to highly irritating products. After the accumulation of more data, an attempt will be made to describe irritation on a unitage basis. At present, however, it is recommended that comparisons of irritation be made within categories of compounds, since there seems to be no universal agreement on a standard irritant. The same practice would probably apply to studies of the anti-inflammatory compounds.

In the course of this investigation, alternate sites of injection and modifications of the procedure have been used. The rat ear, rat testes, rat abdominal skin, and the longitudinal back muscle of the rabbit have been used successfully as an injection site for the irritant. In each case, the procedure of dye removal was modified slightly, but essentially the method remained the same.

Summary. An objective quantitative method to measure the degree of irritation has been described. It has been used to obtain a valid dose-response curve on a known irritant, formaldehyde, and to measure the irritating properties of various categories of injectables. The method has been modified to permit measurement of anti-inflammatory activity and topical irritation.

1. Ebbecke, U., *Klin. Wchnschr.*, 1923, v2, 1725.
2. Hirschfelder, A. D., *Am. J. Physiol.*, 1924, v70, 507.
3. Tainter, M. L., and Hanzlik, P. J., *J. Pharmacol. Exp. Therap.*, 1924, v24, 179.
4. Spagnol, G., *Arch. exp. Path. Pharmakol.*, 1928, v137, 250.
5. Menkin, V., *J. Exp. Med.*, 1929, v50, 171.
6. Tainter, M. L., Throndson, A. H., and Lehman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1937, v36, 584.
7. Weatherby, J. H., *J. Lab. Clin. Med.*, 1940, v25, 1199.
8. Rocha e Silva, M., and Dragstedt, C. A., *J. Pharmacol. Exp. Therap.*, 1941, v73, 405.
9. Last, M. R., and Loew, E. R., *ibid.*, 1947, v89, 81.
10. Hoppe, J. O., Alexander, E. B., and Miller, L. C., *J. Am. Pharm. Assn.*, 1950, v39, 147.
11. Caster, W. O., Simon, A. B., and Armstrong, W. D., *J. Lab. Clin. Med.*, 1953, v42, 493.
12. ———, *J. Applied Physiol.*, 1954, v6, 724.
13. ———, *Anal. Chem.*, 1954, v26, 713.

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Effect of Cyanide on Growth and Respiration in Earle's Strain L Cells.* (22100)

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Respiration in most tissues is inhibited by 3×10^{-3}M cyanide(3,8,12,14). Cyanide has been used very little as an inhibitor of cells grown *in vitro*. Danes and Leinfelder(2) noted that 0.1×10^{-4} to $.38 \times 10^{-4}$ M HCN inhibited growth of chick embryo tissue 73%. Experiments reported below were designed primarily to ascertain to what extent Earle's strain L cells, which have been grown in culture for more than 12 years(4), are cyanide sensitive.

Materials and methods. Strain L cells were obtained through the generosity of Dr. Wilton R. Earle and were cultured on glass (D-3.5 Carrel flasks) in Earle's solution consisting of 40% balanced saline, 40% horse serum and 20% chick embryo extract (in Earle's saline solution, 1:1)(4,5). The culture medium was renewed 3 times weekly and cultures gassed with 5% CO_2 in air. In experiments to test the effect of cyanide on growth, a cell suspension was obtained by gentle agitation of stock cultures. The cells were concentrated by centrifugation at 1000 rpm(7), resuspended in fresh medium after

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TABLE I. Effect of Sodium Cyanide on Growth in Earle's Strain L Cells. Growth of cells in control cultures is taken as 100.

Conc. of NaCN (molar)	No. of cultures	Avg No. of cell nuclei per culture	Growth (% of control)
1×10^{-3}	7	0	.0
1×10^{-4}	16	1.43×10^{5}	2.6
5×10^{-5}	9	2.19×10^{6}	99.6
1×10^{-5}	5	3.79×10^{5}	90.2
1×10^{-6}	20	1.88×10^{6}	95.5
1×10^{-8}	34	1.98 × "	117.3
1×10^{-10}	20	1.96 × "	116.2
1×10^{-12}	17	2.21 × "	94.6

which 1 ml aliquots were inoculated into Carrel flasks. After 2-3 days the cultures were divided into experimental and control groups. Sodium cyanide was added directly to the medium in experimental cultures which were carried for an additional week, at which time the number of nuclei in each culture was ascertained by sample counts on a hemacytometer(13). Cells were collected for respiration experiments in the same way as for growth experiments but they were resuspended in neutralized horse serum. Neutralized horse serum contains bound bicarbonate and remains physiologically neutral when equilibrated with a CO_2 free atmosphere(16) as is employed in Warburg's direct method (15). One ml aliquots were placed in each Warburg flask. Number of cell nuclei was determined in same manner as previously indicated. Calcium cyanide was prepared and its concentration determined according to Robbie(9,10). Constant source of HCN in medium during experimentation was obtained by using $Ca(CN)_2$-$Ca(OH)_2$ mixtures in center well of the Warburg flasks(11).

Results. Since number of cells used from one experiment to another was not exactly the same, determinations were made in pairs for both growth and respiration.

