration of L-asparagine present in homogenates of mouse organs was measured by an enzymatic radiometric technique.[9] Briefly, a 3-hr incubation at 37° with the bacterial enzymes L-aspartate-β-decarboxylase and L-glutamate decarboxylase served to remove any L-aspartic acid and L-glutamic acid present in a 5-μl sample of the heated supernatant. A 10-min incubation at 95° was used to inactivate the decarboxylases. L-Asparagine was then hydrolyzed with low concentrations of bacterial L-asparaginase, and the L-aspartic acid so generated was quantitatively transaminated with 1-[^{14}C]-α-ketoglutaric acid. At the termination of this reaction, the remaining 1-[^{14}C]-α-ketoglutaric acid was decarboxylated with 1% H_2O_2 in 1 N HCl and the residue, consisting of 1-[^{14}C]-L-glutamic acid, counted in a scintillation spectrometer at 50 per cent efficiency.

Measurement of L-*glutamine.* L-Glutamine was measured in 50-μl aliquots of the supernatant from the heated samples by an enzymatic spectrophotometric technique.[10] No attempts were made to remove the free NH_3 present in these samples by evaporation or lyophilization. Instead, this NH_3 was condensed with α-ketoglutarate through the catalytic action of L-glutamate dehydrogenase before addition of concentrated L-asparaginase to the cuvette.

Measurement of L-*asparagine synthetase.* Soluble L-asparagine synthetase was measured by a technique which will be published in full elsewhere. Briefly, [^{14}C]-L-aspartic acid ($1{\cdot}125 \times 10^{-3}$ M) was used as the source of the carbon skeleton and ATP, magnesium chloride and L-glutamine at final concentrations of 0·01, 0·01 and 0·02 M, respectively, were the additional substrates. After suitable incubation, residual L-

aspartic acid was removed by transamination to oxaloacetate and reduction with malate dehydrogenase in the presence of DPNH. L-Asparagine was measured subsequently in the L-aspartate-free incubation mixture by hydrolysis with L-asparaginase and transamination with α-ketoglutarate to yield 4-[^{14}C]-oxaloacetate, which was then β-decarboxylated, at pH 5·0, with zinc ions. Vessels which received L-asparaginase immediately after incubation served as blanks.

Measurement of L-*glutamine synthetase.* L-Glutamine synthetase was measured radiometrically. The reaction mixture allows the synthesis of L-glutamine from 1-[^{14}C]-L-glutamic acid in the presence of a mixture of Mg^{2+}, NH_4Cl and ATP. Five μl of 1:10 (w/v) tissue homogenate was incubated at 37° for 10 min with 5 μl of a substrate mixture containing 0·02 M ATP, 0·02 M $MgCl_2$, 0·04 M L-glutamic acid (0·25 μCi) and 0·16 M NH_4Cl. The reaction was terminated by heating at 95° for 3 min; then, the reaction vessels were centrifuged for 3 min at 12,000 *g* to separate any denatured protein. Unreacted L-glutamic acid was removed by incubation in open vessels with 1 i.u. of L-glutamate decarboxylase in 0·33 M acetate buffer, pH 4·2, for 2 hr at 37°. Thereafter, 1-[^{14}C]-L-glutamine was hydrolyzed to 1-[^{14}C]-L-glutamate by the addition of 20 i.u. of L-asparaginase to each vessel. The [^{14}C]O_2 was collected in 5-μl droplets of 40% KOH placed on the underside of the lid.[9] Appropriate reagent blanks and standards were included in each experiment. After 3 hr of incubation at 37°, the lids were removed and counted in an ethanol–toluene (1:2, v/v) scintillant, containing Liquifluor from New England Nuclear, Boston, Mass., U.S.A.

Measurement of L-*glutaminase.* Freshly excised organs were homogenized (1:3, w/v) in 0·05 M sodium phosphate, pH 7·4, and centrifuged at 50,000 *g* for 30 min, 0·1 ml of the supernatant solution was added to 0·4 ml of 0·01 M L-glutamine in 0·05 M phosphate buffer, pH 7·4, and incubated at 37° for 30 min. Ammoniagenesis was quantitated by an enzymatic spectrophotometric technique.[10]

Measurement of intermediary metabolites. Measurements of intermediary metabolites were carried out as described in the following citations: D-glucose,[11] total lipids,[12] glycogen,[13] L-alanine,[14] L-glutamic acid,[15] L-aspartic acid,[16] GABA;[17] ATP and DPN were measured by new radiochemical techniques, the full details of which will be published elsewhere.

Measurement of protein synthesis. At zero time, mice were given Azotomycin, 900 mg/kg, intravenously; 2, 4 and 7 hr later, 4 μCi UL-[^{14}C]-L-valine was injected intravenously through a caudal vein. One hr later, the mice were sacrificed; organs were removed rapidly and frozen on dry ice. After homogenization in 3 vol. of 5% perchloric acid, the tissue pellets were washed three times with 80% ethanol, then with acetone and finally with water. Lyophilization rather than heat was used to dry the pellets in order to facilitate weighing and subsequent dissolution. The solids were taken up and digested for 1 day in 1 ml of NCS solubilizer then bleached with 10 μl of 30% H_2O_2. A suitable aliquot of the digest was taken for scintillation spectrometry.

Measurement of incorporation of formate into DNA. At time zero, mice were given an intravenous injection of 900 mg/kg of Azotomycin or 0·25 ml of physiologic saline followed in 1 hr by 20 μCi [^{14}C]-Na formate (sp. act., 56 mCi/m-mole). Thirty min later, the organs were removed, rinsed with saline, blotted dry, homogenized in 10 ml of 80% ethanol and centrifuged at 90,000 *g* for 30 min. Nucleic acids were extracted from the neutral pellets by three 30-min incubations with 0·5 ml of 10%

NaCl. Sodium nucleates were precipitated from the salt extracts with 2 vol. of ice-cold 80% ethanol and frozen overnight. The nucleates were collected by centrifugation and the pellets incubated at 37° for 2 hr with 1 ml Pronase, 1 mg/ml of 0·05 M Tris–HCl, pH 8·4, and 0·001 M $CaCl_2$. DNA was precipitated from the resultant solution with an equal volume of 2% cetyl trimethyl ammonium bromide and 0·2% sodium acetate. After centrifugation, the pellets were washed twice with 80% ethanol, taken up in 1 ml of 0·1 N HCl and depurinated by heating at 95° for 0·5 hr. An aliquot was taken for measurement of absorbance at 260 and 280 nm, and the remainder was resuspended in 10 μl water and subjected to paper electrophoresis (4000 V, 0·1 M formic acid) on Whatman 3M chromatography paper in the 5KV unit from Savant Instruments, Inc., Hicksville, New York, U.S.A. The spots of adenine and guanine were detected under ultraviolet light, encircled, excised, and quantitatively eluted with 2 ml of 0·1 N HCl for 2 hr. After centrifugation of the eluate, absorbance was read at 280 and 260 nm.

RESULTS

The results of lethality studies of Azotomycin in mice are given in Table 1. When the drug was administered as a single intraperitoneal dose, the LD_{50} was found to be 262 mg/kg, with a median lethal time (LT_{50}) of about 5 days. When five daily injections of Azotomycin were given, the LD_{50} fell to 0·86 mg/kg and the LT_{50} fell on the fifth day after the initial dose. Addition of a single dose of L-asparaginase, 600 i.u./kg, to the regimen of treatment did not alter the incidence or timing of mortality when single doses of Azotomycin were given; however, five daily intraperitoneal injections of the enzyme given in conjunction with the antibiotic did lower the LD_{50} from 0·86 to 0·48 mg/kg in the 5-day repeated dose studies.

