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INIT 87/14/94

74I-0794-001211



Mr. Martin Greif
Executive Secretary
TSCA Interagency Testing Committee
Environmental Protection Agency TS-792
401 M Street, S.W.
Washington, D.C. 20460



84940000275

Contains No GAF

Re: OPTS 41012 BH-FRL 2462-1,
Chemicals to be reviewed by the
Toxic Substances Act Interagency
Testing Committee; Request for
Information (48 FR 51519, November 9, 1983)

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Dear Mr. Greif:

GAF Corporation (GAF), a basic chemical manufacturer, is the major U.S. producer of 2-Pyrrolidinone (CAS #616-45-5), and is providing these comments and data in response to the request for information on the inclusion of 2-Pyrrolidinone among candidates for possible recommendation to the Administrator of the U.S. Environmental Protection Agency (EPA), to be given priority consideration for the promulgation of testing rules pursuant to Section 4(a) of TSCA.

According to the scoring sheet detailing the results of the Fifth ITC Scoring Exercise, 2-Pyrrolidinone was designated as "No experimental evidence, but no reason for suspicion," for Mutagenicity, Carcinogenicity, Reproductive effects, Bioaccumulation, and Environmental effects. 2-Pyrrolidinone was designated as, "Needs testing; markedly suspect based on possible functional relationship to known teratogen" for teratogenicity, and as "Needs testing; some reason for suspicion" for Other Toxic Effects. Additionally, environmental exposure indices of 0.51 (occ), 0.33 (GP), and 0.33 (ECO) were assigned.

GAF believes that sufficient data does exist to remove 2-Pyrrolidinone from the 1983 List of Chemicals, and that exposure has been overestimated.

Although over 10,000,000 lbs. of 2-Pyrrolidinone were produced in the U.S. during 1983, it should be designated as a 'Chemical of low exposure potential.' Fully 94% of this production is utilized by GAF for the syntheses of N-substituted analogues and their polymers, and should, therefore, be considered as a site limited intermediate. Less than 2% is actually sold in the U.S., and over half of this is for a pharmaceutical application. This regulated use, outside the EPA's jurisdiction, cannot result in general population exposure. Of the remaining material, an insignificant amount (less than 0.25%) ultimately finds its way out of an occupational setting into those uses listed in the Effects Information Profile (floor waxes and inks). 2-Pyrrolidinone should not have made the working list based on this volume.

2-Pyrrolidinone is an endogenous chemical and found as a natural constituent of foods (1-5). The available toxicity data coupled with the extensive data available on N-substituted analogues, all indicate a low level of concern for 2-Pyrrolidinone. Indeed, this low level is reflected in the scores given by the ITC's fifth scoring exercise. The two areas questioned have been adequately investigated, as discussed later, and no significant toxicity demonstrated.

GAF respectfully submits that a sufficiently detailed toxicological profile already exists for the volume and application of 2-Pyrrolidinone, and that this material should be dropped from further consideration.

I. Exposure

GAF Corporation manufactures and supplies approximately 98% of the 2-Pyrrolidinone used in the U.S. and is, therefore, in a position to make accurate estimates of exposure. Although more than 10,000,000 lbs. are produced annually, 94% is used as a site limited intermediate. Of the remaining, over 5% is exported or used in FDA regulated products outside of EPA jurisdiction. Thus, less than 0.5% is used in EPA regulated areas. GAF estimates that one-half of this is used as a solvent in an industrial setting, and one-half in consumer products.

A. Occupational Exposure

2-Pyrrolidinone is manufactured and processed by GAF in entirely closed systems -- partially under positive pressure, and partially under vacuum conditions. Because of the closed processing, occupation's exposure to 2-Pyrrolidinone is limited to the filling of containers, sampling, and equipment maintenance. Less than 25 employees are directly involved in the manufacture or downstream processing of 2-Pyrrolidinone. Were each operator, lab employee, maintenance and warehouse worker to be included, the total still would not exceed 300.

B. General Population Exposure

General population exposure to this product is quite low. Only 30,000 lbs. of the total production of 2-Pyrrolidinone is sold into areas where the general population could be exposed. The remainder is site limited for the production of downstream products, used in pharmaceuticals with no general consumer exposure, or as an industrial solvent.

C. Environmental Exposure

The fraction of 2-Pyrrolidinone released to the environment is insignificant. Potential release points are monitored during production to assure the absence of 2-Pyrrolidinone.

Reaction residues remaining are discharged to either waste disposal wells, or treated in an activated sludge treatment plant. Reactor cleaning water is similarly routed to the respective treatment systems.

In summary, the Environmental Exposure to 2-Pyrrolidinone is minor. The closed process, limited number of employee potential exposure, the sound operational practices, and the primary use as a site limited intermediate, support the designation of lower level exposure potential and should not have resulted in selection of 2-Pyrrolidinone for biological effects scoring.

II. Biological Effects

The data available to GAF and supplied as part of this submission, sufficiently support 2-Pyrrolidinone as presenting a low hazard potential for which sufficient data already exists.

A/B. Mutagenicity/Carcinogenicity

2-Pyrrolidinone was given a score of -1 for both categories, indicating no experimental data, but no reason for suspicion. GAF agrees that there is no reason for suspicion. 2-Pyrrolidinone is an endogenous compound isolated from mouse brains, and shown to be a metabolite of putresane, which is a precursor of spermidine and spermine (3). In addition, 2-Pyrrolidinone is both a precursor and a metabolite of γ -amino butyric

acid (GABA) (4), an important inhibitory transmitter in the central nervous system (5). 2-Pyrrolidinone is found as a natural component of foods, as well (2). These data suggest that the "lack of suspicion" is highly reliable. The lack of specific data should not be considered a negative point in an area where there is no reasonable anticipation of activity. Additionally, GAF Corporation has an extensive background in N-substituted pyrrolidinones. Pharmacokinetic data support the N-dealkylation to form 2-Pyrrolidinone as the metabolic pathway (6). In vitro mutagenicity batteries on these materials show no activity for these materials (7).

C. Teratogenicity

2-Pyrrolidinone was given a numerical score of -2 indicating "needs testing." 2-Pyrrolidinone has been tested for teratogenic effects in mice by interperitoneal and oral administration, and in rats after oral administration (8).

In the first study, NMRI mice were exposed to the test agent intraperitoneally or orally from the 11th-15th day of pregnancy. On the 19th day, all treated and untreated mice were sacrificed, the uteri removed, the implantation and fetal resorption sites recorded, the number of live and dead fetuses, and their body lengths recorded, and weight and sex were determined. The fetuses were then subjected to gross and microscopic examination.

I.P.

Two test groups consisting of 14 dames each received grade dose levels of 2-Pyrrolidinone on the 11th to 15th day post coitum for a total of 5 injections. The high dose group received a dose equivalent to one-half the ALD_{50} , and

the low dose group received a dose equivalent to one-fifth the ALD_{50} (1750 mg/kg and 700 mg/kg respectively). Each group was compared to an individual untreated control. The resorption rate was increased in both test groups (10.8% and 6.8% increase over control respectively), but no difference was evident for fetus weight, length, or number of runts. The number of deformities, at 3.4% and 2.9%, was a small increase over controls of 1.4% and 0.8%, but no other deformities were seen in comparison to controls.

These changes, however, are attributable to non specific effects associated with this route of administration. The increase in cleft palates is a known spontaneous deformity in the mice used, and is a function of stress and ambient conditions.

Oral:

Again, two test groups of 12 and 13 dams received doses corresponding to one-half and one-fifth of the oral LD_{50} (2900 mg/kg and 1160 mg/kg, respectively).

These doses were well tolerated. The only deviations from control are not considered biologically significant.

In summary, 2-Pyrrolidinone has an embryotoxic effect at these doses, but should not be considered as presenting any teratogenic hazard based on these studies in mice.

In a third study, 20 Sprague-Dawley rats were given an oral dose of 1700 cm^3/kg daily from the 6th-15th day post coitum. The 10 doses were well tolerated and no dose related effects were observed. It was, therefore, concluded that 2-Pyrrolidinone did not demonstrate a teratogenic effect in rats.

Based on these data, 2-Pyrrolidinone should be re-scored and given a 0 for teratogenicity.

D. Reproductive Effects

2-Pyrrolidinone was given a score of -1 indicating "Needs testing, no reason for suspicion." GAF agrees that there is no reason for suspicion, but feels there is also no need for testing. The teratogenicity study run in two species clearly indicates that reproductive effects are not seen. Again, GAF maintains that not conducting a specific assay which both experimental and literature data indicate as unnecessary, and for which there is no reasonable suspicion, should not be given a negative connotation.

E. Acute Toxicity

2-Pyrrolidinone was given a score of 0, designating this material as very slightly toxic. GAF agrees with this designation.

F. Subchronic and Other Toxic Effects

2-Pyrrolidinone was given a score of -2, indicating "Needs testing; some reason for suspicion." GAF does not believe subchronic testing is necessary, or would yield any significant data. Again, 2-Pyrrolidinone is an endogenous chemical whose uncyclized congener GAF is well studied. Subchronic studies are available on N-methylpyrrolidone (NMP), whose probable metabolism yields 2-Pyrrolidinone (6). When NMP was fed to wistar-derived rats at levels up to 5000 ppm of their diet, no biologically significant differences between the test group and controls were observed. An EPA

evaluation of this data resulted in a conclusion that the no effect level in male and female rats was 1000 ppm (9).

A similar study run on Charles River mice showed only statistically significant, but biologically irrelevant changes in spleen weight at the highest dose (2500 ppm) for females, and at the two highest levels for the males (1000, 2500 ppm). No histopathologic changes were noted in the spleen, nor in any tissue examined.

When male and female beagle dogs were exposed to NMP at levels of 25, 79, and 250 mg/kg, no statistically significant treatment-related effects were seen in any parameter examined (10).

Application of a 100 fold safety factor for change in structure would still result in a score of 0 for 2-Pyrrolidinone following the ITC scoring criteria.

Although the specific areas for which the scoring team has expressed "some reason for suspicion" are not known to GAF, it is felt that the applications of the 2-Pyrrolidinone, coupled with the available data on the N-Methyl analogue do not support the need for additional testing (10).

G. Bioaccumulation Potential

2-Pyrrolidinone was given a score of -1, indicating "no data; bioaccumulation not expected." GAF agrees with this assessment. Measurements made on the N-vinyl analogue following a single IV injection, yielded a half-life for intact compound in the plasma of 1.9 hrs (6). Additionally, 2-Pyrrolidinone is completely miscible in water. These data suggest a score of 0 is more reflective of the bio-accumulation potential.

H. Biological Effects

2-Pyrrolidinone was given a score of -1, indicating again "No data, but no reason for suspicion." However, data does exist in the literature which show 2-Pyrrolidinone to be readily biodegradable, and to have a low degree of aquatic toxicity (11).

$BOD_5/COD = 0.720$ and BOD_5 (acclimated) was equal to 1.39.

Aquatic toxicity assays show 2-Pyrrolidinone to have no effect on the algae *Selenastrum capricornutum* when tested at levels as high as 100 mg/l. Additionally, the aquatic LC_{50} for the crustacean *Daphnia magna* was determined to be 3.4 mg/l, and the LC_{50} for the Fish *Pimephales promelas* was greater than 100 mg/l.

Following the scoring system, 2-Pyrrolidinone should be given a 0 based on these data.

In conclusion, GAF believes that 2-Pyrrolidinone should not be recommended to the Administrator of the U.S. EPA for promulgation of testing rules pursuant to Section 4(a) of TSCA, as it is a small volume chemical in commerce and has a sufficiently detailed toxicological profile available to demonstrate its safety for its limited uses.

Sincerely yours,



J. N. Ansell, Ph.D.
Manager, Regulatory Science

JMA:bf

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FLUE-CURED TOBACCO FLAVOR. I. ESSENCE AND ESSENTIAL OIL COMPONENTS¹

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In a continuing investigation of tobacco constituents, a chloroform extract of aged flue-cured tobacco was examined. The extract after removal of the bases was molecularly distilled to provide a "flue-cured essence." The flue-cured essence was separated by distillation and chromatography. Isolation of the essence components was accomplished by gas chromatography on packed columns. Individual GC peaks were trapped and identified by comparison with IR, NMR and/or mass spectra and GC retention times of authentic samples when available.

In a second study, an essential oil, obtained by extraction of the condensate from steaming of flue-cured tobacco, was investigated. High-resolution glass capillary gas chromatography was employed to separate the complex fractions obtained by column chromatography of the essential oil. The effluent from the capillary columns was passed directly into a mass spectrometer for structural analysis. Identifications were made from mass spectra and in almost all cases were confirmed by comparison of GC retention times to those of authentic samples.

A total of 323 compounds was identified in flue-cured tobacco. Of these 275 have not been previously reported as flue-cured constituents, and 132 are new to all tobacco types. Compounds previously unreported in the literature but not identified compounds were adequate to characterize tobacco as tobacco. The identification of sufficient quantities

INTRODUCTION

In recent years there has been considerable work on elucidating the constituents of various types of tobacco. S. J. ...'s review (37) listed a large number of tobacco constituents. More recently, Roberts and Rhode (30), and Demole and Berthet, (7,8) have published isolation studies on burley tobacco. Likewise, Schumacher and Vestal (35) and Kimland, Aasen *et al.*, (1-4, 21-24) have reported extensive investigations of Turkish and Greek tobaccos.

In this paper we are reporting the results of a thorough study of the volatile components of a high quality flue-cured tobacco. The two materials studied, an extract of flue-cured tobacco and an essential oil from steaming flue-cured tobacco, possessed the characteristic flue-cured flavor; their breakdown was undertaken to better understand the nature of the flue-cured tobacco flavor.

METHODS AND INSTRUMENTATION

Gas chromatographic separations were carried out on Varian Aerograph 2800 series instruments equipped for flame ionization or thermal conductivity detection, and subambient temperature programming. Packed column gas chromatography was carried out on 2 mm i.d. x 10 ft glass columns (Varian) packed with 15% FFAP or 10% OV-101 batch coated (11) on 100-120 mesh Chromosorb W-AWDMCS (Applied Science Laboratories) which had been previously fluidized in a hot nitrogen stream to remove fines. Surface preparation of glass capillary columns was carried out by variations of the methods of Grob (13) and Novotny (25, 26). Columns were coated by the dynamic method (26) with 10% OV-101 or SF-96 in toluene and 10% FFAP in methylene chloride.

Varian 1700 and 2800 gas chromatographs were coupled directly to a Varian Mat CH-5 Mass Spectrometer. Glass capillary analyses were carried out without a separator with the column effluent split approximately 1:1 to the mass spectrometer and to atmospheric vent.

EXPERIMENTAL

Investigation of a Flue-Cured Tobacco Extract

The chloroform extract obtained from 829 lb of cigarette cut B4F flue-cured tobacco (1967 South Carolina crop) was partitioned against 2% sulfuric acid to remove the bases and alkaloids. The base-free extract concentrate (19.8 lb) was molecularly distilled to yield ca. 580 g of flue-cured essence. Crystalline scopoletin was removed from the essence by centrifugation. Figure 1 diagrams the separation scheme for the essence.

The components of the fractions shown in Figure 1 were separated by repetitive liquid column and gas chromatography and characterized by their spectral properties.

All compounds identified from the essence are indicated in Table 2.

The following thirteen compounds have not been previously reported in the literature. They were isolated from flue-cured tobacco essence obtained as described and identified by spectral methods. Spectral data are given for each compound.

1,9-Dimethyl-12-isopropyl-5-methylene-3,8,13-cyclotetradecatrien-1-ol (53)

MS: MW 288; 41(100), 54(50), 43(47), 91(34), 81(33)

IR: 3450, 1600, 1257, 966, 880 cm^{-1}

NMR: δ 5.83(4), 5.55(1), 4.98(2), 2.30(2), 1.52(3), 1.28(3), 0.85(6)

3-Methylene-7,11,15-trimethylhexadecan-1-ol (68)

¹ Presented at the CORESTA Symposium 1974, Montreux, Switzerland, September 24-27, 1974. Contribution received September 5, 1975; *Tob. Sci. XX: 43-51, 1976.*

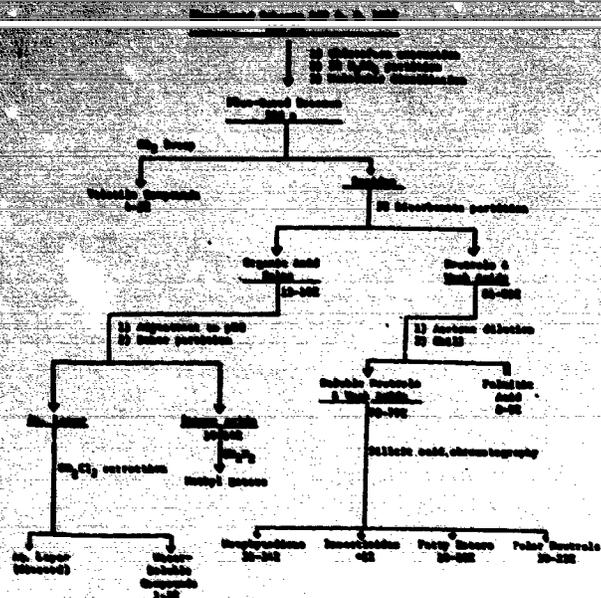


Figure 1. Separation Scheme for Fine-Cured Tobacco Essence

- MS: MW 296; 43(100), 58(99.9), 57(99.6), 41(81), 71(79)
 IR: 3270, 1590, 1020, 890 cm^{-1}
6-Methyl-6,9-oxido-4-nonen-2-ol (72)
 MS: MW 170; 111(100), 43(94), 45(57), 69(51), 41(48)
 IR: 3400, 2950, 1365, 1120, 1076, 1030, 970 cm^{-1}
 NMR: δ 5.5(2), 3.76(3), 1.88(3), 1.17(3)
3,7,11,15-Tetramethyl-1-hexadecen-3-ol (77)
 MS: MW 296; 43(100), 123(98), 57(98), 95(93), 52(92)
 IR: 3350, 1146, 990, 913 cm^{-1}
4,8,12-Trimethyltridec-3-ene-2,8-diol (81)
 MS: MW 256; 43, 55, 69, 81, 93, 67, 79, 91, 107, 109, 105, 123, 218
 IR: 3350, 1666, 1445, 1365, 1042, 972, 900, 840 cm^{-1}
 NMR: δ 5.65(1), 2.05(2), 1.72(3), 1.28(3), 1.15(3), 0.85(6)
2-Ethylidene-3-methylsuccinimide (173)
 MS: MW 139; 96, 68, 67, 53, 139
 IR: 1770, 1720, 1680, 1340, 1250, 1205, 1140, 1035 cm^{-1}
 NMR: δ 6.78(1), 3.31(1), 1.95(3), 1.45(3)
4-(2-Buten-3-on-1-yl)-3,5,5-trimethylcyclohexanone (291)
 MS: MW 208; 43(100), 123(82), 41(25), 109(18), 124(15)
 IR: 2950, 1710, 1675, 1628, 1360, 1250, 1165, 1105, 1050, 980 cm^{-1}
4-(2-Hydroxybutylidene)-3,5,5-trimethyl-2-cyclohexene-1-one (211)
 MS: MW 208; 45(100), 149(98), 164(48), 43(44), 42(33)
 IR: 3450, 2950, 1665, 1595, 1460, 1390, 1325, 1125, 945 cm^{-1}
 NMR: δ 6.08(1), 5.86(1), 4.00(1), 2.55(2), 2.30(3), 2.14(2), 1.26(9)
2-Isopropenyl-3,5,5-trimethyl-2-cyclohexen-1-one (216)

- 30(33)
 IR: 1688, 1608, 1579, 1506, 1254, 1249, 898 cm^{-1}
 NMR: δ 5.83(2), 2.28(2), 2.15(2), 1.89(6), 1.06(6)
4-(1-Propenyl)-3,5,5-trimethyl-2-cyclohexen-1-one (246)
 MS: MW 178; 43(100), 135(44), 178(27), 105(27), 121(23)
 IR: 1680, 1280, 1242, 1205, 872, 835 cm^{-1}
3,7,11,15-Tetramethyl-7-hexadecen-6-one (243)
 MS: MW 294; 43, 41, 84, 57, 55, 69, 71, 294
 IR: 2900, 1670, 1460, 1375, 1190 cm^{-1}
2,2-Dimethyl-4-hydroxy-6-oxoheptanoic acid lactone (262)
 MS: MW 170; 43(100), 41(32), 55(23), 39(20), 58(15)
 IR: 3400, 2940, 1765, 1710, 1250, 1200, 1145, 1120, 1050, 910 cm^{-1}
 NMR: δ 4.67(1), 2.80(2), 2.15(3), 2.00(2), 1.20(6)
2,6-Dioxa-5-methylbicyclo[3.3.0]octan-3-one (267)
 MS: MW 142; 71(100), 43(99), 114(78), 41(40), 27(35)
 IR: 3400, 2950, 1170, 1120, 1060, 945 cm^{-1}
 NMR: δ 4.7(1), 4.37(2), 2.90(2), 2.40(2), 1.65(3)

Investigation of a Fine-Cured Tobacco Essential Oil

Two hundred seventy-five gallons of condensate from steam distillation of 4200 lb of blended fine-cured tobacco were collected. The aqueous condensate was saturated with sodium sulfate and partitioned against 2 x 50 gal of distilled chloroform. The aqueous layer was discarded and the chloroform solution was concentrated under vacuum at 50°C to a volume of about 14 l. This solution was partitioned against two 2-liter portions of 10% tartaric acid solution to remove nicotine and other bases. The alkaloid-free chloroform solution was treated with anhydrous sodium sulfate and concentrated to a residue of 347.8 g (278.2 g solvent-free basis).

During final concentration of the chloroform solution on a rotary evaporator, some of the more volatile material was entrained with the solvent vapor into the condensed chloroform. The last three liters of chloroform removed were redistilled under vacuum through a Vigreux column, and the residue was subjected to direct GC/MS analysis.

Two hundred grams (160 g solvent-free basis) of the base-free condensate extract were chromatographed over pH 7 silicic acid with pentane-ether mixtures to give seven main fractions, data for which appear in Table 1.

Table 1. Essential Oil Main Fractions

Fraction No.	Percent of Total "Dry Weight"
1	11.23
2	2.31
3	14.04
4	4.23
5	48.21
6	2.28
7	1.54
	64.37% Recovered

By repeated liquid chromatography and fractional distillation, these seven fractions were separated into a total of 107 subfractions, which were analyzed by direct capillary GC/MS. Table 2 shows compounds identified as components of this essential oil.

Table 2. Standard List of Compounds

Number	Compound Name	Common Name	Sweetness 1 = Slightly 2 = Essential Off	Smoke Flavor	Product Leaf Cigarette References
1	ACIDS				
2	Acetic		1, 2	Pungent, acidic	7, 27, 33, 37
3	Benzoic		1, 2	Weak, smothering	6, 7, 25, 33, 37
4	2-Furanoic	Formic	1, 2	Caramel, buttery, adds body	6, 23, 37
5	2-Furanoic	Formic	1, 2	Fatty	6, 23, 37
6	3,4-Di-methyl-2,5-hexanedioic	4-Dimethylsuccinic	1	Fruity	
7	3,4-Di-methyl-2,6-heptanedioic	6-Heptanoic	1	Spicy, nutty	8
8	3,4-Di-methyl-2,6-heptanedioic	6-Heptanoic	1	Fatty, adds body	16
9	2-(2-oxopropyl)-4,5-dimethylsuccinic	2-Methylglutamic	1		
10	2-(2-oxopropyl)-4,5-dimethylsuccinic	2-Methylglutamic	1	Soothy, sweet, slightly floral, fresh	16
11	2-(2-oxopropyl)-4,5-dimethylsuccinic	2-Methylglutamic	1	Smooth, sweet, nutty	19
12	2-Furanoic	Margeric	1	Waxy, sweet, nutty	23, 37
13	Heptanoic	Margeric	2	Waxy, waxy	23
14	Nonanoic	Polynic	2	Weak, nutty, butter, cream	6, 7, 23, 27, 30, 33, 37
15	11-Undecanoic	Polynic	2	Sweet, nutty, adds body	23, 27, 30, 33, 37
16	13-Tridecanoic	Polynic	2	Waxy, sweet, maple	6, 7, 23, 27, 30, 33, 37
17	2-Hydroxybenzoic	Salicylic	1	Weak	23
18	4-Hydroxy-3-methoxybenzoic	Vanillic	1	Weak, vanilla	30, 35, 37
19	2-Methylsuccinic	n-Valeric	1	Sweet, mellowing	8, 27
20	3-Methylsuccinic	n-Valeric	1	Sweet, nutty, spicy	
21	2-Methylbutyric	n-Valeric	1, 2	Green, smothering	23
22	3-Methylbutyric	n-Valeric	1, 2	Smothering, cream-butter, nutty	6, 7, 23, 30, 37
23	2-Methylbutyric	n-Valeric	1, 2	Sweet, winy, nutty, cheese	6, 23, 27, 30, 33, 35, 37
24	3-Methylbutyric	n-Valeric	1, 2	Smothering	
25	2-Methylpentanoic	n-Valeric	1, 2	Clove, spicy	7, 23, 37
26	3-Methylpentanoic	n-Valeric	1, 2	Sweet, cherry, fruity	6, 23, 27, 30, 33, 37
27	2-Methylhexadecanoic	n-Valeric	1, 2	Smothering, cheese, fruity	6, 23, 27, 35, 37
28	Nonadecanoic	n-Valeric	1	Sweet, adds body	23
29	2,6-Nonadecanoic	n-Valeric	1	Fatty, waxy	8, 23
30	Nonanoic	Pelargonic	1	Waxy, adds harshness	4, 7, 23, 27, 30, 34, 35, 37
31	9,12-Octadecadienoic	Pelargonic	1	Nutty, waxy	23, 30, 38
32	Octadecanoic	Stearic	1	Waxy, adds body, harshness	23, 30, 35, 37, 38
33	9,12-Octadecatrienoic	Linolenic	1	Waxy, waxy	23, 37, 38
34	9-Octadecenoic	Oleic	1	Spicy, waxy, smothering	23, 35, 37, 38
35	Octanoic	Caprylic	1, 2	Sweet, waxy, smothering	7, 23, 27, 30, 33, 35, 37
36	2-Octenoic	Caprylic	1, 2	Hot, bitter	23
37	3-Octenoic	3-Ketovaleric	1		37
38	2-Octenoic	Pyruvic	1		37
39	4-Oxononanoic	Pyruvic	1	Pungent, earthy, mushroom, adds body	8, 23, 30
40	Pentadecanoic	Valeric	1	Sweet, fruity, phosphate, buttery	27, 27
41	Pentanoic	Valeric	1, 2	Weak, nutty	6, 7, 23, 27, 30, 33, 37
42	Phenylacetic	Cinnamic	1	Smothering, weak, balsamic	6, 7, 23, 27, 30, 37
43	trans-3-Phenyl-2-propenoic	Cinnamic	1, 2	Pungent, acidic	15, 24
44	Propenoic	Myristic	1, 2	Smothering, sweet, waxy	6, 7, 23, 27, 30, 37
45	Tetradecanoic	Myristic	1, 2	Hot, nutty	23, 27, 30, 37, 38
46	3-(3,4,5-Trimethoxyphenyl)-2-propenoic	3,4,5-Trimethoxycinnamic	1	Hot, nutty, sour note	8
47	2,4,6-Trimethylcyclohexanecarboxylic	Undecanoic	1	Fatty, waxy, smothering	3, 27
48	Undecanoic	Undecanoic	1		
49	ALCOHOLS				
50	Benzyl alcohol		1, 2	Weak, floral, smothering	7, 23, 30, 35, 37
51	2,3-Butanediol		2	Green, chemical off-taste	
52	4,4-Dimethylcyclo[3.1.1]hept-7-yl methanol	Myristol	2		
53	alpha-1,5-Dimethyl-12-isopropyl-9-oxo-2,13-dioxo-7,8-oxide-2,13-cyclohexadecanone-1-ol	alpha-5,8-Oxido-2,9(17)-13-davatrien-1-ol	1		30, 37, 38
54	1,9-Dimethyl-12-isopropyl-5-methyl-3,8,13-cyclohexadecatrien-1-ol	3,5(16),8,13-Duvatrien-1-ol	1		
55	trans-3,7-Dimethyl-2,6-octadien-1-ol	Geraniol	2	Floral-soapy, green	7
56	3,7-Dimethyl-1,6-octadien-3-ol	Linalool	1, 2	Sweet, floral, citrus	7, 30, 37
57	3,7-Dimethylocten-3-ol	Tetrahydro-linalool	2	Floral, sweet	
58	2,4-Dimethyl-3,6-oxide-7-octen-2-ol	Linalool oxide	1	Sweet, smooth, adds body, valerian	7
59	Decanoal		1		30, 37
60	Eicosanal		1		37
61	Furfuryl alcohol		1, 2	Cereal, bran, oily, adds body	7, 37
62	Heptanal		2	Sweet, floral, winy	
63	cis-3-Hexen-1-ol	Leaf alcohol	1, 2	Green, leafy	7, 50
64	5-Hydroxy-4,7-dimethylbenzofuran	Menthol	2	Cooling	35, 37
65	2-Isopropyl-5-methylcyclohexanol	Solenol	1, 2	Sweet, floral, smothering	7, 30, 35
66	5-Isopropyl-8-methyl-6,8-oxodien-2-ol	Solenol	1, 2		30, 32
67	12-Isopropyl-5,8-oxide-1,8,9-trimethyl-3,9,13-cyclohexadecatrien-1-ol (alpha and beta isomers)	alpha-8,9,5,8-Oxido-3,9,13-duvatrien-1-ol	1		
68	2-(4-Methyl-3-cyclohexen-1-yl)-2-propanol	alpha-Terpineol	2	Pine, sweet, nutty	7
69	3-Methyl-7,11,15-trimethylhexadecan-1-ol	p-Cymen-8-ol	2		
70	2-(4-Methylphenyl)-2-propanol	p-Cymen-8-ol	2	Green, sweet	7, 30
71	4-Methyl-5-hepten-2-ol		1		
72	2-Methylhexan-2,5-diol		1		
73	4-Methyl-4,9-oxide-4-nonen-2-ol		2	Floral, fruity	
74	1-Octanol		2	Sweet, nutty, harsh	7, 24, 30, 35, 37
75	1-Phenylethanol		1, 2	Floral-rose	
76	2-Phenylethanol		1, 2	Hay, adds body	
77	Tetrahydrofurfuryl alcohol	Phytol isomer	1		
78	3,7,11,15-Tetramethyl-hexadecan-3-ol	Phytol	1	Green, slightly peppery	
79	3,7,11,15-Tetramethyl-3-hexadecan-1-ol	Phytol	1		

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TOBACCO INTERNATIONAL VOLUME 170 No. 7. Established in 1886 and published every other Friday by the Lockwood Trade Journal Company, Inc., 551 Fifth Avenue, New York, New York 10017, U.S.A. Second class postage paid at New York, N.Y. and at additional mailing offices. Publication Office at Lockwood Trade Journal Co., Inc., 20 McEvoy Ave., Llanarth, Pa., 17644. Officers: George E. Lockwood, President and Treasurer; George E. Lockwood, Jr., Vice President; Robert M. Lockwood, Vice President; Frederick E. Winkler, Secretary. Single copy price of regular issue: U.S.A., Canada, Mexico—\$3.25; all other countries—\$3.50. Subscription price: U.S.A., Canada and Mexico, \$62.50 per year; all other countries, \$65.00. Copyright 1975 by the Lockwood Trade Journal Company, Inc. The contents of TOBACCO INTERNATIONAL, including tobacco science and all articles, illustrations, etc., are copyrighted and may not be reproduced except by permission. Cable Address: LOCKNIN. Phone: Area Code 212-663-3000. Telex: 257623 LOCK 00.

