**EXPERIMENTAL BIOLOGY OF AMMONIA-RICH ENVIRONMENTS:
OPTICAL AND ISOTOPIC EVIDENCE FOR VITAL ACTIVITY IN
PENICILLIUM IN LIQUID AMMONIA-GLYCEROL MEDIA AT -40°C^***

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Recently, it has been pointed out that in spite of the intimate relationship between water and the evolution of life on earth, a critical examination of the properties of water has failed to yield compelling evidence that water is uniquely suitable as a biological solvent.¹ This challenge to a long-standing biological axiom has been given support at the enzymological level recently in a demonstration of urease-catalyzed urea-hydrolysis at low-water activities and of peroxidase and catalase activities in alcohols.²

Among the substances often considered as a potential substitute for water in biological systems, liquid ammonia ranks high. A comparison of the two liquids (NH_3 at -33.4°C and H_2O at 100°C) reveals points of gross similarity in dielectric constant, viscosity, surface tension, and heat of vaporization.³ Furthermore, the "ammono" analogues of many compounds from the usual "aquo" system are obtained by simple replacement of their acidic and basic functions. The fundamental acid-base chemistry of the "aquo" system derives from self-dissociation of water.

By analogy,

Hence, HCl becomes NH_4Cl , NaOH becomes NaNH_2 , and their reaction products will be NaCl and NH_3 . The ammono analogues of acetic acid are acetamide and acetamidine. Those of carbonic acid are carbamic acid, urea, and guanidine. Obviously, the "aquo" system as we know it is well supplied with ammono compounds and aquo-ammono hybrids.

Most significantly, saturated aqueous ammonia itself contains little NH_4^+ and OH^- and consists principally of a hydrate, $\text{H}_3\text{N} \cdots \text{H} - \text{OH}$, plus excess H_2O .

Liquid ammonia (-40°C) containing small amounts of water will consist of the same hydrate plus excess NH_3 .³ The two liquids have more in common than is ordinarily recognized by biologists.

Following demonstration that the system NH_3 (gas)/ $\text{NH}_3 \cdot \text{H}_2\text{O}$ (liq.) at ordinary temperatures (e.g., 25°C) will support growth of bacteria, fungi, and even germination of some seeds,⁴ we undertook to examine the feasibility of culturing an ordinary organism in a liquid NH_3 -rich medium. The organism selected was a mutant of *Penicillium notatum* (our designation UC/SAL-2) previously characterized for its ability to grow in harsh and exotic chemical environments.⁵

Methods.—Conidia of UC/SAL-2 were inoculated into a glycerol-NH₃ medium held at -40°C . This medium consisted of: glycerol, 380 ml; liquid NH₃, 600 ml; water, 12 ml; yeast extract, 10 gm; Bacto-Peptone, 10 gm; glucose, 50 gm; adenylic acid, 1 gm.

In experiment 1, 75 mg of dry conidia were introduced into replicate 100-ml vol of medium, and the whole was "incubated" at -40°C for 4 months. Samples were then withdrawn and examined microscopically at $\times 1250$ for evidence of germination. Germination was counted only when the presumptive germ tube was greater in length than one spore in diameter.

Glycerol media containing one half the solids content of the liquid NH₃ composition were saturated with gaseous NH₃ and inoculated with conidia. These partial controls were maintained at 25°C in stoppered vessels.

In a second experiment, conidia of UC/SAL-2 were inoculated into the glycerol-liquid NH₃ composition described, but 100 ml of media contained 1.0 mc of thymidine-H³, uridine-5,6-H³, or a mixture of L-phenylalanine-2,3-H³ + L-leucine-H³ (0.5 mc each). Replicate vessels received freshly harvested conidia, conidia exposed to 125°C for 24 hr, or conidia exposed to 5 Mrad of Co⁶⁰ γ -radiation. Both thermal and radiation treatments reduced viability from the normal 90+ % level to less than 10% as judged by plate dilution assay on Sabaroud's agar at 25°C .

Other experiments that were designed to test tolerance involved storages of spores or mycelium under liquid NH₃ at -40°C for up to 30 days followed by plating at 25°C on Sabaroud's agar.

FIG. 1.—Germinating conidiospore of *Penicillium* after four months of incubation in glycerol-NH₃ medium at -40°C . Note germ tube and branch initial. Spore diameter ca. $2\ \mu$, tube length ca. $10\ \mu$. Over-all magnification ca. $\times 2300$.

Results and Discussion.—The immersion of neither mycelium nor conidia in liquid NH₃ for 30 days impaired viability; spores produced macroscopic mycelium within 36 hours after transfer to ordinary culture conditions.