The present results confirm the powerful cumulative lethal action of Azotomycin in mice: very large single doses can be tolerated, whereas very small repeated doses

TABLE 1. LETHALITY OF AZOTOMYCIN ALONE AND IN COMBINATION WITH L-ASPARAGINASE*

Drugs	LD_{50} (mg/kg)	Approximate lethal time at LD_{50} (days)	Overt signs
Azotomycin			
Single dose	Mean: 262 Range: 244–280	~ 5	Diarrhea, unkemptness, ataxia, convulsions, fatty changes in liver
5 Daily doses	Mean: 0·86 Range: 0·77–0·95	~ 5	Diarrhea, tremors, somnolence, fatty changes in liver
Azotomycin plus L-Asparaginase (600 i.u./kg)			
Single dose	Mean: 259 Range: 198–300	~ 5	Lethargy, diarrhea, fatty changes in liver
5 Daily doses	Mean: 0·48 Range: 0·44–0·51	~ 6	Diarrhea

* The drugs, formulated in sterile water, were administered to BDF_1 mice by the intraperitoneal route. Animals receiving Azotomycin in a single dose received L-asparaginase 30 min later; animals receiving Azotomycin in five daily doses received L-asparaginase 30 min after each injection.

are lethal. A pharmacologic explanation for this effect will be adduced below. It is relevant to point out that for most of the experiments described in the remaining sections of this paper, comparatively high roughly equimolar concentrations of Azotomycin (900 mg/kg) and DON (600 mg/kg) were used in order to produce reliably severe toxicosis. Most mice given these doses survived 48 hr.

Action of Azotomycin on the CNS

In the single dose lethality studies, summarized in Table 1, it was observed that mice receiving Azotomycin at doses of 400 mg/kg or greater exhibited ataxia and tonic–clonic convulsions from 4 to 24 hr after administration of the drug. Sound and other simple stimuli appeared to provoke these convulsions, which are illustrated electroencephalographically in Fig. 1.

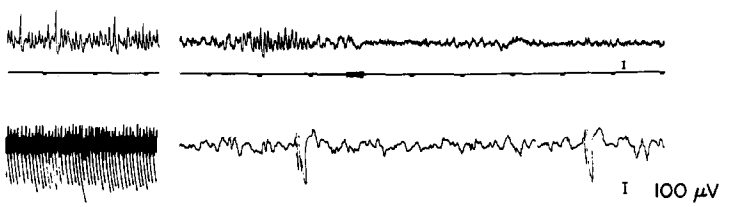

FIG. 1. Electrocorticogram (ECoG) of the mouse before and 7 hr after the intravenous injection of Azotomycin. For recording of the electrocorticogram (ECoG), three mice were implanted with cortical electrodes under pentobarbital sodium anesthesia (65–70 mg/kg, i.p.). Stainless steel screws (00–90, 1/8 in.) with attached connector pins (Amphenol 220–501) served as electrodes and were placed epidurally bilateral and 1 mm anterior of bregma and lambda. An indifferent electrode was placed in the frontal bone. Electrodes and pins were attached to the skull with dental resin (Meyerson's Tooth Corp., Cambridge, Mass. 02138). Postoperatively, the mice were treated with benzathine penicillin, 50,000 units. The mice were, thereafter, housed individually in plastic cages to minimize mechanical loss of electrodes. The ECoG was monitored in bipolar fashion by cable connectors leading to a Grass polygraph (model 7). At least two control recording sessions were performed prior to treatment, and the ECoG was recorded while the mice were awake, drowsy or asleep. Each mouse was treated with a single i.v. injection of Azotomycin (NSC-56654), 900 mg/kg.

Top record: At the left is shown the pretreatment ECoG during synchronization; at the right, during arousal. The time marker is set at 1-sec intervals; the calibration marking is 100 μV. The horizontal bar indicates stimulation by noise. Bipolar recordings. Bottom record: At the right is shown the ECoG 7 hr after treatment with Azotomycin, 900 mg/kg. Note the appearance of spikes and slow waves. At the left a slower paper speed better demonstrates the rhythmic spike discharges during approximately 6-sec intervals.

Three hr after the intravenous injection of Azotomycin, 900 mg/kg, the electrocorticogram (ECoG) showed a few isolated spikes and slow waves. These changes were followed by occasional trains of repetitive spikes with a frequency of 9–11 c/s. Four hr after treatment, the ECoG exhibited frequent sharp waves and epileptic slow waves, while at 5 hr, touching the mice triggered a pattern of spikes and slow waves. At this time, affected mice also showed spontaneous jerky body movements and jumping, together with ECoG seizures lasting 1·5 sec. Seven hr after treatment, repetitive spikes at 7-sec intervals occurred, and 25 hr after treatment, the ECoG showed slow wave activity, interrupted by occasional low voltage seizures and burst discharges. This neurotoxic action of Azotomycin contrasts with that of previous studies in which DON was given intracerebrally to rats (50 μg/rat) without evidence

of central nervous damage.[18] Azaserine, on the other hand, is well known for its neurotoxicity, intracerebral injections producing paraplegia.[19]

It is instructive to recall that L-methionine-*dl*-sulfoximine, (MS) was discovered on the basis of its convulsant properties. In order to determine the basis for this neurotoxicity, the enzymes of the CNS utilizing L-glutamine were examined in experimental subjects given MS. Eventually it was discovered that L-glutamine synthetase of brain was inhibited *in vivo* by L-methionine-*dl*-sulfoximine given at convulsant doses;[20] moreover, the inhibition was irreversible by virtue of the fact that L-methionine-*dl*-sulfoximine was covalently bound at the catalytic site of the enzyme.[21] As a consequence of the inactivation of the enzyme, the concentration of cerebral L-glutamine fell precipitously.[10]

When the convulsant action of Azotomycin was first observed, it was logical to suppose that the drug also was inhibiting cerebral L-glutamine synthetase. However, direct measurements of the enzyme in brain homogenates from mice convulsing as a consequence of high doses of Azotomycin failed to show inhibition (Table 2). Separate experiments with DON given to mice intraperitoneally at doses ranging from 500 to 1500 mg/kg also failed to provide evidence of inhibition of cerebral L-glutamine synthetase.

It was therefore decided to monitor the influence of Azotomycin on the concentration of L-glutamine in the cerebrum of intoxicated mice, in the event that the drug was interfering with the utilization of L-glutamine, as opposed to its production. L-Glutamine is known to donate its amide to a plentitude of metabolically important receptors (see Chart 1), and DON is known to inhibit virtually all of these donations. It seemed likely that cerebral L-glutamine might be elevated during Azotomycin-induced convulsions, especially in view of the failure of the drug to inhibit the synthesis of L-glutamine.