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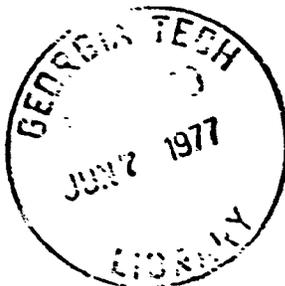
Circulation address:

TOBACCO INTERNATIONAL
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Volatile constituents of white bread crust

D. J. FOLKES* AND J. W. GRAMSHAW†



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Summary

A total of 190 volatile components were identified in essences prepared from white bread crust (in twenty cases the identification is provisional only); of these compounds, ninety-seven have not previously been reported as constituents of white bread. A limited number only, of the compounds now reported, may be partially responsible for the crusty notes which are present in bread aroma.

Introduction

In view of continuing interest in the volatile constituents of foodstuffs, we wish to record the preliminary results of our investigations into bread aroma. A detailed report, together with an assessment of the aroma of bread crust essences and the contribution which identified components make to the collective aroma, will appear later.

Freshly baked bread derives its characteristic aroma and, to a lesser extent, its flavour from volatile compounds formed during fermentation and baking. Indeed as Baker, Parker & Fortmann (1953) emphasized, fermentation followed by the formation of a brown crust is essential to the development of a full aroma and flavour. Thus, a conventionally fermented dough may be 'baked', by internal electrical resistance heating, without formation of a crust, but the product has a mild yeasty flavour, deficient in aroma (Baker & Mize, 1939). Similarly, chemically leavened doughs, when subjected to normal oven baking, have been reported to yield breads having very bland flavours (Miller, McWilliams & Matz, 1959; Wiseblatt & Kohn, 1960; Jackel & Ersoy, 1961) or no flavour (Chichester, Sharrah & Simone, 1960; Simone, Sharrah & Chichester, 1962). These, and other aspects of bread flavour formation are fully described in a comprehensive review by Collyer (1964).

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Compounds formed during fermentation and by Maillard-type reactions in the crust during baking doubtless contribute directly to the overall aroma of bread. However, it seems almost certain that organoleptically important constituents are formed by reactions which occur in the browning crust and involve precursors derived by fermentation. Experiments involving model systems (S.K. Berry & J.W. Gramshaw unpublished) support this view and have indicated that both acidic and non-acidic constituents of dough are important in the generation of crusty aromas.

Modern plant-produced bread is often considered to be somewhat deficient in flavour and aroma, this probably being a reflection of the short baking period normally employed which is insufficient for full flavour development, although adequate quantities of the relevant precursors may well be present. This investigation was therefore undertaken in an attempt to identify volatile compounds present in bread crust and, in particular, those responsible for crusty bread-like aroma notes. Such knowledge is essential before the formation of bread aroma can properly be understood and any logical attempt made to increase the flavour of bread produced in plant bakeries. It was thus appropriate to choose baking conditions as close as possible to those used for a traditional loaf.

Crust rather than whole bread was chosen for the preparation of essences, because, as the site at which organoleptically important constituents of the aroma complex form during baking, it contains these materials in highest initial concentration and thus much less material needs to be processed. It is necessary, however, to separate the crust before an appreciable proportion of these volatiles have an opportunity to migrate into the crumb. Use of crust alone gives a further advantage in that it produces essences which contain a much smaller proportion of the major fermentation volatiles, although ethanol and amyl alcohol nevertheless remain very prominent constituents of crust essences.

Materials and methods

Preparation of bread crust essences

Bread was produced using a standard formulation specifying flour, water, yeast and salt only. Initially, bleached flour was used, but in later experiments the flour was unbleached. The dough was bulk fermented, split, proved and baked as Coburgs (which are large round crusty loaves, normally baked on the oven sole or on a flat metal tray), rather than in a deep metal baking container, so that the maximum proportion of crust was formed. Baking time, in steam, was 40–45 min. i.e. well beyond the 25–35 min of commercial practice, in order to provide a thick dark brown crust of optimum flavour. Volatile constituents were isolated in a number of ways, of which the following was strongly preferred. As soon as possible after baking, the crust was separated from the crumb, finely comminuted and extracted with purified diethyl ether. Acidic

components, which otherwise complicate the GLC pattern and interfere in the subsequent mass spectrometric examination, were removed from the ethereal extract and examined separately. Removal of acids and water was achieved simultaneously by stirring with a mixture of anhydrous sodium carbonate and anhydrous sodium sulphate. Solids were removed by filtration and the extract was freed of ether and subjected to vacuum distillation in a closed system at a pressure of 0.01 Torr using the apparatus described by Lea, Swoboda & Hobson-Frohock (1967).

The distillate thus obtained was a pale yellow oil which retained its powerful crusty, bread-like aroma for considerable periods when stored at -20°C .

Absence of acids from the essence produced only a minor change in the overall aroma, although the isolated acidic fraction possessed a rich burnt note which probably contributes to a small extent to the total aroma of bread.

Instrumental examination of bread crust essences

In the early stages of the investigation, bread essences were subjected to gas-liquid chromatography (GLC), using a variety of stationary phases. A linearly temperature programmed oven and either a flame ionization detector (FID) or an electron capture detector (ECD) were employed and identification of constituents was made by comparison of their retention data with those of reference compounds. Certain major components were recovered from column effluents by trapping and the identities of these were confirmed from infrared spectra. A double beam grating instrument (Perkin-Elmer 237) was used and each sample was presented in solution (microcell) or as a film on a sodium chloride plate.

Later, essences were examined by linked gas-liquid chromatography-mass spectrometry (GLC-MS), using a fast scanning low-resolution mass spectrometer (Cronin, Nursten & Woolfe, 1972-73) and the identities of a considerable number of components were thus established (or confirmed) by comparison of their mass spectra and retention data with those of reference compounds. In this connection, porous layer open tubular (PLOT) columns (Cronin, 1970) and wide bore capillary columns, coated with a selection of stationary phases, were extensively employed, usually in conjunction with a FID. Further valuable information was gained by the use of element-specific chromatographic detectors. A nitrogen-specific alkali FID (AFID) and a flame photometric detector (FPD), used in the sulphur mode, were separately employed in conjunction with GLC-MS. GLC was also performed using both detectors simultaneously by splitting the effluent between them.

Sensory examination of bread crust essences

In addition to instrumental examination, effluents from the columns most frequently used were submitted to 'nasal appraisal'. This was performed by

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splitting the effluent between the instrumental detector and an odour port and writing descriptions of odour notes, as they emerged, on the chromatogram. In this way, particular odour notes were associated with the appropriate regions of the chromatogram and the most important regions were selected for more intensive study.

Nasal appraisal was also carried out in conjunction with the AFID and the FPD in order to correlate the elution of compounds containing nitrogen and/or sulphur with regions of bread-like aroma.

Results

Constituents identified in bread crust essences

The compounds which were detected in bread crust essences are listed in Table 1. Identities were assigned on the basis of retention data, positive responses (where relevant) to element-specific detectors and, in almost all cases except carboxylic acids and phenol, by mass spectra. A few major components (19, 23-25, 68, 70, 75, 77, 83, 112, 119, 120, 122) were also recognized on the basis of their infrared spectra. In a few cases identification is tentative, either because authentic compounds were not available for comparison or because the constituents were present at levels too low to give an unequivocal mass spectrum. Such exceptions are indicated in Table 1.

Sensory evaluation of essences

When the essences were subjected to nasal appraisal, a very large number of distinct aroma notes were perceived, very many of which had no obvious relationship to bread aroma although in total they may exert a minor influence on the overall aroma. However, twelve regions of the chromatograms were clearly associated with distinct and strong bread-like aroma notes. These, and the compounds associated with them, will be described in a later publication.

Discussion and conclusion

Several isolation techniques were examined in addition to that described above. This method was selected because it gave the optimum recovery of volatiles important to bread crust aroma, as judged on the basis of the following criteria:

- (a) the resulting essence had the strongest overall crusty aroma;
- (b) the resulting essence revealed the greatest number of bread-like aroma notes upon nasal appraisal and these notes were also more intense than those from essences prepared in other ways;

36	cyclopentanone (*) (a)	134	2-ethyl-6-methylpyrazine	176	dimethyl sulphide
37	2,3-butanedione (diacetyl)	135	2-propylpyrazine (+)	177	dimethyl disulphide
38	2,3-pentanedione	136	tetramethylpyrazine (*)	178	thiophene (*)
39	1-hydroxy-2-propanone (acetol) (*)	139	2,3-diethylpyrazine (*) (b)	179	2-methylthiophene (*)
40	2-hydroxy-3-butanone (acetoin)	140	2,5-dimethyl-2-ethylpyrazine (*) (b)	180	3-methylthiopropanal (*) (d)
41	3-penten-2-one	141	2,6-diethylpyrazine (*) (b)	181	2,3-dimethylthiophene (*)
42	2-cyclopenten-1-one	142	2,5-dimethyl-2-ethylpyrazine (*) (b)	182	2-acetylthiophene (*)
43	3-methylcyclopent-2-en-2-ol-1-one (*)	143	2,6-dimethyl-3-ethylpyrazine (*) (b)	183	2-formylthiophene
	<i>Aldehydes</i>	144	2-methyl-6-(and/or 5)-propylpyrazine (*) (b)	184	2-formyl-5-methylthiophene (*)
44	propanal	145	2,5-dimethyl-3-isopropylpyrazine (*) (b)	185	2-thiophenemethanol (*) (d)
45	butanal	146	vinylpyrazine (+)	186	benzthiophene (*)
46	pentanal	147	propenylpyrazine (*) (b)		<i>Heterocyclic compounds containing nitrogen and sulphur</i>
47	hexanal	148	2-methyl-6-(and/or 5)-vinylpyrazine (*) (b)	187	benzthiazole (*)
48	heptanal	149	2-methyl-6-(and/or 5)-propenylpyrazine (*) (b)	188	2-methylbenzthiazole (*)
49	octanal	150	6,7-dihydro-(5H)-cyclopentapyrazine (*) (b)	189	2-acetylthiazoline (*) (c)
50	nonanal			190	5,7-dihydrothieno-[3,4b]-pyrazine (*) (b)
51	dodecanal (+)				
52	2-methylpropanal				
53	3-methylbutanal (+2-methylbutanal)				
54	2-ethylbutanal (*)				

- (*) Not previously identified in wheat or rye breads;
 (+) previously only identified in rye bread or rye crispbread.
 (a) Previously identified as a wheat flour constituent;
 (b) tentative; authentic sample not available for comparison;
 (c) tentative; insufficient material present to give unambiguous mass spectrum;
 (d) tentative; mass spectra of bread constituent and authentic compound could not be obtained due to losses in spectrometer inlet system;
 (e) very tentative; insufficient material present to penetrate into spectrometer (authentic compound gave adequate spectrum at concentrations much higher than those possibly present in bread crust essences);
 (f) also identified via its spectrum.

ANALYTICAL CHEMISTRY

1,4-DIAMINOBUTANE (PUTRESCINE), SPERMIDINE, AND SPERMINE

•918

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INTRODUCTION

1,4-Diaminobutane (putrescine), spermidine, spermine, and closely related derivatives are found in a wide variety of animals, bacteria, yeasts, and plants. During the

past 25 years' the biosynthetic pathways have been elucidated, and many studies have been carried out that clearly indicate that these amines are physiologically important. Convincing evidence for their significance is the observation that several microorganisms have an absolute requirement for diamines or polyamines for growth. In addition, the concentration of these amines or polyamines for growth is high in actively proliferating animal tissues and their biosynthesis increases with differentiation in resting cells. These changes usually precede increases in DNA, RNA, and protein. Furthermore, these amines, because of their polycationic nature, bind tightly to nucleic acids and have a variety of other nucleic acid biosynthesis and metabolism in vitro.

On the basis of these and other observations, it is generally accepted that polyamine biosynthesis is intimately interrelated with the synthesis of nucleic acids and proteins. Some investigators have postulated that polyamines specifically catalyze control nucleic acid biosynthesis and that they are directly responsible for the increased macromolecular synthesis that occurs during growth and neoplasia. Despite the efforts of many investigators, and the accumulation of a large amount of experimental data, there is still no conclusive evidence to support any of these proposals.

In this review we summarize our present knowledge of diamines and polyamines, and evaluate the various theories proposed for their biochemical role in vivo.

DISTRIBUTION AND CONCENTRATION OF POLYAMINES

Polyamines are ubiquitous in biological materials, although the relative amounts of 1,4-diaminobutane, spermidine, and spermine differ markedly in different cells. In general, prokaryotes have a higher concentration of 1,4-diaminobutane than eukaryotes and lack spermine. Eukaryotes usually have little 1,4-diaminobutane, and have spermine as well as spermidine (Table 1).

Since the earlier history of this area has been reviewed previously (1, 2), we are not giving a detailed historical review here, except to give credit to a few of the original investigators. The synthesis of spermine phosphate was first discovered in 1877 by Lewenhoeck in human semen at 41 in 1926. Although there were a number of subsequent investigations, the field was essentially reopened around 1930 by the investigations of Herbst and Snell and of S. M.

There have been considerably more than a thousand articles in this area since our review in *Annual Review of Biochemistry* in 1961, with S. M. Rosenthal (3). Consequently, because of the limited space allocated for the present review, we have not been able to cite most of these references, but have frequently referred to review articles that include these citations. Complete bibliographies since 1969 can be obtained from the MEDLINE computer service of the National Library of Medicine, Bethesda, Maryland, under the headings polyamines, diamines, ornithine decarboxylase, and arginine decarboxylase.

In addition the reader is directed to the reviews listed in (1-17) for a detailed survey of the literature, and to (10) for a recent review on polyamines in plants. The literature survey for this review was completed in August 1975.

1,4-DIAMINOBUTANE, SPERMIDINE, AND SPERMINE 287

In the past, the assays for amines have been cumbersome, insensitive, or non-specific. However, improved techniques have recently been developed, which are based on automated ion-exchange chromatography (18, 19, 27-31), gas chromatography (32-34), combined gas chromatography-mass spectroscopy (35, 36), and thin-layer chromatography of amines and of their fluorescent derivatives (37-41). The sensitivity of the ion-exchange method has been markedly increased by the use of fluorescamine (41b) or ortho-phthalaldehyde (41c) for the quantitation of the amines. Procedures have also been described which use high pressure liquid chromatography (42, 43) or radioimmuno assays (44, 45). Of these techniques, thin-layer chromatography of dansylated derivatives has been used most extensively because of its sensitivity and convenience. In our laboratory (18) we have found that automated ion-exchange chromatography, though time-consuming, is particularly useful in separating and quantitating a large number of closely related amines.

BIOSYNTHESIS OF 1,4-DIAMINOBUTANE, SPERMIDINE, AND SPERMINE

The pathway for the biosynthesis of 1,4-diaminobutane and spermidine was first established in microorganisms (2-6, 46-49) and was later found to be very similar in animal cells (7, 14, 16, 17) (Figure 1).

Ornithine and Arginine Decarboxylases

In bacteria, 1,4-diaminobutane may be formed either by ornithine decarboxylase or by arginine decarboxylase via agmatine (9, 48, 49). Both biosynthetic enzymes are normally present in *Escherichia coli*, although ornithine decarboxylation is usually the major pathway (49). Both decarboxylases have been purified from *E. coli* and have been shown to require pyridoxal phosphate (50-52). These enzymes are subject to feedback inhibition and repression by 1,4-diaminobutane or spermidine (53, 54). Involvement of the *rel* gene in the control of the decarboxylases is indicated by the higher amine content of relaxed strains (5, 52), compared to stringent strains, after deprivation of a required amino acid, and by the inhibitory action of ppGpp on ornithine decarboxylase (55). GTP, on the other hand, has a strong activating effect (52, 55). In addition to these two biosynthetic enzymes, two biodegradative enzymes can be induced under special growth conditions and have been highly purified (50, 56, 57). Both biodegradative enzymes require pyridoxal phosphate.

In animal tissues amines are derived by decarboxylation of ornithine, rather than by decarboxylation of arginine (7, 8, 14-17, 58). Purification of ornithine decarboxylase has been hindered by the very low enzyme activity normally present. However, as discussed in the section on the role of polyamines in growth, ornithine decarboxylase levels increase dramatically after a variety of stimuli, and the enzyme has been

We are not reviewing these enzymes in detail as the amino acid decarboxylases have been reviewed extensively by Morris & Fillingame in the *Annual Review of Biochemistry* for 1974 (9).

Table 1 Concentration of polyamines^{a, b, c}

	1,4-Diaminobutane	Spermidine	Spermine	References
<i>Escherichia coli</i> ^d	13.1	4.7	0	12
Liver (mouse)	0.01	0.61	0.82	18
Brain (human):				
frontal lobe	0.015	0.23	0.10	19
Pons (rat)	— ^e	7.73	4.77	20
Pons (rat)	— ^e	8.62	0.80	21
Seminal plasma (human)	0.23	0.11	3.04	21
Hepatitis (rat)	0.29	0.92	0.69	22

^a A complete list of all of the reported polyamine levels in various biological materials cannot be included because of limited space. Therefore, only a few representative values are given here. Review articles 1-17 should be consulted for other assays and references, but one should note that different investigators have used different analytical techniques and that some of these techniques have been rather nonspecific.

^b In a few animal and microbial materials, small amounts of 1,3-diaminopropane and 1,5-diaminopentane (cadaverine) have been reported. Complex materials containing diamine or polyamine structures are also found occasionally, especially in plants (see 10, 11).

^c These data were obtained from *E. coli* grown in purified media. One should note that polyamines can be transported into the cells from the medium (23), and consequently the amounts found in the cells reflect the amine content of the medium as well as aminobiosynthesis. Acetylated derivatives have also been described in bacteria (5, 24, 25), the concentration depending on the growth medium and on the temperature during harvesting (26).

^d Not determined.

purified to apparent homogeneity from regenerating liver (59) and from the livers of rats treated with thiocetamide (60). Pyridoxal phosphate appears to be a required cofactor (58, 61).

Adosylmethionine Decarboxylase

Adosylmethionine decarboxylase has been purified to homogeneity from *E. coli* and has pyruvate, rather than pyridoxal phosphate, as a cofactor (62). Mg²⁺ is required for activity (46, 63). The same reaction occurs in eukaryotes, but the enzyme has markedly different characteristics. The eukaryotic enzyme requires 1,4-diaminobutane or spermidine for activation and is not stimulated by Mg²⁺ (63-70). It is markedly inhibited by methylglyoxal bis(guananyldrazone) (64, 71-76). This inhibitor also stabilizes the decarboxylase in vivo, resulting in a greatly enhanced enzyme concentration (72, 73, 75). The rat liver and yeast adosylmethionine decarboxylases have been purified to homogeneity (65, 77), and, in contrast to earlier reports (64, 70, 78), have been completely separated from the aminopropyl transferase activity (65, 77, 79). The cofactor requirements are not clear (67), despite earlier reports (63, 66) that pyridoxal phosphate is involved.

1,4-DIAMINOBUTANE, SPERMIDINE, AND SPERMINE

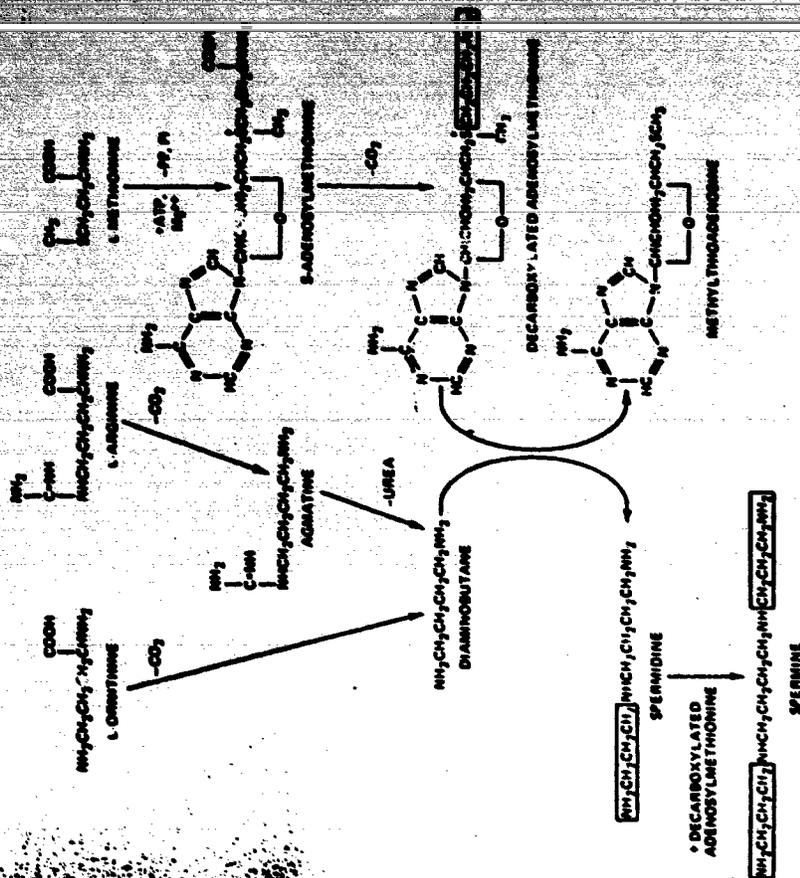


Figure 1 Biosynthesis of 1,4-diaminobutane, spermidine, and spermine.

Aminopropyl Transferase

Aminopropyl transferase has been purified to homogeneity from *E. coli* and has no known cofactors (80). The aminopropyl group of decarboxylated adosylmethionine is transferred to 1,4-diaminobutane to form spermidine; at a more alkaline pH and with high spermidine concentration, it can also form spermine, which is not a normal constituent of *E. coli*.

Two different aminopropyl transferases have been partially purified from mammalian tissue; one enzyme acts with 1,4- β -aminobutane to form spermidine, and the other forms spermine from spermidine (81, 82).

THE ROLE OF POLYAMINES IN GROWTH

From among the wide array of data now in the literature, several observations stand out as evidence that polyamines have an essential biochemical role, and these are

discussed below. Most significant is the finding of microbial mutants that require these amines for growth. A close physical relationship of the polyamines to nucleic acids is demonstrated by the packaging of amines with the DNA in bacteriophage T4 and in herpesvirus. In animal systems the rapid increase in the amine content after the stimulation of growth, plus the very rapid and large increases in ornithine decarboxylase under these conditions, strongly suggest that the polyamines are physiologically important. Other studies in cell-free systems have shown that polyamines stimulate many of the reactions involved in nucleic acid and protein synthesis, and affect membrane stability, but the significance of these effects *in vivo* is difficult to evaluate.

Microbiological Studies

The first evidence that diamines or polyamines might be an essential requirement was the finding of Herbst & Snell that *Haemophilus influenzae* requires 1,4-diaminobutane, 1,3-diaminopropane, or spermidine for growth (83). Subsequently, mutants of *Aspergillus nidulans* (84) and *Neurospora crassa* (85) were described that require 1,4-diaminobutane.

E. coli has been a particularly useful organism for the study of polyamines since the biosynthetic pathways have been described in detail and the amine concentration is high (17.5 mM for 1,4-diaminobutane and 6.3 mM for spermidine) (18). These values are only slightly less than the intracellular concentration of Mg^{2+} (24 mM) (86). The importance of these amines to the bacteria may be assumed from the observations that large amounts of amines are synthesized even when bacteria are grown on minimal media (18) and that bacteria form substantial quantities of spermidine even when the precursor amino acids (ornithine or arginine) are markedly restricted by slow growth of an arginine auxotroph in a chemostat (87).

A promising development has been the isolation of mutants of *E. coli* that require 1,4-diaminobutane or spermidine for growth at a normal rate (88-91). The requirement is the result of mutations in the genes for biosynthetic arginine decarboxylase (*speA*), agmatine ureohydrolase (*speB*), or biosynthetic ornithine decarboxylase (*speC*). These mutations map at 56-57 min on the *E. coli* map, very near *metK*, the gene for S-adenosylmethionine synthetase. We have recently isolated a mutant of *E. coli* deficient in adenosylmethionine decarboxylase (unpublished findings). The genetic locus is located at 0.5-1.5 min on the *E. coli* map. No mutants for aminopyl transferase have been described.

Effects of these mutations on the synthesis of various macromolecules are referred to later. A limitation of these mutations is that they show only a partial requirement for 1,4-diaminobutane or spermidine, and spermidine can be detected in the bacteria even in the absence of any supplementation. Furthermore, in 1,4-diaminobutane-limited bacteria, 1,5-diaminopentane and its aminopropyl derivative accumulate and presumably can, in part, satisfy the growth requirements (91, 92).

Studies in Eukaryotes

CHANGES IN ORNITHINE DECARBOXYLASE. Ornithine decarboxylase has been described in many tissues and species, and provides the only biosynthetic route in

animal tissues for 1,4-diaminobutane and spermidine.² Enzyme activity is normally very low in quiescent tissues, but is dramatically higher in rapidly proliferating cells and in many other conditions, most of which involve increase in protein synthesis, e.g. liver regeneration after hepatectomy (7, 93-98), renal and cardiac hypertrophy (99, 100), embryonic (70, 93, 101, 102) and malignant growth (7, 93, 103), exposure to cold and other stresses (96, 104, 105), sham operation (96), feeding of diets high in amino acids (95), the administration of growth hormone (106-109), cortisone (107, 108, 110), insulin (107, 111, 112), prolactin (113), dibutyl cyclic AMP (104, 112, 114), theophylline (104, 114), epidermal growth factor (115), isoproterenol (116), and thioacetamide (95). In almost all of these instances, a peak of ornithine decarboxylase activity occurred 4-24 hr after the stimulus and lasted only a few hours. In some cases a biphasic increase has been observed, with peaks at 4 hr and 12 hr. In view of the marked variation in the nature of the stimuli, the fact that the enzyme uniformly responds by a marked increase at about 4 hr after many of the stimuli is striking. The increase in activity appears to represent synthesis of new protein (93). In most cases the rise in ornithine decarboxylase is followed by a rise in 1,4-diaminobutane content, and, together with the increases in adenosylmethionine decarboxylase activity (see next section), probably accounts for the earlier observations (8) that polyamines increase during liver regeneration and embryonic growth (14, 117-119).

In cell cultures and cell suspensions a similar increase in ornithine decarboxylase (sometimes biphasic) occurs after dilution into fresh medium (120-122) or after the addition of fresh serum (120, 123, 124), amino acids (1-2, 125), insulin (123, 126), pituitary growth factors (127), mitogenic agents (125, 128, 129), dibutyl cyclic AMP (123, 126, 130, 131), theophylline (123, 131), epidermal growth factor (115, 126), or a serum factor (132). Ornithine decarboxylase also increases after virus infection (133) and after virus-induced transformation (134, 135). Changes have also been observed during the cell cycle in synchronous cultures (120, 136, 137).

The dramatic early increase in ornithine decarboxylase observed after so many stimuli, particularly in conditions associated with rapid growth or increased protein synthesis, strongly implies that this enzyme has an important function related to cell growth. The mechanism of the increase in ornithine decarboxylase is probably partly explained by its high rate of turnover, i.e. its rapid synthesis and degradation. Thus, in regenerating liver the half-life of ornithine decarboxylase is 10 min (136), much shorter than that reported for any other enzyme. 8-Aminolevulinic synthetase and tyrosine aminotransferase, which are usually considered to turn over rapidly, have half-life values of 1 and 2 hr, respectively, and most other enzymes have much slower turnover rates (139, 140). Berlin & Schimke (139) and Schimke & Doyle (140) have pointed out that any general increase in protein synthesis will manifest itself first in those enzymes that turn over rapidly, i.e. those enzymes with a high rate of both synthesis and degradation. Thus if all protein synthesis were to increase by a factor of 4, the concentration of ornithine decarboxylase would double in about

²Study of the mammalian ornithine decarboxylase is the most active area in polyamine biochemistry. Because of the large number of papers, references can only be made to a small number. The reader is referred to the references cited in previous review articles (4-9, 14-17).

