mate given 10 minutes before the strychnine protected 7 out of 10 mice given 0.05 mg strychnine. Fifty mg and higher doses prolonged the life of mice given 0.1 mg strychnine, but they eventually died within a period of 90 minutes. The lives of mice given lethal doses of metrazol or picrotoxin were prolonged by anthranilic hydroxamate but there was no protection.

A dog was lightly anesthetized with nembutal. One g/kg of anthranilic hydroxamate was injected intravenously in a period of 30 minutes. During the next 2 hours, there were no changes in blood pressure, heart rate or EKG. The respiratory rate was slightly depressed at first, then became regular and shallow.

The hydroxamates of salicylic and benzoic acids in equivalent doses produced only transient depression, failed to abolish the conditioned avoidance response and could be given daily for 10 days without apparent ill effect as indicated by loss of body weight. They showed no protection against strychnine, metrazol or picrotoxin. The hydroxamate of phthallic acid is toxic; 0.25 mg/g is the lethal dose. When half this dose was given, tremors occurred at intervals for several hours, and the rat appeared very sensitive to touch. Respiration was of the Cheyne-Stokes type. Anthranilic hydroxamate antagonized these effects.

Discussion. Salicylic and anthranilic hydroxamates should be almost equally effective as chelating agents. Since their pharmacological actions are so different, chelation probably does not play an important part in the mechanism of action of anthranilic hydroxamate. This compound appears to penetrate the blood-brain barrier rapidly and the data suggest that its action is central. Despite the salivation, the absence of effect on blood pressure and heart rate indicate that the autonomic system is not affected centrally or peripherally. It will be of interest to study its effect on brain metabolism.

Summary. The hydroxamate of anthranilic acid injected intraperitoneally into rats in a dose of 1.0 mg/g causes anesthesia lasting 10 hours or more. Recovery is apparently complete. One half this dose causes tranquilization and suppression of the conditioned avoidance response. The compound protects mice against twice the lethal dose of strychnine. It merely prolongs the life of animals given metrazol or picrotoxin. A dose of 1.0 g/kg injected intravenously in a dog has no effect on heart rate, EKG or blood pressure and only minor effects on respiration. Equivalent amounts of the hydroxamates of salicylic and benzoic acids have none of the pharmacological effects.

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Effect of Arsenic on Growth of Mammalian Cells *in vitro*.* (26170)

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Arsenicals are useful in veterinary medicine (1,2), and in the past decade they have also acquired importance in animal nutrition as growth stimulants(3). Interest in the metabolic turnover of the growth promoting arsenicals and the question of tissue residues has spurred the need for additional basic information on the physiologic role of this class of compounds. The application of tissue culture methods to determine the direct response

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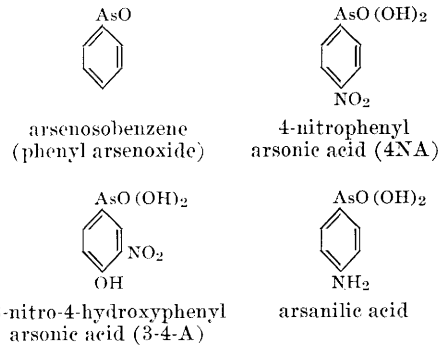
of mammalian cells to a variety of arsenicals was the immediate aim of the present study.

Through the standard technics of tissue culture based on the classical tissue explant-plasma clot preparation, arsenic has been reported to exert both growth promoting(4,5,6) and toxic(7,8,9) effects. Subsequent advances in tissue culture have made it possible to grow large numbers of homogeneous cells descended from a common ancestor,—a single isolated cell. Cultivating tissue cells dispersed in readily prepared fluid media of reproducible composition has overcome some of the limitations of the plasma clot and has improved the quantitative aspects of cell culture. In the present work the effect of arsenicals in the immediate chemical environment of living cells was ascertained quantitatively by measuring survival and proliferation of human and animal cells in replicate glass monolayer cultures.