However, as is shown in Table 2, this was found not to be the case: the concentration of L-glutamine was not significantly altered in the brains of mice given convulsant doses of DON or of Azotomycin. Also evident from the data of Table 2 is the observation that cerebral L-glutaminase is rather resistant to inhibition by Azotomycin *in vivo* following doses of 900 mg/kg. However, *in vitro*, the drug did prove to be a moderate inhibitor of the amidohydrolysis of L-glutamine catalyzed by crude cerebral supernatants. Thus, when incubated along with 0·01 M L-glutamine and a 50,000 *g* supernatant prepared from mouse brain, 0·2 M Azotomycin brought about a 38 per cent inhibition of soluble cerebral L-glutaminase; 0·02 M Azotomycin brought about only 24·3 per cent inhibition. Under the same circumstances, DON at 0·2 M inhibited the amidohydrolysis of L-glutamine by 84 per cent; 0·02 M DON produced only 30 per cent inhibition. In absolute terms, the inhibitory actions of these diazoketones toward mammalian cerebral L-glutaminase stand in marked contrast to the interaction of DON with the comparable enzyme from *Escherichia coli*. Hartman[22] has reported that DON, at 1×10^{-3} M, titrates and totally inhibits bacterial L-glutaminase in the absence of substrate. It is thus possible that the relatively high levels of L-glutamine present during the incubations described in the present studies have operated to diminish the apparent inhibitory potency of DON and Azotomycin. A shielding action of L-glutamine also would be operative *in vivo* where the concentrations of the amide range from 6×10^{-4} M in plasma to 2×10^{-3} M in liver. It should also be pointed out that the techniques used to prepare

TABLE 2. EFFECT OF PARENTERAL AZOTOMYCIN AND DON ON THE SYNTHESIS AND CONCENTRATION OF THE AMIDES OF THE DICARBOXYLIC AMINO ACIDS IN MOUSE BRAIN HOMOGENATES*

Drug	L-Asparagine synthetase (nmoles/g/hr)	L-Glutamine synthetase (μmoles/g/hr)	L-Glutaminase (μmoles/g/hr)	L-Asparagine (nmoles/g)	L-Glutamine (μmoles/g)	L-Aspartic acid (μmoles/g)	L-Glutamic acid (μmoles/g)	Protein synthesis (% Inhibition) time after dosing (2 hr)	(4 hr)	(7 hr)
Saline (0·25 ml)	66·00 ± 18·30	255·00 ± 25·00	94·80 ± 4·80	132·30 ± 36·00	4·48 ± 1·00	4·00 ± 0·43	11·00 ± 1·00	0	0	0
Azotomycin† (900 mg/kg)	27·45 ± 16·80‡	299·40 ± 66·00	97·60 ± 8·60	46·56 ± 21·00‡	3·65 ± 1·10	6·47 ± 1·90	12·80 ± 1·90	26	20	20
DON (600 mg/kg)	19·04 ± 13·90‡	298·12 ± 33·00	95·20 ± 4·20	83·60 ± 57·00‡	4·54 ± 1·40	5·90 ± 1·30	10·70 ± 1·90			

* Groups of ten mice were sacrificed 24 hr after administration of saline or the drugs. Measurements of enzymes and substrates were carried out as described in Methods. Each value represents the mean ± standard deviation of duplicate determinations on the brains of ten mice.

† Doses of Azotomycin of 90, 9 and 0·9 mg/kg produced 51, 47 and 23 per cent inhibition of cerebral L-asparagine synthetase.

‡ This group significantly different from the controls; $P < 0·01$.

organ extracts have largely eliminated mitochondria, so that the interaction of Azotomycin with the amidohydrolase from these organelles remains to be assessed.

From the data in Table 2 it can also be appreciated that convulsant doses of Azotomycin significantly inhibited the synthesis of L-asparagine in the brain. So far as is known, this is the first demonstration of an action of the drug *in vivo* against L-asparagine synthetase. As a consequence of this inhibition, levels of L-asparagine in the brain fell significantly and the levels of L-aspartic acid tended to increase.

L-Asparagine has no known neurotransmitter function. Nevertheless, it is possible that a diminished pool size of L-asparagine prevents normal neuronal protein synthesis. This situation would be analogous to that produced in many organs by the action of L-asparaginase. Enzymes responsible for the production of neuroinhibitors might be among those whose synthesis would be impaired by a scarcity of L-asparagine. An excitable state would be the logical sequel of this postulated chain of events. It was beyond the scope of the present study to determine whether Azotomycin specifically inhibited the synthesis of enzymes responsible for elaborating neuroinhibitors; nevertheless, single convulsant doses of the drug were found to inhibit the incorporation of radioactive L-valine into cerebral proteins by ~ 26 per cent at the time of a convulsive episode. The inhibition was persistent; cerebral protein synthesis had not normalized by 7 hr after dosing. Other L-glutamine antagonists, L-methionine-*dl*-sulfoximine and δ-hydroxylysine, are also known for their ability to inhibit protein synthesis.[23]

In an attempt to counteract the neurological toxicity of Azotomycin, several putative antidotes were tested: (1) L-asparagine, because of the depressed levels of this amide seen in convulsing subjects; (2) L-glutamine, because of the known interaction of Azotomycin and DON with amidotransferases; and (3) adenine, in the hope of circumventing the blockade of purine biosynthesis brought about by Azotomycin. Additionally, L-asparaginase was administered to mice given Azotomycin in order that the effect of more profound depletion of the concentration of L-asparagine might be examined. In Table 3, the incidence of overt neurologic dysfunction in recipients of drugs and antidotes has been tabulated. L-Glutamine, given subcutaneously at a dose of 1 g/kg, appeared to antagonize the convulsive action of Azotomycin, although neurotoxicity in the form of ataxia was still present. It is noteworthy, though, that the subcutaneous administration of L-glutamine to control subjects failed to elevate the concentration of L-glutamine in the brains of the recipients, probably because this amino acid cannot penetrate the CNS to an important degree.[24] The similarity in structure and polarity of Azotomycin and DON to L-glutamine makes it likely that these diazoketones would also be excluded by the blood–brain barrier. Our observation that Azotomycin fails to inhibit cerebral L-glutaminase significantly *in vivo*, as opposed to its inhibitory action *in vitro*, supports this contention. Of the other putative antidotes or synergists studied, none interacted in a pronounced way with either the pharmacologic or biochemical actions of Azotomycin on the CNS.

In order to determine whether the derangement of L-asparagine metabolism produced by Azotomycin would disturb the homeostasis of any other amino acids, an analysis of brain homogenates from mice given the drug was carried out. L-Glutamic acid, a putative neurotransmitter, increased significantly in the brains of intoxicated mice from a mean of 10·3 μmoles/g wet wt to a mean of 13·5 μmoles/g wet

TABLE 3. CONCENTRATION OF L-GLUTAMINE IN MOUSE BRAIN AND LIVER AFTER PRETREATMENT WITH POTENTIAL ANTAGONISTS OF AZOTOMYCIN AND DON*