10 min, while an enzyme with a turnover time of 24 hr would only increase about 1% in 10 min. In addition, any decrease in the rate of degradation of ornithine decarboxylase, as previously described for other enzymes (139-141), would markedly increase the enzyme levels observed, and some evidence for such a decrease in the rate of degradation has been published (122, 125, 128). The secondary fall in the enzyme level can be explained by repression of the synthesis of ornithine decarboxylase by the amines generated by the high levels of this enzyme; such repression has already been demonstrated by the addition of polyamines to cell cultures (129, 130) and by the administration of polyamines to animals during liver regeneration (97).

These mechanisms are probably sufficient to account for the response of ornithine decarboxylase to the various stimuli. The high turnover rate of this enzyme is the way in which the cell insures its ability to change the level of ornithine decarboxylase and of the polyamines rapidly. Some investigators have postulated other mechanisms for the specific stimulation of ornithine decarboxylase, however, possibly mediated by a rise in cyclic AMP (104, 131, 136), but more definitive studies with isotopic and immunological techniques in cell cultures are needed before these putative mechanisms can be accepted (140).

Because the rise in ornithine decarboxylase often precedes the increase in DNA and RNA synthesis, some workers (7) have suggested that the polyamines directly control RNA and DNA synthesis. There is no evidence for such direct control. It seems more likely that the increase in ornithine decarboxylase, as well as the rise in RNA and DNA, represent separate and perhaps coordinated increases in several systems that are important for cell growth.

CHANGES IN ADENOSYLMETHIONINE DECARBOXYLASE AND AMINOPROPYL TRANSFERASE Similar but less marked changes in adenosylmethionine decarboxylase occur after various stimuli (7, 128, 142-144). This enzyme has a half-life of 60 min in regenerating liver. Even though this half-life is longer than that of ornithine decarboxylase, it is still much shorter than that of other enzymes. The changes in aminopropyl transferase levels have not been studied extensively; the enzyme appears to have a long half-life, but nevertheless shows a significant increase after partial hepatectomy (144).

HORMONAL EFFECTS Important studies in this area have been those of Oka (145) and Oka & Perry (146), using the mammary gland system described by Topper (147), in which mammary gland explants can make milk protein in the presence of hydrocortisone, insulin, and prolactin. Involvement of spermidine has been shown by the following observations: (a) administration of spermidine has been shown to increase adenosylmethionine decarboxylase level and the spermidine content; (b) in the absence of hydrocortisone, the spermidine content of these cells (normally 20-180 nmole/g) drops markedly; (c) added spermidine can replace hydrocortisone in maintaining synthesis of milk proteins. Furthermore, addition of methylglyoxal bis(guanethylhydrazine) inhibits the synthesis of milk protein; this effect was attributed to the inhibition of adenosylmethionine decarboxylase, since the inhibition is reversed by spermidine.

POLYAMINES AND NUCLEIC ACIDS

One of the earliest demonstrations of a physical association of polyamines and DNA was the finding that the polyamines are packaged with DNA in *E. coli* bacteriophage T4 and represent about 40% of the cations in the bacteriophage (148). Subsequently it was shown that polyamines have a high affinity for nucleic acids and stabilize them against denaturation and shearing (4-6, 12, 149-151); the physical chemical nature of the polyamine-nucleic acid interaction has been studied (viewed in 4-6, 12). These effects are thought to be due to neutralization of the phosphate groups of the nucleic acid reducing their repulsive action.

Studies In Vivo

Numerous studies followed these observations, attempting to determine whether DNA and RNA are associated with the amines in vivo. Unfortunately, in most instances it has not been possible to answer this question, even though one might intuitively assume that the high affinity observed in vitro means that there is a similar association in vivo. After cellular disruption, redistribution of the intracellular amines among the subcellular fractions can take place, and the various fractions associated with those polyanions that bind them most strongly. Thus, although many reports indicate association of polyamines with nucleic acids or ribosomes, such data do not necessarily prove that such association occurs in vivo. Two exceptions are the studies on the packaging of polyamines with DNA in bacteriophage T4 (149) and in herpesvirus (152). These viruses were shown to be impermeable to external amines, and thus the amines found in the isolated intact particles could not have been taken up after the viruses had been packaged. Several attempts have been made to localize the amines in cells, with the use of autoradiographic techniques; although the results have been relatively inconclusive, there was some indication that added 1,4-diaminobutane is associated with RNA in oocytes of locusts (153) and in cultured *Xenopus* liver cells (154), and that spermidine is associated with the nuclei in the salivary gland of *Drosophila* (155).

Other studies, beginning with those of Reina, Herbst, Calderara, and their colleagues (14, 117, 119), approached this problem from another direction; they compared the rise in polyamines and in the biosynthetic enzymes with the increase in DNA and RNA synthesis under various conditions (5, 7, 92, 93, 110, 111, 116, 120, 174, 128, 137, 156-164). In most of these experiments, a rise in 1,4-diaminobutane and spermidine, and their biosynthetic enzymes, preceded or coincided with the increase in DNA and RNA. These observations led to suggestions that polyamines directly control DNA and RNA synthesis. However, the available evidence, although fragmentary, is against such a specific control. When polyamine synthesis is decreased by the addition of the ornithine decarboxylase inhibitor, α -hydrazinornithine, or by the adenosylmethionine decarboxylase inhibitor, methylglyoxal bis(guanethylhydrazine), RNA synthesis continues without change (165-167). There is one report (165) that there is also no change in DNA synthesis, but other investigators have reported a decrease (166, 167). Although there have been experiments showing that bacteriophage nucleic acid is not formed after infection of

polyamine-deficient bacteria unless polyamines are added (92, 168a), the data do not prove that polyamines are specifically involved in DNA synthesis, since some other phage or host functions might be the site of action. Also conflicting with the postulation that polyamines directly control DNA and RNA synthesis is the finding that addition of 1,4-diaminobutane to a polyamine-depleted mutant of *E. coli* results in an increase in the rate of protein synthesis before any increase in DNA or RNA synthesis occurs (168b).

Although at first there was some controversy as to whether polyamines can modify the ability of the *rel* gene to affect RNA synthesis (5, 169), recent data indicate that this is not the case. Studies with polyamine-deficient mutants of *E. coli*, which are either *rel⁺* or *rel⁻* (170a), and with a 1,4-diaminobutane-requiring strain of *Aspergillus nidulans*, which is *rel⁺* (170b), showed that, upon amino acid deprivation, the synthesis of RNA is not affected by the addition of polyamines.

Although polyamines do not affect the expression of the *rel* gene, the *rel* gene does affect polyamine synthesis in *E. coli* (56, 162), and ppGpp inhibits ornithine decarboxylase (56). This control is similar to that described for the effect of the *rel* gene on several other biosynthetic systems, such as the synthesis of stable RNA and of phospholipids.

Thus, despite a large number of papers, the evidence from in vivo experiments for a causal interrelationship between polyamines and nucleic acid biosynthesis is not conclusive.

Studies In Vitro

Numerous studies (4-6, 12, 14) have shown that polyamines interact with nucleic acids. The most recent study is that of Gosule & Schellman (171a), who showed that, in the presence of spermidine, T7 DNA exists in a compact form. Flink & Pettijohn (171b) have recently shown that spermidine affects the nucleic acid in isolated nucleoids of *E. coli*, stabilizing a folded configuration.

DNA synthesis is stimulated by spermidine in both crude and purified systems in vitro (172-181). In relatively purified systems, either spermidine or *E. coli* DNA-binding protein stimulates DNA synthesis with a primed single-stranded template (176-179); there are conflicting reports on whether both spermidine and DNA-binding protein are required with an unprimed ϕ X174 template (178, 179, 182). However, while the complete replicative form of DNA was synthesized in the presence of DNA-binding protein, only small DNA fragments were formed if spermidine was substituted (178, 183).

Spermidine is required for the integration of λ DNA into *E. coli* chromosomal DNA and stimulates the excision of λ prophage DNA in vitro (184, 185). The first step in the packaging of λ DNA into heads requires spermidine (186). In addition, λ phage heads are stabilized by 1,4-diaminobutane, but are destabilized by spermidine and spermine (187, 188).

Polyamines also affect several enzymatic reactions involving RNA. Spermidine and 1,4-diaminobutane markedly increase the methylation of certain nucleotides in tRNA in vitro (189-192). The effect varies with the specific tRNA molecules studied and has been attributed to effects of the amines on the tertiary structure of each

RNA (192). Spermidine also affects the methylation of 23S RNA by an *E. coli* methylase (193). Several amines stimulate DNA-dependent RNA polymerases from rat liver (194, 195), bovine brain cell nuclei (195), and calf thymus (197), while an RNA-dependent RNA polymerase from foot-and-mouth disease virus is inhibited (198). Polyamines strikingly alter the specificity and stimulate the activity of bacterial and mammalian ribonucleases (199-201).

Most of these experiments are difficult to interpret, even when relatively purified components are used. The amines can bind to the template, the primer, the product, or to the various nucleoside triphosphates, resulting in changes in conformation, size, and charge; with large concentrations of polyamines, actual precipitation may occur. Such changes can affect the availability of substrates, or can alter the affinity of substrates or products for the enzymes. If any nucleases are present, the situation is complicated further, since polyamines can affect their activity, possibly by interacting either with the substrates or with the enzymes.

Other cations, such as Mg^{2+} , can usually mimic the effects of polyamines in vitro, even though a higher concentration may be needed. In view of the high concentration of both divalent cations and polyamines in the cell, it is not possible to decide which cation is effective for any given system in vivo. In addition, considerable uncertainty exists as to the concentration of available polyamines in the intracellular water, since most of them may be bound to intracellular anions, such as nucleic acid and phospholipids.

POLYAMINES AND PROTEIN SYNTHESIS

Many previous studies have shown that polyamines (a) stimulate protein synthesis in vitro and in vivo, (b) stimulate many of the intermediate steps in protein synthesis in vitro, (c) bind tightly to substrates such as tRNA and ATP, and (d) bind to ribosomes and stabilize ribosomal structure and function. These studies have already been reviewed (4-6, 12, 14).

The complexity of the effects and the multicomponent reaction mixtures necessary make it impossible to identify definitively any specific step as the primary site of action of the polyamines in vivo. A particular complication is the ability of polyamines to interact with the nucleic acids present in the reaction mixtures or to inhibit contaminating nucleases. Conclusions regarding the in vivo role of polyamines are rendered even more difficult by the observations that Mg^{2+} can replace the polyamines in most of the systems studied, even though in a few instances polyamines have stimulating effects in the presence of optimal Mg^{2+} . Recently, however, the importance of the polyamines for protein biosynthesis was strongly supported by the finding that protein biosynthesis increases before DNA or RNA synthesis when 1,4-diaminobutane is added to a polyamine-depleted mutant (*speB⁻*) of *E. coli* (168b).

Gesteland and his co-workers (202) have recently shown that the larger proteins normally found in an S40 adenoovirus hybrid can be synthesized in vitro only if polyamines were added. In their absence, only shorter polypeptides are made. Thus, even though polyamines are not required for the synthesis of some proteins, they

are required for the completion of the larger protein molecules. These effects of polyamines were observed, even in the presence of optimum Mg^{2+} . The mechanism of these effects is not understood.

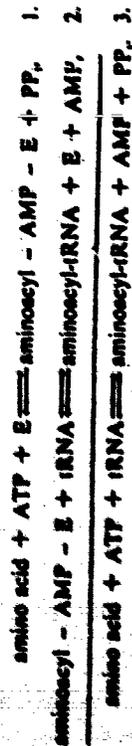
Transfer RNA

There has been special interest in the binding of polyamines to tRNA because of the stimulatory effect of the polyamines on aminoacyl-tRNA synthetase and on protein synthesis. Most striking is the very tight binding of 2 to 5 molecules of spermidine to a molecule of tRNA, and the cooperativity of this binding; additional molecules (up to a total of about 16) are bound less tightly and no cooperative effect is seen (5, 203-205). The cooperativity observed in the binding studies is very similar to that observed for Mg^{2+} and Mn^{2+} and, as is the case with the divalent cations, suggests that the binding of the first few molecules of spermidine facilitates the formation of the tertiary structure of the tRNA (206, 207). This effect has been confirmed by fluorescence studies that measure the effect of conformational changes on the environment around 4-thiouridine and the Y base in certain tRNA molecules (203, 208). These data do not necessarily indicate the exact site at which spermidine binds, however, since binding of polyamines anywhere in the tRNA might result in conformational changes that would affect the environment around specific nucleoside residues. This effect of spermidine is of special interest since changes in the configuration of tRNA could affect its activity in protein biosynthesis.

The affinity of spermine and spermidine for tRNA is utilized in the preparation of crystals of tRNA for X-ray crystallographic studies. Addition of spermine to the tRNA solution influences the type of crystal obtained (209-211) and is essential for the preparation of certain crystal forms.

Aminoacyl-tRNA Synthetase Reaction

There has been considerable controversy on the ability of spermidine to completely substitute for Mg^{2+} in the aminoacylation of tRNA by aminoacyl-tRNA synthetase:



Mg^{2+} is required for both reaction 1 (pyrophosphate exchange) and reaction 3 (overall acylation of tRNA). Surprisingly, spermine was found to substitute for the Mg^{2+} requirement of the overall reaction, but could not substitute at all in reaction 1 (212-216).

These findings were considered as evidence that the entire reaction occurs as a concerted one-step reaction instead of the two-step reaction indicated above (214, 217). Chakrabarty et al (218) and Senti & Webster (219) claim that these results are artifactual since, even though additional Mg^{2+} was not added to the reaction mixtures, Mg^{2+} was present as a contaminant in the added tRNA and was displaced by the added spermine. When reaction 1 is studied separately, no tRNA is added,

and this source of Mg^{2+} is not present. There is still some disagreement in the literature, however, since different groups obtain contradictory results when Mg^{2+} -free tRNA is used (218-221). There is also disagreement as to whether a one-step or two-step reaction is involved, as there are kinetic experiments in favor of both concepts (222-224).

Although there is a controversy on whether or not the enzyme is active in the complete absence of Mg^{2+} , there is ample evidence that spermine can stimulate, or under some circumstances inhibit, the activity of the aminoacyl-tRNA synthetase when added in the presence of some Mg^{2+} (219, 220, 225). There are a number of possible sites for these effects: directly on the enzyme (226); by formation of an inactive spermine-ATP complex (226); by preventing the binding of the added Mg^{2+} to tRNA (226); and by stimulation of both the enzymatic and nonenzymatic decylation of the aminoacyl-tRNA (227-229).

Polyamines may affect the aminoacylation reaction *in vivo* since a preliminary report (230) has shown that an *E. coli* mutant deficient in polyamines contains a higher fraction of nonacylated tRNA than when supplemented with polyamines. However, extracts from such deprived mutants do not show a deficiency in the formation of phenylalanyl-tRNA (231).

Amines and Ribosomes

Polyamines have been found in ribosomes isolated from both bacterial and animal cells (4-6, 12, 232-235). On this basis, it has been widely accepted that ribosomes *in vivo* also contain polyamines, and that polyamines are essential for ribosomal structure and function. However, it has been clearly demonstrated that ribosomes can take up amines from the cytoplasm after cells are disrupted (232-235). The binding of polyamines to ribosomes is reversible, and consequently the amine content of the ribosome changes during isolation procedures, such as gradient centrifugation (232, 235). Thus, it is impossible to be certain that ribosomes, *in fact*, bind polyamines *in vivo*.

Polyamines, like Mg^{2+} , cause 30S and 50S subunits to associate to form 70S monomers (5, 236). However, polyamines cannot replace all of the ribosomal Mg^{2+} (237, 238). Studies with isolated 30S and 50S subunits have shown that 10-20% of the phosphate groups of RNA must be occupied by Mg^{2+} or a closely related divalent metal ion in order to maintain both the active structure and the activity of the subunits. Spermidine also promotes 30S and 50S ribosomal subunit association in the presence of initiation factor IF-3 or tetraacycline (239) and also stabilizes 70S particles by inhibiting subunit exchange in a cell-free protein-synthesizing system treated with puromycin (240).

In contrast to an earlier report (241), the *in vitro* assembly of functionally active 50S ribosomal subunits from *r*-associated RNA and protein components does not require polyamines when optimal Mg^{2+} is present (242).

Several papers suggest that the polyamines play a role in peptide initiation, possibly by altering the ribosomal conformation; the amines decrease the requirement for Mg^{2+} for the formation of an initiation complex between aminoacyl-tRNA, messenger RNA, and ribosomes (243-249). Spermine may also affect peptidyl chain elongation, since in low Mg^{2+} concentrations it stimulates the peptidyl transferase activity

ity of ribosomes (250). In addition, polyphenylalanine synthesis is decreased in poly (U)-supplemented extracts from polyamine-deficient auxotrophs of *E. coli* (231); this appears to result from an abnormality of the 30S subunit.

UNUSUAL AMINE DERIVATIVES

Glutathionylspermidine (γ -glutamylcysteinylglycylspermidine) (251-253) is found in *E. coli* in stationary cells and accounts for all of the spermidine and a large fraction of the intracellular glutathione. Glutathionylspermidine is in equilibrium with intracellular spermidine and is rapidly converted to spermidine when the stationary cells are diluted into fresh medium. Synthesis *in vitro* has been described. The possible role of this derivative in *E. coli* is intriguing since it contains, in covalent linkage, two compounds that are normally present in high concentration in many cells, and whose physiological functions are still unknown, despite extensive investigations over many years.

Edeine A and edeine B are complex antibiotics that are obtained from *Bacillus brevis* Vm 4 and that contain spermidine or guanylspermidine in covalent linkage to a pentapeptide (6, 254, 255). Edeine A inhibits DNA synthesis in bacteria and in cell-free preparations from polymerase I-deficient mutants of *E. coli* (256). At high concentrations, it inhibits initiation of protein synthesis (257). Preliminary studies have been reported on the synthesis of edeine *in vitro* (258).

N-Carboxyethyl-1,4-diaminobutane [putrescine ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{COOH}$)] was first described in brain (19, 259, 260a) but is present in smaller amounts in liver. Isotopic evidence indicates that this compound is derived from spermidine (260a).

$\text{N}_2\text{N}'$ -bis-(2-carboxyethyl)-1,4-diaminobutane (spermic acid) has been isolated from bovine brain (260b).

Thermine [$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$], an analog of spermine, has been found in an extreme thermophile, *Thermus thermophilus* (261).

3-Aminopropyl-1,5-diaminopentane has been found in 1,4-diaminobutane-deficient mutants of *E. coli* (92).

2-Hydroxyputrescine has been found to be a major diamine in two *Pseudomonas* strains (262-264).

Hypusine [N^6 -(4-amino-2-hydroxybutyl)-2,6-diaminohexanoic acid] (265, 266), a derivative of hydroxyputrescine and α -aminoaldipate- β -semialdehyde, has been found in very low concentration in various animal tissues. Abnormally high excretion of hypusine in the urine was described in a child with congenital hyperlysinemia (267).

α -Putrescinylythymine [5-(4-aminobutylaminomethyl)-uracil] constitutes 12% of the bases of the DNA of a *Pseudomonas* phage ϕ W-14 (268, 269).

A large number of new amine derivatives that occur in plants have been described in reviews by Smith (10, 11).

CLINICAL STUDIES

The most active clinical area concerns changes in polyamines in malignancy (7). Some, but not all, human and animal tumors contain high levels of ornithine

decarboxylase and high concentrations of polyamines. At present, however, there is no reason to conclude that the increase in amines is specifically related to the neoplastic process, other than as a reflection of a relatively rapid growth rate. Studies have also been carried out on cell cultures after virus transformation (134, 135, 270). In general, the 1,4-diaminobutane content is higher in the transformed cells, but it seems likely that these results are the effect of transformation on growth and contact inhibition rather than specifically related to the neoplastic process.

Increased amine levels have been described in blood and urine of some patients with leukemia or several other types of malignancy (7, 271-274). Interpretation of this type of clinical data is difficult. Some of these increases may be related to destruction of tumor or normal tissue, especially during therapy (275, 276). Other factors such as weight loss, simultaneous infection, variations in food intake, and bacterial contamination of urine may also alter the amount of amines found in the urine. Much of the early data are of questionable value since they were obtained with less specific assays; newer and more specific assays have yielded markedly lower values (14, 31).

A low ornithine decarboxylase activity has been reported in cultures of epidermal cells of a patient with a congenital syndrome, hyperornithinemia (277).

The ratio of spermidine/spermine in the whole blood of male cystic fibrosis patients and in heterozygotes is increased, compared with normals (278, 279). The changes are too small to permit any conclusions on their significance, however, especially since no changes are seen in female patients. Who: blood levels are particularly difficult to evaluate, since most of the amines are present in the blood cells, and any variation in the cell pattern may affect the results.

MISCELLANEOUS

Membrane stability A role for polyamines in the stabilization of membranes has been proposed on the basis of their ability to stabilize both *E. coli* spheroplasts (4, 280-282) and halophilic organisms (283) when placed in dilute salt solutions, and to stabilize isolated mitochondria (284). Probably related to these effects on membranes is the recent observation that spermine facilitates transfection by phage DNA of *E. coli* spheroplasts that have been stored in the cold (285). Although polyamines have been found in isolated membranes and in lipopolysaccharides, there is no definitive evidence that polyamines are located in membranes *in vivo* (286-288).

Effect of K^+ concentration and ionic strength An inverse relationship of the 1,4-diaminobutane and K^+ concentration has been shown in *E. coli* grown in high K^+ -high osmolar medium and in both K^+ -deficient *E. coli* (289-291) and K^+ -deficient plants (10, 11). In addition, the growth rate is slowed when 1,4-diaminobutane-deficient *E. coli* is placed in a low osmolar medium (292).

Metabolism Metabolism is not discussed, as only a few papers have appeared since our last review (4, 6, 7). Because of its importance for cell culture studies, however, it is worth repeating the warning (4) that bovine serum albumin contains an amine oxidase that converts spermine and spermidine to an aminoaldehyde, which is toxic, possibly by its conversion to acrolein.

Antimutator Effect Bacterial mutations, either spontaneous or induced by a variety of mutagenic agents, were moderately depressed when spermine was present. The role of spermine as an antimutagenic agent was reviewed recently (293).

CONCLUSION

As is evident from the above summary of the recent literature, plus many other references not cited here, there is an extensive literature indicating the physiological significance of these amines. The most important studies can be summarized as follows:

- (a) Polyamines and their biosynthetic enzymes are ubiquitous.
- (b) Microbiological mutants have been described in which there is a definite requirement of polyamines for growth.
- (c) The concentrations of polyamines and their biosynthetic enzymes increase when the growth rate increases. The α increases usually precede or are simultaneous with increases in RNA, DNA, and protein levels.
- (d) Ornithine decarboxylase has a remarkably fast turnover rate in animal cells, and the level of this enzyme rapidly changes after a variety of growth stimuli.
- (e) Polyamines have a high affinity for nucleic acids and stabilize their secondary structure. They are found associated with DNA in bacteriophages and have a variety of stimulatory effects on DNA and RNA biosynthesis and have a variety of stimulatory effects on DNA and RNA biosynthesis and have a variety of stimulatory effects on DNA and RNA biosynthesis.
- (f) Polyamines protect spheroplasts and halophilic organisms from lysis, indicating their ability to stabilize membranes.

Despite these observations, no specific mechanism has been firmly established for the action of the polyamines in vivo. It is clear that these compounds are physiologically important, however, and further work is necessary to establish the mechanism of their action.

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CONCEPTS AND PERSPECTIVES IN ENZYME STEREOCHEMISTRY

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PERSPECTIVES AND SUMMARY

The last twenty years have produced a vast quantity of information about enzyme stereochemistry. This has been recorded in monographs (1, 2), general reviews (3-17), discussions of hydrogen transfer and isotope effects (18, 19), and surveys of specific areas of metabolism. New additions to the literature have been noted in the *Annual Reports on the Progress of Chemistry, Section B* (e.g. 20) and in *Specialized Periodical Reports* (e.g. 21). This essay, although it concentrates on the literature since 1970, cannot possibly cover all the material available. Instead, several approaches to the subject are explored.

Sections 1 and 2 are concerned with the evolution of a consistent set of stereochemical concepts that meet the present needs of both biochemists and organic chemists. Although the major concepts for understanding enzyme stereospecificity

The Pharmacologica

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ogical Basis of Therapeutics

FIFTH EDITION

MACMILLAN PUBLISHING CO., INC.

New York

COLLIER MACMILLAN CANADA, LTD.

Toronto

HILLIÈRE TINDALL

London

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PRINTED IN THE UNITED STATES OF AMERICA

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**MACMILLAN PUBLISHING Co., Inc.
866 Third Avenue • New York, N.Y. 10022**

**COLLIER MACMILLAN CANADA, LTD.
BARRIÈRE TINDALL • London**

**Library of Congress catalog card number 75-13903
ISBN 0-02-344781-8
Barrère Tindall SBN 0 7020 0584 3**

Printing: 2345678

Year: 678901

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In this textbook, reference to proprietary names of drugs is ordinarily made only in chapter sections dealing with preparations. Such names are given in SMALL-CAP TYPE, usually immediately following the official or nonproprietary titles. Proprietary names of drugs also appear in the index.

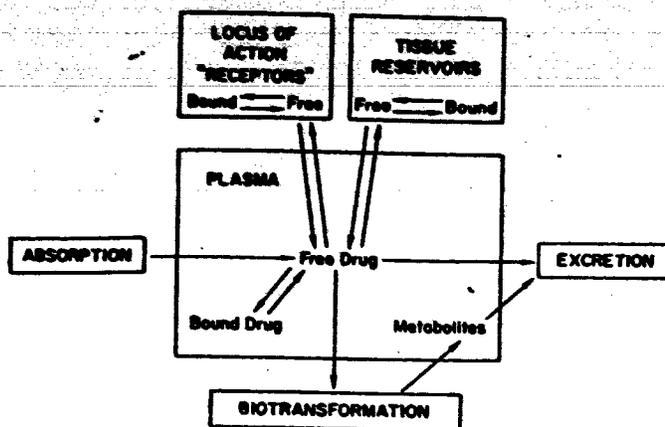


Figure 1-1. Schematic representation of the interrelationship of the absorption, distribution, binding, biotransformation, and excretion of a drug and its concentration at its locus of action.

Possible distribution and binding of metabolites are not depicted.

lium) or several layers of cells (skin). Despite these structural differences, the diffusion and transport of drugs across these various boundaries have many common characteristics, since drugs in general pass through cells rather than between them. The cellular plasma membrane thus represents the common barrier.

Cell Membranes. The classical observations by Overton and by Collander and Bårlund led to the theory that the cell (plasma) membrane was a thin layer of lipid material interspersed with minute water-filled channels. Subsequent studies suggested that the plasma membrane consisted of a bimolecular lipid sheet bound on both sides by protein, but this hypothesis has been broadened to a more dynamic model in which lipids and intrinsic and extrinsic proteins are viewed as being organized in a mosaic structure (Symposium, 1972). The intrinsic (integral) proteins are embedded or intercalated, in ordered or disordered arrangement, into a discontinuous lipid bilayer that forms the matrix of the mosaic. The intrinsic proteins are globular and bimodal, with their ionic and highly polar groups located largely on the membrane surfaces in contact with the extracellular and intracellular aqueous media, and with their nonpolar residues sequestered from contact with water in the membrane interior. Extrinsic proteins are bound to the exposed surfaces of the intrinsic proteins by electrostatic or hydrophobic interactions, but they are not involved in lipid-protein interactions that are critical to the membrane structure and its functions. Channels appear to be present in the central axes of the globular intrinsic proteins. Cell membranes are approximately 80 Å thick.

Passive Processes. Drugs cross membranes by either passive processes or by mechanisms involving the participation of components of the membrane. In the former, the drug molecules penetrate either by passage through aqueous channels in the membrane or by dissolving in the membrane. Both non-polar lipid-soluble compounds and polar water-soluble substances that retain sufficient lipid solubility can cross the lipid portion of the membrane by *passive diffusion*. Such transfer is directly proportional to the concentration gradient across the membrane and the lipid:water partition coefficient of the drug. The greater the partition coefficient, the higher is the concentration of drug in the membrane and the faster is its diffusion. However, after a steady state is attained, the concentration of the free drug is the same on both sides of the membrane, if the drug is a nonelectrolyte. For ionic compounds, the steady-state concentrations will be dependent on the transmembrane potential and may be influenced by the state of ionization of the molecule on each side of the membrane. Passage through channels is called *filtration*, since it involves bulk flow of water as a result of a hydrostatic or osmotic difference across the membrane. The bulk flow of water carries with it any water-soluble molecule that is small enough to pass through the channels. Filtration is a common mechanism for transfer of many small, water-soluble, polar and nonpolar substances. The size of the membrane channels differs in the various body membranes. Capillary endothelial cells have large channels (40 Å), and molecules as large as albumin may pass to a limited extent from the plasma to the extracellular fluid. In contrast, the channels in the red-cell membrane, the intestinal epithelium, and most cell membranes are about 4 Å in diameter and permit passage only of water, urea, and other small, water-soluble molecules. Such substances generally do not pass through channels in cell membranes if

Most molecular weights are greater than 100 to 1000. Most inorganic ions are sufficiently small to penetrate the channels in membranes, but their concentration gradient across the cell membrane is generally determined by the transmembrane potential (e.g., chloride ion) or by active transport (e.g., sodium and potassium ions).

Weak Electrolytes and Influence of pH. Most drugs are weak acids or bases and are present in solution as both the nonionized and ionized species. The nonionized portion is usually lipid soluble and can readily diffuse across the cell membrane. In contrast, the ionized fraction is often unable to penetrate the lipid membrane because of its low lipid solubility, or to traverse the membrane channels because of its size. If the ionized portion of a weak electrolyte can pass through the channels, or through the membrane, it will distribute according to the transmembrane potential in the same manner as an inorganic ion. For example, chloride, bicarbonate, bromide, and the ionized form of drugs, such as 5,5-dimethyl-2,4-oxazolinedione, are distributed unequally across the red-blood-cell membrane.

