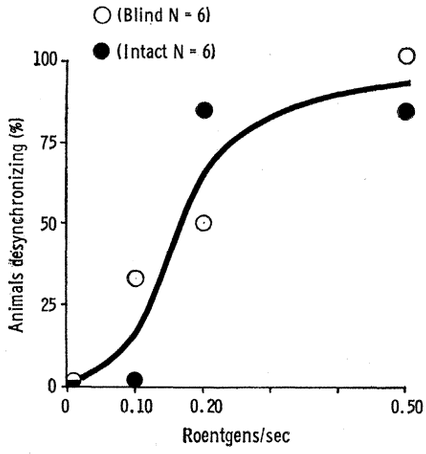

Fig. 2. Threshold determinations based on frequency of animals exhibiting EEG desynchronization (activation) as a function of x-ray dose rate during a 10-second exposure.

attributed to extraneous stimuli associated with the x-ray machine and the shutter.

We studied habituation of the response by subjecting other animals to repeated 10-second exposures at 0.2 r/sec during a single experimental run. After each exposure the animal was allowed to return to sleep for several minutes before it was exposed again. This procedure was repeated until the animal no longer responded to the x-rays. Five days later the animals were tested again. The results (Fig. 3) indicate the animals become habituated rather rapidly to the stimulus of radiation at this dosage. After several days, spontaneous recovery of the response occurs, followed by a more rapid habituation. This pattern is characteristic of responses to repeated mild stimulation through a peripheral afferent system. Electroencephalographic desynchronization, again, appeared to be a more sensitive measure than behavioral arousal, since it lingered in the records after all behavioral manifestations of arousal were absent.

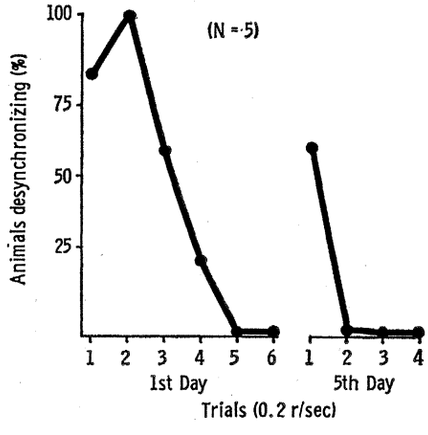

Fig. 3. Habituation of the EEG desynchronization (activation) to repeated 10-second exposure to x-rays (0.2 r/sec). Spontaneous recovery of the response occurred after a 4-day "rest" period.

We also explored the possibility that exposures of longer than 10 seconds' duration might have a summation effect. Another group of four animals was exposed to 10 minutes of 0.1 r/sec radiation, a dose which appeared to have little arousal effect with a 10-second exposure. Continued exposure at this "subthreshold intensity" increased the mean amount of desynchronization in the EEG record from a pre-exposure level of approximately 16 percent to 34 percent during the 10-minute exposure period. In the 5 minutes immediately following radiation exposure, the mean amount of desynchronization increased even further (48 percent). The amount of behavioral arousal observed during these periods substantiated the EEG evidence indicating that prolonged exposure produces a summation effect. Furthermore, these data are in accord with previous work which indicates that chronic exposure to extremely low intensities (0.0007 r/sec for 4 hours) will produce a conditioned aversion in the rat (*5*).

Russian investigators have reported EEG changes resulting from low levels of radiation. These have been interpreted as "depressions" of cortical electrical activity apparently due to a direct effect upon the central nervous system (*6*). In a recent article entitled "Evidence for direct stimulation of the mammalian nervous system with ionizing radiation" (*7*), Hunt and Kimeldorf reported behavioral arousal from sleep within 12 seconds in response to x-rays (0.25 r/sec). In general our data agree with the empirical findings reported in these papers. Our lower thresholds and faster reaction times are probably due to: a greater sensitivity of the EEG when compared with behavioral indices and depth of sleep, which is an important factor, for we have demonstrated even lower thresholds (0.05 r/sec) in active alert animals with the conditioned suppression technique (*2*).