Pretreatment drug (dose and route)	Challenging drug (dose and route)	Condition (at 6 hr)	Incidence of convulsions	Condition (at 24 hr)	Incidence of convulsions	L-Glutamine (μmoles/g) Liver	Brain
L-Asparaginase	Saline	Normal	0/10	Normal	0/10	1·35 ± 0·03	3·34 ± 1·09
(10^5 i.u./kg, i.v.)	Azotomycin (900 mg/kg, i.v.)	Ataxia	0/10	Ataxia, diarrhea	0/10	5·79 ± 0·83	3·40 ± 0·46
	DON (600 mg/kg i.v.)	Ataxia	0/10	Irritability, ataxia, unkemptness, diarrhea, spasticity	2/10	3·06 ± 0·95	3·95 ± 0·99
L-Asparagine	Saline	Normal	0/10	Normal	0/10	1·51 ± 0·89	3·34 ± 1·09
(1 g/kg, s.c.)	Azotomycin (900 mg/kg, i.v.)	Ataxia	3/10	Ataxia, diarrhea	0/10	4·72 ± 1·67	3·17 ± 1·03
	DON (600 mg/kg, i.v.)	Ataxia, paralysis	5/10	Ataxia, irritability	1/10	7·67 ± 2·34	4·21 ± 1·03
L-Glutamine	Saline	Normal	0/10	Normal	0/10	1·26 ± 0·44	2·37 ± 0·34
(1 g/kg, s.c.)	Azotomycin (900 mg/kg, i.v.)	Ataxia	0/10	Slight ataxia, diarrhea	0/10	5·57 ± 1·21	3·32 ± 0·55
	DON (600 mg/kg, i.v.)	Ataxia	1/5	Spasticity, ataxia	4/5	4·69 ± 0·81	3·91 ± 0·75
Adenine-HCl	Saline	Normal	0/10	Normal	0/10	1·41 ± 0·65	2·21 ± 0·41
(100 mg/kg, s.c.)	Azotomycin (900 mg/kg, i.v.)	Severe ataxia	2/10	Slight ataxia, diarrhea	0/10	4·53 ± 0·24	3·24 ± 1·66
	DON (600 mg/kg, i.v.)	Ataxia	3/10	Spasticity, ataxia, diarrhea	4/10	6·90 ± 1·44	4·01 ± 1·41
Saline	Saline	Normal	0/10	Normal	0/10	1·47 ± 0·24	2·42 ± 0·45
(0·25 ml, i.v.)	Azotomycin (900 mg/kg, i.v.)	Ataxia	7/10	Spasticity, ataxia, diarrhea	2/10	4·34± 1·41	3·69 ± 0·74
	DON (600 mg/kg, i.v.)	Ataxia	7/10	Ataxia, diarrhea	3/10	3·31 ± 1·05	4·21 ± 1·01

* One hr before administration of the challenging drugs, groups of ten male Swiss mice were given the pretreatment drugs at the doses and by the routes indicated. L-Asparagine, L-glutamine and adenine-HCl were suspended in steroid suspending vehicle. Twenty-four hr after the challenge, animals were killed by cervical dislocation and the concentration of L-glutamine in brain and liver was determined by the methodology given in Methods.

wt; gamma amino butyric acid, the decarboxylation product of L-glutamic acid, also increased from a mean of 0·46 μmole/g wet wt to a mean of 0·54 μmole/g wet wt, but this increase was not significant.

Stewart *et al.*[25] have recently stressed that the administration of L-glutamate to rats at a dose of 20 m-moles/kg gave rise to vigorous epileptiform convulsions. After such a dose, the immediate plasmatic concentration of drug would approximate 0·02 M. Since, however, only a small fraction of this lipid-insoluble injectate would penetrate the cerebrum, it is likely that the concentration of L-glutamate in the nervous system would not be elevated greatly from its basal level by such a challenge. In the present studies, Azotomycin augmented the concentration of cerebral L-glutamate from 0·010 to 0·0135 M. Thus, the observed alterations in the concentrations of this dicarboxylic acid may be implicated in the pathogenesis of Azotomycin's toxicity.

Hepatotoxicity of Azotomycin

In the course of acute lethality studies in mice, it was observed that Azotomycin, at the LD_{50} or above, induced a pronounced yellow discoloration of the liver. This finding suggested that the drug was steatogenic in mice. Biochemical analyses confirmed this supposition. Total hepatic lipids had risen dramatically 24 hr after a single dose of 300 mg/kg and remained elevated for another 72 hr (Table 4). Simultaneous with these fatty changes, hepatic glycogen also was found to increase in concentration, whereas hepatic free glucose was depressed on the first day after administration of the drug.

Ogura *et al.*[26] have reported that selective amino acid imbalance can give rise to hepatic steatosis. L-Asparaginase is also known to produce fatty liver in the monkey. In view of these precedents, and because of the derangements in the metabolism of the dicarboxylic amino acids which Azotomycin produces in the CNS, the levels of L-asparagine, L-glutamine and their synthetases were studied in treated mice with steatosis (Table 5).

Hepatic L-glutamine rose dramatically after a single dose of 900 mg/kg of Azotomycin (Tables 3 and 5); on the other hand, hepatic concentration of L-asparagine was not significantly altered by the drug, a finding which contrasts with the cerebral action of Azotomycin. Inhibition of soluble hepatic L-glutaminase was demonstrated (Table 5) *in vivo* as well as *in vitro*. In both cases, Azotomycin inhibited the enzyme of liver far more strongly than it did that of the CNS. *In vitro*, a 0·2 M concentration of drug reduced amidohydrolysis to 10 per cent of that seen in the uninhibited system, while 0·02 M Azotomycin halved hepatic L-glutaminase activity. Quantitatively similar patterns of inhibition were produced by DON.

Not shown in Table 5 is the effect of graded intravenous doses of Azotomycin on hepatic L-glutaminase: in mice receiving only saline, the mean rate of hydrolysis of L-glutamine by liver supernatants was 32 μmoles deamidated per g, wet wt per hr; doses of Azotomycin of 900, 450, 225 and 112 mg/kg reduced hydrolysis to 35, 60, 75 and 97 per cent respectively, of this control rate.

In liver, as in brain, no evidence for inhibition of L-glutamine synthetase was found. On the other hand, hepatic L-asparagine synthetase was strongly inhibited by Azotomycin, to a degree similar to that seen with the cerebral enzyme. It is perplexing that Azotomycin also was found to produce significant inhibition of hepatic L-asparaginase 24 hr after a single dose of 900 mg/kg, inasmuch as the drug fails to

TABLE 4. LIPID AND CARBOHYDRATE LEVELS IN THE LIVERS OF MICE TREATED WITH AZOTOMYCIN AND DON*

Drug (dose)	Total lipid Day 1 (mg/g)	(% of control)	Day 4 (mg/g)	(% of control)	Glucose (μmoles/g)	(% of control)	Glycogen (mg/g)	(% of control)
Saline (0·25 ml)	38·4 ± 6·7	100	42·3 ± 11·2	100	9·9 ± 3·4	100	251·0 ± 144·7	100
Azotomycin (300 mg/kg)	79·8 ± 13·7	207·8	167·2 ± 36·2	395·3				
Azotomycin (600 mg/kg)	85·3 ± 11·3	222·1	141·5 ± 38·1	334·5				
Azotomycin (900 mg/kg)	69·6 ± 21·6	181·3			4·9 ± 0·9	49·5	419·0 ± 188·3	166·9
DON (600 mg/kg)	72·4 ± 31·3	188·5			5·3 ± 1·0	53·6	405·5 ± 153·0	161·5

* On days 1 and 4 after the intravenous injection of Azotomycin, DON or saline at the doses indicated, livers were removed and processed as described in Methods for the measurement of lipids and carbohydrates.