Methods. The growth response of 4 strains of mammalian cells was determined after a period of continuous exposure to various arsenicals. Solutions of the arsenicals, sterilized by filtration through fritted glass, were added to suspensions of the cells in growth medium composed of 90% Eagle's basal medium‡ and 10% horse serum. The medium also contained penicillin (100 units/ml), streptomycin (100 mmg/ml), tetracycline (10 mmg/ml), and nystatin (50 units/ml). There were 4 to 6 replicate cultures for each dose of arsenical. The cell strains were human skin of substrain 2198(10), HeLa S-3, Chang's human liver, and Earle's L-929 mouse fibroblast. Cells were grown for 4 days in stationary cultures containing 2 ml of nutrient in T-9 flasks(11), 10 flasks to a rack,§ and incubated at 37°. At the end of 4 days the cells were harvested by trypsinization, washed with saline, acid hydrolyzed, and assayed for deoxyribonucleic acid (DNA), which is synthesized in the cell nuclei.

In addition to sodium arsenite and sodium arsenate, 4 organic arsenicals used as feed supplements(12) were also tested. One of these was arsenosobenzene, a potent coccidiostat containing trivalent arsenic. The remaining compounds were derivatives of phenyl arsonic acid (As^V), *viz.*, 4-nitrophenyl arsonic acid (4NA), 3-nitro-4-hydroxyphenyl arsonic acid (3-4-A), and arsanilic acid.



arsenosobenzene (phenyl arsenoxide)

4-nitrophenyl arsonic acid (4NA)

3-nitro-4-hydroxyphenyl arsonic acid (3-4-A)

arsanilic acid

Since the low pH of solutions of 3-4-A acidified the culture medium, this compound was neutralized before filter sterilization. Arsanilic acid was tested as the sodium salt (atoxyl). Arsenosobenzene was brought into solution by first neutralizing with a drop of alkali followed by several hours of magnetic stirring. Studies of sodium arsenite and arsenate were carried out simultaneously, the same controls serving both series.

Throughout the present studies, measurement of growth response was based on the demonstration that DNA serves as a reliable index of size of cell populations cultivated either in natural or synthetic media(13). The DNA content of a culture gives a relative estimate of total cell number and avoids the problem of obtaining an accurate count of degenerating cells in aliquots of suspensions from cultures with high growth inhibition or mortality. The procedure selected for assaying DNA was an adaptation of a quantitative colorimetric test for deoxyribose(14). This method required some modification for application to tissue culture, and the variations are therefore described briefly.

DNA Assay. The contents (2 ml) of each T-9 culture flask are transferred to a 15-ml conical centrifuge tube. The flasks are rinsed with saline after removing the adherent cell layer with 0.125% trypsin. The harvested cells are centrifuged down, and supernatant is discarded. The cells are then resus-

‡ Microbiological Assoc., Inc., Bethesda, Md.

§ Kontes Glass Co., Vineland, N. J.

pended in saline. The centrifuging is repeated, following which the saline is replaced with 3 ml of 5% trichloroacetic acid (TCA). The cells are hydrolyzed with TCA for 30 minutes in a gently boiling water bath while the centrifuge tubes are loosely stoppered with sealed ampoules. After cooling in water, 1 ml more of TCA solution is added to each tube and the acid-insoluble precipitate is centrifuged down. The hydrolysate is transferred to a 15-ml graduated, glass-stoppered, centrifuge tube, and the volume is adjusted to 4.0 ml. The unknowns, reference solutions containing from 10 to 100 mmg DNA‖ in 4 ml of TCA, and a blank consisting of 4 ml of 5% TCA, each receive 0.2 ml of a 0.4% alcoholic solution of p-nitrophenylhydrazine. With sealed ampoules as condensers, the tubes are heated for 20 minutes in a gently boiling water bath. After cooling, 9 ml of *n*-butyl acetate are added to each tube, glass stoppers are applied and the tubes well shaken. The stoppers are removed, the tubes centrifuged and the upper layer aspirated off. With finger in place over the end of the pipet as the tip passes through the organic phase, 3 ml of the lower (aqueous) phase are pipetted into a 5-ml volumetric flask, and 1 ml of *2N* NaOH is added for color development. The solution is made up to volume with water, mixed, and read in a colorimeter at 560 mμ. A Spectronic 20 has been found suitable for this purpose.