However, we do not believe that these electrographic changes (and the associated behavioral events) imply a direct effect upon neural tissue. On the contrary, such changes are usually the result of arousing action of stimuli operating via a peripheral receptor. Our data demonstrate a prompt response to radiation which is functionally related to intensity (dose-rate) and which habituates rapidly. This suggests to us the intriguing hypothesis that sensitive radiation receptors may exist within the mammalian system (*8*).

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8 February 1963

In vitro Culture of the Flagellate Protozoan Hexamita salmonis

Abstract. *Trophozoites of* Hexamita salmonis, *asserted pathogen of juvenile salmonid fishes, were isolated from two species of Pacific salmon hosts and cultured repeatedly in an organic medium saturated with nitrogen. Primary isolates and serial subcultures usually exhibited five- to tenfold population increases per passage.*

The culture technique reported here was developed as an essential prelude to experimental evaluation of the polymastigine flagellate *Hexamita salmonis* (Moore, 1922), a common resident of the intestinal tract of trouts and salmons less than a year old. The basic objectives of this study were to produce routinely vigorous, populous, and axenic primary and secondary cultures which could be harvested for inoculation into disease-free test fishes. These objectives were met, and *H. salmonis*, under the

experimental conditions employed, was found to be nonpathogenic. The method described below may prove applicable to the cultivation of other fastidious organisms which require microaerophilic or anaerobic environments.

The genus *Hexamita* Dujardin, 1841, is represented by a wide array of free-living, symbiotic, commensalistic, and parasitic species. Few protozoan genera enjoy such wide zoological distribution; aside from free-living forms, species of *Hexamita* have been recorded as intimate associates of trematodes, univalve and bivalve mollusks, leeches, arthropods, fishes, frogs, salamanders, snakes, turtles, birds, rodents and monkeys.

Hexamitiasis has been considered economically important in the husbandry of oysters, juvenile salmonid fishes (*1*), and turkeys (*2*). Convincing evidence of this has been presented by Mackin *et al.* (*3*) and Stein *et al.* (*4*) in the case of oyster hexamitiasis. The significance of hexamitiasis in salmonid fishes and turkeys is open to question despite the implications of a voluminous literature which followed the discovery of *Hexamita* in these vertebrate hosts. Uzmann and Jesse (*5*) challenged the asserted pathogenicity of *H. salmonis* and cautioned against routine practice of chemical prophylaxis or chemotherapy until it had been justified by controlled experimental studies. Zander (*6*) was unable to induce experimentally significant morbidity or mortality in turkey poults with axenic, egg-cultured *H. meleagridis*.

Attempts by others to culture *Hexamita* spp. have met with varying degrees of success (*7, 8*). Only Bishop (*8*), who cultured *H. gigas* from the gut of the horse leech, *Haemopis sanguisugae*, met the classical requirements of in vitro cultivation. Early in our investigation we found that aerobic environments are inimical to survival of *H. salmonis;* isolates invariably declined to extinction within 96 hours regardless of the medium employed or other physicochemical conditions. Subsequent to these findings, we explored various techniques for reduction of oxygen tension in fluid media. Supersaturation of medium with nitrogen gas followed by a nitrogen atmosphere overlay led to successful culture in a balanced medium fortified with antibiotics. Although oxygen tension of the medium was not measured, it was conservatively estimated to range below 10 mm-Hg following equilibrium of the system.