The distribution of a weak electrolyte is usually determined by its pK_a and the pH gradient across the membrane. To illustrate the effect of pH on distribution of drugs, the partitioning of a weak acid ($pK_a = 4.4$) between plasma (pH = 7.4) and gastric juice (pH = 1.4) is depicted in Figure 1-2. It is assumed that the gastric mucosal membrane behaves as a simple lipid barrier that is permeable only to the lipid-soluble, nonionized form of the acid. The ratio of nonionized to ionized drug at each pH can be calculated from the Henderson-Hasselbalch equation. Thus, in plasma, the ratio of nonionized to ionized drug is 1:1000; in gastric juice, the ratio is 1:0.001. The total concentration ratio between the plasma and the gastric juice would therefore be 1000:1 if such a system came to a steady state. For a weak base with a pK_b of 4.4 ($BH^+ \rightleftharpoons B + H^+$), the ratio would be reversed. These considerations have obvious implications for the absorption and excretion of drugs, as will be discussed more specifically below. The establishment of concentration gradients of weak electrolytes across membranes with a pH gradient is a purely physical process and does not require an active transport system. All that is necessary is a membrane preferentially permeable to one form of the weak electrolyte and a pH gradient across the membrane. The establishment of the pH gradient is, however, an active process.

Carrier-Mediated Membrane Transport. *Active Transport.* Passive processes do not explain the passage of all drugs across cell membranes. Active transport is responsible for the rapid transfer of many organic acids and bases across the renal tubule, choroid plexus, and hepatic cells. *Active transport* differs from a passive process in that it exhibits selectivity, saturability, and a requirement for energy. The transported substance is transferred against an electrochemical gradient (uphill transport). Active transport is thought to be mediated by

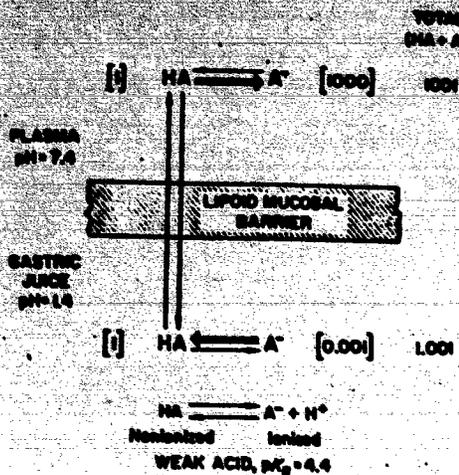


Figure 1-2. Influence of pH on the distribution of a weak acid between plasma and gastric juice, separated by a lipid barrier.

Only the nonionized moiety can readily penetrate the membrane; hence, at equilibrium its concentration is the same in both compartments. The degree of dissociation of the acid on each side depends on the pH of the plasma and gastric juice. The total concentration difference between the two sides is a direct function of the pH gradient across the membrane.

The values in brackets represent relative concentrations of the ionized and nonionized forms on each side of the membrane. The thick horizontal arrows point in the direction of the predominant form of the weak acid at the indicated pH.

carriers—membrane components that form a complex with the substance to be transported. These processes are dependent in part upon the Na^+ gradient across the membrane and are influenced by drugs that modify Na^+ - K^+ transport.

Transcellular fluids are formed by the active transport of Na^+ across epithelial cells. So-called tight intercellular junctions between these cells prevent diffusion of fluids and solutes in both directions. Proteins and other macromolecules slowly cross epithelial cells by *pinocytosis*, a form of vesicular transport.

Facilitated Diffusion. Carrier-mediated transport that exhibits selectivity and saturability but in which the substance does not move against a concentration gradient is called *facilitated diffusion*. This is not an energy-dependent process. Glucose, for example, is transported into most cells by this process. Transport is facilitated by attachment to a carrier and is more rapid than simple diffusion. The transport of glucose across the gastrointestinal mucosa and by the kidney, however, is active and can proceed against a concentration gradient.

ABSORPTION

It is of manner in rate of absorption of drug in determining choice of administered preparations of

Factors variables, i factors dissolution of absorption of administered solubility. I are more r: in oily sol: For those : dissolution: absorption. pH of the are absorbed in the fluid: occurs ver: stances ma: mentary tr: drug influ: ingested or

ROUTE

Intravenous

Subcutaneous

Intramuscular

Oral ingestion

* See text for

ever, some of these receptors may be located in glial cells, the functions of which are largely unknown (Gilman and Nirenberg, 1971).

Blom and associates have investigated the mechanism of the inhibitory effect of a tract of norepinephrine-containing fibers that arise in the locus ceruleus of the pons and terminate on the dendrites of the Purkinje cells of the cerebellar cortex of the rat (see Hoffer *et al.*, 1973). Stimulation of the locus ceruleus, or microiontophoretic application of norepinephrine or cyclic AMP to the Purkinje cells, produces inhibition of the latter, characterized in each case by (1) decreased rate of spontaneous discharge, (2) hyperpolarization with decrease in membrane conductance (in contrast to the increased conductance that generally accompanies the IPSP), (3) delayed onset and decline of the inhibitory response, (4) enhancement of such by inhibitors of cyclic AMP degradation, and (5) antagonism by β -adrenergic blocking agents of the foregoing effects following electrical stimulation of the nerve tracts or application of norepinephrine, but not following application of cyclic AMP. Thus, these findings appear not only to fulfill most of the standard criteria for identifying norepinephrine as the inhibitory transmitter at the site studied but also to provide strong evidence that its inhibitory action is mediated by cyclic AMP.

OTHER NEUROHUMORAL TRANSMITTERS

From the foregoing account, it is evident that ACh has been established as the neurohumoral transmitter agent at most efferent sites in the peripheral nervous system; the major exception is the terminals of most postganglionic sympathetic fibers, where norepinephrine has this function. In addition, ACh, norepinephrine, and dopamine probably serve as transmitters at certain sites within the CNS. Accordingly, there remain to be defined the transmitting agents of the primary afferent fibers, of certain exceptional postganglionic autonomic fibers (discussed below), and of the presumed majority of various excitatory and inhibitory fibers of the CNS that are neither cholinergic nor adrenergic. Although many naturally occurring compounds have been proposed for these roles, none has as yet been definitely established as a transmitter substance. The most likely candidates are discussed briefly below.

Amino Acids. By means of microiontophoretic application of compounds to neurons in various regions of the CNS, exhaustive searches for agents that might simulate the effects of impinging excitatory and inhibitory nerve impulses were conducted by Curtis and Watkins (1960), Krnjević and

Phillis (1963), and others. A major outcome of these collective efforts was the focusing of attention on a group of amino acids, which appear to fulfill to a considerable degree the criteria established for the identification of neurohumoral transmitters. Parallel investigations of synaptic and neuromuscular transmission in crustaceans have provided even stronger evidence that two amino acids, gamma-aminobutyric acid (GABA) and L-glutamic acid, function there as inhibitory and excitatory transmitters, respectively. At present, the two most promising candidates of this class for roles as inhibitory transmitters in the mammalian CNS are the monocarboxylic amino acids, GABA and glycine. Two dicarboxylic amino acids, L-glutamic acid and L-aspartic acid, qualify most closely as excitatory transmitters. (For documentation, see reviews by Curtis and Crawford, 1969; McLennan, 1970; Phillis, 1970; Curtis and Johnston, 1974; Krnjević, 1974.)

GABA was first proposed as an inhibitory transmitter or modulator of the mammalian CNS by Hayashi (1954) on the basis of its known presence in the brain and its anticonvulsant activity. In the same year, Florey (1954) extracted a synaptic inhibitory substance from various parts of the CNS, which was later identified as GABA (Bazemore *et al.*, 1957). Subsequently GABA was shown to produce inhibition accompanied by hyperpolarization, through an increase in chloride conductance, at cortical neurons and at cerebellar neurons of Deiter's nucleus; the latter effect was identical with that obtained by stimulation of inhibitory axons arising from Purkinje cells in the cerebellar cortex. By means of a remarkably sensitive enzymatic cycling method that permits determination of the GABA content of single mammalian neurons, Otsuka and Miyata (1972) have obtained evidence that the inhibitory terminations of the Purkinje cell axons on the Deiter's neurons contain extremely high concentrations of GABA. GABA may also produce presynaptic inhibition by depolarization of afferent terminals in the spinal cord (Davidoff, 1972).

The identification of GABA as an inhibitory transmitter has been strengthened further by the more or less selective blocking action obtained with picrotoxin, the alkaloid bicuculline, and more recently penicillin against both GABA and inhibitory nerve impulses at various sites. At the same time, these observations have provided an explanation for the mechanism of the convulsant action of these drugs. Semicarbazide and other pyridoxal-complexing hydrazides may produce convulsions through inhibition of the enzyme that synthesizes GABA, glutamate decarboxylase, resulting in a lowering of the level of GABA in the brain.

In summary, GABA has been established as an

DISPOSITION OF N-VINYLPYRROLIDINONE IN THE RAT

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ABSTRACT

The disposition of N-[¹⁴C-vinyl]-2-pyrrolidinone was studied in male Sprague-Dawley rats following a single iv injection. Plasma levels of the intact compound dropped rapidly within the first 6.0 hr after dosing with a half-life of 1.5 hrs. Urinary excretion by 6.0 hr represented 40 to 65% of the total radioactivity dosed while the biliary excretion ranged from 17 to 20%. ¹⁴C-activity attributed to the intact compound was found to be less than 0.2% of the dose in the urine and less than 0.34% in the bile. Tissue distribution studies showed that the liver and small intestines contained the highest accumulation of ¹⁴C-activity at 0.25, 0.5, 1.5 and 6.0 hr after administration of N-[¹⁴C-vinyl]-2-pyrrolidinone. Urine analyses performed for metabolite elucidation showed that 12% of the radioactivity dosed was incorporated into acetate and the major remaining portion in species which appeared to be water soluble, acidic compounds.

INTRODUCTION

N-Vinyl-2-pyrrolidinone (NVP) is the monomer from which polyvinylpyrrolidinone (PVP) is synthesized utilizing a method involving thermal polymerization of NVP in the presence of hydrogen peroxide and ammonia. Polymer preparations arising from this synthesis contain a small percentage of NVP, generally less than 0.5%. Worldwide production of the monomer approaches 30 million pounds per year with almost 90% utilized in production of the polymer¹.

PVP is employed widely in pharmaceutical and medicinal applications as well as in cosmetics, beer, and foodstuffs. It is used as a binder for tableting, for coating and sub-coating tablets, and for film coating and is incorporated in antibiotic suspensions as a stabilizer. A primary cosmetic application of this polymer is its use as a film former in hair sprays. Use of PVP is officially approved by the FDA in the USA for clarifying beer, for stabilizing vinegar, and as a stabilizer and binder in the manufacture of vitamin preparations (1).

To date, no data on the *in vivo* disposition of NVP has been reported. In view of this fact and in consideration of the high level of NVP production, the wide usage of PVP, and the presence of the monomer in the polymer products, the present study was undertaken to elucidate the *in vivo* disposition of NVP in rats. Additionally, NVP represents a novel system to study due to its unique N-(exocyclic vinyl)-lactam structure, and thus the data may serve as a precedent for determinations of the metabolic fate of similar chemical entities.

MATERIALS AND METHODS

Reagents

N-[¹⁴C-Vinyl]-2-pyrrolidinone with a specific activity of 1.7 mCi/mmol was obtained from Pathfinder Laboratories Inc. (St. Louis, Missouri). Utilizing normal saline as the solvent vehicle, a dosing solution of 5.0 μCi/0.1 ml was prepared. During the course of the studies, purity of the labeled compound in the solution injected was periodically checked by thin layer chromatography (TLC). This was accomplished by application of the solution injected to TLC plates (Whatman K3, Whatman Inc., Clifton, New Jersey) and subsequent elution with chloroform. Following development, the alumina gel was scraped from the plate in 1.0 cm sections, each of which was suspended separately in 0.8 to 0.9 g of a suspending agent (Cab-O-Sil, Cabot Corporation, Boston, Massachusetts) and 15 ml of a scintillation cocktail (ACS, Amersham Corporation, Arlington, Illinois). The

samples were counted in a liquid scintillation counter (Packard 3255, Packard Instrument Co., Inc., Downers Grove, Illinois) for ^{14}C -activity determination. Sample quenching was compensated for by use of quench curves and external standardization. Purification of [^{14}C] NVP, when necessary, was accomplished by TLC as above. However, after plate development, only the section of the plate corresponding to intact authentic unlabeled NVP was scraped from the plate. The alumina gel was extracted twice after centrifugation with water which had been adjusted to pH 8.0 with K_2CO_3 . This solution then served as the purified injection solution. The specific activity of the ^{14}C -labeled NVP was not altered during the purification procedure.

Analyses of Radioactive Samples

In those experiments involving determination of the ^{14}C -activity attributed to [^{14}C] NVP as well as total ^{14}C -activity in plasma, the 0.4 ml blood samples collected were centrifuged to precipitate red blood cells. The supernatant plasma was collected and a 50 μl aliquot from this fraction was combusted using a sample oxidizer (Packard Tri-Carb Model 306). The combusted samples were counted in a liquid scintillation counter (Packard 3255). A second 50 μl aliquot from the plasma fraction was applied to a TLC plate (Whatman K3) which was developed with chloroform. The area of the plate corresponding to the known r.f. value of unlabeled NVP (detected by iodine) was scraped from the plate. The alumina gel was suspended in 0.8 to 0.9 g of Cab-O-Sil and 15 ml of scintillation cocktail (ACS), followed by counting of radioactivity.

^{14}C -activity attributed to intact [^{14}C] NVP and total ^{14}C -activity in urine and bile samples were determined by measuring the total volume of all samples and then analyzing aliquots. Urine aliquots of 50 μl and bile aliquots of 25 μl were treated like the supernatant plasma aliquots.

Three, four, and five ml aliquots of each CO_2 -trapping solution (Carbosorb, Packard Chemical Company, Downers Grove, Illinois) used during the metabolism cages studies were mixed with 15 ml of scintillation cocktail (ACS) and counted.

Feces collected from the animals were lyophilized and then weighed. The freeze-dried feces from each collection time were pulverized separately with a mortar and pestle. Aliquots were combusted in a sample oxidizer and then counted.

Organs and protein precipitates weighing greater than 1.0 g were either divided into less than 1.0 g portions, if the entire sample was to be combusted in parts, or were homogenized for sample homogeneity before combusting an

aliquot. The stomach and contents (or an aliquot) were combusted together. The large and small intestines were separated from their contents prior to combustion.

Animal Studies

Determination of intact [^{14}C] NVP and total ^{14}C -activity in plasma, urine, and tissues 6.0 hr after iv administration of [^{14}C]NVP. Male Sprague-Dawley rats (Harlan Industries, Indianapolis, Indiana) weighing 250 to 350 g were used. The rats were anesthetized with urethane (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin), 1200 mg/kg ip, and remained anesthetized until sacrificed. The body temperature of the animals was maintained at 37° with a heat lamp. The jugular vein and carotid artery were cannulated with PE-10 and PE-50 tubing (Clay Adams, Division of Becton, Dickinson and Company, Parsippany, New Jersey), respectively, and each rat was dosed by an iv injection of 5.0 μCi of [^{14}C] NVP into the cannulated jugular vein. Blood samples (0.4 ml) were withdrawn at 10, 20, 30, 45, 60, 90, 120, 240, and 360 min from the cannulated carotid artery. The rats were sacrificed at 6.0 hr and organs and urine samples (taken by bladder puncture) were collected. Total ^{14}C -activity and ^{14}C -activity attributed to intact NVP were determined for the blood and urine samples while only the total ^{14}C -activity was determined for the organs.

In a separate experiment, rats were anesthetized with Nembutal (Abbott Laboratories, North Chicago, Illinois), 45 mg/kg ip, and remained anesthetized until sacrificed. The jugular vein was cannulated with PE-10 tubing and each rat dosed as previously described. One rat was sacrificed at each of three time points: 15, 30, and 90 min. Organs were removed and total ^{14}C -activity was determined.

Determination of total ^{14}C -activity in CO_2 , urine, and feces six days after iv administration of [^{14}C] NVP. Six male Sprague-Dawley rats weighing 250 to 350 g were anesthetized temporarily with Nembutal, 45 mg/kg ip, and the jugular vein was cannulated with PE-10 tubing. Two rats were likewise dosed with 5.0 μCi , two with 2.86 μCi , and two with 1.16 μCi of [^{14}C] NVP. The vein was tied off at both ends of the incision area after dosing and the muscle and skin layers of the incision site were sutured. Each animal was then housed individually for periods ranging to six days in glass metabolism cages (MC 300 series, Crown Glass Company, Somerville, New Jersey) which allowed separate collection of the urine and feces. Expired CO_2 was collected in a CO_2 trapping solution (Carbosorb). Urine, feces, and CO_2 samples from these animals were then analyzed.

Biliary excretion of intact [^{14}C] NVP and total ^{14}C -activity after an iv dose of [^{14}C] NVP. Two rats weighing 300 g were anesthetized with urethane, 1200 mg/kg ip, and remained anesthetized throughout the study. The jugular vein and bile duct were cannulated with PE-10 tubing and each rat was dosed with 5.0 μCi of [^{14}C] NVP. Bile samples were collected at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 hr from the cannulated bile duct. Total ^{14}C -activity and that attributed to the parent compound were determined for each sample collected.

Protein Binding of ^{14}C -Activity from [^{14}C] NVP Incubation With Rat Liver Microsomal Fractions.

Two male Sprague-Dawley rats weighing 192 and 236 g were anesthetized with Nembutal, 45 mg/kg ip. removed, minced, and washed with a $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (Fisher Gram - Pac Buffer, Fisher Scientific Company, Fairlawn, New Jersey), pH 7.41, at 0.0 to 4.0° using ice cold solutions, instruments, and glassware. After washing, the livers were separately homogenized (Tek Mar SDT-182 Tissueizer, Tekmar Company, Cincinnati, Ohio) and were centrifuged at 0.0 to 4.0° for 20 min at 9000 g (Beckman J2-21 Centrifuge, Beckman Instruments, Inc., Fullerton, California). The resulting supernatants containing the microsomal fractions of the liver homogenates were collected after discarding the fatty tissue floating atop the supernatant². Four aliquots of the microsomal fraction from the rat weighing 236 g (#1) in volumes of 0.5, 1.0, 1.5, and 2.0 ml were each diluted with the previous buffer to a total volume of 2.0 ml. Four 1.0 ml aliquots of the microsomal fraction from the rat weighing 192 g (#2) were diluted to a total volume of 2.0 ml each with buffer. All eight aliquots were placed in an incubator (Dubnoff Metabolic Shaking Incubator, Precision Scientific Co., Chicago, Illinois) and allowed to equilibrate for 2.0 min, after which 5.284×10^4 dpm of [^{14}C] NVP were added to each of the four aliquots from rat #1 while 1.321×10^4 , 2.642×10^4 , 3.963×10^4 , and 5.284×10^4 dpm were added to the four aliquots from the second rat. All eight preparations were incubated for 15 min at 37° and then terminated by addition of 1.0 ml of 3.0 N trichloroacetic acid to each. The resulting protein precipitated from each incubation was washed successively with 0.6 N trichloroacetic acid and acetone until the wash solutions contained background ^{14}C levels. The protein was analyzed for ^{14}C content.

Analyses of Urine Samples Following Intravenous
Intravenous Injection of [¹⁴C] NVP

Three hundred and eighty mg of acetaldehyde were added to a flask containing 20 ml of distilled water. The contents of the flask were distilled into an ice chilled flask containing 5.0 ml of methanol. The methanol trapped distillate was treated with a solution prepared by dissolving 2.0 g of 2,4-dinitrophenylhydrazine in 100 ml of methanol and 4.0 ml of concentrated sulfuric acid and refrigerated overnight. Crystals of the derivative were then collected and washed with cold methanol. The phenylhydrazone, confirmed by NMR, was produced in 77% yield based on the amount of acetaldehyde put in the distillation flask. A 1.0 ml sample of urine collected during the first 6.0 hr following a 2.86 μ Ci dose was treated as above and the phenylhydrazone crystals were collected in 76% yield. These crystals, along with the methanol wash, were analyzed for radioactivity.

A 1.0 ml sample of urine collected during the first 6.0 hr following a 2.86 μ Ci dose was utilized to analyze for the presence of ¹⁴C-labeled acetic acid as a metabolite of [¹⁴C] NVP. To the urine sample was added 0.3 g of carrier acetic acid, two drops of concentrated sulfuric acid, and enough water to obtain a total volume of 25 ml. The mixture was distilled at 120° and the distillate was then collected. The distillation was repeated twice, with carrier acetic acid and water added prior to the second and third distillations. The distillates obtained from these distillations were analyzed individually for radioactivity.

A urine sample collected from 6.0 to 18.0 hr following a 2.86 μ Ci dose was acidified with 1.0 ml of concentrated hydrochloric acid and passed through a cation exchange column (Bio Rad type AG50N-X2 Hydrogen form, 100-200 mesh size, Bio Rad Laboratories, Richmond, California). The acidified urine sample was followed by two 100 ml water washes and then 100 ml of a 1.0 N NaOH solution. All washes were analyzed for ¹⁴C content. A urine sample collected during the first 7.0 hr after a 1.16 μ Ci dose was basified with 6.0 N NaOH and passed through an anion exchange column (Bio-Rad Type AG 1-X2, 100-200 mesh size, Bio Rad Laboratories, Richmond, California). The alkaline urine sample was passed through the column, followed by 100 ml of distilled water and 100 ml of 1.0 N HCl. The solutions collected from the column were individually analyzed for radioactivity.

A urine sample collected during the first 6.0 hr after a 2.86 μ Ci dose was extracted with chloroform and then refluxed in 10% NaOH for 12 hr. The basic solution

was acidified and again extracted with chloroform. Both chloroform extracts were analyzed for ^{14}C -activity. Subsequently the acidified hydrolysate was distilled and the radioactivity in the distillate was counted. The residue of acidified hydrolysate remaining after distillation was chromatographed on a cation exchange column as described above. All fractions collected were analyzed individually for ^{14}C -activity.

In a separate experiment, a 40 μl sample of urine collected during the first 6.0 hr following a 2.86 μCi dose of [^{14}C] NVP was applied to a TLC plate (Whatman K3) which was developed using CHCl_3 as the eluent. The plate was then analyzed for distribution of radioactivity by the method described for analysis of the dosing solution. The presence or absence of 1,1-bis-(N-2-oxopyrrolidinyl)-ethane was determined by comparison of the chromatogram with that of authentic non-radioactive compound.

Fat from the greater omentum was removed at 42 hr and 145 hr after dosing with 2.86 μCi and 1.16 μCi of NVP respectively and was analyzed for ^{14}C -activity.

RESULTS

Radioactivity in the Plasma, Urine, and Tissues of Rats After a Dose of [^{14}C] NVP.

Plasma levels of intact NVP in three rats decreased rapidly over a 6.0 hr period following a 5.0 μCi iv injection with a $t_{1/2}$ of 1.5 hr. A plot of this data resulted in a biphasic curve (Figure 1). Forty to 65% of the total radioactivity dosed was excreted in the urine during this 6.0 hr study. However, intact NVP in the urine at this time point accounted for less than 0.2% of the administered radioactivity. At 15, 30, and 90 min as well as 6.0 hr (Table I) following an iv dose of [^{14}C] NVP, the liver, small intestines and contents, and kidneys were the organs of highest ^{14}C -activity accumulation. If the distribution is expressed as relative concentration (2) (Table I), the small intestine, liver, and kidneys exhibited the highest ^{14}C -accumulation at 15, 30, and 90 min while the small intestine and contents, adrenal gland, kidney and liver had the highest ^{14}C -activity at 6.0 hr.

Radioactivity in the Urine, Feces, and Expired CO_2 of Rats After a Dose of [^{14}C] NVP.

By 24 hr after administration of 5.0 μCi of [^{14}C] NVP to two rats, 73.4 and 64.5% of the administered dose had been excreted in the urine and 2.4 and 5.5% in the feces. At this time point, two rats receiving a 2.86 μCi dose eliminated 70.2 and 66.1% of the administered ^{14}C -activity in the urine, 7.8 and 1.9% in the feces, and 2.7 and 0.81% in

the expired CO_2 . By 24 hr, the two animals receiving a 1.16 μCi dose eliminated 92.5 and 75.1% of the activity dosed into the urine, 1.0 and 1.6% in the feces, and 2.0 and 2.6% in the expired CO_2 . Collection of urine, feces, and expired CO_2 samples at daily intervals after the first 24 hr showed that excretion by these three routes was the most rapid during the initial 24 hr period following dosing (Table II).

Biliary Excretion of Radioactivity After a Dose of [^{14}C] NVP.

By 6.0 hr following administration of a 5.0 μCi dose of [^{14}C] NVP to two rats, 17.2 and 20.0% of the ^{14}C -activity administered was excreted into the bile. Biliary excretion of intact NVP during this 6.0 hr study accounted for 0.34 and 0.14% of the administered dose. Cumulative excretion of radioactivity by this route in one rat is shown in Figure 2.

Protein Binding of ^{14}C -Activity Following Incubation of [^{14}C] NVP with Rat Liver Microsomal Fractions.

Results from protein binding studies utilizing a constant activity of [^{14}C] NVP incubated with varying amounts of rat liver microsomal fraction are shown in Table III. As the quantity of microsomal fraction utilized was increased, the percent of the ^{14}C -activity associated with precipitated protein increased from 3.0 to 11.9% of the activity used. Following incubation of variable amounts of [^{14}C] NVP with a constant volume of rat microsomal fraction, the radioactivity associated with precipitated protein remained within a range of 5.3 to 6.8% of the ^{14}C -activity originally added (Table IV).

Analysis of Metabolites in Urine from Rats Following a Dose of [^{14}C] NVP.

Practically no [^{14}C] acetaldehyde was found in the 1.0 ml aliquot of the urine collected during the first 6.0 hr following a 2.86 μCi dose as determined by addition of non-radioactive acetaldehyde to the urine, distillation, and finding only a negligible amount of radioactivity in the 2,4-dinitrophenylhydrazone derivative collected. Twelve percent of the total ^{14}C -activity in the 1.0 ml aliquot of the urine collected during the first 6.0 hr following a 2.86 μCi dose was present in the form of acetate, as determined by distillation of the acetic acid treated urine. Cation exchange chromatography showed that 1.7% of the radioactivity in urine collected between 6.0 and 18 hr following a 2.86 μCi dose was in the form of a basic molecule as shown by elution in the final NaOH wash, while the

remaining activity was an acidic or neutral species eluted either in the HCl or water fractions. Use of an anion exchange column for analyzing a urine sample collected during the first 7.0 hr after a 1.16 μCi dose of [^{14}C] NVP showed that 11.0% of the ^{14}C -activity in the urine was recovered as basic or neutral material while 88.9% was found to be acidic species.

Chloroform extraction of urine collected during the first 6.0 hr following a 2.86 μCi dose yielded no radioactivity in this extract. The aqueous phase was refluxed in 10% NaOH for 12 hr following which the hydrolysate solution was acidified, and a second chloroform extraction recovered no radioactivity. The acidified hydrolysate was distilled and 12% of the initial radioactivity in the sample was found in the distillate. The residue of the acidified hydrolysate remaining in the distillation flask was introduced onto a cation exchange column and 73% of the original ^{14}C -activity in the sample was eluted as a neutral or acidic species in the HCl and water washes. Basic moiety(s) eluted by the NaOH wash accounted for 15% of the radioactivity in the urine sample.

Thin layer chromatography analysis of a urine sample collected during the first 6.0 hr after a 2.86 μCi dose of [^{14}C] NVP resulted in no detection of radioactivity at the position of the Rf. value of 1,1-bis-(N-2-oxopyrrolidinyl)ethane. Most of the radioactivity on the plate was found to reside at the origin, indicating the presence of a highly polar fraction.

Analysis of fatty tissues excised at 42 hr after a 2.86 μCi dose of [^{14}C] NVP and 145 hr after a 1.16 μCi dose resulted in $2.28 \times 10^{-3}\%$ of the dose per gram of fat and $8.65 \times 10^{-3}\%$ respectively.

DISCUSSION

Following iv administration of [^{14}C] NVP to the rats, the rate of urinary excretion of ^{14}C -activity was the highest during the initial 6 hr and the majority of the radioactivity was eliminated by this route within 24 hours. Only a negligible portion of the ^{14}C -activity found in the urine samples was due to intact [^{14}C] NVP. Thus, the rapid decline of the plasma levels of [^{14}C] NVP shown in Figure 1 is attributed to extensive metabolism and rapid urinary elimination of the metabolites. Additionally, biliary excretion appears to be an important factor in the loss of ^{14}C -activity from the plasma. In contrast, however, ^{14}C -activity eliminated in fecal and expired CO_2 samples accounted for only a minor portion of the total loss of ^{14}C -activity from the rat.

The observed high biliary excretion of ^{14}C -activity (17

to 20% in 6 hr) following iv administration of [^{14}C] NVP and the low elimination of ^{14}C -label in the feces (2.3% in 6 hr), relative to the amount found in the small intestines, suggest that enterohepatic recycling of NVP metabolites is occurring to a significant extent. It is noteworthy that more than 98% of the total ^{14}C -activity in the bile was found to be in species other than intact NVP at the end of 6 hr.

The relatively low percent of radioactivity associated with the precipitated microsomal fraction (Tables III and IV) indicates that NVP is probably not converted to an alkylating species in the course of its catabolism. This is partially supported by the fact that NVP failed to exhibit positive results in the Ames and Mouse Lymphoma tests. Furthermore, NVP did not display any mutagenic activity in *in vitro* transformation with BALB/3T3 cells and was shown to be inactive in an unscheduled DNA synthesis assay. Additional experiments are needed to conclusively eliminate the possibility of formation of an exocyclic epoxide species of NVP or any other metabolite of NVP with alkylating ability. Such experiments are currently being conducted in this laboratory.