Substances interfering at 560 mμ, such as the serum and the phenol red of the basal medium, were absent in the second supernatant when checked with the Beckman DK-1 spectrophotometer. Excesses of p-nitrophenylhydrazine causing occasional colorimetric errors are averted by preparing the dye as a 0.4 instead of the 0.5% solution originally prescribed(14). The use of glass-stoppered centrifuge tubes eliminates the possibility of color extraction from rubber stoppers by butyl acetate. The standard error of a representative series of 5 control cultures with a mean DNA content of 47.7 mmg (Table I, arsonic acid) was ± 0.8 mmg of DNA.

‖ California Corp. for Biochemical Research, Los Angeles, Calif.

TABLE I. Inhibition of Mammalian Cell Growth by Arsenicals as Measured by DNA Content of Cell Cultures.

Arsenosobenzene (phenyl arsenoxide) Cell strain L-929			3-NO_2-4-OH-phenyl arsonic acid (3-4-A) Human skin, substrain 2198		
As^{III} (ppm)	DNA content (mmg)	Growth inhibition (%)	As^{V} (ppm)	DNA content (mmg)	Growth inhibition (%)
.00 *	17.1		0*	20.7	
.00 †	53.8	.0	0†	47.7	.0
.005	52.0	4.9	10	44.8	10.7
.015	47.6	16.9	30	42.2	20.4
.025	38.6	41.4	50	39.2	31.5
.035	30.7	62.9	100	32.5	56.3
.05	17.0	100.0	300	24.4	89.6
			500	22.0	95.2

* 0-day controls (inoculum).
† 4-day control cultures.

Results and discussion. The magnitude and range of variation in DNA over the 4-day test period in 2 typical experiments are indicated in Table I. The difference in magnitude of the concentration range of these 2 arsenicals is reflected in the difference in slopes of the respective dose-response curves. A plot of percent growth inhibition of skin cells by 3-4-A *vs.* logarithm of arsenic concentration gives a relatively gradual slope, or increase in growth inhibition with progressive increase in concentration of arsenic. Concentrations of arsenic corresponding to a 50% suppression of cellular proliferation (Table II) were derived from either log-probability(15) or semilogarithmic plots,—the respective values being almost identical in most instances. The magnitude of the plotted slopes bore no consistent relation either to valence of arsenic or to cell strain.

The reaction of all 4 strains of tissue cells to any single arsenical was fairly similar. All cells were decidedly most sensitive to arsenosobenzene, more so than to inorganic arsenite (Table II). Arsenosobenzene is the nitrogen-free, reduced prototype of the arsonic acids. A possible mechanism for the strong lethal action of arsenosobenzene may lie primarily in the greater permeability of the cell wall to this compound, following which arsenosobenzene may block more enzyme systems than

TABLE II. Concentration of Arsenic Effecting 50% Growth Inhibition of Mammalian Cells.

Compound		Cell strain L-929	Skin	HeLa	Liver
Arsenosobenzene	(As^{III})	.029	.029	.044	.022
Sodium arsenite	(As^{III})	.19	.22	.13	.14
" arsenate	(As^{V})	1.6	4.7	2.0	4.0
4-NO_2-Ø arsonic acid	(As^{V})	10.3	28.6	26.1	18.7
3-NO_2-4-OH-Ø arsonic acid	(As^{V})	46.2	84.	92.	44.7
Sodium arsanilate (atoxyl)	(As^{V})	73.9	122.	64.8	117.

Values are expressed in parts/million of elementary arsenic.

do other arsenicals. Eagle and Doak(16) have reviewed the permeability theory, but did not attempt to identify the factors determining which compounds can penetrate the cell wall and which cannot.