TABLE 5. EFFECT OF AZOTOMYCIN AND DON ON CONCENTRATION AND SYNTHESIS OF THE DICARBOXYLIC AMINO ACIDS AND THEIR AMIDES IN MOUSE LIVER HOMOGENATES*

Drug dose	L-Asparagine synthetase (nmoles/g/hr)	L-Glutamine synthetase (μmoles/g/hr)	L-Glutaminase (μmoles/g/hr)	L-Asparaginase (μmoles/g/hr)	L-Asparagine (nmoles/g)	L-Glutamine (μmoles/g)	L-Aspartic acid (μmoles/g)	L-Glutamic acid (μmoles/g)	L-Alanine (μmoles/g)
Saline (0·25 ml)	48·9 ± 40·4	3·52 ± 0·70	52·8 ± 11·5	72·08	148·0 ± 20·0	4·17 ± 0·66	1·09 ± 0·20	2·27 ± 0·40	4·74 ± 1·10
Azotomycin† (900 mg/kg)	27·1 ± 21·4‡	3·91 ± 0·73	7·98 ± 5·6‡	35·68‡	115·0 ± 35·0	6·70 ± 0·61	0·84 ± 0·30	1·34 ± 0·30	3·71 ± 0·40
DON (600 mg/kg)	21·9 ± 8·6‡	3·61 ± 0·41	9·68 ± 5·3‡		120·2 ± 39·0	16·73 ± 7·21‡	0·77 ± 0·10	2·41 ± 0·90	3·62 ± 0·70

* Measurements of enzymes and amino acids were performed as described in Methods. Animals were sacrificed 24 hr after exposure to the respective drugs. Means and standard deviations have been calculated for each group containing ten mice.

† In BDF_1 mice bearing subcutaneous leukemia 5178Y resistant to L-asparaginase, intraperitoneal doses of Azotomycin of 900, 90, 9 and 0·9 mg/kg produced 90, 88, 76 and 76 per cent inhibition of hepatic L-asparagine synthetase. The possibility that a fraction of the activity measured in this instance might have been due to metastasis was not ruled out.

‡ This group is significantly different from the saline control group; $P < 0·01$.

inhibit the same enzyme *in vitro*.[4] This discrepancy might be explained by postulating that the biosynthesis of L-asparaginase had been interrupted by Azotomycin as a consequence of its inhibition of protein synthesis in general.

Because Azotomycin produced such a striking increase in hepatic free L-glutamine, it seemed important to determine the influence of the drug on the remaining free amino acids of the liver. Chromatographic analysis on the amino acid analyzer confirmed the marked elevation of L-glutamine which had been seen in enzymatic assays. The concentration of L-glutamate was also doubled. Apart from these findings, however, and despite the marked steatosis seen in these livers, the concentrations of the remaining amino acids were surprisingly little affected. The concentration of NAD in these fatty livers was also unchanged. Thus, in 5% perchloric acid extracts of the livers of mice receiving saline, the mean hepatic concentration of NAD was 476·6 nmoles/g; while in animals given Azotomycin, 900 mg/kg 24 hr earlier, the mean concentration of this pyridine nucleotide was 494·9 nmoles/g. On the other hand, Azotomycin did significantly depress hepatic ATP from a mean of 1·07 ± 0·10 μmoles/g in the control group to 0·58 ± 0·06 μmoles/g in the treated group.

Action of Azotomycin on the gastrointestinal tract

Another salient manifestation of Azotomycin toxicity is the profuse diarrhea which begins about 18 hr after large single intravenous doses and persists to death. Histopathologically, the intestines of intoxicated mice show acute colitis with dilatation of glandular crypts, crypt abscesses, loss of goblet cells, and a pseudomembrane composed of polymorphonuclear leukocytes covering the denuded mucosa. In an attempt to investigate the causes of the intestinal lesions in mice, biochemical studies analogous to those described above were carried out on homogenates of the duodenum and colon. To avoid contamination in making measurements of intestinal L-asparagine synthetase, care was taken to strip off as much as possible of the pancreas, which in contrast to the bowel proper, carries out a very vigorous synthesis of L-asparagine.[27] The intestine also was irrigated with saline in an effort to remove bacteria.

Azotomycin was found to inhibit small and large intestinal L-asparagine synthetase, but not to a significant degree (Table 6), and no significant depression of intestinal L-asparagine ensued as a consequence of the inhibition. In this last regard, then, the bowel behaved like the liver rather than the brain. Several explanations for this failure of Azotomycin to depress intestinal L-asparagine can be given: (1) absorption of amino acids from the bowel lumen can very likely counteract any tendency of the drug to depress the intracellular concentration of L-asparagine; (2) circulating L-asparagine doubtless gains access to the cells of the bowel (and of the liver) more readily than to the neurons of the CNS. Thus, whereas the blood–brain barrier might impede the entry of L-asparagine into the brain (and it is pertinent to recall that the concentration of L-asparagine in CSF is approximately one-fifth that in the plasma[16]), no such barrier is likely to be operative in the case of the bowel. The dynamic flux of amino acids across the bowel probably also masked the effect of Azotomycin on the gastrointestinal concentration of free L-glutamine.

Attempts to reverse the gastrointestinal toxicity of Azotomycin with subcutaneous injections of L-asparagine or L-glutamine at doses of up to 1 g/kg were unsuccessful (Table 3).

Table 6. Effect of Azotomycin on the concentration and synthesis of the amides of the dicarboxylic acids in mouse intestines*

Drug	Dose	L-Asparagine synthetase (nmoles/g/hr)		L-Glutamine synthetase (μmoles/g/hr)		L-Asparagine (nmoles/g)		L-Glutamine (μmoles/g)	
		Large intestine	Small intestine	Large intestine	Small intestine	Large intestine	Small intestine	Large intestine	Small intestine
Saline	0·25 ml	32·52 ± 15·8	15·22 ± 5·82	10·26 ± 7·9	4·78 ± 0·6	109 ± 90	689 ± 230	1·58 ± 0·5	4·19 ± 0·8
Azotomycin†	900 mg/kg	10·28 ± 3·7	9·46 ± 4·19	10·32 ± 6·9	4·98 ± 0·6	122 ± 60	453 ± 380	2·12 ± 0·7	2·79 ± 1·5

* Animals were sacrificed 24 hr after administration of Azotomycin or saline. Enzymes and amino acids were measured as described in Methods.

† Doses of Azotomycin of 90, 9 and 0·9 mg/kg produced 45, 51 and 42 per cent inhibition of large intestinal and 58, 56 and 52 per cent inhibition of small intestinal L-asparagine synthetase.

TABLE 7. EFFECT OF AZOTOMYCIN ON THE BIOSYNTHESIS OF NUCLEIC ACIDS IN THE SPLEEN, LARGE AND SMALL INTESTINES OF THE MOUSE*

Treatment	Specific activity of nucleic acids: Nucleic acid (cpm/mg)	Inhibition (%)	Specific activity of isolated purines: Adenine (pCi/nmole)	Inhibition (%)	Guanine (pCi/nmole)	Inhibition (%)
Spleen						
Saline	55,610 ± 5130	0	4590 ± 1470	0	11,180 ± 1560	0
Azotomycin (900 mg/kg)	2790 ± 300†	95·0	130 ± 50†	97·1	9 ± 10†	99·9
Azotomycin (100 mg/kg)	5210 ± 2190†	90·7				
Azotomycin (20 mg/kg)	3070 ± 1010†	94·5				
Azotomycin (1 mg/kg)	7520 ± 380†	86·5				
Azotomycin (0·1 mg/kg)	42,940 ± 8020	22·8				
Azotomycin (0·01 mg/kg)	47,140 ± 3750	15·3				
Large intestine						
Saline	10,050 ± 7030	0	1670 ± 1580	0	1640 ± 1380	0
Azotomycin (900 mg/kg)	360 ± 270†	96·5	230 ± 30†	86·5	100 ± 140†	93·9
Azotomycin (100 mg/kg)	460 ± 110†	95·4				
Azotomycin (20 mg/kg)	730 ± 160†	92·7				
Azotomycin (1 mg/kg)	1070 ± 60†	89·4				
Small intestine						
Saline	45,770 ± 5600	0	1850 ± 30	0	6490 ± 5160	0
Azotomycin (900 mg/kg)	2020 ± 460†	95·6	20 ± 30†	99	65 ± 40†	99·0
Azotomycin (100 mg/kg)	1030 ± 260†	97·8				
Azotomycin (20 mg/kg)	650 ± 160†	98·6				
Azotomycin (1 mg/kg)	1010 ± 910†	97·8				

* Injection of mice, sample removal and isolation of nucleic acids were performed as outlined in Methods; values given are ± the standard deviation.