In the first glycerol-NH₃ experiment at low temperature, four-month samplings of triplicate cultures gave the following—*germ tubes seen*: 8, 3, and 4; *spores counted*: 386, 496, and 491.

The over-all yield of germinated conidia average 1.2 ± 0.8 per cent. Among the 15 germinated spores, most germ tubes were only $2\text{--}4\ \mu$ in length, but in a few cases tubes of $7\text{--}10\ \mu$ were noted (Fig. 1).

In ammoniated glycerol media at 25°C , no germination was in evidence in over 2000 conidia sampled from six replicate tubes. The spores were not uncommonly greatly enlarged, and frequently ruptured.

Glycerol-liquid NH₃ cultures containing H³-labeled precursors of DNA, RNA, and protein were held at -40°C for six months. Entire vessels were then degassed, and the

glycerol-nutrient residue was made up to 1 *M* in trichloroacetic acid (TCA) and centrifuged rapidly at $10,000 \times g$. The pellet was resuspended and washed in 1 *M* TCA followed by saline, suspended in methanol-toluene, and counted with

TABLE 1. *TCA-insoluble H³-β activity in conidia incubated in glycerol-NH₃ media at -40°C with H³-labeled metabolites.*

	Pretreatment of Spores (β-Activity, cpm/mg)					
	125°C/24 Hr		Co ⁶⁰ , 5 Mrads		Untreated	
	Cpm per mg	Per cent untreated	Cpm per mg	Per cent untreated	Cpm per mg	Per cent untreated
Thymidine-H ³	68 ± 8	48	10 ± 2	7	143 ± 10	100
Uridine-5,6-H ³	48 ± 6	29	19 ± 3	12	162 ± 12	100
L-Phenylalanine-2,3-H ³ + L-Leucine-H ³	23 ± 3	16	28 ± 3	20	134 ± 10	100

liquiflor in the Nuclear-Chicago "Unilux" scintillation counter. Background levels of *ca.* 25–40 cpm were observed.

TCA-insoluble H³-β activity in the spores was present in all cases, but reduced when they had been preheated or preirradiated (Table 1).

These data show that the ability of *Penicillium* UC/SAL-2 spores to incorporate constituents of DNA, RNA, and protein can be sustained in an environment that contains water only as a minor, possibly unavailable component. Furthermore, incorporation occurred at a temperature not ordinarily considered capable of supporting active life processes and showed the sensitivity to heat and radiation expected of a biochemical process. These data do not prove that "normal" DNA, RNA, and protein were being synthesized, but autoradiography of the thymidine-H³ group suggested strictly nuclear localization of the label.

The incorporation of both thymidine and uridine was depressed substantially and more or less similarly by dry heat but left appreciable residual activity. Intense Co⁶⁰ radiation was far more effective, as expected, depressing activity by about 90 per cent in both cases. In contrast, amino acid incorporation, although more heat-sensitive, was somewhat more radiation-tolerant, resulting in approximately the same amount of depression, *ca.* 80 per cent, in both treatments.

In 1962, the late L. Baas Becking⁶ described an attempt that he had made some 20 years earlier to cultivate organisms from a desert soil in NH₃ at -50°C. He also noted technical difficulties in this experimentation. Although his own results were apparently indecisive, he urged the repetition and extension of such experiments, concluding that "If hydrobionts could also live as ammonobionts, the outcome of this future experiment might be positive. . . . And with such an experiment we would elevate life from the Ptolemaic (geocentric) to the Copernican status."

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² Siegel, S., and K. Roberts, *Space Life Sci.*, in press.

³ Sienko, M., and R. Plane, *Physical Inorganic Chemistry* (New York: W. A. Benjamin, 1964); Jolly, W., *The Inorganic Chemistry of Nitrogen* (New York: W. A. Benjamin, 1964).

⁴ Siegel, S., and C. Giunarro, *Icarus*, **4**, 37–40 (1965); Siegel, S., and C. Giunarro, these PROCEEDINGS, **55**, 349–353 (1966); Siegel, S., and O. Daly, *Plant Physiol.*, **41**, 1218–1221 (1966); Siegel, S., K. Roberts, H. Nathan, and O. Daly, *Science*, **156**, 1231 (1967).

⁵ Siegel, S., K. Roberts, M. Lederman, and O. Daly, *Plant Physiol.*, **42**, 201–204 (1967);

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⁶ Baas Becking, L., in *The Evolution of Living Organisms*, ed. G. Leeper (Victoria, Australia: Melbourne Univ. Press, 1962), p. 37.