In a recent publication (3), the kinetics of the *in vitro* acid-catalyzed decomposition of NVP were reported. The proposed mechanism involved the production of acetaldehyde and 2-pyrrolidinone, with subsequent formation of 1,1-bis-(N-2-oxopyrrolidinyl)-ethane (Scheme I, structure X). The possibility was considered of non-enzymatic formation of the 1,1-bis-(N-2-oxopyrrolidinyl)-ethane adduct upon contact with the low pH environment of the stomach or the slightly acidic urine. Nuclear magnetic resonance (NMR) studies in our laboratory showed that NVP was susceptible to acid decomposition at pH 1.2 (the approximate pH of the stomach), with formation of the bis-adduct. Additionally, the compound was seen to be unstable to TLC on silica gel plates (Whatman LK5), due to the acidity of the silica gel used as the sorbent.

Partially in view of the acid susceptibility of NVP, an initial objective of the metabolic studies was to determine if ^{14}C -labeled acetaldehyde was present in the urine of rats as a metabolite, following a dose of [^{14}C] NVP. Derivatization, the method chosen to accomplish this objective, produced only a negligible amount of ^{14}C -labeled acetaldehyde derivative in the urine sample. Since it is known that acetaldehyde is oxidized *in vivo* to acetic acid, an attempt was made to isolate ^{14}C -labeled acetate in the urine of a rat dosed with [^{14}C] NVP. It was found that 12% of the total ^{14}C -activity in the urine was due to acetic acid. TLC analysis of the urine used for the acetaldehyde and acetic acid determinations revealed no

1,1-bis(N-2-oxopyrrolidinyl)-ethane (Scheme I, structure X). The formation of the ^{14}C -labeled two-carbon fragment is probably enzymatic and could occur at any point in the metabolism of [^{14}C] NVP, giving rise to acetic acid, but would not necessarily result in the formation of the bis adduct. Additionally, the adduct may have been formed and then further metabolized.

Occurrence of a ^{14}C -labeled two-carbon molecule in the urine led to analysis of the fat tissues to check for incorporation of labeled two-carbon fragments into fatty biomolecules, but negligible ^{14}C -activity was found in the excised adipose tissue.

Studies using ion exchange chromatography to obtain information on the functional groups of the ^{14}C -species excreted into the urine after a dose of [^{14}C] NVP showed that 1.7% of the radioactivity in the urine represented a basic material, 88.9% represented acidic species (12% acetic acid), and approximately 11.3% appeared to be neutral. However, when the urine sample was hydrolyzed prior to ion exchange analysis, the percent of ^{14}C -activity corresponding to basic species increased from 1.7 to 15%, while the remaining activity was due to acidic or neutral material. Recovery of the chromatographed ^{14}C -activity from the columns using aqueous eluents was essentially quantitative. This, in addition to the fact that ^{14}C -activity could not be extracted from the urine into chloroform, emphasizes the high water solubility of the metabolites.

Some proposed routes of metabolism for NVP are shown in Scheme I. From this, it should be noted that a metabolite arising from [^{14}C] NVP must contain at least one of the ^{14}C -labeled vinyl carbons in order to be detected. Thus, if NVP undergoes fragmentation during metabolism, the fate of the ring portion of the molecule would not be determined. Due to the chemical reactivity of the enamide, transformation of NVP (I) to 2-pyrrolidinone (II) is suspected to occur readily. If this is indeed the case, the interpretation of results or any conclusions drawn would have to concentrate on the possibility that the large majority of the activity being analyzed arises from the initial production of ^{14}C -labeled acetaldehyde with concomitant formation of an unlabeled pyrrolidinone ring.

If 2-pyrrolidinone (II) is formed, it may be converted to the naturally occurring gamma-aminobutyric acid (GABA) (III), a transformation known to occur in the brain of the mouse (4), and in bacteria (5). GABA (III) is shown to be metabolized to succinic acid (VII) via succinic semi-aldehyde (IV). Alternatively, 2-pyrrolidinone (II) could be converted to succinimide (VI) which is known to be

metabolized to succinic acid (VII) in the rat and dog (6). Succinic acid is a constituent of the tricarboxylic acid cycle.

An alternate route for the metabolism of [^{14}C] NVP may be its conversion to N-vinyl-succinimide (IX), with subsequent production of succinic acid and ^{14}C -labeled acetamide.

Metabolic studies with doubly labeled NVP ([$4\text{-}^3\text{H}$] and [vinyl- ^{14}C]) are underway in this laboratory in order to distinguish between the metabolic pathways of NVP which involve separation of the two-carbon (vinyl) moiety from the ring, and routes which result in retention of the carbons of the vinyl group.

FOOTNOTES

1. Private communication from GAF Corporation.
2. Procedure from: Mazel, P., "General Principles and Procedures for Drug Metabolism In Vitro," in Fundamentals of Drug Metabolism and Drug Disposition, B.N. La Du, H.G. Mandel, and E.L. Way, eds., Robert E. Krieger Publishing Company, Huntington, New York, pp. 531-535 (1979).

Table I Tissue Distribution of ¹⁴C-Activity in Rats Following a 5 μ Ci iv Injection of [¹⁴C] MVP, Expressed as Percent of the Dose Administered and Relative Concentration^a.

Tissue	Time after dosing (hr)		
	0.25 ^b	0.5 ^b	1.5 ^b
Heart	0.34 (1.24) ^a	0.32 (0.93) ^a	0.32 (1.01) ^a
Lungs	0.78 (1.89)	0.79 (1.26)	0.66 (1.50)
Spleen	0.30 (1.27)	0.27 (1.02)	0.26 (0.98)
Kidneys	2.19 (2.84)	1.70 (2.21)	2.36 (3.01)
Liver	11.45 (2.90)	19.78 (5.51)	8.93 (2.27)
Pancreas	0.44 (1.01)	0.40 (0.96)	0.45 (1.12)
Testes	0.56 (1.11)	0.53 (0.75)	0.50 (0.80)
Muscle	-	-	-
Skin	-	-	-
Adrenal gland	0.04 (1.66)	0.03 (0.79)	0.03 (0.16)
Thymus	0.14 (1.21)	0.10 (0.90)	0.02+0.01 (1.82+0.97)
Thyroid gland	0.03 (1.47)	0.02 (1.09)	0.04+0.02 (0.34+0.07)
Brain	0.41 (0.95)	0.32 (0.57)	0.00 (0.59+0.09)
Stomach and contents	0.73 (0.77)	0.48 (0.65)	0.16+0.03 (0.31+0.05)
Sm. intestine	5.60 (5.12)	12.80 (9.19)	0.92 (0.73)
Sm. intestine contents	-	-	3.30 (3.56)
Large intestine	0.461(1.57)	0.370(0.83)	4.96+1.24 (2.31+0.58)
intestined	-	-	1.46 (1.06)
			0.77+0.23 (0.99+0.29)

^aRelative Concentration = $\frac{\mu\text{Ci found per g specimen}}{\mu\text{Ci administered per g body weight}}$

^bvalues for one rat

^cvalues are the means \pm S.D. of three determinations

^dExcluding contents

Table II ¹⁴C-Activity Distribution in the Urine, Feces, and Expired CO₂ of Rats Following a Single iv Injection of [¹⁴C] MVP, Expressed as Percent of the Dose Administered.

Dose	Days post administration	Urine	Rat 1 Feces	CO ₂	Urine	Rat 2 Feces	CO ₂
5 μCi	1	73.36	2.38	-	64.45	5.51	-
	2	0.99	0.42	-	10.44	4.02	-
	3	0.21	0.23	-	1.49	0.24	-
	4	0.01	0.10	-	0.08	0.22	-
	5	0.10	0.04	-	0.18	0.12	-
	6	0.05	0.07	-	0.41	0.22	-
2.86 μCi	1	70.23	7.84	2.67	66.05	1.89	0.81
	2	0.52	0.36	0.40	0.35	0.49	0.42
	3	-	-	-	0.30	0.16	0.20
	4	-	-	-	0.01	0.08	0.15
	5	-	-	-	0.09	0.06	0.18
	6	-	-	-	0.06	0.07	0.11
1.16 μCi	1	92.53	1.03	1.96	75.07	1.56	2.63
	2	-	-	-	0.64	0.35	0.47
	3	-	-	-	0.21	0.19	0.24
	4	-	-	-	0.12	0.13	0.16
	5	-	-	-	0.13	0.13	0.14
	6	-	-	-	0.11	0.07	0.30

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Table III. Effect of Incubation of a Constant Amount of [¹⁴C] MVP with Varying Volumes of Rat Liver Microsomal Fraction on Protein Binding of ¹⁴C-Activity.

Volume of microsomal fraction ^a	DPM of [¹⁴ C] MVP incubated	Precipitated protein		Dose in precipitated protein	DPM/g of precipitated protein
		Weight	DPM		
0.5 ml	5.284 x 10 ⁴	0.08 g	1582.6	3.00	19,782.5
1.0 ml	5.284 x 10 ⁴	0.24 g	4215.3	7.98	17,563.8
1.5 ml	5.284 x 10 ⁴	0.36 g	5277.4	10.00	14,659.4
2.0 ml	5.284 x 10 ⁴	0.48 g	6275.6	11.88	13,074.2

^aBuffer added to total 2.0 ml in all cases.

Table IV: Effect of Incubation of Varying Amounts of [¹⁴C] MVP with a Constant Volume of Rat Liver Microsomal Fraction on Protein Binding of ¹⁴C-Activity.

Volume of microsomal fraction ^a	DPM of [¹⁴ C] MVP incubated	Precipitated protein		Dose in precipitated protein	DPM/g of precipitated protein
		Weight	DPM		
1.0 ml	1.321 x 10 ⁴	0.36 g	898.5	6.80	2,495.8
1.0 ml	2.642 x 10 ⁴	0.37 g	1553.1	5.88	4,197.6
1.0 ml	3.963 x 10 ⁴	0.39 g	2623.4	6.62	6,726.7
1.0 ml	5.284 x 10 ⁴	0.38 g	2809.8	5.32	7,394.2

^aBuffer added to total 2.0 ml in all cases.

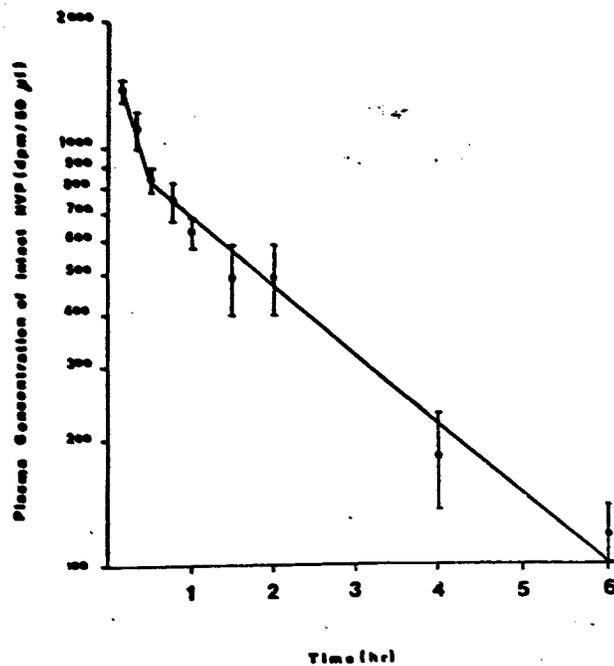


Fig. 1. Plasma Levels of Intact [^{14}C] NVP in Rats Following a Single iv Injection of 5 μCi . Results are the means (\pm S.D) of Three Determinations.

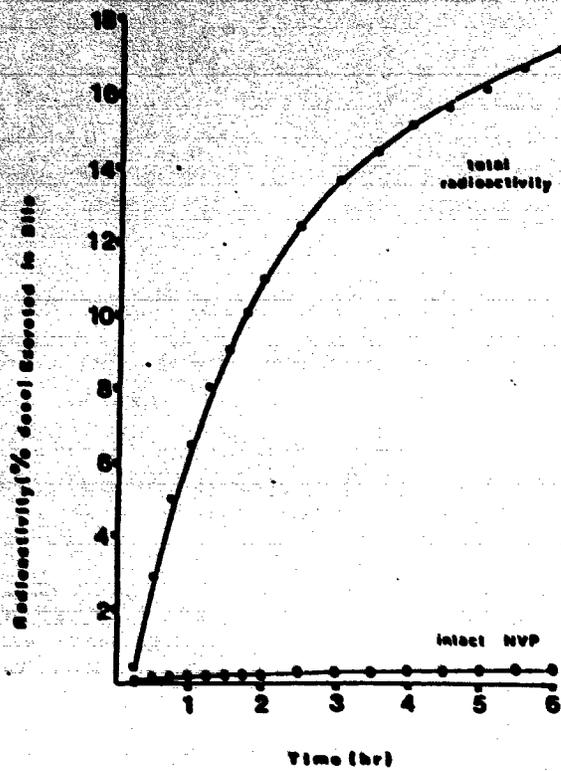
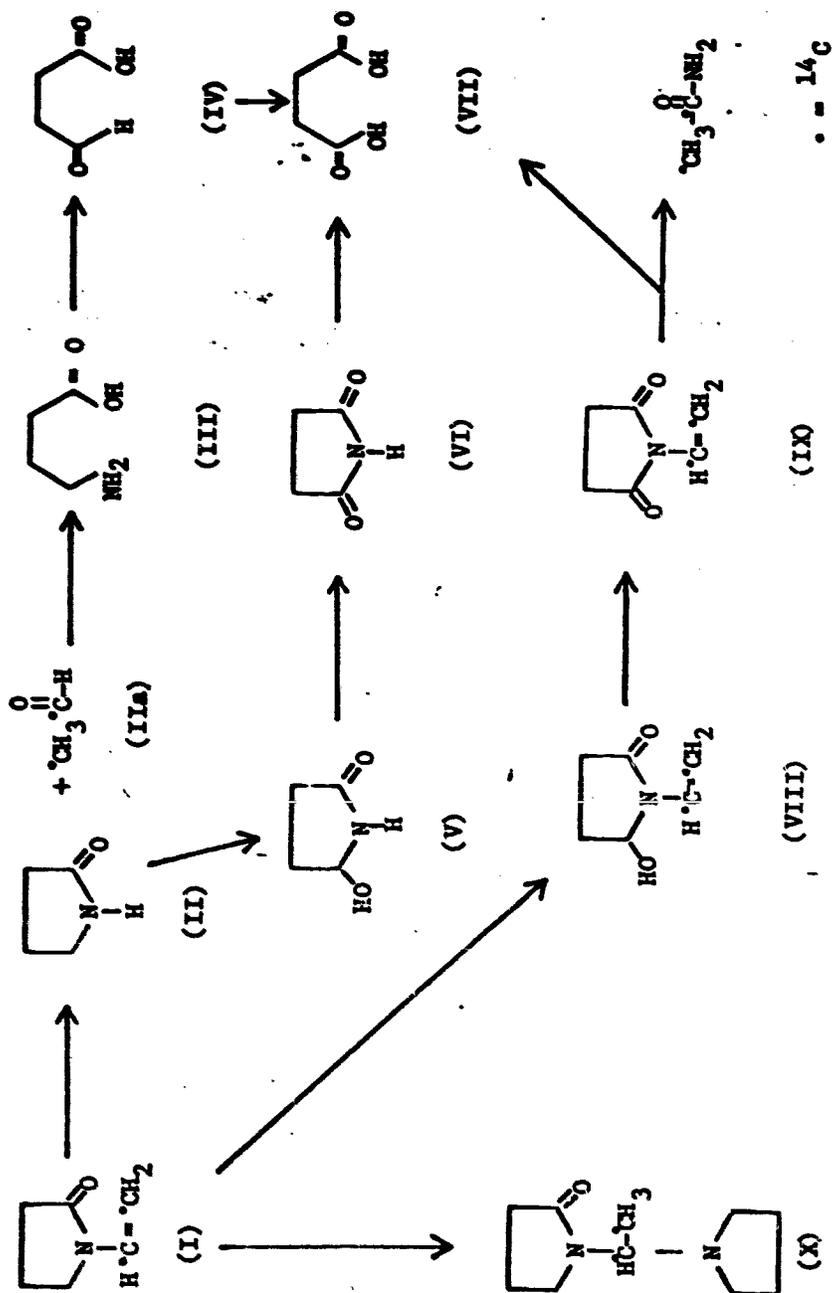


Fig. 2. Cumulative Biliary Excretion of ¹⁴C-Activity in One Rat Following a Single iv Injection of 5 µCi of [¹⁴C] NVP.

Scheme I



REFERENCES

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GENETICS ASSAY NO. 4899

LBI SAFETY NO. 4816

MUTAGENICITY EVALUATION OF
V - PYROL[®] (N-VINYL-2-PYRROLIDONE)

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

FINAL REPORT

SUBMITTED TO:

GAF CORPORATION
1361 Alps Road
Wayne, New Jersey
07470

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20989

REPORT DATE: FEBRUARY, 1980



BIONETICS

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I - IX. Items I - IV provide sponsor and compound identification information, type of assay, and the assay design reference number. All assay design references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled Assay Design, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.



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- I. SPONSOR: GAF Corporation
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO. 4899
 - A. Identification: V-Pyrol[®] (N-Vinyl-2-Pyrrolidone)
 - B. Date Received: January 4, 1980
 - C. Physical Description: clear colorless liquid
- III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay
- IV. ASSAY DESIGN NUMBER: 431 (DMT-106)
- V. STUDY DATES:
 - A. Initiation: January 14, 1980
 - B. Completion: January 28, 1980
- VI. SUPERVISORY PERSONNEL:
 - A. Study Director: Brian C. Myhr, Ph.D.
 - B. Laboratory Supervisor: Jane Fisher
- VII. RESULTS:

The data are presented in Table 1 on page 4.
- VIII. INTERPRETATION OF RESULTS:

The test material, V-Pyrol[®] (N-Vinyl-2-Pyrrolidone), was completely miscible with water at a concentration of 100 $\mu\text{l/ml}$. This solution was serially diluted with water in two-fold steps to provide a series of 10 concentrations down to 0.195 $\mu\text{l/ml}$. These stocks were then diluted 1:10 into culture medium containing the cells to yield a final concentration range of 10 $\mu\text{l/ml}$ to 0.0195 $\mu\text{l/ml}$ for the preliminary cytotoxicity assay. No precipitation in the culture medium was noted. Twenty-four hours after treatment, the cell count was considerably reduced by the exposure to 5 $\mu\text{l/ml}$, and 10 $\mu\text{l/ml}$ was completely lethal. Therefore, in order to cover a wide toxicity range, the mutation assay was initiated with a series of concentrations from 10 $\mu\text{l/ml}$ to 0.39 $\mu\text{l/ml}$.

The results of the mutation assay are presented in Table 1.

Under nonactivation conditions, the mutant frequencies in the treated cultures were all comparable to the background frequency (average of the solvent and untreated negative control values).



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VIII. INTERPRETATION OF RESULTS (continued)

The minimum criterion for demonstrating mutagenesis by a given treatment in this assay was a mutant frequency exceeding 44.5×10^{-6} . No indication of an increase in mutant frequency was obtained over the assayed concentration range of $0.313 \mu\text{l/ml}$ to $5.0 \mu\text{l/ml}$. Moderate toxicity was achieved with the relative growth dropping to 33.9% for the $5.0 \mu\text{l/ml}$ treatment. A further small increase in concentration to $7.5 \mu\text{l/ml}$ (not shown) was completely lethal to the cells. Therefore, the test material did not exhibit mutagenic activity under nonactivation conditions for concentrations closely approaching those causing excessive lethality.

In the presence of the S9 microsomal activation mix, the test material was not toxic from $0.313 \mu\text{l/ml}$ to $1.25 \mu\text{l/ml}$, and the $2.5 \mu\text{l/ml}$ and $5.0 \mu\text{l/ml}$ treatments were only weakly-to-moderately toxic. Treatment with $7.5 \mu\text{l/ml}$ (not shown) killed nearly all the cells within twenty-four hours. Except for the one culture exposed to $2.5 \mu\text{l/ml}$, the mutant frequencies remained below the minimum value of 52.9×10^{-6} considered necessary to indicate mutagenesis. The observed increase at $2.5 \mu\text{l/ml}$ was too small to provide sufficient evidence for mutagenesis and could only be interpreted as an anomaly, since the more toxic treatment with $5.0 \mu\text{l/ml}$ did not yield a similar or even larger increase. These results were therefore evaluated as showing the lack of mutagenesis by the test material for concentrations closely approaching excessive lethality.

The average cloning efficiencies for the solvent and untreated negative controls varied from 84% without activation to 72% with activation, which demonstrated good culturing conditions for the assays. The negative control mutant frequencies were normal, and the positive control compounds yielded normal mutant frequencies that were greatly in excess of the backgrounds.

IX. CONCLUSIONS:

The test material, V-Pyrol[®] (N-Vinyl-2-Pyrrolidone), did not induce a significant change in the mutant frequency at the TK locus in L5178Y mouse lymphoma cells in the absence or presence of rat liver S9 microsomal activation. Concentrations up to 5.0 μ l/ml were assayed; 7.5 μ l/ml was extremely toxic and impossible to assay. Therefore, the test material is considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

Submitted by:

Study Director

Brian C. Myhr
Brian C. Myhr, Ph.D.
Section Chief
Mammalian Genetics
Department of Genetics
and Cell Biology

2-13-80
date

Reviewed by:

David J. Brusick
David J. Brusick, Ph.D.
Director
Department of Genetics
and Cell Biology

2/13/80
date

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8. SUBSTITUTED MOUSE LYMPHOMA CELL LINE RESULTS

TABLE

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: V-PYROL (N-VINYL-2-PYRROLIDONE)
 B. LOT CODE #: 4899
 C. SOLVENT: WATER
 D. TEST DATE: 01/15/80

TEST NONACTIVATION	SOURCE	ISSUE	DAILY COUNTS			RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH	MUTANT FREQUENCY IN LIVER
			1	2	3						
SOLVENT CONTROL	---	---	11.0	12.1	100.0	61.0	222.0+	100.0	100.0	27.3	
SOLVENT CONTROL	---	---	6.3	10.6	100.0	46.0	221.0	100.0	100.0	26.3	
UNTREATED CONTROL	---	---	7.7	9.3	71.7	65.0	316.0	142.7	102.2	28.1	
EMS .5 UL/ML	---	---	5.3	5.7	30.2	564.0	60.0	27.1	0.2	900.0	
TEST COMPOUND	---	---	11.9	7.2	65.7	45.0	187.0	84.4	72.4	24.1	
0.313 UL/ML	---	---	13.3	5.1	67.9	46.0	278.0	129.5	85.2	16.3	
0.625 UL/ML	---	---	9.4	6.2	58.3	50.0	204.0	93.0	94.2	24.1	
1.250 UL/ML	---	---	9.1	7.8	71.0	53.0	189.0	85.3	60.6	28.0	
2.500 UL/ML	---	---	6.3	6.3	39.7	51.0	189.0	85.3	33.9	27.0	
5.000 UL/ML	---	---									

ACTIVATION

SOLVENT CONTROL	PAT	LIVER	9.3	10.1	100.0	64.0	166.0	100.0	100.0	34.0
SOLVENT CONTROL	RAT	LIVER	8.5	12.5	100.0	68.0	237.0	100.0	100.0	28.0
UNTREATED CONTROL	RAT	LIVER	10.5	13.2	138.5	52.0	229.0	108.3	149.9	22.0
DMW .3 UL/ML	RAT	LIVER	5.8	3.4+++	19.7	105.0	13.0	5.4	1.1	807.7
TEST COMPOUND	RAT	LIVER	7.6	16.4	124.5	72.0	206.0+	97.4	121.3	35.0
0.313 UL/ML	RAT	LIVER	10.3	10.3	106.0	79.0	222.0	105.0	111.3	35.0
0.625 UL/ML	RAT	LIVER	11.7	10.3	120.4	90.0	180.0+	85.1	102.5	50.0
1.250 UL/ML	RAT	LIVER	7.9	11.9	93.9	104.0	170.0+	80.4	79.9	41.0
2.500 UL/ML	RAT	LIVER	6.6	8.5	56.0	123.0	247.0	116.8	65.5	49.8

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
 ** THE RATIO OF CELLS SEDED FOR MUTANT SELECTION TO CELLS SEDED FOR CLONING EFFICIENCY IS 10E+4.
 *** THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES) * 10E+6.
 † THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

‡ ONE PLATE CONTAMINATED; VALUE BASED ON REMAINING TWO PLATES.
 +++ CULTURE NOT SPLIT BACK TO 3.0 X 10⁶ CELLS; THE ENTIRE CULTURE WAS ASSAYED AND 340 CELLS SEDED FOR VIABLE CLONE COUNT.
 ††† VALUES FOR RELATIVE CLONING EFFICIENCY AND PERCENT RELATIVE GROWTH WERE ADJUSTED ACCORDINGLY.

ASSAY DESIGN (NO. 431)

1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK+/- mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU) or 5-trifluorothymidine (TFT).

2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single step forward mutation to the TK -/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK-/- mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK-/- mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK-/- genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK+/-, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 100 µg/ml of BrdU or 3 µg/ml of TFT.



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3. MATERIALS (continued)

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 μ l/ml as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 μ l/ml as a positive control for assays performed with activation.

D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.



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B. Mutagenicity Testing

1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period, 3×10^6 cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50 μ l S9/ml.

C. Preparation of 9,000 x g Supernatant (S9)

Fischer 344 male rats are normally used as the source of hepatic microsomes. Induction with Aroclor 1254 or other agents is performed by injections five days prior to sacrifice. After decapitation and bleeding, the liver is immediately dissected from the animal using aseptic technique and placed in ice cold 0.25M sucrose buffered with Tris at pH 7.4. When an adequate number of livers is obtained, the collection is washed twice with fresh buffered sucrose and completely homogenized. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge and the supernatant (S9) from this centrifuged sample is retained and frozen at -80°C until used in the activation system. The S9 fraction may be obtained from induced or noninduced rats or other species, as requested.



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5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

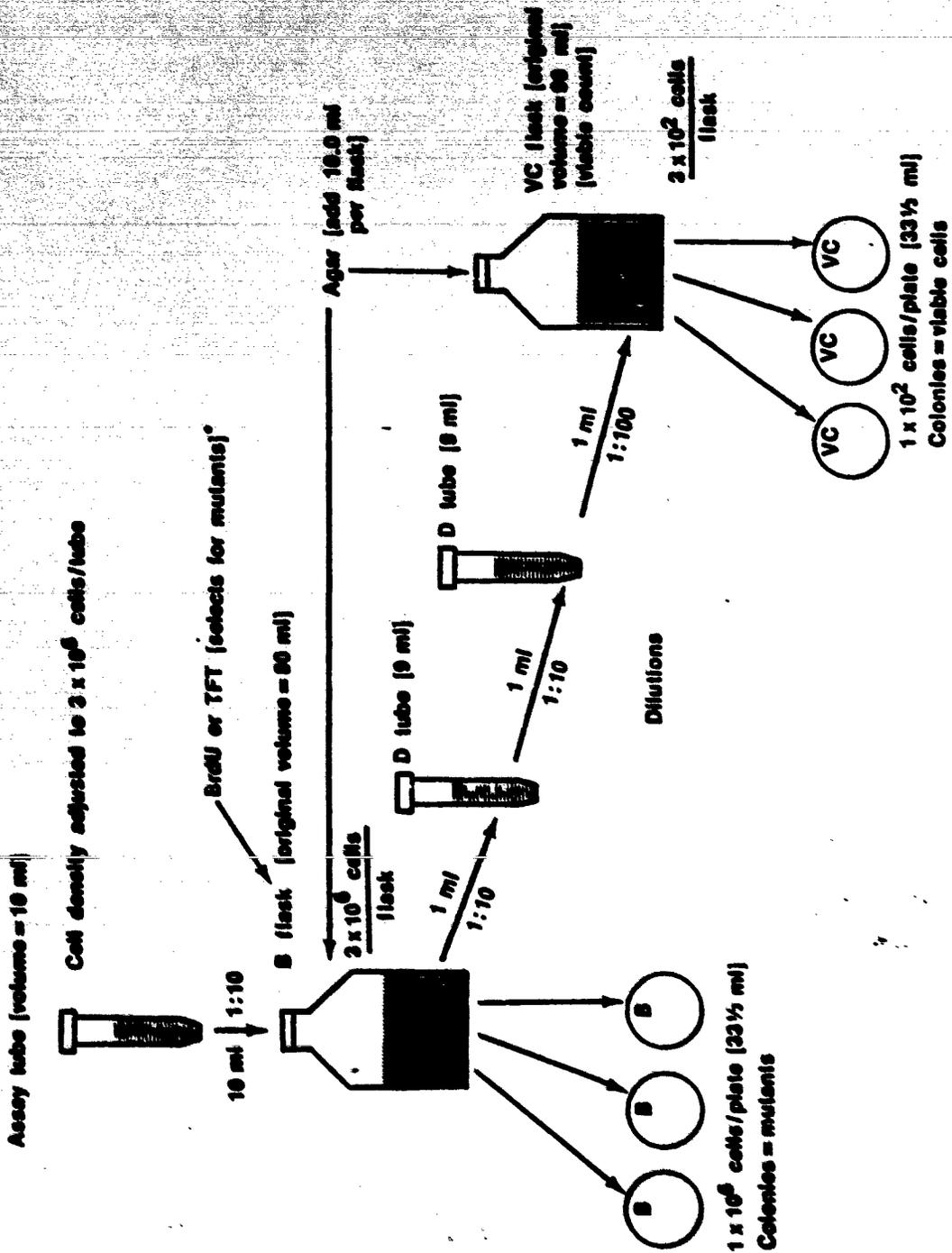
6. REFERENCE

Clive, D. and Spector, J.F.S.: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutation Res.*, 31:17-29, 1975.