† This group is significantly different from the saline controls; P = 0·01.

In addition to these actions on amide metabolism, Azotomycin also was found to inhibit purine biosynthesis in both intestines to a highly significant extent, even at doses of 1 mg/kg (Table 7). It therefore participates in the generalization: agents which interrupt the synthesis of nucleic acids tend to be toxic to the gut. It is pertinent to recall that gastrointestinal toxicity in man is a formidable side effect of chemotherapy with this family of diazoketones.

Influence of Azotomycin on splenic structure and function

The spleens of animals dying in the acute and subacute lethality studies were found to be markedly atrophic. Histopathologically the splenic lesions were charac-

terized as focal necrosis of the Malpighian corpuscles and depression of the extramedullary hematopoiesis which is ordinarily active in the spleen of the mouse. These effects of the drug *in vivo* confirm the lymphotoxic action of Azotomycin which Hersh *et al.*[28] have described *in vitro* using mitogen-stimulated lymphocytes. In an effort to understand the biochemical basis of such effects, measurements were made of the pool size of L-glutamine in the spleens of mice given large intravenous doses of the drug. A modest elevation of splenic L-glutamine was demonstrated 24 hr after dosing (Table 8). The enzymatic synthesis of L-glutamine was found to be unaltered; conversely, the synthesis of L-asparagine was inhibited powerfully, as a consequence of which the concentration of L-asparagine fell. This fall probably contributes to the depression of protein synthesis seen in the spleen at 2 and 4 hr after administration of Azotomycin. Chromatographic analysis demonstrated an increase in the concentration of virtually all the free amino acids in the spleens of treated mice. Most dramatically elevated was L-threonine, whose concentration rose from a mean of 343 nmoles/g wet wt in the controls to 880 nmoles/g wet wt in the mice given Azotomycin. This result is consonant with the inhibition of protein synthesis in this organ.

TABLE 8. INFLUENCE OF AZOTOMYCIN AND DON ON THE CONCENTRATION AND SYNTHESIS OF THE AMIDES OF THE DICARBOXYLIC ACIDS IN MOUSE SPLEEN*

Drug (dose)	L-Asparagine synthetase (nmoles/g/hr)	L-Glutamine synthetase (μmoles/g/hr)	L-Asparagine (nmoles/g)	L-Glutamine (μmoles/g)	Protein synthesis (% inhibition) Time after dosing (2 hr)	(4 hr)
Saline (0·25 ml)	84·0 ± 4·0	2·0 ± 0·3	225·8 ± 82·0	1·4 ± 0·5	0	0
Azotomycin† (900 mg/kg)	18·0 ± 7·0‡	2·2 ± 0·2	172·4 ± 9·0	1·0 ± 0·9	43	47
DON (600 mg/kg)	14·0 ± 7·0‡	2·2 ± 0·2	209·1 ± 17·0	0·9 ± 0·5		

* Animals were sacrificed 24 hr after administration of Azotomycin, DON or saline. Enzymes and amino acids were measured as described in Methods. Means and standard deviations have been calculated for each group containing ten mice.

† Doses of Azotomycin of 90 and 0·9 mg/kg produced 44 and 40 per cent inhibition of splenic L-asparagine synthetase under comparable conditions.

‡ This group is significantly different from the control; $P < 0·005$.

Since purine biosynthesis also is known to proceed at an active rate in the mouse spleen, attempts were made to assess it in animals given graded single doses of Azotomycin. At all doses down to 0·01 mg/kg, perceptible impairment of the incorporation of [^{14}C]-Na formate into DNA was demonstrable (Table 7). This extraordinary potency is analogous to that of Duazomycin A.[29]

Cumulative toxicity of Azotomycin

The cumulative toxicity of Azotomycin is extreme; the LD_{50} of 262 mg/kg for a single dose falls to 0·86 mg/kg for five daily doses (Table 1) and to 0·6 mg/kg for six daily doses (not shown). The maximum molarity of drug reached *in vivo* after the last-mentioned dose would be approximately 3×10^{-6} M. In the studies reported above, large single doses of Azotomycin proved to be effective inhibitors of L-asparagine synthetase. In order to determine whether inhibition of this enzyme could be

considered to play a pathogenetic role in subacute intoxication with Azotomycin, the influence of repeated small daily doses of the drug on L-asparagine synthetase of brain and pancreas was studied. The latter organ is included because its content of synthetase is 10–100 times higher than that of the brain, so that smaller degrees of inhibition become more readily and reliably perceptible. The results of these studies are summarized in Table 9. It can be seen that one dose of 0·86 mg/kg given intravenously did not materially alter the L-asparagine synthetase of the brain, but did depress the pancreatic enzyme by greater than 60 per cent. When the dose was repeated for four additional daily injections, the synthesis of L-asparagine in the mouse pancreas was brought to a virtual standstill; the cerebral synthetase remained resistant.

TABLE 9. INFLUENCE OF REPEATED DOSES OF AZOTOMYCIN ON L-ASPARAGINE SYNTHETASE IN THE BRAIN AND PANCREAS OF MICE*

	Day of sacrifice	L-Asparagine synthetase (nmoles/g/hr) Brain	Pancreas
Saline (0·25 ml × 1)	2	136 ± 10	3410 ± 490
Azotomycin (0·8 mg/kg × 1)	2	148 ± 30	1210 ± 380
Saline (0·25 ml × 5)	5	181 ± 30	3340 ± 370
Azotomycin (0·8 mg/kg × 5)	5	236 ± 100	238 ± 80
Saline (0·25 ml × 1)	5	184 ± 80	4175 ± 960
Azotomycin (400 mg/kg × 1)	2	101 ± 10	223 ± 20
Azotomycin (400 mg/kg × 1)	5	177 ± 20	865 ± 100

* Azotomycin was administered to six mice per group via a caudal vein on what was designated day 1. Animals were sacrificed on the days indicated and assays performed as outlined in Methods.

Degradation of Azotomycin

Each mole of Azotomycin contains 2 moles DON. In comparative studies, the two drugs have been found to produce similar biochemical and toxicological effects. This qualitative similarity raised the possibility that Azotomycin was, in fact, liberating DON *in vivo*, and that DON was the proximate cause of the several pharmacologic effects described above. Other workers have argued that this was the case: thus, Brockman *et al.*[1] showed that mouse plasma and the proteolytic enzyme, Pronase, can both decompose Azotomycin.