Each 100 ml of culture medium contained 80 ml of single strength tissue culture medium 199 (*9*), 10 ml of human cord serum (*10*), 10 ml of a 5.0 percent solution of lactalbumin hydrolysate (*11*), and 0.2 g of Sigma 7–9 buffer (*12*). This medium was dispensed aseptically in 2.0-ml amounts into screw-cap culture tubes (125 by 16 mm). Tubes were transferred from filling racks to beakers containing a chilling mixture of ice and water. Commercially prepared nitrogen (oil-free) was passed through a 24- by 1.5-inch round, autoclavable glass column packed with moist bone charcoal; the filtered nitrogen was delivered through sterile rubber tubing with a Pasteur pipette terminus to the surface of the chilled medium for several seconds to displace the overlayer of air; next, the pipette tip was submerged to the bottom of the tube and a fine stream of nitrogen was bubbled through the medium for 1 minute; bubble size and flow rate were adjusted carefully to prevent excess foaming or overflow of the medium. The tube was quickly and firmly capped and transferred temporarily to another beaker of coolant. If the prepared tubes were not used immediately, they were stored under refrigeration. Just prior to inoculation, medium intended for primary isolations was protected against bacterial and mycotic contamination by addition (per milliliter of medium) of 2000 units each of penicillin and streptomycin, and 250 units of Mycostatin. In subsequent passages of isolates, antibiotic fortification was limited to the indicated levels of penicillin and streptomycin.

Hatchery-reared juveniles (less than 6 months old) of silver salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) were the regular sources of *H. salmonis* in this study. Fish lots were first routinely sampled and examined to find the approximate population density of their *Hexamita* burden. Only fish stocks which harbored moderate to heavy concentrations of *Hexamita* were used. The caecal intestine of a selected fish was dissected free with aseptic precautions, and its contents were expressed onto a cooled (10°C), sterile slide into a 0.1-ml pool of culture medium. The mixture of medium and caecal contents was quickly drawn up with a sterile Pasteur pipette and inoculated into the tubed medium. Contents of a second intestine were introduced in a like manner to enhance the probability of success with the starter culture. After completion of all inoculations, the tubes were stacked in a beaker of water at 10°C, and the medium was gently overlaid with a fresh nitrogen atmosphere. No bubbling or foaming was permitted at this stage of procedure. The tubes were capped tightly again and racked in vertical position in a refrigerated (10°C), darkened water bath and left undisturbed for 48 hours.

Preliminary estimates of population increase and vigor were determined by direct microscopic examination of tube contents. Tubes were placed horizontally in a test tube cradle and the culture was viewed through the side wall of the tube. Cultures which showed obviously high concentrations of *Hexamita* were sampled with a sterile Pasteur pipette, and population estimates were made from hemocytometer counts. As a standard, subculturing was attempted only when a culture approximated 10^5 organisms per milliliter; normally, four subcultures were made from each prior culture. Thus, with 0.5 ml of standard culture as a minimum inoculum per 2.0 ml of medium, subcultures would be started at approximately 2×10^4 organisms per milliliter.

During the course of this study 95 primary isolations and 271 subcultures were initiated. Several variations of the technique and medium reported above were evaluated simultaneously. Among the cultures which attained or exceeded the 10^5 concentration of organisms, 88 percent achieved this population density within 8 days. Although little emphasis was placed on indefinite passaging of isolates, up to nine passages were effected with some isolates to attain a maximum age of 98 days. The definitive method prescribed was applied in 48 culture trials; among these 32 attained a mean population density of 10^5 cells per milliliter in a mean period of 6.9 days.

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21 February 1963

"Applejack" Technique: New Application of an Old Approach to Solute Concentration

Abstract. *Freezing and slow thawing is a simple and inexpensive means for preparing concentrated solutions from large volumes of water-soluble compounds. Particularly appropriate for colored compounds, it can be adapted easily to colorless solutes for which quantitative tests are available.*

The purification of compounds from plant or animal sources often uses techniques such as dialysis, chromatography, and electrophoresis. These procedures dilute the products in the course of their separation from contaminants in the crude extracts.

The extremely simple technique described here has been used to concentrate the blue-green plant pigment, allagochrome (*1*). Solutions which have been completely frozen in plastic bottles in a deep freeze or dry-ice chest are transferred to a cold room (3° to 4°C) and supported in an inverted position above a collecting container. A several-fold concentration of the solute is obtained in the first effluent. When containers are placed on blocks of dry-ice, freezing is more rapid than in a deep freeze and samples freeze from the bottom up. The solute is thus most concentrated in the last portions of the solution to freeze where it is in a position to drain off first on melting. Maintaining a low constant temperature during thawing permits sufficient equilibration between the outside and the inside of the container so that melting (accompanied by extensive channeling) occurs throughout the sample rather than just in those parts adjacent to the bottle wall.