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*Added after removal of 1 ml for viable count dilutions.

FIGURE 1. LYMPHOMA CLONING FLOW CHART

ASSAY ACCEPTANCE CRITERIA

An assay will normally be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and negative controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

- 1) The average absolute cloning efficiency of the negative controls (average of the solvent and untreated controls) should be between 70% and 130%. A value greater than 100% is possible because of errors in cell counts (usually $\pm 10\%$) and cell division during unavoidable delays between the counting and cloning of many cell cultures. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50% to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range are conditionally acceptable and dependent on the scientific judgement of the study director. All assays below 50% cloning efficiency are unacceptable.
- 2) The solvent and untreated negative controls normally have the same growth rates and cloning efficiencies within experimental error. An unusual effect by the solvent therefore indicates an abnormal cell state or excessive amount of solvent in the growth medium. An assay will be unacceptable if the average percent relative growth of the solvent controls is less than about 70% of the untreated control value.
- 3) The average negative control suspension growth factor should not be less than about 15. The optimal value is 25, which corresponds to 5-fold increases in cell number for each of the two days following treatment of the experimental cultures.
- 4) The background mutant frequency (average frequency of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 5×10^{-6} to 50×10^{-6} . Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for the evaluation of a test material. These assays can provide supporting evidence.



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5) A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by 0.5 μ l/ml EMS (nonactivation assay) is 300 to 800 $\times 10^{-6}$; for 0.3 μ l/ml DMN (activation assay) the normal range is 200 to 800 $\times 10^{-6}$. The concurrent background frequencies have been subtracted from these values. These ranges are broad primarily because the effective treatment with these agents is variable between assays. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test material clearly shows mutagenic activity as described in the evaluation criteria. If the test material appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency above the lower limits of the normal range. Assays in which the normal range is exceeded may require further interpretation by the study director.

6) For test materials with little or no mutagenic activity, an assay must include applied concentrations that reduce the suspension growth to 5% to 10% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. Suspension growth is a combined measure of cell death and reduced growth rates. A 5% relative suspension growth therefore could correspond to 90% killing followed by growth of the survivors at one-half the normal rate for one day and normal growth for the second day. At the other extreme, this condition could be obtained by no killing and complete inhibition of growth for two days. A reasonable limit to testing for the presence of mutagenic action is about 80% to 90% killing of cells. Because of the uncertainty in the actual lethality of treatment in the assay and the fact that mutant frequencies increase as a function of lethality, an acceptable assay for the lack of mutagenic activity must extend to the 5% to 10% relative suspension growth range. There is no maximum toxicity requirement for test materials which clearly show mutagenic activity.

7) An experimental treatment that results in fewer than 2.5×10^6 cells by the end of the two-day growth period will not be cloned for mutant analysis.

8) An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 20. These limits avoid problems with the statistical distribution of scoreable colonies among dishes and allows factors no larger than 10 in the adjustment of the observed number of mutant clones to a unit number of cells (10^6) able to form colonies.



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9) Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about 3-fold.

10) The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test material.



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ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test material that is expected, on the basis of preliminary toxicity studies, to span the cellular responses of no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

The minimum condition considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that exceeds 150% of the concurrent background frequency by at least 10×10^{-6} . The background frequency is defined as the average mutant frequency of the solvent and untreated negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds and the calculated minimum increase as defined above is often a repeatable result; statistical analysis for the confidence limits is not yet available.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test material as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.
- An increase in mutant frequency may be followed by only small or no further increases at higher concentrations or toxicities. However, a decrease in mutant frequency to values below the minimum criterion is not acceptable in a single assay for classifying the test material as a mutagen. If the mutagenic activity at lower concentrations or toxicities was large, a repeat assay will be performed to confirm the mutagenic activity.
- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in the Assay Acceptance Criteria, the test material will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.



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For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied material that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent relative growth), can be used to establish whether the mutagenic activity is related to an increase in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

A test material will be evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing 5% to 10% relative suspension growth. If the test material is relatively nontoxic, the maximum applied concentrations will normally be 10 mg/ml (or 10 μ l/ml) for water-soluble materials or 1 mg/ml (or 1 μ l/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response, as discussed above, the test material will be evaluated as nonmutagenic in this assay system.

The ASSAY ACCEPTANCE AND EVALUATION CRITERIA are presented to acquaint the sponsor with the considerations used by the study director to determine assay validity and the mutagenic activity of the test material. This presentation may not encompass all test situations, and the study director may use other criteria, especially when data from several repeat assays are available, to arrive at a conclusion. The report will provide the reasoning involved when departures from the above descriptions occur.



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Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 20989

LBI Assay No. 4899

TYPE OF STUDY Money Laundering Inward Protection Assay

This final study report was reviewed by the LBI Quality Assurance Unit on 2/20/80. A report of findings was submitted to the Study Director and to Management on 2/26/80.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately every three months to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Patrick J. O'Malley
Auditor, Quality Assurance Unit

GENETICS ASSAY NO. 4899

LBI SAFETY NO. 4816

EVALUATION OF
V-PYROL[®]
(N-VINYL-2-PYRROLIDONE)
IN THE
IN VITRO TRANSFORMATION
OF BALB/3T3 CELLS ASSAY

FINAL REPORT

SUBMITTED TO:

GAF CORPORATION
1361 ALPS ROAD
WAYNE, NEW JERSEY 07470

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20975

LBI PROJECT NO. 20992

REPORT DATE: APRIL 1980



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PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IX. Items I-IV provide sponsor and compound identification information, type of assay, and the assay design reference number. All assay design references indicate a standard procedure described in the Litton Bionetics, Inc. Manual for the Identification of Potential Mutagens and Carcinogens. Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled Assay Design, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.



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- I. SPONSOR: GAF Corporation
- II. MATERIAL (TEST COMPOUND): Genetics Assay Number 4899
- A. Identification: V-Pyrol[®] (N-Vinyl-2-Pyrrolidone)
- B. Date Received: January 4, 1980
- C. Physical Description: Clear liquid
- III. TYPE OF ASSAY: In Vitro Transformation of Balb/3T3 Cells Assay
- IV. ASSAY DESIGN NUMBER: 441 (DMT-107)
- V. STUDY DATES:
- A. Initiation: January 28, 1980
- B. Completion: March 25, 1980
- VI. SUPERVISORY PERSONNEL:
- A. Study Director: John O. Rundell, Ph.D.
- B. Laboratory Supervisor: G. Murthi

VII. RESULTS:

The results of the assay are presented in Tables 1 and 2 on pages 4 and 5.

VIII. INTERPRETATION OF RESULTS:

The test material, N-vinyl-2-pyrrolidone, was soluble in culture medium at a concentration of 1.0 μ l/ml. Dilutions were performed with culture medium to obtain a series of 15 concentrations in 2-fold dilution steps for the preliminary cytotoxicity test. The cytotoxicity test determines the effect of the test material on the ability of 3T3 cells to form colonies after 72 hours exposure and is used to select test concentrations for the transformation assay.

The results of the cytotoxicity test are shown in Table 1.

The relative survivals of the treated cultures ranged from 37.0% at 1.0 μ l/ml to 85.3% at 0.061 nl/ml. The transformation assay is applied to treatments that cause between 50% and 100% survival and is considered to be most sensitive near 70% survival, since the observed frequency of transformed foci is not corrected for the



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VIII. INTERPRETATION OF RESULTS (continued)

number of cells surviving the treatment. Therefore, the wide concentration range of 0.5 μ l/ml to 0.1 nl/ml, corresponding to a survival range of approximately 52.3% to 83% (estimated graphically) was selected for the assay.

The results of the transformation assay are presented in Table 2.

The historical negative control for the subclone of 3T3 cells used in this assay consists of 145 flasks containing a total of 12 transformed foci for an average of 0.08 foci/flask. In this assay, 4 foci were observed among 15 negative control flasks, giving an average frequency of 0.27 foci/flask. This spontaneous frequency was significantly different ($p < 0.05$) from the historical control value, using the Kastenbaum-Bowman Tables². Therefore, the assay results were evaluated by comparison with the internal negative control.

A sudden increase in the background frequency of transformed cells is a characteristic of the 3T3 line that seems to occur randomly but with increased probability as the number of passages in culture increases. When the increase occurs, the frequency remains high and it is necessary to return to frozen stock cells to maintain a low background for future assays. The increase seems to represent a rapid accumulation of transformed cells rather than a change in the sensitivity of the cells to transformation by treatment. Thus, the MCA positive control in this assay yielded 4.26 foci/flask above the background frequency, and this result was similar to other assays in which MCA induced 3.5 to 5 foci/flask with cells that had a background frequency within the historical baseline for clone I13C14. The validity of the assay, therefore, does not seem to be influenced by the increase in background; the primary disadvantage is the loss in statistical resolution between the treated cultures and a limited set of negative control flasks.

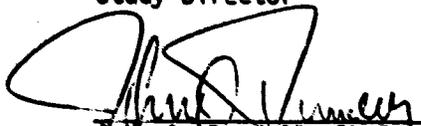
As shown in Table 2, treatment with the test material at concentrations from 0.1 nl/ml to 0.5 μ l/ml caused a small, progressive increase in the average number of transformed foci/flask. The largest increase, however, represented a change in total number of foci from 4 in the negative controls to only 9 in the cultures exposed to 0.5 μ l/ml of test material. This increase was not significant at the 95% confidence level, using the Kastenbaum-Bowman Tables², so the test material was considered to be inactive as a transforming agent.

IX. CONCLUSIONS:

The test material, N-vinyl-2-pyrrolidone, did not induce a significant increase in transformed foci over the applied concentration range of 0.5 μ l/ml to 0.1 nl/ml. This concentration range corresponded to approximately 52.3% to 83% survival in the cytotoxicity test. Therefore, the test material is considered to be inactive in the Balb/3T3 In Vitro Transformation Assay.

Submitted by:

Study Director


John O. Rundell, Ph.D.
Assistant Section Chief
Department of Genetics
and Cell Biology

5/28/80
date

Reviewed by:


David J. Brusick, Ph.D.
Director
Department of Genetics
and Cell Biology

3/31/80
date



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TABLE 1

TOXICITY TEST IN BALB 3T3 CELLSCLIENT: GAF GENETICS ASSAY NO. 4899 DATE: February 13, 1980COMPOUND CODE: N-Vinyl-2-pyrrolidoneSOLVENT: Medium

<u>TEST COMPOUND DOSES TESTED</u>	<u>AVERAGE NUMBER OF COLONIES/PLATE</u>	<u>% SURVIVAL RELATIVE TO CONTROL</u>
1. 0.000061 μ l/ml	107.7	85.3%
2. 0.000122 μ l/ml	102.0	80.8%
3. 0.000244 μ l/ml	93.3	73.9%
4. 0.000488 μ l/ml	93.7	74.2%
5. 0.000977 μ l/ml	92.0	72.8%
6. 0.00195 μ l/ml	92.0	72.8%
7. 0.00391 μ l/ml	86.7	68.6%
8. 0.00781 μ l/ml	81.7	64.7%
9. 0.0156 μ l/ml	80.0	63.3%
10. 0.0313 μ l/ml	80.0	63.3%
11. 0.0625 μ l/ml	76.7	60.7%
12. 0.125 μ l/ml	72.7	57.6%
13. 0.250 μ l/ml	69.0	54.6%
14. 0.500 μ l/ml	66.0	52.3%
15. 1.000 μ l/ml	46.7	37.0%
16. 0 (control)	126.3	100%



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TABLE 2
SUMMARY OF DATA FROM TRANSFORMATION ASSAY

CLIENT: GAF GENETICS ASSAY NO. 4899 TEST DATE: February 18, 1980
 CLIENT'S COMPOUND CODE: N-vinyl-2-pyrrolidone SOLVENT: Medium 3T3 CLONE: IL3C1*

TEST	DOSES TESTED	NUMBER OF FOCI PER FLASK SCORED															TOTAL NO. OF FOCI	NO. OF FOCI/FLASK	NO. OF FOCI/FLASK CORRECTED FOR SPONTANEOUS
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
Solvent Control		0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	4	0.27	
Positive Control (MCA) 5 µg/ml		6	5	5	5	5	4	3	5	3	5	4	5	4	4	68**	4.53	4.26	
Test Material:																			
4899 0.0001 µl/ml		0	0	1	0	0	0	1	0	0	0	1	0	0	0	3	0.20	0	
4899 0.001 µl/ml		0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0.07	0	
4899 0.01 µl/ml		0	0	1	0	0	0	0	0	0	0	1	0	1	0	3	0.20	0	
4899 0.1 µl/ml		0	0	1	0	0	1	1	0	0	0	1	0	0	1	5	0.33	0.06	
4899 0.5 µl/ml		1	0	1	1	1	0	1	1	1	0	0	0	1	0	9	0.60	0.33	

MCA = 3-Methylcholanthrene

** = Data significant from the negative control at $p \leq 0.01$.

ASSAY DESIGN (NO. 441)

1. OBJECTIVE

This assay evaluates the carcinogenic potential of test materials using mouse BALB/3T3 cells in culture. The objective of this semi-quantitative assay is to evaluate the test material for its ability to induce foci of transformed cells, recognized by dense, piled-up colonies on a monolayer of normal cells.

2. RATIONALE

BALB/3T3 mouse cells will multiply in culture until a monolayer is achieved and will then cease further division. These cells, if injected into immunosuppressed, syngeneic host animals, will not produce neoplastic tumors. However, cells treated in vitro with chemical carcinogens will give rise to foci of cellular growth super-imposed on the cell monolayer. If these foci are picked from the cultures, grown to larger numbers and injected into animals, a malignant tumor will in most cases be obtained. Thus, the appearance of piled-up colonies in treated cell cultures at a higher frequency than in control cultures is highly correlated with malignant transformation.

3. MATERIALS

A. Indicator Cells

Clone 13a of BALB/3T3 mouse cells was obtained from Dr. Takeo Kakunaga. Further subclones, selected for low spontaneous frequencies of foci formation, are used for assays. Stocks are maintained in liquid nitrogen and laboratory cultures are checked periodically to ensure the absence of mycoplasma contamination. Cultures are grown and passaged weekly in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum.

B. Control Compounds

1. Negative Controls

A negative control consisting of assays performed on untreated cells is performed. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are assayed as the solvent negative control to determine any effects on survival or transformation caused by the solvent alone. Fifteen flasks of the



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3. MATERIALS (continued)

B. Control Compounds

1. Negative Controls

appropriate type of negative control are prepared for each assay.

2. Positive Control

3-methylcholanthrene (MCA) is a known carcinogen and is used as a positive control for the transformation of 3T3 cells. Fifteen flasks are treated with 5 µg MCA per milliliter for each assay.

C. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection

The solubility of the test chemical in growth medium, DMSO or other solvent is first determined. Fifteen dose levels of the test compound are then chosen, starting with a maximum applied dose of 1 mg/ml for solid compounds or 1 µl/ml for liquid samples and decreasing in twofold-dilution steps. Each dose is applied to three culture dishes seeded 24 hours earlier with 200 cells per dish. After an exposure period of three days, the cells are washed and incubated in growth medium for an additional four days. The surviving colonies are stained and counted by an automatic colony counter. A relative survival for each dose is obtained by comparing the number of colonies surviving treatment to the colony counts in negative control dishes. The highest dose chosen for subsequent transformation assays should cause no more than a 50% reduction in colony forming ability and is best located near 30% reduction. Four lower doses (including one or two doses with low or no apparent toxicity) are also selected for the transformation assay.

B. Transformation Assay

The procedure used at LRI is based on that reported by Kakunaga (1973)^{1, 2}. Twenty-four hours prior to treatment, a series of 25-cm² flasks is seeded with 10⁴ cells/flask



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4. EXPERIMENTAL DESIGN (continued)

B. Transformation Assay

and incubated. Fifteen flasks are then treated for each of the following conditions: Five preselected doses of test chemical; positive control; and solvent negative control, if applicable. The flasks are incubated for a three-day exposure period; the cells are then washed and incubation is continued for four weeks with refeeding twice a week. The assay is terminated by fixing the cell monolayers with methanol and staining with Giemsa. The stained flasks are examined by eye and by microscope to determine the number of foci of transformed cells.

5. SCORING OF TRANSFORMED FOCI

At the end of the four-week incubation period, cultures of normal cells yield a uniformly stained monolayer of round, closely-packed cells. Transformed cells form a dense mass (focus or colony) that stains deeply (usually blue) and is superimposed on the surrounding monolayer of normal cells. The foci are variable in size.

Scored foci have several variations in morphological features. Most foci consist of a dense piling up of cells and exhibit a random, criss-cross orientation of fibroblastic cells at the periphery of the focus. Other scored foci are composed of more rounded cells with little criss-crossing at the periphery but with necrosis at the center caused by the dense piling up of a large number of cells. A third variation is a focus without the necrotic center and large number of cells but which exhibit the criss-cross pattern of overlapping cells throughout most of the colony.

Some foci are not scored. These include small foci of transformed morphology that are found in close proximity to larger foci; these foci are regarded as being formed from cells which migrated from the larger colony. Other unscored foci are small areas where some piling up of rounded cells has occurred but the random orientation of fibroblastic cells is not observed. Microscopic examination is employed for scored foci and in the final judgement of transformed character for any marginal foci.

6. CONFIRMATION OF TUMORIGENICITY OF TRANSFORMED CLONES

Most transformed clones will produce malignant tumors when collected from an unstained transformation plate and injected into syngeneic host animals. Although not routinely performed, this confirmation step can be conducted at additional cost.



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ASSAY ACCEPTANCE CRITERIA

The assay will be considered acceptable for evaluation of the test results if the following criteria are met:

- 1) The negative control flasks consist of a contiguous monolayer of cells which may or may not contain transformed foci. The lack of a contiguous sheet of cells indicates growth conditions too poor to allow the reliable detection of weak transforming agents
- 2) The negative control transformation frequency does not exceed an average of about 2 foci/flask. Attempts are made to isolate and maintain cell stocks (subclones of BALB/3T3 I13) with a very low spontaneous frequency of transformation.
- 3) The positive control yields an average number of foci/flask that is significantly different from the negative control at the 99% confidence level.²
- 4) A minimum of 8 flasks per test condition are available for analysis. At least 4 dose levels of test substance are assayed.
- 5) The dose range of test substance assayed falls within the 50-100% survival range as determined by the preliminary toxicity test, which measures relative cloning efficiencies.



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EVALUATION CRITERIA

In many cases, no transformed foci will be observed in the set of flasks comprising the negative control. This does not necessarily mean that any foci found in the treated flasks constitutes a positive response in this assay. In order to determine what minimum number of foci will allow a conclusion that the frequency of transformed foci has been elevated over the negative control, a historical negative control data base is used. This data base consists of the ten most recent assays in which 100 to 150 negative control flasks have been scored. The total number of flasks and transformed foci in this set will be provided in each report.

The statistical tables provided by Kastenbaum and Bowman² are used to determine whether the results at each dose level are significantly different from the historical control at the 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing the probabilities in the tails of two binomial distributions. The 95% confidence level must be met to consider the test substance active at a particular dose level.

If the negative control is found by the same test to be significantly different from the historical control ($p \leq 0.05$), the assay will be evaluated independently. Comparisons between the current negative control and tested dose levels will be analyzed by the Kastenbaum-Bowman tables.

The number of induced foci usually does not increase proportionately with the applied dose in this assay. In fact, above a minimum dose level where the number of foci is elevated, further increases in dose may result in little or no increase in the number of foci. The number of foci can be reduced at the highest dose assayed if the toxicity is too high. A response at only one dose level (other than the highest tested dose) that just meets the 95% confidence level will normally not be considered sufficient evidence for activity in this assay. All other degrees of response will usually provide evidence for classifying a test substance as active, although the study director exercises scientific judgement and may obtain expert opinion in the evaluation of each test substance.



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7. REFERENCES

1. Kakunaga, T.: A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3T3. *Int. J. Cancer*, 12:463-473, 1973.
2. Kastenbaum, M.A. and Bowman, K.O.: Tables for determining the statistical significance of mutation frequencies. *Mutation Res.*, 9:527-549, 1970.



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Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 20992

LBI Assay No. 4899

TYPE OF STUDY In V.tro Transformation of BALB/3T3 Cells

This final study report was reviewed by the LBI Quality Assurance Unit on 7/2/80. A report of findings was submitted to the Study Director and to Management on 4/2/80.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately every three months to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Mitchell S. Ehrlich
Auditor, Quality Assurance Unit

GENETICS ASSAY NO. 4899

LBI SAFETY NO. 4816

EVALUATION OF
V-Pyrol[®] (N-vinyl-2-pyrrolidone)
IN THE
PRIMARY RAT HEPATOCYTE
UNSCHEDULED DNA SYNTHESIS ASSAY

FINAL REPORT

SUBMITTED TO:

GAF CORPORATION
T361 ALPS ROAD
WAYNE, NEW JERSEY 07470

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20991

REPORT DATE: APRIL, 1980



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PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IX. Items I-IV provide sponsor and compound identification information, type of assay, and the assay design reference number. All assay design references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled Assay Design, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.



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I. SPONSOR: GAF Corporation

II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO. 4899

A. Identification: V-Pyrol[®] (N-vinyl-2-pyrrolidone)

B. Date Received: January 4, 1980

C. Physical Description: Clear, colorless liquid

III. TYPE OF ASSAY: Primary Rat Hepatocyte Unscheduled DNA Synthesis Assay

IV. ASSAY DESIGN NO.: 447

V. STUDY DATES:

A. Initiation: January 16, 1980

B. Completion: April 11, 1980

VI. SUPERVISORY PERSONNEL:

A. Study Director: Brian C. Myhr, Ph.D.

B. Laboratory Supervisor: Marie McKeon

VII. RESULTS:

The data are presented in Table 1 on page 4.

VIII. INTERPRETATION OF RESULTS:

The test material, V-Pyrol[®], was soluble in the LME (1% serum) culture medium at a concentration of 500 μ l/ml, although the medium turned pink, indicating an alkaline pH by the phenol red component of the medium. This stock was serially diluted in 2-fold steps and 25 μ l quantities were added to the 2.5 ml cell cultures to achieve a dose range of 5 μ l/ml to 0.305 μ l/ml for the preliminary cytotoxicity testing. No pH changes were noted in the cell cultures as a result of treatment. Two hours after a 45 min-exposure period, the number of viable cells per unit area of the culture flasks was determined. Only the 5 μ l/ml treatment caused apparent toxicity (81.9% survival). By 24 hours treatment, however, the toxic action of the test material became more evident. The survival to the 5 μ l/ml treatment was approximately 15%, and the survival increased steadily as the concentration decreased until 97.7% survival was obtained at 0.156 μ l/ml. These results suggested a concentration range of about 20 μ l/ml to 0.3 μ l/ml for the UDS assay in order to cover wide range of toxic effects on the cells.



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VIII. INTERPRETATION OF RESULTS, continued:

In the UDS assay, 1 hour treatments in WME containing 1% serum were followed by a 3 hour labeling period with ^3H -thymidine in complete WME (10% serum). Six cultures were exposed to each concentration of test material in order to have three for labeling, one for a 2-hour viable cell count, and two for determining a 24-hour average viable cell count. The assayed concentrations (Table 1) were not whole numbers because the 2.5 ml cultures were diluted with 0.25 ml of stock solutions, which were freshly prepared in WME (1% serum) medium. The survival data in Table 1 shows that a wide range of toxicity was manifest during the labeling period. The exposure to 18.2 $\mu\text{l/ml}$ was completely lethal, and as the concentration decreased from 9.09 $\mu\text{l/ml}$ to 0.284 $\mu\text{l/ml}$, the survival increased from 25.2% to 100.0%. Further cell death occurred with additional time following treatment such that a survival range of 6.2% to 84.5% was observed 24 hours later. These results show that an appropriate treatment range was assayed for the possibility of different degrees of DNA damage, detectable by measuring unscheduled DNA synthesis (UDS).

The assay did not detect the presence of UDS activity caused by the treatments with test material. None of the parameters used to assess UDS activity were greater than the solvent control values. One of the solvent control coverslips had an unusually high level of nuclear labeling with a mean grain count of 5.02 grains/nucleus and 32% of the nuclei having 6 or more grains. If this culture had been excluded because of some technical fault, the solvent control values in Table 1 would have been 1.88 grains/nucleus and 9.0% nuclei with 6 or more grains. Only the high dose of test material (9.09 $\mu\text{l/ml}$) gave UDS values exceeding this adjusted solvent control level, but the increase was insignificant and well below the criteria for a positive response. In contrast to the test material, the 2-AAF positive control clearly induced a response that met all of the criteria for UDS. Therefore, the assay provided convincing evidence that the test material did not interact with cellular DNA in a way that resulted in UDS activity.

The number of heavily-labeled nuclei (blackened with numerous grains) was low in this assay. Among the 12,000 cells screened in the entire assay (500 per coverslip), an average of 0.06% (7 cells) were heavily labeled. Therefore, very few hepatocytes were undergoing DNA replication and this process did not interfere with the measurement of UDS.

IX. CONCLUSIONS:

The test material, V-Pyrol, did not induce detectable UDS in primary rat hepatocytes over an applied concentration range of 9.09 $\mu\text{l/ml}$ to 0.284 $\mu\text{l/ml}$. This concentration range corresponded to a cell survival range of 6.2% to 84.5% at 24 hours after treatment. Exposure to 18.2 $\mu\text{l/ml}$ caused complete lethality. Therefore, the test material is considered to be inactive in the Primary Rat Hepatocyte UDS Assay.

SUBMITTED BY:

STUDY DIRECTOR

Brian C. Myhr

Brian C. Myhr, Ph.D.
Section Chief
Mammalian Genetics
Department of Genetics
and Cell Biology

4-14-80
date

Reviewed by:

David J. Brusick

David J. Brusick, Ph.D.
Director
Department of Genetics
and Cell Biology

4/15/80
date



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TABLE 1

SUMMARY OF DATA FROM RAT HEPATOCYTE UDS ASSAY

CLIENT: GAF Corporation LBI ASSAY NO. 4899 ASSAY INITIATION DATE: January 16, 1980
 CLIENT'S CODE: V-Pyrol[®] SOLVENT: WME medium TECH: M. McKeon

Test Condition	Concentration	UDS* grains/nucleus	Avg.† % nuclei with ≥ 6 grains	Avg.† % nuclei with ≥ 20 grains	Survival†† at 2 hr, %	Survival†† at 24 hr, %
Solvent Control (WME medium)	---	2.93	16.7	0	100.0	100.0
Positive Control 2-AAF	400 µg/ml	10.09	70.0	11.3	58.3	36.3
<u>Test Material:</u>						
V-Pyrol [®]	18.2 µl/ml	Not analyzable (no cellular labeling)				
V-Pyrol [®]	^a 9.09 µl/ml	2.11	11.3	0	0	0
V-Pyrol [®]	4.55 µl/ml	1.38	4.0	0	25.2	6.2
V-Pyrol [®]	2.27 µl/ml	1.26	4.0	0	47.4	21.9
V-Pyrol [®]	1.14 µl/ml	1.43	7.3	0	73.2	50.1
V-Pyrol [®]	0.568 µl/ml	1.50	5.3	0	60.2	62.3
V-Pyrol [®]	0.284 µl/ml	1.37	6.7	0	90.4	63.4
					100.0	84.5

*UDS = Average of net nuclear grain counts on triplicate coverslips (150 total cells).
 †Average values for triplicate coverslips.

††Survival = Number of viable cells per unit area relative to the solvent control value x 100%, determined 2 hrs and 24 hrs after the treatment period.

^a2-AAF = 2-acetyl aminofluorene

One coverslip not analyzable; for the remaining two coverslips, 100 cells were scored on one and 50 cells on the other.

ASSAY DESIGN (NO. 447)

1. OBJECTIVE

The objective of this assay is to detect DNA damage caused by the test material or an active metabolite by measuring UDS in primary rat hepatocytes in vitro. The existence and degree of DNA damage will be inferred from an increase in nuclear grain counts compared to untreated hepatocytes. The types of detectable DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^3H -thymidine) into the DNA.

2. RATIONALE

Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continue to metabolize for several days without undergoing cell division. Less than 0.2% of the cells enter S phase (replicative DNA synthesis). Therefore, if ^3H -thymidine is introduced in the culture medium, little or no label will be incorporated into nuclear DNA. The addition of a test material that interacts with the DNA often stimulates a repair response in which the altered portion of DNA is excised and the missing region replaced by DNA synthesis. This synthesis of DNA by non-dividing cells is known as UDS and can be measured by determining the amount of ^3H -thymidine incorporated into DNA. In this assay, an autoradiographic technique is used to determine the number of grains per nucleus caused by ^3H -thymidine incorporation. This UDS measurement of DNA repair is highly sensitive and appears to correlate very well with the known mutagenic or carcinogenic activities of chemicals (Williams, 1977). Furthermore, the use of primary hepatocytes has the advantage that these cells have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system.

3. MATERIALS

A. Indicator Cells

The indicator cells for this assay are hepatocytes obtained from adult male Fischer 344 rats (150-300 g), which are purchased from Charles River Breeding Laboratories, Inc. The animals are fed Purina Regular Rodent Chow (Formula 5001) and water ad libitum. One animal, identified by cage card, is used for the assay after a minimum quarantine period of one week.



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3. MATERIALS (Continued)

A. Indicator Cells

The cells are obtained by perfusion of the liver in situ with a collagenase solution, as described in Experimental Design. Monolayer cultures are established on plastic coverslips in culture dishes and are used the next day for the UDS assay.

B. Media

The cells are cultured in Williams' Medium E (WME) supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 125 µg/ml gentamycin. This medium is referred to as complete WME; incomplete WME contains no serum.