In our attempts to investigate this possibility, two experimental approaches were taken; in the first of these, a search was made for enzymes capable of degrading Azotomycin to its constituent parts. Advantage was taken of the finding that DON can be resolved readily from Azotomycin by high voltage electrophoresis as well as of the finding that L-glutamic acid, arising from the hydrolysis of Azotomycin, can be measured spectrophotometrically.[15] When the drug was exposed to the plasma of the mouse *in vitro*, a significant hydrolysis of Azotomycin to DON was observed

(Fig. 2) as judged by the more rapid decrease of Azotomycin than of antimicrobial activity. Under these conditions, the half-life of Azotomycin *in vitro* approximated 15 min; *in vivo*, the half-life of the drug is at least ten times shorter than this. For example, in the BDF_1 mouse 1·2 min after after the intravenous injection of Azotomycin, 100 mg/kg, a concentration of 10 μg/ml of blood was detected by microbiological techniques, whereas no drug was present 5 min after dosing. Binding to plasma proteins was demonstrated to be insignificant both by ultrafiltration experiments and by incubation with bovine serum albumin. It follows that the major part of an injection of Azotomycin would be distributed to spaces other than the plasma before significant hydrolysis could occur. Urine is one such space (Fig. 2, insert), feces is another (Fig. 2, legend), and the intracellular compartment is a third.

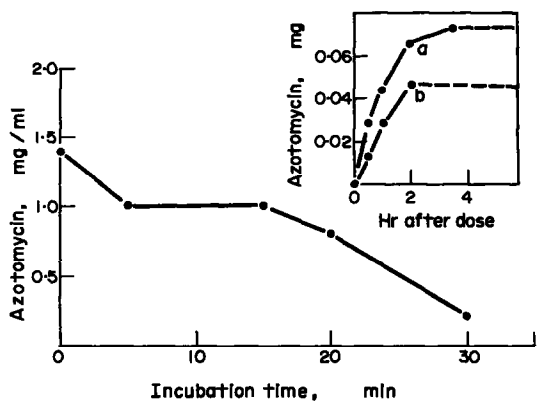

FIG. 2. Destruction of the antimicrobial activity of Azotomycin by the plasma of the BDF_1 Mouse. Azotomycin, 1 mg, was added to 1 ml of heparinized mouse plasma and the mixture incubated at 37° for 30 min. At the intervals indicated, aliquots were removed, subdiluted serially with sterile saline and tested for "antimicrobial activity" against a strain of *E. coli* sensitive to Azotomycin and, to a lesser extent, DON. Results are expressed as mg/ml Azotomycin equivalents. Azotomycin was first separated by electrophoresis before being quantitated microbiologically. The apparent increase in the concentration of Azotomycin antimicrobial activity at zero time results from a nonspecific enhancement of the activity of Azotomycin in this microbiological assay by uncharacterized factors present in plasma.

Insert: Urinary excretion of Azotomycin in the mouse. The urine from five BDF_1 male mice was pooled after the intravenous administration of 100 mg/kg of Azotomycin. "Antimicrobial activity" (a) was determined on an aliquot of the pooled urine, and Azotomycin (b) was determined as above. The cumulative urinary excretion of Azotomycin in this experiment did not exceed 1 per cent of the injectate; an additional 2·2 per cent of the administered dose of the drug was found in the feces.

It therefore became important to determine whether the organs which are most sensitive to the toxic effects of Azotomycin would also be capable of degrading the drug to DON. Extracts of brain, liver, spleen and intestine all exhibited this capability (Table 10). Of these, the duodenum proved to be the most vigorous catalyst. When the organ extracts and plasma samples from various species were heated at 80° for 30 sec, their ability to decompose Azotomycin was destroyed, a finding which furnished presumptive evidence that the mechanism is enzymatic. Further evidence was provided by the observation that purified hog intestinal peptidase was able to destroy greater than 80 per cent of a 1 mg/ml solution of Azotomycin in 0·1 M phosphate buffer at pH 8·0 during a 30-min incubation at 37°.

The relative ability of various organ homogenates to degrade Azotomycin *in vitro* was closely reflected in the concentration of drug measurable *in vivo* in the several tissues surveyed shortly after the intravenous administration of doses of 100 mg/kg. Thus, in liver, at 1·2, 1·7 and 2·5 min after injection of the drug, antimicrobial activity equivalent to 4, 2 and 1 μg Azotomycin per liver was detected, yielding an approximate hepatic half-life of about 0·5 min. In the small intestine, peak levels at 1·2 min were roughly half those found in the liver, and no drug whatsoever could be measured at the latter two time periods, so that the half-life must have been less than 0·25 min.

TABLE 10. DECOMPOSITION OF AZOTOMYCIN BY MURINE ORGAN HOMOGENATES*

	Generation of L-glutamic acid (nmoles/min/g wet wt)
Small intestine	24·10
Liver	19·90
Spleen	14·40
Brain	7·19
Large intestine	6·80

* Freshly excised organs of adult male Swiss mice were homogenized 1:10 (w/v) in 0·1 M Tris–HCl (pH 7·6) rendered 0·5 mM in EDTA and 0·001 M in dithiothreitol. The homogenates were dialyzed against 1 l. of homogenizing medium for 2 hr; then, 20 μl homogenate was incubated with 500 μl of 0·02 M Azotomycin for 5, 10 and 15-min. The amount of L-glutamic acid generated was determined spectrophotometrically. The rate of hydrolysis was calculated from the linear segment of the time course.

When Azotomycin was degraded by a crude murine hepatic supernatant, and electrophoresed on paper, quantitative measurements of the principal spots revealed that each mole of Azotomycin gave rise only to 1·7 moles DON instead of the 2 moles required by theory. Two explanations might be offered for this sub-stoichiometric effect: (1) there is selective loss of DON in the analysis, as for example by an electrolytic process (indeed the diazoketones are known to be labile under a variety of conditions); or (2) DON is destroyed by the crude enzyme system to a small extent. The former possibility was not explored extensively in the present study. However, attempts to demonstrate destruction of DON by hepatic extracts, using disappearance of the 274 ultraviolet maximum of the compound as the criterion of hydrolysis, did reveal a slow decline in absorbance. It is highly likely, too, that DON, as a primary acid whose stereochemistry resembles that of both L-glutamate and L-glutamine, can enter into intermediary metabolic pathways. Evidence has already been presented which indicates that the compound is a substrate for one of the hepatic transaminases.[4] Moreover, it is possible that DON, like gamma glutamylhydrazide, is itself incorporated into proteins as a fraudulent amino acid.[30] This utilization of one of the products of the hydrolysis of Azotomycin would, of course, account for the observed stoichiometrics. Such an effect also might be responsible for some of the cumulative toxic actions of the drug reported here.

While the aforementioned studies make it likely that Azotomycin undergoes biotransformation to DON, they do not rule out the possibility that Azotomycin, of itself, is capable of exerting pharmacologic effects. In order to explore adequately this possibility, it would be necessary to compare the interaction of both Azotomycin and of DON with purified preparations of the numerous enzymes catalyzing amido donation from L-glutamine, because these are the systems which DON is known to inhibit (cf. below). Since such purified enzymes would presumably be free of the peptidase activity which decomposes Azotomycin to DON, any inhibition seen could be ascribed to the intact drug as opposed to a product thereof. Brockman *et al.*,[1] for example, studied the interaction of DON and Azotomycin with a purified phosphoribosylamine synthetase and demonstrated that the tripeptide was ineffective, whereas the diazoketone was a powerful inhibitor.