Trivalent inorganic arsenic was as inhibitory at 0.1 to 0.2 ppm as was the pentavalent form at 2 to 5 ppm. At least 5 to 10 times as much organic pentavalent arsenic (4NA) was required to effect the degree of growth suppression produced by the element in the form of the arsenate. Skin and liver cells showed the widest range of sensitivity to the arsenicals. To suppress growth 50%, all cells required from 1500 to 5000 times as much arsenic in the form of atoxyl as in the form of arsenosobenzene. It is interesting that 3-4-A was exceptionally well tolerated by HeLa cells. At 300 to 500 ppm of 3-4-A growth of HeLa cells was suppressed only 60%. Results with cultured mammalian cells in many respects parallel the findings with *Lactobacillus leichmannii* reported from this laboratory (17). The microbiological studies likewise demonstrated higher growth suppression by the trivalent arsenicals and showed arsanilic acid to be least inhibitory of the compounds tested.

Arsenic content of the medium and detoxifying ability of the serum were not determined. It is possible that a formulation of some protein-free medium may demonstrate a growth stimulating influence of a specific arsenical on a particular pure cell line. In one of our preliminary trials a growth increment 50% above that of control skin cultures was noted in the presence of arsenate (1.5 ppm As^{V}). This observation was not confirmed by subsequent tests. The arsenate may have substituted for or replaced phosphate in some enzymatic reactions and increased enzyme activity, oxidation, or overall rate of reaction. The subject of arsenolysis is outlined in a recent review of the biochemistry of arsenic(2).

Wide variation in the potency of arsenicals *in vivo* from a pharmacologic standpoint is generally considered to be related to the difference in valence of arsenic. This variation was emphasized by the response of rapidly multiplying mammalian or bacterial cells *in vitro*. Both types of cell reacted to the inorganic and organic trivalent compounds by a reduction of cellular proliferation exceeding that produced by the pentavalent arsenicals. From a nutritional and metabolic standpoint it would appear, therefore, that throughout the course of absorption, distribution, uptake, retention, and elimination of arsenicals in the animal body the arsenic content of the compounds may be of far less consequence than the valence of the element.

Summary. Under continuous exposure to arsenicals *in vitro*, mammalian cells showed a remarkably wide range of tolerance to arsenic. To suppress growth of cells by 50% the absolute quantity of arsenic administered as atoxyl was up to 5000 times that in the form of arsenosobenzene. Pentavalent organic arsenicals with nitrogen groups were comparatively well tolerated in cell culture. In cells originating from different tissues and cultured in the same medium, degree of growth inhibition by a particular arsenical was fairly uniform. An adaptation of a DNA assay, useful in estimating cell populations in tissue culture, is described.

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A Study of the Relationship Between Canine Distemper and Measles in the Dog.* (26171)

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Evidence has been presented for(1-3) and against(4,5) the suggestion that the viruses of human measles and canine distemper are antigenically related. This report describes the results of inoculation of dogs with live measles virus and subsequent challenge with virulent canine distemper virus. Despite the minimal canine distemper antibody response which followed the measles inoculation, all these dogs resisted challenge with distemper virus.

Materials and methods. The Edmonston strain of measles virus(6) was obtained from Dr. John F. Enders, after 28 transfers in human kidney tissue culture. Two pools of live measles virus were used. Pool 1 had been passaged 3 times in rhesus monkey kidney tissue culture and had an infectivity titer of $10^{4.0}$/0.1 ml in that system. Pool 2 was in the 6th tissue culture passage and titered $10^{4.5}$/0.1 ml. Control material consisted of uninoculated monkey kidney tissue culture fluid derived from the same batch of cells in which the measles virus was produced.

The Snyder Hill strain of distemper virus, maintained by serial intracerebral transfer in dogs, was previously reported(7) to produce convulsions and/or death in dogs with regularity, when given intracerebrally. This same strain, when administered as spleen suspension by the intravenous route, produced the classical disease described by Dunkin and Laidlaw(8).

The dogs used in these studies came from a purebred colony of beagles, reared and maintained in isolation by the Veterinary Virus Research Inst. The 19 dogs represented 6 different litters, ranging in age from 5 to 24 weeks at time of measles injection. Each litter was placed together in a separate isolation unit for observation a few days prior to inoculation.

Three groups of dogs were inoculated with measles virus or control tissue culture fluid, then challenged with distemper virus, the time and route of challenge varying with each group. Group A (Tables I and II) included 9 dogs, consisting of 3 separate experiments with litters of 3 dogs each. In Group A, 2 dogs of each litter were injected intravenously

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