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test material is not soluble in water, a stock solution in an organic solvent (normally DMSO) is prepared; the final concentration of solvent in the growth medium will be 1% or less in the treated cultures and the negative (solvent) control.

2. Positive Controls

The positive control compounds are known to induce UDS in rat hepatocyte primary cell cultures. 2-Acetyl aminofluorene (2-AAF) at $2 \times 10^{-3}M$ (400 µg/ml) is normally used as the positive control. Aflatoxin B₁ (AFB₁) at $2 \times 10^{-4}M$ (60 µg/ml) or a sponsor-specified positive control may also be added or substituted for 2-AAF.

4. EXPERIMENTAL DESIGN

A. Dose Selection

A preliminary cytotoxicity test is initiated with a series of applied concentrations of test material, starting at a maximum concentration of 5000 µg/ml (or 5 µl/ml) and diluting in two-fold steps to about 0.6 µg/ml (0.6 nl/ml). The cells are exposed for a period of 1 hour about 2 hours after initiation of the primary cultures. After removal of the test material, the cells are incubated an additional 2 hours in WME. A viable cell count (trypan blue exclusion) is obtained and those treatments that reduced the number of viable cells below about 50%, relative to the negative control, will be eliminated from further testing. A second viable cell count is obtained at about 24 hours for the remaining treated cultures. At least five doses which span the range from no apparent toxicity to complete loss of viable cells in about 24 hours will be chosen for the UDS assay.



BIONETICS

4. EXPERIMENTAL DESIGN (Continued)

B. UDS Assay

This assay is based on the procedures described by Williams (1977). The hepatocytes are obtained by perfusion of livers in situ for 4 min with Hanks' balanced salts (Ca^{++} - and Mg^{++} - free) containing 0.5M ethyleneglycol-bis (β -aminoethyl ether)- $\text{N,N}'$ -tetraacetic acid (EGTA) and HEPES buffer at pH 7.0. Then incomplete WME with 100 units/ml of Type I collagenase is perfused through the liver for 10 min. The hepatocytes are dispersed by scraping the excised livers with a sterile steel comb in a culture dish containing incomplete WME and collagenase. After centrifugation to remove the collagenase, the cells are resuspended in complete WME and are counted. A series of 35 mm culture dishes, each containing a 25 mm round plastic coverslip, is inoculated with about 0.5×10^6 viable cells in 3 ml complete WME per dish.

An attachment period of 1.5 hours at 37°C in a humidified atmosphere containing 5% CO_2 is allowed. Unattached cells are then removed and the cultures are refed with 2.5 ml complete WME. Some of the cultures are then used for the preliminary cytotoxicity test, while the remaining cultures are incubated at 37°C until the next day for the UDS assay.

The UDS assay is initiated by replacing the media in the culture dishes with 2.5 ml WME containing only 1.0% fetal bovine serum and the test material at the desired concentration. If the test material is dissolved in DMSO, 25 μl aliquots of appropriate stock solutions are added to 2.5 ml of media (1.0% serum) in the culture dishes. Each treatment, including the positive and negative controls, is performed on six cultures. After treatment for one hour, the test material is removed and the cell monolayers are washed twice with incomplete WME. Three of the cultures for each treatment are used to monitor the toxicity of treatment; these cultures are refed with complete WME and returned to the incubator. The other three cultures from each treatment are refed with 2.5 ml complete WME containing 1 $\mu\text{Ci/ml}$ of ^3H -thymidine and incubated for 3 hours. The labeling is terminated by washing the cultures with complete WME containing 1mM thymidine. The toxicity of each treatment is monitored by performing viable cell counts on one culture 2 hours after treatment and on two cultures about 24 hours later.



BIONETICS

4. EXPERIMENTAL DESIGN (Continued)

B. UDS Assay

The nuclei in the labeled cells are swollen by placement of the coverslips in 1% sodium citrate for 10 min, and then the cells are fixed in acetic acid:ethanol (1:3) and dried for at least 3 days. The coverslips are mounted on glass slides (cells up), dipped in Kodak NTB2 emulsion, and dried. The coated slides are stored for 2 weeks at 4°C in light-tight boxes containing packets of Drierite. The emulsions are then developed in D19, fixed, and stained with Williams' modified hematoxylin and eosin.

The cells are examined microscopically at approximately 1500x magnification under oil immersion and the field is displayed on the video screen of an automatic counter. UDS is measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the net nuclear grain count. The coverslips are coded to prevent bias in grain counting.

5. EVALUATION CRITERIA

The net nuclear grain count is determined for 50 randomly selected cells on each coverslip, whether or not the nuclei contain grains. Only normal-appearing nuclei are scored, and any occasional nuclei blackened by grains too numerous to count are excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. If the actual count for any nucleus is less than zero (i.e., cytoplasmic count is greater than nuclear count), a net value of zero is used in the calculation of the mean value. The mean net nuclear grain count is determined from the triplicate coverslips (150 total nuclei) for each treatment condition.

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. These criteria are formulated on the basis of published results and laboratory experience and are used in lieu of a statistical treatment at this time to indicate a positive response. While the criteria are arbitrary guidelines that may not be applicable to all assays and may need revision as the data base increases, they represent a reasonable approach to the evaluation of the test material.



BIONETICS

5. EVALUATION CRITERIA (Continued)

The test material is considered active in the UDS assay at applied concentrations that cause:

- 1) An increase in the mean nuclear grain count to at least 6 grains/nucleus in excess of the concurrent negative control value, and/or
- 2) The percent of nuclei with 6 or more grains to increase above 10% of the examined population, in excess of the concurrent negative control, and/or
- 3) The percent of nuclei with 20 or more grains to reach or exceed 2% of the examined population.

Generally, if the first condition is satisfied, the second and often the third conditions will also be met. However, satisfaction of only the second or third conditions can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns and weak agents may strongly affect only a small minority of the cells. Therefore, all three of the above conditions are considered in an evaluation. If the negative control shows an average of 6 grains/nucleus or 1% of the cells have 20 grains/nucleus, the assay will normally be considered invalid.

A dose related increase in UDS for at least two consecutive applied concentrations is also desirable to evaluate a test material as active in this assay. In some cases, UDS can increase with dose and then decrease to near-zero with successively higher doses. If this behavior is associated with increased toxicity, the test material can be evaluated as active. If an isolated increase occurs for a treatment far removed from the toxic doses, the UDS will be considered spurious.

The test material is considered inactive in this assay if none of the above conditions are met and if the assay includes the maximum applied dose or other doses that are shown to be toxic by the survival measurements. If no toxicity is demonstrated for doses below the maximum applied dose, the assay is considered inconclusive and will be repeated with higher doses.

The positive control values are not used as reference points to measure the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.



BIONETICS

6. REFERENCE

Williams, G.M.: Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture. *Cancer Res.*, 37: 1845-1851, 1977.



BIONETICS

Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 20991

LBI Assay No. 4899

TYPE OF STUDY Human Cat Hepatic Unscheduled ~~Met~~ Synthesis

This final study report was reviewed by the LBI Quality Assurance Unit on 1-21-80. A report of findings was submitted to the Study Director and to Management on 1-21-80.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately every three months to assure that no significant problems exist that are likely to affect the integrity of this type of study.

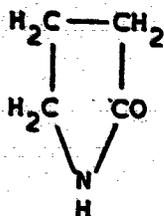
Michael P. Phil
Auditor, Quality Assurance Unit

August 4, 1971

Report on the testing of pyrrolidone for any teratogenic effect in the rat after oral administration

Pyrrolidone

Our test No.
XIX/421



Formulation of question

Pyrrolidone has already been tested for any teratogenic effect in the mouse after repeated intraperitoneal and oral administrations (see Report of May 29, 1970). There follows a report on corresponding tests with the product on the rat after oral administration.

Experimental procedure

Sprague-Dawley rats (SPF breed supplied by Gassner, Ottobrunn, FRG) were used for the investigations. The animals received Altromin-R (Altrogge, Lage/Lippe, FRG) as feed and water ad libitum.

The animals were housed in Makrolon cages, type D 3, (2 animals/cage) in an air-conditioned room ($22 \pm 2^{\circ}\text{C}$, $50 \pm 5\%$ humidity).

The investigations were carried out in accordance with the FDA guidelines (Guidelines for reproduction studies for safety evaluation of drugs for human use. Food and Drug Administration, Washington, January 1966).

Pregnant animals were weighed on day 0 of pregnancy (= positive evidence of sperm after mating at night) and split up into groups at random. The dose of the test substance administered from the 6th-15th day post coitum was based on the weight of the rat on day 0. The concentration of the solutions was adjusted in such a way that the amount of test substance to be administered for 100 g rat was contained in a volume of 0.5 ml. On the 20th day post coitum all the treated and untreated rats were sacrificed, the uteri were removed, the insertion and fetal resorption sites were recorded, the number of live and dead fetuses, their body length, their weight, and the sex and weight of the placentas were determined, and the fetuses were examined macroscopically for any deformities. Then a third of the fetuses of each dam were fixed in Bouin's solution and transversal sections were prepared and assessed according to WILSON's method (Wilson, Warkany: Teratology, Principles and Techniques, 1965). For the assessment of the skeletal system, the remaining fetuses were fixed in 96% strength alcohol, clarified with potassium hydroxide solution and stained with Alizarin red-S according to a modified DAWSON method (DAWSON, Stain Technol. 1, 123 (1926)). The uteri of the apparently non-pregnant animals or the empty uterine horns in the case of single-horn pregnancy were placed in 10% strength ammonium sulfide solution, left there for approximately 5 minutes and then assessed again in order to determine early resorptions. (Salewski, Arch. exp. Path. Pharmacol. 247, p. 367 (1964)).

Test protocol

Acute oral toxicity

The approximative median lethal dose, with an observation period of 14 days, was

about 8500 mm³/kg for the rat, orally.

The test substance was administered to the rats in the form of 30 - 60% strength solutions in aqua dest.

Test group	Amount of test substance	Number of pregnant dams
I	1700 mm ³ /kg	20
II	control, untreated	22

The amount of test substance specified in the table was administered orally to the dams daily from the 6th - 15th day post coitum.

All the animals were checked each day for any clinical symptoms of toxicity and weighed three times a week and before being sacrificed on the 20th day post coitum.

The treatment of the animals was carried out daily between 11.00 and 12.00 hours. The solutions were prepared freshly each day.

A total of 141 and 72 in test group I and 145 and 72 fetuses in test group II were examined by DAWSON's and WILSON's methods respectively.

RESULTS (see Tables 1 - 8)

1. Effect on the dams (Tables 1 - 5)

All the pregnant rats tolerated the 10 oral administrations of pyrrolidone at a dose of 1700 mm³/kg without visible signs of toxicity. One dam (No. 11) died intercurrently on the 17th day post coitum. The animal proved to be not pregnant. No substance-induced changes could be observed macroscopically.

The mean number of implantations and the percentage of resorptions with reference to implantations in the test group corresponded to the values of the control group.

2. Effect on the fetuses (Table 6)

The mean weight of the fetuses and their length in the test group did not differ from the values in the control group. The mean weights of the placentas in the test group and untreated control group were also almost the same.

The percentage of deformed fetuses with reference to live fetuses was 2.8 in both groups; similarly, the number of runts was of the same percentage in the test and control groups.

3. Skeleton assessment (Table 7)

Fetuses of dams Nos. 1 - 25^{x)} (1700 mm³/kg)

One fetus of dam No. 6 had a bipartite 12th thoracic vertebral centrum. One fetus of dam No. 10 was observed to have anasarca and two further fetuses of this dam had a cleavage of the 11th thoracic vertebral centrum. Dam No. 22

x) Original records can be inspected

had one deformed fetus. The tail of this fetus was missing and atresia ani was also recorded. One fetus of dam No. 24 had a bipartite 11th thoracic vertebral centrum.

Fetuses of dams Nos. 26 - 51^x) (Control untreated)

One fetus of dam No. 30 had a bipartite 11th thoracic vertebral centrum. One fetus of dam No. 33 had a bipartite 12th thoracic vertebral centrum. One fetus of each of dams Nos. 34 and 35 had a bipartite 11th thoracic vertebral centrum. The presphenoid was missing in one fetus of dam No. 44. One fetus of dam No. 47 had a bipartite 12th thoracic vertebral centrum.

4. Transversal sections (Table 8)

Fetuses of dams 1 - 25^x) (1700 mm³/kg)

No deformities were found in the fetuses of these dams.

Fetuses of dams 26 - 51^x) (Control, untreated)

No deformities were found in the fetuses of these dams.

Summary

Pyrrolidone was tested for any teratogenic effect on Sprague-Dawley rats.

In accordance with the FDA recommendations, pyrrolidone was administered to pregnant rats from the 6th to 15th day post coitum.

The dose was 1700 mm³/kg, corresponding to 1/5 ALD 50. Control animals remained untreated as control.

The following criteria were used for evaluating the study:

Effect of the test substance on the dams

Macroscopically evident changes in the dams at necropsy

Determination of the length and weight of the fetuses
and weight of the placentas

Macroscopic assessment of the fetuses

Assessment of the skeletal system after staining

Assessment of transversal sections.

The pregnant dams tolerated the 10 oral administrations of pyrrolidone without any visible symptoms of toxicity or any macroscopically evident pathological changes. The deformities or anomalies found in the fetuses of the test group corresponded in type and number to those of the changes occurring spontaneously in Sprague-Dawley rats. Pyrrolidone does not therefore have a teratogenic effect in Sprague-Dawley rats.

sign.

(Dr. med. H. ZELLER)

sign.

(Dr. med. vet. J. PEH)

Annexes

Tables 1 - 8

Mean litter data

Animal species: rat

Test substance: pyrrolidone

Treatment: 5th - 15th day post coitum

	1700 mm ³ /kg	Control (untreated)
Total number of animals	25	26
Pregnant animals	20	22
Conception rate %	80	84.6
Died spontaneously	1	-
Animals with deformed fetuses	4	6
in %	20	27.3
Implantations, total	233	232
Implantations per dam	11.7	10.5
Live fetuses, total	213	217
Live fetuses per dam	10.7	9.7
Dead fetuses	-	3
Resorptions	20	12
% with reference to implantations	8.6	5.2
Dams with:		
1 resorption	3	7
2 resorptions	7	1
3 resorptions	1	1
more than 3 resorptions	-	-
all embryos resorbed	-	-

Table 2

Animal species: rat

Individual litter data

Test substance: pyrrolidone

Treatment: 6th - 15th day post coitum

Dose: 1700 mm³/kg

Litter No.	Implantations	Live fetuses			dead fetuses	Resorptions			Deformities	
		total	male	female		total	early	late	skeleton	organs
1	14	12	3	9	-	2	-	-	-	-
2	9	8	3	5	-	1	-	1	-	-
3	4	1	-	1	-	3	-	-	-	-
4	12	10	5	5	-	2	-	-	-	-
5	12	12	4	8	-	-	-	-	-	-
6	17	16	7	9	-	-	-	-	-	-
7	13	11	6	5	-	1	1	-	1	-
8	10	8	1	7	-	2	2	-	-	-
9	11	11	5	6	-	2	2	-	-	-
10	17	17	9	8	-	-	-	-	-	-
11	not pregnant/died				-	-	-	-	3	-
12	14	12	6	6	-	2	2	-	-	-
13	7	6	2	4	-	1	1	-	-	-
14	11	9	4	5	-	2	2	-	-	-
15	not pregnant									
16	not pregnant									
17	7	7	2	5	-	-	-	-	-	-
18	15	13	7	6	-	-	-	-	-	-
19	9	9	5	4	-	2	1	1	-	-

Table 4

Animal species: rat

Individual litter data

Test substance: pyrrolidone

Dose: control (intraperitoneal)

Treatment: 6th - 15th day post coitum

Litter No.	Implantations	Live fetuses			dead fetuses	Resorptions		Deformities	
		total	male	female		total	early	late	skeleton
26	9	3	3	-	3	-	-	-	-
27	9	8	6	2	-	1	-	-	-
28	12	11	6	5	-	1	-	-	-
29	not pregnant								
30	11	11	5	6	-	-	-	1	-
31	not pregnant								
32	13	12	5	7	-	1	-	-	-
33	12	12	3	9	-	-	-	1	-
34	7	6	3	3	-	1	-	1	-
35	6	6	4	2	-	-	-	1	-
36	2	2	1	1	-	-	-	-	-
37	12	12	8	4	-	-	-	-	-
38	not pregnant								
39	10	10	6	4	-	-	-	-	-
40	12	12	5	7	-	-	-	-	-
41	12	10	4	6	-	-	-	-	-
42	not pregnant				2	1	1	-	-
43	13	12	6	6	-	1	-	-	-
44	15	15	4	11	-	-	-	1	-

Table 5

Animal species: rat

Test substance: pyrrolidone

Route of administration: oral

Treatment: 5th - 15th day post coitum

	1700 mm ³ /kg	Control (untreated)
Live fetuses, total	213	217
Ø	10.7	9.7
Dead fetuses, total	-	3
Weight in g Ø	3.27	3.30
Length in cm Ø	3.5	3.5
Weight of the placentas Ø	0.46	0.53
Fetuses with deformities	6	6
% with reference to live fetuses	2.3	2.8
Number of runts (%)	19 (8.9)	19 (8.8)

Animal species: rat

Test substance: pyrrolidone

Treatment: 5th - 15th day post coitum

Route of administration: oral

Assessment of the fetuses according to DAWSON (skeleton)

	1700 mm ³ /kg	Control (untreated)
Number of fetuses examined	141	145
<u>Type and number of</u> <u>deformities (%)</u>		
Anasarca	1 (0.7)	-
Atresia ani	1 (0.7)	-
Cleavage of thoracic vertebral centra	4 (2.8)	5 (3.4)
Ecaudate	1 (0.7)	-
Aplasia of the presphenoid	-	1 (0.7)
Aplasia of the sternum	-	1 (0.7)
<u>Anomalies</u>		
Sternum	2 (1.4)	1 (0.7)
Aplasia of individual sternabrae	59 (41.8)	44 (30.3)
<u>Insufficient ossification</u>		
General retardation	3 (2.1)	2 (1.3)
Skull	5 (3.5)	-

Table 8

Animal species: rat

Test substance: pyrrolidone

Treatment: 6th - 15th day post coitum

Route of administration: oral

Assessment of the fetuses according to WILSON (transversal sections)

	1700 mm ³ /kg	Control (untreated)
Number of fetuses examined	72	72
<u>Anomalies (%)</u>		
Slight dilatation of the lateral ventricle	2 (2.8)	-
Incomplete descent of the testis	1 (1.4)	-

Our reference
XIX/421

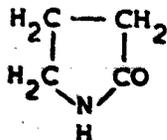
6700 Ludwigshafen
May 29, 1970

Report on the testing of pyrrolidone for any teratogenic effect in the mouse

The Monomer Department (Dr. Hofmann) sent us a sample to be tested for any teratogenic effect

Pyrrolidone

Our test No.
XIX/421



Formulation of question

The testing of pyrrolidone for any teratogenic effect proved to be necessary because an analgesic containing a component substituted with the pyrrolidone ring had been found to have a teratogenic effect in rats which a comparative product containing the component without a pyrrolidone ring did not have.

The tests described below were initially carried out only on mice since, at the time, corresponding tests on rats were not possible because of lack of space. It was investigated whether the repeated intraperitoneal or oral administration of pyrrolidone to dams in the sensitive phase of pregnancy caused deformities in the fetuses.

TESTS

The investigations were carried out with NMRI mice (breed of Gassner, Ottobrunn, FRG). At the beginning of the test the mice had a mean weight of 27 g.

Method

Since, when compounds are being tested for any teratogenic effect, not only the amount of test substance administered is of decisive importance but in particular the timing of the administration, several hundred sexually mature females were placed together with males (5 ♀ / 1 ♂) for two hours in these tests in order to keep the cohabitation period and thus the fertilizing period as short as possible. Then all female animals that had a vaginal plug and were thus considered to be very probably pregnant were housed individually. The animals were weighed and split up into groups of equal weights. The dose of the test substance that was administered from the 11th - 15th day of pregnancy was based on this mean initial weight. Thus each mouse was administered the same amount of test substance in an equal volume. On the 19th day of pregnancy all treated and untreated mice were sacrificed, the uteri were removed, the implantation and fetal resorption sites were recorded, the number of live and dead fetuses and their body length, weight and sex were determined, and the fetuses were examined macroscopically for any deformities. Then the fetuses were fixed in 96% strength alcohol, clarified with potassium hydroxide solution and stained with Alizarin red-S according to a modified DAWSON method for a better assessment of the skeletal system.

Pyrrolidone was administered to the dams intraperitoneally or orally in the middle of pregnancy, i.e. from the 11th - 15th day of pregnancy. Aqua dest. was used as the solvent. The solutions were prepared freshly each day. The volume administered was 0.2 ml/mouse both orally and intraperitoneally.

I. Testing pyrrolidone for any teratogenic effect when it is administered intraperitoneally from the 11th to 15th day of pregnancy

a) Acute intraperitoneal toxicity

The approximative median lethal dose (ALD 50), with an observation period of 14 days, was

about 3500 cmm/kg for the mouse, intraperitoneally

b) Treatment of the test groups

Group 1 : 5 x 1/2 ALD 50 = 5 x 1750 cmm/kg
= 5 x 0.2 ml of a 23.65% strength aqueous solution

Group 2 : control untreated

Group 3 : 5 x 1/5 ALD 50 = 5 x 700 cmm/kg
= 5 x 0.2 ml of a 9.46% strength aqueous solution

Group 4 : control untreated

Result (compare Tables 1 and 2)

The 5 intraperitoneal administrations of 1/2 and 1/5 ALD 50 from the 11th to 15th day of pregnancy were tolerated by all the dams without any symptoms.

In the case of the 5 intraperitoneal administrations of 1/2 ALD 50 (= 1750 cmm/kg) the resorption rate of 19.7% was increased in comparison with that of the controls (8.9%). The mean fetus weight, the mean length and the number of runts did not differ from the corresponding spontaneous values or the corresponding values in the untreated control group. The number of deformities at 3.4% was somewhat above

Table 1

Testing pyrrolidone for any teratogenic effect when it is administered intraperitoneally to the mouse.
 Animal species: NMRI mice supplied by Gassner, Ottobrunn
 Treatment: 11th to 15th day of pregnancy

Dose 1/2 ALD 50 = 1750 cmm/kg	Control untreated	1/5 ALD 50 = 700 cmm/kg	Control untreated
Dams, total	13	14	12
with deformed fetuses	1	2	1
in %	7.7	14.3	8.3
Implantations, total	156	160	133
∅	12.0	11.4	11.1
Resorptions, total	13	18	6
% with reference to implantations	8.9	11.3	4.5
Dams with 1 resorption	5	5	6
" 2 resorptions	4	4	-
" 3 resorptions	-	1	-
more than 3 resorptions	-	1	-
all embryos resorbed	-	-	-

Table 2

Testing pyrrolidone for any teratogenic effect when it is administered intraperitoneally to the mouse
 Animal species: NMRI mice supplied by Gaszner, Ottobrunn
 Treatment: 11th to 15th day of pregnancy

	Dose 1/2 ALD 50 = 1750 cmm/kg	Control untreated	1/5 ALD 50 = 700 cmm/kg	Control untreated
Live fetuses, total	118	143	140	127
Ø litter size	8.4	11.0	10.0	10.6
Weight g Ø	1.18	1.24	1.29	1.27
Length cm Ø	2.3	2.3	2.4	2.3
Dead fetuses, total	-	-	-	-
Number of runts (%)	3 (2.6)	1 (0.8)	-	-
<u>Deformities, total</u> In %	4 3.4	2 1.4	4 2.9	1 0.8
<u>Type and number of individual deformities</u>				
Cleft palates (%)	4 (3.4)	2 (1.4)	1 (0.7)	1 (0.7)
Atresia ani (%)			1 (0.7)	1 (0.7)
Scoliosis (%)			1 (0.7)	1 (0.7)
Clubfoot (%)			1 (0.7)	1 (0.7)
<u>Insufficient ossification</u>				
Skull (%)	2 (1.7)	2 (1.4)		
Sternum (%)	3 (2.5)	9 (6.3)	8 (5.7)	
<u>Anomalies</u>				
Sternum (%)	13 (11.0)	12 (8.4)	11 (7.9)	5 (3.9)
5th sternebra missing (%)	1 (0.8)			
Zygosyle missing (%)				
Kinked tail (%)				
14th rib, right (%)	2 (1.7)	1 (0.7)	1 (0.7)	9 (7.1)
14th rib, left (%)	7 (5.9)	-	2 (1.4)	4 (3.1)
14th rib, bilaterally (%)	23 (19.5)	3 (2.1)	3 (2.1)	7 (5.5)

that of the spontaneous values (1.7%). Four of the 118 live fetuses (3.4%) had cleft palates; no further deformities were seen in comparison to the controls.

Even after 5 intraperitoneal administrations of 1/5 LD 50 (= 700 cmm/kg) the resorption rate at 11.3% was still higher than that of the controls (4.5%). Litter size, mean fetus weight and mean length of the fetuses did not differ from those of the controls.

The overall deformity rate at 2.9% was somewhat higher than the corresponding spontaneous values (0.8%).

The various individual deformities are listed in Table 2. The slightly increased resorption rate observed in both treated groups (group 1 = 5 x 1750 cmm/kg, intraperitoneally = 19.7% and group 3 = 5 x 700 cmm/kg, intraperitoneally = 11.3%) is attributable to the intraperitoneal route of administration selected, although it is not clear whether the action is mechanical or substance-induced. The volume and the concentration of the solution administered, the pH of the solution and the resulting change of the physiological environment in the abdomen, the pressure caused by the administration of the solution, and the direct effect of the administered substance on the uterus and thus on the placenta or the vessels supplying the fetuses may increase the resorption rate when there is repeated intraperitoneal administration. This is supported by the fact that no increased resorption rate was observed with the repeated oral administration of the product.

The percentage of deformities at 3.4% in test group 1 (5 x 1750 cmm/kg, intraperitoneally) was somewhat higher than the spontaneous values (1.7%); only cleft palates were involved. This cannot, however, be assessed as a teratogenic effect of the product since cleft palates occur spontaneously as a deformity in the mice used and

can fluctuate in their frequency as the result of various external influences. It has emerged from our own previous experience that the increased incidence of cleft palates can depend on the absence of standardized conditions (humidity, constant temperature, etc.) and on the season. PETERS and STRASSBURG ("Stress als teratogener Faktor", Arzneimittel-Forschung, 19, 1106 (1969) also observed that a stress situation created for the dam produces a significant increase in cleft palates in the fetuses. They concluded from their investigations that any unphysiological external stimulus can exert a teratogenic influence as soon as it produces a stress situation in the dam.

II. Testing pyrrolidone for any teratogenic effect when it is administered orally from the 11th to 15th day of pregnancy

a) Acute oral toxicity

The median lethal dose (LD 50, calculated according to LITCHFIELD & WILCOXON), with an observation period of 14 days, was

5800 cmm/kg (5200-6470) for the mouse, orally.

b) Treatment of the test groups

- Group 1 : 5 x 1/2 LD 50 = 5 x 2900 cmm/kg
= 5 x 0.2 ml of a 38.72% strength aqueous solution
- Group 2 : control untreated
- Group 3 : 5 x 1/5 LD 50 = 5 x 1160 cmm/kg
= 5 x 0.2 ml of a 15.49% strength aqueous solution
- Group 4 : control untreated

Result (compare Tables 3 and 4)

The 5 oral administrations of 1/2 LD 50 and 1/5 LD 50 from the 11th to 15th day of pregnancy were tolerated by all the dams without any symptoms.

In the case of the 5 oral administrations of 1/2 LD 50 the resorption rate, mean number of implantations and the mean litter size corresponded to the control values.

The mean weight and the mean length of the fetuses were as a whole somewhat lower than the comparative values, although the total number of runts (2) was very low. Deformed fetuses were observed in the case of 2 of 12 dams in the test group (16.7%) and two of 14 dams in the control group (14.3%).

Of the total of 4 deformities (3.3%) in the test group, three were exhibited by one fetus (cleft palate, micrognathia and fused ribs). This fetus was observed to have general retardation of the ossification.

The corresponding control group was also observed to have 4 deformities (2.6%), 3 of which were similarly found in one fetus (cleft palate, hypoplasia of the extremities and hypoplasia of the ribs).

The mean resorption rate was not increased after 5 oral administrations of 1/5 LD 50 (test group 3). The mean number of implantations and the mean litter size were somewhat reduced in this test group. However, this is not the effect of the test substance administered, since the nidation of the egg had already taken place by the time (11th to 15th day of pregnancy) the treatment of the dams was carried

Table 3

Testing pyrrolidone for any teratogenic effect when it is administered orally to the mouse
 Animal species: NMRI mice supplied by Gassner, Ottobrunn
 Treatment: 11th to 15th day of pregnancy

	Dose 1/2 LD 50 = 2900 cmm/kg	Control untreated	1/5 LD 50 = 1160 cmm/kg
Dams, total	12	14	13
with deformed fetuses	2	2	3
in %	16.7	14.3	23.1
Implantations, total	123	175	134
resorptions, total	10.7	12.6	10.3
% with reference to implantations	7	22	8
Dams with 1 resorption	5.4	12.6	5.9
" 2 resorptions	3	4	4
" 3 resorptions	-	1	2
more than 3 resorptions	1	4	-
all embryos resorbed	-	1	-

Table 4

Testing pyrrolidone for any teratogenic effect when it is administered orally to the mouse
 Animal species: NMRI mice supplied by Gassner, Ottobrunn
 Treatment: 11th to 15th day of pregnancy

	Dose 1/2 LD 50 = 2900 cmm/kg	Control untreated	1/5 LD 50 = 1160 cmm/kg	Control untreated
Live fetuses, total	121	153	126	130
Ø litter size	10.1	10.9	9.7	10.8
Weight in g Ø	1.15	1.25	1.24	1.25
Length in cm Ø	2.2	2.3	2.3	2.4
Dead fetuses, total	-	-	-	-
Number of runts (%)	2 (1.6)	1 (0.7)	4 (3.2)	1 (0.8)
Deformities, total in %	4 3.3	4 2.6	6 4.9	0
Type and number of individual deformities				
Cleft palates (%)	2 ^x) (1.6)	2 ^x) (1.3)	4 (3.2)	
Malocclusion (%)	1 ^x) (0.8)			
Malocclusion (%)	1 ^x) (0.8)			
Shortening of the extremities (%)		1 ^x) (0.7)	1 (0.8)	
Shortening of the ribs (%)		1 ^x) (0.7)		
Malocclusion (%)				
Insufficient ossification				
Sternum (%)	17 (14.0)	1 (0.7)	2 (1.6)	4 (3.1)
Gen. retardation (%)	1 ^x) (0.8)	1 (0.7)	2 (1.6)	1 (0.8)
Anomalies				
Sternum (%)	7 (5.8)	12 (7.9)	10 (7.9)	7 (5.4)
5th sternebra missing (%)	1 ^x) (0.8)			
14th rib, right (%)	3 (2.5)	2 (1.3)	3 (2.4)	2 (1.6)
14th rib, left (%)	5 (4.1)	12 (7.9)	5 (3.9)	5 (3.9)
14th rib, bilaterally (%)	26 (21.5)	23 (15.0)	23 (18.2)	21 (16.2)

^x) = 1 deformity per fetus

out. The nidation of the egg takes place between the 5th and 6th day of pregnancy and was thus not affected by the treatment.