Inasmuch as Azotomycin can inhibit crude L-asparagine synthetase, and in view of the fact that a purified preparation of mammalian synthetase was available, this system was selected for further study. Crude L-asparagine synthetase of tumor was included for purposes of comparison, since this preparation has been shown to decompose the antibiotic. Azotomycin was found to be a relatively ineffective inhibitor of the purified enzyme but an active inhibitor of the crude; DON was inhibitory toward both (Table 11).

TABLE 11. INTERACTIONS OF AZOTOMYCIN AND DON WITH CRUDE AND PURIFIED PREPARATIONS OF L-ASPARAGINE SYNTHETASE*

Source	Amide donor	Azotomycin (% inhibition)	DON (% inhibition)
Crude L-asparagine synthetase	L-Glutamine	75	75
	Ammonia	8	8
Purified L-asparagine synthetase	L-Glutamine	16	88
	Ammonia	21	26

* Azotomycin (1×10^{-3} M) and DON (1×10^{-3} M) were incubated with crude L-asparagine synthetase from 100,000 *g* supernatants of the 6C3HED-AR lymphosarcoma. Ammonia and L-glutamine were present in concentrations of 0·05 and 0·02 M respectively.[4] The L-asparagine synthetase was 45-fold purified from Novikoff hepatoma to a specific activity of 1084 nmoles L-asparagine synthesized/mg protein/hr.

DISCUSSION

L-Glutamine is known to donate its amide to at least 11 important receptors. The pertinent reactions are presented in Chart 1, along with the concentration of DON necessary to bring about significant inhibition of each. In most cases, comparable data on Azotomycin were unavailable.

In mammals, the reaction most sensitive to DON is usually considered to be the conversion of formylglycinamide ribonucleotide to formylglycinamidine ribonucleotide; the K_i is roughly 1×10^{-6} M. In bacteria sensitive to DON, the synthesis of guanylic acid appears to be most susceptible to diazoketone inhibition, with a K_i of 1×10^{-9} M. From the tabulation, it is clear then that this family of drugs can exert a profound effect on a broad variety of biosynthetic reactions, the inhibition of any single one of which could produce lethal toxicity. However, when these reactions are collectively inhibited, the toxicologic effect becomes devastating.

CHART 1. HYDROLASE AND AMIDOTRANSFERASE REACTIONS UTILIZING L-GLUTAMINE—INHIBITION BY DIAZOKETONE ANTIBIOTICS

Enzyme or reaction	EC No.	Enzyme source	% Inhibition of Don	Reference
Carbamyl phosphate synthetase	6.3.5.x	Fetal rat liver Ehrlich ascites Walker carcinoma	72 at 1×10^{-4} M	Yip and Knox[31]
Guanosine monophosphate synthetase	6.3.5.2	Rabbit erythrocyte	97 at 6×10^{-4} M	Lowry *et al.*[32]
Anthranilic acid synthetase	6.3.5.x	*Salmonella typhimurium*	50 at 3×10^{-5} M	Nagano *et al.*[33]
L-Glutaminase	6.3.5.x	*E. coli*	100 at 1×10^{-3} M (absence of substrate)	Hartman[34]
Glucosamine synthetase	6.3.5.x	*E. coli* Rat liver	50 at $1{\cdot}1 \times 10^{-3}$ M* 50 at $4{\cdot}1 \times 10^{-6}$ M	Ghosh *et al.*[35]
α-Ketoglutarate amidotransferase	6.3.5.x	*B. megaterium*	Unknown	Elmerich and Aubert[36]
Amidation of L-Gln-tRNA	6.3.5.x	*B. megaterium*	Unknown	Wilcox and Nirenberg[37]
5-Phosphoribosyl-1-pyrophosphate amidotransferase	6.3.5.x	Adenocarcinoma 755	50 at $1{\cdot}2 \times 10^{-5}$ M	Hill and Bennett[38]
Formylglycinamide amidotransferase	6.3.5.3	Pigeon liver	50 at $1{\cdot}1 \times 10^{-6}$ M†	Levenberg *et al.*[39]
L-Asparagine synthetase	6.3.5.4	Human KB tumor	71 at 1×10^{-3} M‡§	Haskell and Canellos[3]
Cytidine triphosphate synthetase	6.3.5.x	*E. coli*	98 at 2×10^{-3} M	Long *et al.*[40]
Diphosphopyridine nucleotide synthetase	6.3.5.1	Yeast	100 at 1×10^{-4} M	Yu and Dietrich[41]

* Azaserine inhibited this reaction 12% at $9{\cdot}2 \times 10^{-4}$ M.
† Azaserine inhibited this reaction 95% at $2{\cdot}0 \times 10^{-6}$ M.
‡ Azotomycin inhibited this reaction 50% at $1{\cdot}0 \times 10^{-3}$ M.
§ Azotomycin inhibited this reaction 70% at $2{\cdot}5 \times 10^{-3}$ M.

Although it is possible that the marked elevation of L-glutamine in the liver of animals intoxicated with Azotomycin arises in large part from an interruption of the utilization of that amino acid, it is also likely that the inhibition of hepatic L-glutaminase by the drug contributes to this effect. Indeed, the findings that cerebral L-glutamine does not accumulate after the administration of Azotomycin and that cerebral L-glutaminase is unaltered by high parenteral doses of the drug are taken as ancillary evidence in favor of this conclusion.

That L-glutamine is not the only amide whose metabolism is deranged by Azotomycin (and by DON) is indicated by the present studies *in vivo* of L-asparagine synthetase and L-asparagine homeostasis in the organs of mice given large single or small repeated doses of the drug. Both modes of administration brought about a notable curtailment of the biosynthesis of L-asparagine, as a consequence of which the organ concentration of L-asparagine tended to be depressed. This depression is all the more significant in view of the finding that hepatic soluble L-asparaginase, which is a prime candidate for the role of negative regulator of the level of free L-asparagine, was inhibited *in vivo* by Azotomycin.

The inhibition of the biosynthesis of L-asparagine was especially dramatic in the pancreas, most likely because this is an organ which synthesizes L-asparagine at a preeminently vigorous rate in mammals. It can be speculated that the ability of Azotomycin to depress the intracellular concentration of L-asparagine provides a partial explanation for the oncolytic action of the drug against tumors with feeble L-asparagine synthetase.

While the present experiments clearly indicate that Azotomycin, like Duazomycin A and DON, produces powerful cumulative toxicity, they have not identified the biochemical lesion principally responsible for this toxicity. Nevertheless, it is warranted to suggest that the interruption of purine synthesis is probably instrumental in the production of damage to organs with rapidly dividing cells, and that the interruption of L-asparagine biosynthesis is probably contributory to the damage produced by Azotomycin in nondividing organs such as the liver and brain.

Lastly, our studies of the enzymatic degradation of Azotomycin to DON make it probable that Azotomycin is functioning as a stable "pro-drug" from which the active and proximate antimetabolite, DON, is generated both intracellularly and extracellularly as a consequence of peptidasic attack. This conclusion is entirely in accord with that of Brockman *et al.*,[1] who utilized plasma and Pronase to demonstrate the lability of Azotomycin. It is very likely that DON is responsible for interrupting the biosynthesis of the purines and pyrimidines of glucosamine and, as was demonstrated here for the first time *in vivo*, of L-asparagine. Among the fraudulent amino acids, then, these diazoketones must be viewed as exceptionally successful imposters.

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