Table 1 summarizes measurements of the optical density of consecutive 20-ml fractions from three different 500-ml samples of allagochrome of known initial concentrations. Data on the concentration of hemoglobin and flavin mononucleotide are also included. Although the latter compounds exhibited rather unusual patterns of concentration in the effluent fractions, at least a twofold increase in concentration is possible by visually selecting that portion of the melted sample of obviously darker color. Other means would have to be used to estimate the concentrations of colorless solutions.

Between six- and seven-fold concentrations of allagochrome and quantitative recovery of solute have been achieved by this method. Routinely, the melting sample is collected in two fractions: the first 25 percent which contains the major portion of the solute, and the 25 to 80 percent fraction which contains the rest. The second fraction is recycled with a new sample. The ice remaining in the freezing bottle is discarded.

This technique is available to anyone with access to a deep freeze or dry-ice chest and a refrigerator. It provides an economical method for concentrating large volumes of water-soluble materials in laboratories where the facilities for lyophilization are limited.

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Table 1. Relative concentration (optical density) of consecutive 20-ml fractions collected from 500-ml frozen samples. Fractions were collected on an automatic fraction collector with a 20-ml volumetric siphon. Spectra were measured with a Beckman DK-2 recording spectrophotometer. Initial concentrations were as follows: allagochrome, 0.89, 2.18, and 3.01 mg of dried sample per milliliter; flavin mononucleotide (FMN), 0.01 mg/ml; hemoglobin, 0.17 mg/ml.

Allagochrome (673 mμ) at initial concentration			Hemoglobin (406 mμ)	FMN (444 mμ)
0.89	2.18	3.01		
		Initial sample		
0.58	1.42	1.96	1.20	0.24
		Fractions 1 to 7		
2.18	9.50	9.45	0.57	0.39
2.08		6.06	1.20	0.22
	4.56*			
1.99		5.25	1.46	0.26
1.62	2.60	4.85		0.39
			2.62*	
1.35	2.47			0.54
		3.97*		
1.03	2.08		2.66	0.66
0.86	1.77	3.00	0.93	0.61
		Average		
1.59	3.93	5.33	1.72	0.44

* Average for two consecutive fractions.

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15 February 1963

Stratospheric Cloud over Northern Arizona

Abstract. *An unusual ring-shaped cloud was widely observed over northern Arizona near sunset on 28 February 1963. From a large number of observers' reports it is known to have appeared overhead near Flagstaff, Arizona. From initial computations based on four photos taken in Tucson, 190 miles south of the cloud, its altitude was approximately 35 kilometers. The most distant observation reported was made 280 miles from the cloud. The cloud remained sunlit for 28 minutes after local sunset. Iridescence was noted by many observers. Tentatively, the cloud may be regarded as similar to a nacreous cloud; but its unusually great height and unusually low altitude, plus its remarkable shape, suggest that it was a cloud of previously unrecorded type.*

Near sunset, on 28 February 1963, a cloud of unusual configuration and coloration was observed in widely scattered localities in Arizona and some surrounding states. The cloud took the form of a large oval ring (clear in the middle) with the long axis running north and south (Fig. 1 and cover photograph, this issue). It remained brightly illuminated well after the sun had set on high cirrus clouds to the west. From Tucson, 190 miles to the south, its angular elevation appeared to be about 6 degrees. A rough computation of its height, based on sunset geometry (*1*), made immediately after the cloud entered the earth's shadow, led me to appeal by press and radio for confirmatory reports in order to establish the approximate location and to secure descriptions from the largest possible number of other observers.

From approximately 150 reports, many communicated by persons well aware that they had seen a type of cloud unprecedented in years of sky-watching, it was quickly established that the cloud lay overhead in the vicinity of Flagstaff, Arizona, that it exhibited iridescence of the sort asso-