The deformity rate was 4.9% and was thus slightly increased in comparison to that of the control. Four of the 6 deformities observed were cleft palates (3.2%). This slightly increased deformity rate is probably in the range of physiological fluctuations, since no increased incidence of deformities was observed after 5 oral administrations of the altogether 2 1/2-fold LD⁵⁰ (= 5 x 2900 cmm/kg).

ASSESSMENT

5 intraperitoneal administrations of 1/2 ALD 50 and of 1/5 of the LD 50 caused a slight increase in the resorption rate as a sign of an embryotoxic effect; after 5 oral administrations of 1/2 LD 50 the mean weight of the fetuses is lower than that of the controls.

Therefore, although pyrrolidone has an embryotoxic effect in the said doses, no increased deformity rate as compared with the control values can be found according to the present investigations on the fetuses of dams treated with these amounts.

Thus pyrrolidone does not have a teratogenic effect on NMRI mice.

sign.

(Dr. med. H. ZELLER)

sign.

(Dr. med. vet. J. PEH)

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M-METHYLFYRROLIDONE (M-PYROL) (M)SUMMARY OF TOXICITY INFORMATIONAcute Oral Toxicity

In a study using five groups of ten fasted albino rats (Sprague-Dawley; five males, five females) and a 14-day observation period after intubation with graded doses, the approximate acute oral LD₅₀ was calculated to be 4.2 ± 0.8 ml/kg. (4320 ± 820 mg/kg.) Based on this result, M-Pyrol may be classed as mildly to moderately toxic by ingestion.

Skin Irritation

In a repeated insult patch test using 50 human subjects, no irritation was produced during the first 24-hour exposure. However, through repeated and prolonged contact, some mild transient irritation reactions were noted leading to the conclusion that it is a mild fatiguing agent. No evidence pointing to sensitization was noted.

In a modified Draize procedure using six albino rabbits, 0.5 ml portions of 100% M-Pyrol were applied occlusively to both abraded and non-abraded sites for 24 hours. Sites were scored at 24 and 72 hours and resulted in a calculated Primary Irritation Index of 0.5 (indicates very low potential for skin irritation).

Although the above tests indicate the material is mild to the skin, experience over many years has included reports of skin effects, ordinarily associated with continued or repeated gross contact with M-Pyrol such as might be associated with washing by hand of metal or other parts in open containers of the solvent. Based on this experience, repeated or prolonged skin contact should be avoided.

Due to the wide solvent properties of M-Pyrol, choice of protective glove material is restricted. From the solubility characteristics of M-Pyrol, it appears that gloves of polyolefin should be suitable although GAF has not had good experience to report. A PVC-type glove, "Snorkel 4-460" (Edmont-Wilson Co., 1300 Walnut Street, Coshocton, Ohio 43812) has given good service and protection in GAF manufacturing and handling.

Acute Dermal Toxicity (skin absorption)

Tests on albino rabbits showed an Approximate Lethal Dose (ALD) for intact skin greater than 4 and less than 8 gm/kg. For abraded skin, the ALD was observed to be greater than 2 and less than 4 gm/kg.

From this, M-Pyrol does not appear to be in the range ordinarily classified as toxic (for example; the Federal Hazardous Substances Act considers materials with an LD₅₀ of 2 g/Kilo and less as toxic and requiring labeling).

Chronic Dermal Toxicity

A 20-day, sub-acute, dermal toxicity study, using rabbits, has been conducted using M-Pyrol at application levels of 0.4 and 0.8 ml per kilo of body weight per day. Both abraded and normal skin sites were included in the study. A mild local skin irritation was observed on repeated application. Blood studies, weight gain and final histopathological studies on major organs revealed no systemic effects attributable to the treatment. At a higher level of 1.6 ml per kilo per day, one of the four experimental animals (abraded skin) died. Observations on the others in this group were the same as those made at the lower dosage levels.

Inhalation Studies

In tests conducted on white rats exposed to methylpyrrolidone vapors for a single uninterrupted six-hour period, all animals survived the high concentrations studied. Under the most stringent conditions, air was bubbled through a reservoir of methylpyrrolidone held at 110°C and thence into the test chamber holding the animals. Supersaturation was evidenced by considerable condensation on the chamber walls. Close observation over the following two-week period showed no evidence of toxic effects.

Another test exposing 12 rats to air saturated at room temperature (about 1.5 mg methylpyrrolidone/liter) for 10 days (six hrs/day) gave similar results. In this experiment, gross and microscopic tissue examination was conducted to further observe that no incipient effects could be noted.

Eye Irritation

Methylpyrrolidone, 100% was tested in the right eye of each of six albino rabbits. The procedure and evaluation were according to that described in the regulations for the Federal Hazardous Substances Act. The material produced corneal opacity in four animals and conjunctivitis in all. In two of the rabbits, corneal effects persisted through the seven-day observation period. The conjunctivitis cleared before the end of this period in all but one test animal. M-Pyrol should be regarded as a severe eye irritant.

Mutagenicity

Mutagenicity potential was measured by the Ames Test using histidine auxotrophic strains of Salmonella typhimurium (TA-1535, TA-1537, TA-1538, TA-98, TA-100). Tests were conducted in all five strains both non-activated and activated (induced S-9 mouse liver preparation). Inhibition of growth of test strains was no problem even undiluted. No mutagenic activity was observed under any of the conditions used.

Dermal Teratology

Six groups of 25 pregnant female Sprague-Dawley rats received the following treatments on days 6 through 15 of gestation:

- 0.75 ml/kg b.w. deionized water dermally (negative control)
- 10 mg/kg b.w. hexafluoroacetone dermally (positive dermal control)
- 250 mg/kg b.w. asovirin orally (positive oral control)
- 75 mg/kg b.w. N-Methylpyrrolidone dermally (experimental low group)
- 237 mg/kg b.w. N-Methylpyrrolidone dermally (experimental mid group)
- 750 mg/kg b.w. N-Methylpyrrolidone dermally (experimental high group)

Treatment with NMP resulted in dose-dependent brightly colored yellow urine and dry skin. All animals were sacrificed on day 20 of gestation and uterine contents examined.

The control groups exhibited expected findings. No particular teratogenicity was noted in the negative (water-treated) control group. Frank teratogenic effects were noted in both the dermally and orally-treated positive control groups.

In the experimental groups, significantly lower dam weight gains and skeletal variations were observed at 750 mg NMP/kg b.w. These effects could be the result of maternal toxicity. Unequivocal teratogenic effects could not be defined. There was no evidence of teratogenic effects nor effects on the dams at 75 or 237 mg/kg.

Sub-Acute Ninety-Day Feeding Studies, Rats and Mice

Wistar-derived rats: four groups of 50 rats (25 male, 25 female) were fed diets containing levels of M-Pyrol as follows, for a period of 90 days.

<u>Group</u>	<u>ppm in diet</u>
1	0 (control)
2	800 0.08%
3	2000 0.2%
4	5000 0.5%

No gross or behavioral abnormalities in any of the test animals were observed which were of toxicological relevance. There were no gross toxic or pharmacologic effects noted, nor differences in survival. In organ weight and clinical examinations conducted at termination, various minor but statistically significant effects were noted among the test groups which included increased male thyroid weights (only in high test group); minor differences in final urine pH and specific gravity in females, not of toxicological significance; and SGPT enzyme value elevation in Group 3 males. There were no histopathological abnormalities observed attributable to the test diets. An EPA evaluation concluded that the no-effect level in male and female rats was 1000 ppm.

Charles River (CD-1) mice: four groups of 60 mice (30 males, 30 females) were fed diets containing levels of M-Pyrol as follows, for a period of 90 days:

<u>Group</u>	<u>ppm in diet</u>
1	(control)
2	400
3	1000
4	2500

As in the rat study, daily observation, weight recording, clinical studies at 30 and 90 days, and complete gross and histopathological examination (24 organs) on all animals which died during the period or were sacrificed at 90 days were made. No survival rate differences were noted. No treatment related pathological observations, gross or microscopic were noted. Clinical (urine and blood) parameters were within normal limits at termination with the exception of slight dose-related serum chloride elevation. Somewhat depressed spleen weight was observed in females at the highest dosage. In males, this was noted at the two higher levels (1000 and 2500 ppm). No histopathologic changes were noted in the spleens, however, nor in any tissues examined. A conservative value for no-effect level from this study is 400 ppm (for males, 400 ppm; for females, 1000 ppm).

Fish Toxicity

Static bioassays for acute LC50 according to an EPA suggested procedure (1975) were conducted using bluegills (*Lepomis macrochirus*), fathead minnows (*Pimephales promelas*) and rainbow trout (*Salmo gairdneri*). The LC50 values obtained from these studies are given below:

<u>Fish</u>	<u>Temp.</u>	<u>LC50 (96 hr)</u>	<u>Confidence limits</u>
Sunfish	22°C	832 mg/liter	(724-995)
Fathead minnow	22°C	1972 mg/liter	(912-1259)
Trout	12°C	3048 mg/liter	(2692-3388)

Subchronic Feeding Study in Beagle Dogs of *N*-Methylpyrrolidone

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Key words: *N*-methylpyrrolidone, Beagle dogs, subchronic toxicity

The potential toxicity of *N*-methylpyrrolidone was evaluated following dietary administration for 13 weeks to male and female beagle dogs at dosage levels of 25, 79 and 250 mg per kg body weight per day. Body weight gain and food consumption, hematological and clinical chemical data, and ophthalmic, gross and histopathological examinations were used to study possible toxicological or pathological effects. No statistically significant treatment-related effects that were judged to be biologically meaningful were seen in any parameters of either male or female animals exposed to *N*-methylpyrrolidone at any dose level. However, a dose-dependent decrease in body weight and increase in platelet count that correlated with increased megakaryocytes was observed. Serum cholesterol in males decreased with increasing doses.

INTRODUCTION

N-Methylpyrrolidone (CAS 872-50-4) is a solvent used extensively in chemical processing, particularly in petroleum refining. It is also important as a solvent in agricultural chemical formulations and is used as a chemical intermediate in the pharmaceutical industry.

A considerable body of acute and subchronic toxicology information has been developed in support of these uses. The acute oral LD₅₀ in rats was found to be 4.3 ± 0.8 g per kg body weight.¹ The approximate lethal dose via skin absorption in albino rabbits was found to be between 4 and 8 g kg⁻¹ and 2 and 4 g kg⁻¹ for intact and abraded skin, respectively. Results of skin irritation tests in rabbits using a modified Draize procedure indicated a low potential for skin irritation,² tests in guinea pigs produced a moderate response.³ Studies in human subjects confirm that repeated and prolonged skin contact can cause mild transient irritation. However, there was no evidence that *N*-methylpyrrolidone induced contact sensitization.⁴ Attempts to sensitize guinea pigs were unsuccessful.⁵ Results of a standard eye test in albino rabbits indicate that *N*-methylpyrrolidone should be regarded as a severe eye irritant.⁶ When administered dermally to Sprague-Dawley rats at 75, 237, or 750 mg per kg body weight per days on days 6 to 15 of gestation, *N*-methylpyrrolidone produced a reduction in dam body weight gained during gestation, fewer live fetuses per dam, an increase in the percentage of resorption sites, and fetal skeletal abnormalities at the highest dosage level. There was no evidence of teratogenic effects or effects on the dams at the two lower dosage levels. Therefore, *N*-methylpyrrolidone was regarded as a

coeffective teratogen (adversely affecting 10% of litters, but only at dose levels that adversely affect 10% of litters).⁷ No mutagenic activity was observed in *Salmonella typhimurium* strains TA-1535, TA-1537, TA-1538, TA-98 and TA-100, tested with and without S-9 mouse liver activation.⁸

The present study was conducted to evaluate the toxicological effects of *N*-methylpyrrolidone following dietary administration to beagle dogs for 13 consecutive weeks.

EXPERIMENTAL

Animals and materials

Pure-bred beagle dogs, 5-6 months of age at the initiation of the study, were obtained from Marshall Research Animals, North Rose, New York. Animals were individually housed in pens with hard-wood chips as bedding in an environment-controlled room maintained at 18-22 °C and artificially illuminated for 12 h each day. All animals received Purina Dog Chow No. 5006 (Ralston Purina Company, St. Louis, Missouri) for a 1-h period, twice a day, and tap water *ad libitum*.

N-Methylpyrrolidone was provided by GAF Corporation, Wayne, NJ and was 99.9% pure, with a methylanine content of 0.008% and water content of 0.04%. Mazola corn oil, used for diet preparation, was obtained locally.

Design of experiment

The dose levels of *N*-methylpyrrolidone used were 0, 25, 79 and 250 mg per kg body weight per day. The test diets were prepared by dissolving *N*-methylpyrrolidone in corn oil and then incorporating the mixture into dog feed using a Hobart blender. For the control diet, an equivalent volume of corn oil was mixed into the feed. The dogs were fed their diets for 90 consecutive days. The amounts of *N*-methylpyrrolidone in the feed were adjusted weekly following determination of body weight and food consumption.

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Each dosage group consisted of six male and six female dogs. Gross eye examinations were performed prior to the start of dosing and again at the termination of the study. Animals were observed daily for external signs of toxicity. Body weight and food consumption of each dog were measured weekly.

Hematological and clinical chemical evaluations were performed on all dogs prior to the start of treatment and again after 4, 8 and 12 weeks of dosing. Measurements of hematocrit, erythrocyte count, total and differential leukocyte count, platelet count, levels of hemoglobin, urea nitrogen, glucose, total and direct bilirubin, cholesterol, albumin, globulin, calcium, sodium, potassium, chloride, total protein and activities of alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase and lactate dehydrogenase were made. Urine analysis, prior to the start of the study and again after 4 and 12 weeks, provided measurements of pH, specific gravity, glucose, ketones, bilirubin, urobilinogen, protein and microscopic examination of the sediment.

At the termination of the study, all animals were killed using an overdose of pentobarbital. Selected organs (adrenal glands, brain, heart, kidneys, liver, ovaries, pituitary gland, spleen, testes and thyroid with parathyroid glands) from all dogs were weighed. For all animals, the organs or tissues that were taken for histopathological examination included the organs weighed, as well as the following: aorta, epididymides, esophagus, eyes, gallbladder, large and small intestines, lungs, lymph node (mesenteric), mammary glands, pancreas, prostate, salivary glands, sciatic nerve, skeletal muscle, skin, spinal cord, sternum with marrow, stomach, thymus, trachea, urinary bladder, uterus and grossly abnormal tissues.

Statistical analysis

Body weight, food consumption, clinical laboratory and organ weight data were evaluated using analysis of variance.⁶ Difference between the test and control groups were determined using the least significant difference test. Discrete data were analyzed using 2 x 2 contingency tables with Yates' correction for continuity.²

RESULTS

Compound consumption

Group mean compound intake for the 13 week feeding period is given in Table 1. Male animals at the low, middle and high dosage levels of *N*-methylpyrrolidone received 94, 95 and 99% of the intended dose, respectively. Females received 98, 96 and 99% of their intended dose.

Survival, ophthalmic and in-life observations

All animals survived the 13 week feeding period. Ocular evaluations revealed no treatment-related effects. Animal behavior and physical appearance were normal for all dogs throughout the duration of the study.

Body weight, food consumption and food conversion

Body weight data showed no statistically significant differences in weight gain among groups over the course of the study (Table 1). However, body weight gain of male and female high-dose dogs was 54 and 47% of control dogs, respectively. Food consumption, as well as food conversion for dogs that were fed *N*-methylpyrrolidone, were not statistically significantly different from control dogs.

Clinical chemistry

Serum cholesterol levels of male dogs were found to decrease with increasing *N*-methylpyrrolidone dose at all sampling periods after the start of treatment. This decrease was statistically significant at the middle dosage level at 4 and 8 weeks, and at all three sampling periods for the high dosage level dogs. No such effect was noted in female dogs.

Serum total protein was decreased by 7 and 11% in the male middle and high dosage groups, respectively, at week 12. Serum albumin levels for male dogs were decreased by 9% at week 4 (low and high dosage groups) and by 8% at week 12 (middle and high dosage groups). The albumin to

Table 1. Compound consumption, body weight gain and food conversion data of dogs fed diets containing *N*-methylpyrrolidone^a

Sex	Dosage (mg per kg body weight per day)	<i>N</i> -Methylpyrrolidone consumed ^b (mg per kg body weight per day)	Body weight gain (% of initial)	Food conversion ^c
Male	0	0 ± 24	12.8 ± 7.7	4.0 ± 2.4
	25	24 ± 4	13.2 ± 5.8	3.8 ± 1.7
	79	75 ± 9	11.0 ± 11.0	3.2 ± 3.2
	250	246 ± 42	6.9 ± 9.1	1.9 ± 2.7
Female	0	0	10.2 ± 5.7	3.0 ± 1.9
	25	24 ± 3	10.9 ± 5.6	3.1 ± 1.6
	79	76 ± 10	9.5 ± 5.5	2.6 ± 1.6
	250	246 ± 40	3.8 ± 9.3	0.8 ± 2.5

^a Values are group means ± SD of six dogs per sex per group.

^b Based upon actual body weight and food consumption data.

^c Body weight gain (g ± SD) per 100 g of food consumed.

Table 2. Selected clinical chemical data of male dogs fed diets containing *N*-methylpyrrolidone^a

Treatment level (mg per kg body weight per day)	Protein at week (g dl ⁻¹)			Albumin at week (g dl ⁻¹)			Albumin: globulin at week			Cholesterol at week (mg dl ⁻¹)					
	0	4	8	0	4	8	0	4	8	0	4	8	12		
0	6.4±0.4	6.3±0.4	6.2±0.3	6.1±0.2	3.8±0.1	4.2±0.2	4.1±0.3	2.9±0.2	1.7±0.2	2.3±0.6	2.0±0.2	1.8±0.2	184±30	166±37	169±34
25	5.9±0.3	5.9±0.3	6.0±0.2	5.8±0.3	3.8±0.1	3.9±0.1 ^b	4.0±0.2	3.2±0.2	2.0±0.7	2.0±0.3	2.0±0.2	1.8±0.3	180±26	146±27	189±33
75	5.8±0.5	6.0±0.5	6.0±0.6	5.7±0.4 ^b	3.7±0.3	4.0±0.3	4.0±0.2	3.9±0.1 ^b	1.8±0.3	2.3±0.8	2.0±0.4	1.7±0.3	175±23	129±27 ^b	131±30
250	5.7±0.6	5.6±0.2	5.7±0.3	5.4±0.2 ^b	3.7±0.3	3.9±0.2 ^b	3.8±0.2	3.5±0.1 ^b	1.9±0.2	2.4±0.3	2.0±0.1	1.9±0.1	178±19	138±17 ^b	172±16 ^b

^a Values are means ± SD of six dogs per group.

^b Significantly different from respective control (*p* < 0.05).

Table 3. Summary of organ weight data of dogs fed diets containing *N*-methylpyrrolidone^a

Sex	Dose (mg per kg body weight per day)	Body weight (kg)	Organ weights (mg per kg body weight)						Prostate (X 10 ⁻³)		
			Liver	Kidneys	Spleen	Heart	Testes/ovaries (X 10 ⁻³)	Thyroid (X 10 ⁻³)		Adrenals (X 10 ⁻³)	Brain
Male	0	10.2±1.2	3.37±0.83	0.59±0.14	0.69±0.17	0.61±0.07	0.15±0.01	0.76±0.16	1.10±0.34	0.74±0.06	0.89±0.11
	25	10.5±0.9	3.28±0.36	0.52±0.04	0.68±0.36	0.77±0.13	0.15±0.02	0.78±0.14	1.14±0.20	0.69±0.04	0.88±0.04
	75	10.1±0.7	3.49±0.31	0.64±0.07	0.48±0.22	0.78±0.07	0.16±0.04	0.78±0.16	1.38±0.26	0.74±0.06	0.81±0.11
Female	250	9.7±1.4	3.44±0.46	0.58±0.10	0.42±0.26	0.78±0.11	0.16±0.03	0.82±0.21	1.29±0.29	0.79±0.14	0.89±0.10
	0	8.2±1.7	3.22±0.69	0.48±0.07	0.59±0.18	0.77±0.09	0.93±0.28	0.86±0.13	1.30±0.20	0.80±0.14	0.89±0.14
	25	8.0±1.4	3.38±0.53	0.49±0.06	0.50±0.22	0.78±0.14	1.40±0.76	0.76±0.16	1.38±0.33	0.84±0.22	0.89±0.34
	75	8.0±1.3	3.67±0.42	0.48±0.04	0.78±0.26	0.73±0.02	0.85±0.45	0.83±0.11	1.20±0.17	0.91±0.06	0.76±0.11
	250	7.8±1.0	3.43±0.21	0.51±0.06	0.56±0.23	0.84±0.13	0.89±0.17	0.86±0.14	1.63±0.30	0.94±0.13	0.80±0.17

^a All organ values are expressed as per cent of body weight and are group means ± SD of six dogs per sex per group. Absolute organ weights are comparable among groups.

globulin ratio was comparable among groups at all sampling periods.

Other parameters evaluated revealed no significant differences among groups.

Hematology and urine analysis

Platelet count data of male dogs is graphically depicted in Fig. 1. At all sampling periods, platelet counts were found to increase with dose. Compared to control dogs, statistically significant increases in platelet counts were noted for all dosage levels at week 8 and for the middle and high dosage levels at week 12. In addition, mean platelet counts of male control dogs decreased with time. However, the counts at the initiation and termination of the study were not statistically different from each other.

For other hematology parameters evaluated, as well as the urine analysis data, no significant differences among groups were noted.

Organ weight

No significant differences in either organ weights or organ to body weight ratios were noted among groups (Table 3).

Histopathology

The number of megakaryocytes per 200x field (ten fields observed per dog) were counted in sternal marrow of all male dogs to determine if there was a correlation between increased platelet counts and megakaryocyte counts. In male animals fed *N*-methylpyrrolidone, an apparent increase in the number of megakaryocytes was noted with increased dose. The mean number of megakaryocytes per 200x field \pm SD at *N*-methylpyrrolidone dosage levels of 0, 25, 79 and 250 mg per kg body weight per day were 4.4 ± 0.8 , 7.4 ± 1.4 and 10.0 ± 1.4 , respectively.

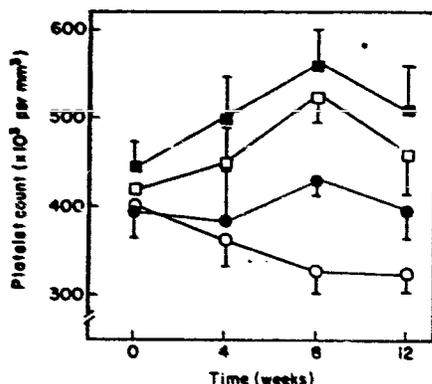


Figure 1. Platelet counts (means \pm SE) of male dogs administered *N*-methylpyrrolidone at a level of 0 (○), 25 (●), 79 (□) and 250 (■) mg per kg body weight per day.

Examination of other tissues revealed no other *N*-methylpyrrolidone-related effects.

DISCUSSION

No significant differences in body weight, food consumption, feed conversion, ophthalmic and organ weight data were noted among groups during the 13-week feeding period. In addition, dietary administration of *N*-methylpyrrolidone did not produce any overt external signs of toxicity or behavioral abnormality.

Intermittent significant differences in mean clinical chemical parameters of total cholesterol, albumin and total protein for male dogs were noted. The possible toxicological significance of these differences was not supported by any histopathological observation. Furthermore, all mean values fall within the normal range for beagle dogs (cholesterol, 105-267 mg dl⁻¹; albumin, 3.5-4.4 g dl⁻¹; protein, 5.2-6.5 g dl⁻¹; FDRL historical data). Thus, the differences were considered incidental and not related to treatment.

Mean platelet counts of male control dogs decreased slightly with time, whereas at the low *N*-methylpyrrolidone dosage level such a decrease was not noted. At the middle and high dosage levels, a slight increase in platelet count was noted. However, when the initial platelet count of each group was compared with the final count of its respective group, no statistically significant differences were found. The mean platelet counts in male dogs were found to be significantly increased at all dosage levels at week 8 and at the middle and high dosage levels at the termination of the study, compared with control male dogs. The trend toward higher platelet counts in the high dosage level male dogs was supported by the finding of an increased occurrence of megakaryocytes in the sternal marrow of these animals. It must be noted, however, that all megakaryocyte counts fall within the normal range for beagle dogs at our laboratories (approximately 4-13 per 200x field) and the mean platelet counts fall within our laboratory's normal range (approximately 200-500 $\times 10^3$ per mm³). Furthermore, the mean platelet counts fall within the range of those published in the literature for normal beagle dogs,^{3,4} 175-500 $\times 10^3$ per mm³ and 155-517 $\times 10^3$ per mm³. When the above considerations are taken into account, the differences in platelet counts are of doubtful significance.

In conclusion, feeding of *N*-methylpyrrolidone at levels up to 250 mg per kg body weight per day resulted in no statistically significant toxic or pathologic effects.

Acknowledgements

We thank our staff for their expert technical assistance, and Carol Antes and Wendy Goble for typing this manuscript.

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Received 25 May 1982; accepted (revised) 14 September 1982

HANDBOOK OF ENVIRONMENTAL DATA ON ORGANIC CHEMICALS

SECOND EDITION 1983

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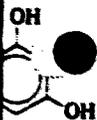
VAN NOSTRAND REINHOLD COMPANY
NEW YORK CINCINNATI TORONTO LONDON MELBOURNE

600 ppm, 6 hr (211)
4 hr/day, 1-2 weeks (211)

ethoxybenzene

ethoxyphenol

oxybenzene; pyrogalllic acid)



w. 126.11; m.p. 133/134°C decomposes; v.p.
C; sp.gr. 1.453 at 4/4°C; solub. 625,000 mg/l

n = 0.194 ppm, 1 ppm = 5.15 mg/cu m
orless

Cr₂O₇) (274)

InO₄) (274)

(274)

0°C-product is sole carbon source: 40% COD

(327)

0 mg/l (998)

/l (81)

by *Pseudomonas fluorescens*: at 3 mg/l

by *E. coli*: at 30 mg/l (293)

(30)

al dose: 1.1 g/kg (211)

18 mg/l; 48 hr (226)

(30)

al dose: 1.1 g/kg (211)

18 mg/l; 48 hr (226)

(30)

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18 mg/l; 48 hr (226)

(30)

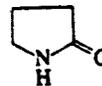
al dose: 1.1 g/kg (211)

18 mg/l; 48 hr (226)

1-PYRROLYLMETHANOIC ACID 1039

2-pyrrolidinone see 2-pyrrolidone

2-pyrrolidone (2-pyrrolidinone; butyrolactam)



Use: plasticizer for acrylic latexes; solvent for polymers, insecticides, special inks etc.

A. PROPERTIES: m.w. 85.11; b.p. 245°C; soluble in water; sp.gr. 1.1

C. WATER POLLUTION FACTORS:

theoretical

TOD = 2.44 -

COD = 1.69 -

NOD = 0.75 -

analytical

reflux COD = 95.3% recovery

rapid COD = 101.8% recovery

TKN = 90.0% recovery

BOD₅ = 1.16

BOD₅/COD = 0.720

BOD₅ (acclimated) = 1.39

-The results of the BOD₅ analyses suggest 2-pyrrolidone to be readily biodegradable. (1828)

-Biodegradation:

	chemical conc. mg/l	effect
unacclimated system	126	no effect
	1,250	no effect
acclimated system	137	biodegradable

In contrast to the BOD₅ analysis, the Warburg analysis, using an unacclimated biomass, did not measure any evidence of biodegradation of the compound. This is probably due to the different characteristics of the seeds used in the two analyses. The BOD₅ seed, from a source servicing a high industrial input, readily assimilated the compound. The Warburg seed, from a domestic waste source, was completely unadapted to the compound. The compound was found, however, to be amenable to high rate biodegradation by the acclimated biomass. (1828)

D. BIOLOGICAL EFFECTS:

-Algae: *Selenastrum capricornutum*: 1 mg/l: no effect

10 mg/l: no effect

100 mg/l: no effect (1828)

-Crustacean: *Daphnia magna*: LC₅₀: 3.4 mg/l

-Fish: *Pimephales promelas*: LC₅₀ > 100 mg/l (1828)

pyrrolylene see 1,3-butadiene

1-pyrrolylmethanoic acid see dl-proline

proline

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