The results obtained on effect of NaCN on growth (Table I) indicate that Earle's strain L cells do not divide nor grow when placed in culture medium containing 10^{-3} M NaCN. After the first day in this solution all cells become detached from the floor of the flask. In 10^{-4} M NaCN the number of nuclei was scant or non-existent. Although some cells were still intact after a week it is doubtful that growth occurred in this concentration of cyanide. In culture media containing 10^{-8} M NaCN growth is stimulated. As checked by the test for observations involving paired groups(6), the accelerated growth in 10^{-8} M NaCN was significantly different from controls (P = about 0.01). It appeared that growth might be stimulated in 10^{-10} M NaCN but statistically the results are not significant.

Results from experiments on effect of cyanide on respiration are presented in Table II. Oxygen uptake is inhibited 85.3% in 7×10^{-4} M HCN. It should be noted that a similar concentration of cyanide inhibited growth. Statistically, there was no evidence for stimulation of respiration with lower concentrations of HCN.

Discussion. Most of strain L cells did not grow in 10^{-4} M NaCN. In contrast, oxygen uptake was not completely inhibited in 2×10^{-4} M HCN; the mean oxygen uptake was 26.9% as much as the control. Probably difference in degree of inhibition of growth as compared to respiration can best be explained by the fact that the cells were exposed to cyanide for a longer period in the growth experiments.

It is known that the bulk of respiration is carried out by the cytochrome system. Cyanide inhibits cytochrome oxidase almost completely at concentrations of the order of 10^{-3} M, and thus inhibits about 80% of the respiration in most tissues(1).

Growth of embryonic chick heart in tissue culture is inhibited 73% in 0.1×10^{-4} to 0.38×10^{-4} M cyanide(2). The same is true for

TABLE II. Effect of Cyanide on Oxygen Consumption in Earle's Strain L Cells. Rate of oxygen consumption in control cultures is taken as 100.

Conc. of HCN (molar)	No. of determinations	Avg μl oxygen consumed/ hr/10^6 cells	Oxygen consumption (% of control)
7×10^{-4}	28	1.2	14.7
2×10^{-4}	28	2.0	26.9
6×10^{-5}	28	4.9	67.0
4×10^{-7}	24	7.7	109.2
2×10^{-8}	21	8.4	101.4
3×10^{-9}	28	7.5	100.4
2×10^{-10}	21	8.5	101.5
1×10^{-12}	24	8.1	105.8

other tissues. With slightly greater cyanide concentration (10^{-4} M NaCN), growth of strain L is inhibited 97.4%. Thus, although concentrations of cyanide in our experiments were slightly different from those used by other workers, the results are in agreement. Strain L is about as sensitive to cyanide as other types of tissue cells.

We noted that growth of strain L cells was stimulated with 10^{-8} M NaCN, as described by many investigators for other cells and tissues but no adequate explanation is available.

Summary. 1. Earle's strain L cells were cultured *in vitro* and the effects of different concentrations of NaCN were studied. Concentrations ranging from 10^{-3} to 10^{-12} M NaCN were tested. 2. In 10^{-4} M NaCN growth was inhibited 97.4% but in 10^{-8} M, growth was enhanced and exceeded that of controls by 17%. 3. The effect of cyanide on oxygen uptake of strain L cells in neutralized horse serum was determined by means of the Warburg Respirometer. 4. At a concentration of 7 x 10^{-4} M HCN, which was slightly stronger than the concentration necessary to stop growth, there was still an oxygen uptake of 14.7% as compared to controls. 5. Strain L cells possess a cyanide sensitive type of respiration similar to that found in normal cells.

1. Baldwin, E., Dynamic Aspects of Biochemistry. Cambridge, Cambridge Univ. Press, 1952.

2. Danes, B., and Leinfelder, P. J., *J. Cell. Comp. Physiol.*, 1951, v37, 427.

3. Dixon, M., Allen, K., and Elliott, C., *Biochem. J.*, 1929, v23, 812.

4. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

5. Earle, W. R., Evans, W. J., Sanford, K. K., Shannon, J. E., and Waltz, H. K., *J. Nat. Cancer Inst.*, 1951, v12, 563.

6. Edwards, A. L., Statistical Analysis. New York, Rinehart and Co., 1946, pp. 172-181.

7. Evans, V. J., Earle, W. R., Sanford, K. K., Shannon, J. E., and Waltz, H. K., *J. Nat. Cancer Inst.*, 1951, v11, 907.

8. Field, J., and Field, S. M., PROC. SOC. EXP. BIOL. AND MED., 1931, v28, 995.

9. Robbie, W. A., *Arch. Biochem.*, 1944, v5, 49.

10. ———, *J. Cell. Comp. Physiol.*, 1946, v27, 181.

11. Robbie, W. A., and Leinfelder, P. J., *J. Indust. Hyg. Toxicol.*, 1945, v27, 269.

12. ———, *Arch. Biochem.*, 1948, v16, 437.

13. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., *J. Nat. Cancer Inst.*, 1951, v11, 773.

14. Stare, F. J., and Elvehjem, C. A. Studies on the respiration of animal tissues. *Am. J. Physiol.*, 1933, v105, 655.

15. Umbreit, W. W., Burris, R. K., and Stauffer, J. F. Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Minneapolis, Burgess Pub. Co., 1949.

16. Warren, C. O., *J. Biol. Chem.*, 1944, v156, 559.

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