centage "takes" of experimental animals over normal control group. This leads to the belief that following marked catabolic stress conditions in tissues of experimental animal are more receptive to the implanted malignant cell than tissues of normal healthy animal.

At first glance the increased resistance of Tannenbaum and Silverstone's animals with chronic malnutrition to development of spontaneous tumors might appear to be at variance with our data showing that acute starvation reduces resistance of the rat to inoculated Walker 256 cells. However, in our estimation there is a marked difference in metabolic status of the 2 groups of animals. We believe that prolonged complete starvation (for 7 days) acts as a severe body "stress" whereas chronic malnutrition does not act as a stress, although the "soil" in malnourished animal's tissue is depleted or changed sufficiently to decrease incidence of formation of spontaneous tumors.

Our findings tend to support those of Buinauskas *et al.*(3) revealing increased susceptibility of animals having the stress of an operation (celiotomy) to inoculated Walker 256 cells.

*Summary.* The effect of acute starvation and dehydration in rats, on "takes" of Walker 256 cells following their injection subcutaneously has been investigated. Our results indicate that starvation and dehydration for 48 hours do not increase susceptibility of the rat to subcutaneous inoculation of Walker tumor, but if starvation is continued for 7 days there is increase in percentage "takes" of the tumor as compared to incidence in normal healthy animals. There is also an increase in "takes" of implanted tumor in young rats 28 days of age compared to more mature rats.

1. Tannenbaum, A., Silverstone H., *Cancer Res.*, 1949, v9, 724; 1950, v10, 577.

2. Talalay, P., Takano, G. M., Huggins, C., *ibid.*, 1950, v12, 834.

3. Buinauskas, P., McDonald, G. O., Cole, W. H., *Ann. Surg.*, 1958, v148, 642.

Received April 20, 1960. P.S.E.B.M., 1960, v104.

## Effects of Respiratory Inhibitors on Glucose and Protein Utilization and Growth in Strain L Cells.* (25876)

D. M. PACE AND L. M. ELROD†
*Dept. of Physiology, University of Nebraska, Lincoln*

Azide, urethan, arsenite and fluoride are well known respiratory inhibitors and have been used as such in innumerable investigations. Except for the work of Cailleau *et al.* (2) on azide, there have been no reports on effects of these substances on strain L cells. Westfall *et al.*(14) studied glucose utilization by strain L cells and although amino acid requirements have been ascertained to some extent, no measurements of total protein utilization by these cells have been reported. In the following report, results of experiments made upon strain L cells by exposing them to these several substances and noting the effects on glucose and protein utilization, as well as upon cell numbers and cell division, are recorded.

*Materials and methods.* Earle's medium, consisting of 40% horse serum, 40% balanced salt solution and 20% (1:1) chicken embryo extract was used. This medium was modified for control cultures by using 39% Earle's balanced salt solution and 1% triple distilled water. The test medium also contained 39% balanced salt solution and 1% of desired concentration of inhibitor solution. For these experiments, 1 ml of cell suspension containing approximately 1 million cells was placed in each of 20 Carrel D-3.5 flasks. The medium was removed 48 hours later and in each of 10 flasks, was replaced with 2 ml of fresh

* This investigation supported in part by research grants from Nat. Science Fn. and U.S.P.H.S.

† Present address: Dept. of Biology, Westminster College, Fulton, Mo.

TABLE I. Effect of Sodium Fluoride, Sodium Azide, Sodium Arsenite and Urethan on Growth in Earle's Strain L Cells. Initial cell count, approximately 1 million cells/ml. Cell numbers are avg obtained for 20 cultures.

| Molar conc. of inhibitor | | Cell numbers $10^6$/ml Control | Test | Growth (% of control) |
|---|---|---|---|---|
| Fluoride | $10^{-2}$ | 2.57 | .00 | 00 |
| | $10^{-3}$ | 3.07 | 2.33* | 76 |
| | $10^{-4}$ | 1.90 | 2.06 | 108 |
| | $10^{-5}$ | 3.45 | 3.60 | 104 |
| Azide | $10^{-3}$ | 3.38 | .00 | 00 |
| | $10^{-4}$ | 2.99 | 1.09* | 37 |
| | $10^{-5}$ | 3.25 | 3.21 | 99 |
| Arsenite | $10^{-4}$ | 3.62 | .00 | 00 |
| | $10^{-5}$ | 3.09 | .58* | 19 |
| | $10^{-6}$ | 3.37 | 3.25 | 96 |
| | $10^{-7}$ | 3.45 | 3.45 | 100 |
| Urethan | $10^{-1}$ | 3.67 | .00 | 00 |
| | $10^{-2}$ | 2.78 | 2.24 | 81 |
| | $10^{-3}$ | 3.51 | 3.50 | 100 |
| | $10^{-4}$ | 3.43 | 3.53 | 103 |

* Highly significant (P equals less than 1%).

medium containing the inhibitor. The 10 control flasks also received 2 ml of medium but without inhibitor. Glucose and protein determinations were made 72 hours later as follows: The medium was removed from 5 test flasks and 5 control flasks and cells separated by centrifugation. Glucose remaining in medium of each flask was determined colorimetrically by means of Somogyi(12) method. Duplicate measurements were made using "Glucostat"‡ enzymatic reagent. Total unused protein was ascertained by Biuret reaction (Cornall *et al.*,(3)). These results were checked by using Tsuchiya's reagent and measuring precipated protein (Simmons and Gentzkow(10)). At the same time, mitotic counts and nuclei counts were made; the latter by nuclei enumeration method of Sanford, Earle *et al.*(9).

*Results.* Counts were made 72 hours after exposure of cells to inhibitors (120 hours after cultures had been started). The results are presented in Table I. Cells in control cultures averaged 3.17 million/ml after 5 days. Sodium fluoride ($10^{-2}$ M), sodium azide ($10^{-3}$ M), sodium arsenite ($10^{-4}$ M) and urethan ($10^{-1}$ M), killed the cells. Slightly lower concentrations retarded proliferation, although $10^{-2}$ M urethan appeared to have no significant effect. Statistical analyses were made by the method of group comparisons of Snedecor(11).

Results of studies on utilization of glucose by strain L cells are presented in Table II. Glucose in control medium was in most cases completely depleted. There is 28% and 23% reduction in glucose uptake in $10^{-2}$ M sodium fluoride and $10^{-4}$M sodium arsenite, respectively, while urethan and azide apparently exert little if any effect in concentrations used.

The effect of inhibitors on protein utilization is shown in Table III. Sodium azide ($10^{-3}$ M) and sodium fluoride ($10^{-3}$ M) inhibit protein uptake by 92 and 39%, respectively. Concentrations of $10^{-5}$ M and $10^{-6}$ M arsenite increase protein uptake by 87 and 110%, respectively. A concentration of $10^{-2}$ M urethan increases protein uptake by 39%. An unusual effect, for which we have no explanation, was noted in $10^{-5}$ M azide, where protein content of the medium increased by 93%.

Examination of cultures in which cells had been stained, revealed a mitotic index of 10.5% for untreated (control) cells. It was much lower for all experimental cultures ex-

TABLE II. Effect of Sodium Fluoride, Sodium Azide, Sodium Arsenite and Urethan on Glucose Utilization in Earle's Strain L Cells. Initial glucose concentration, 1 mg/ml. Each figure for glucose uptake is an avg obtained for 10 cultures.

| Molar conc. of inhibitor | | Glucose utilization, mg/ml Control | Test | % of control |
|---|---|---|---|---|
| Fluoride | $10^{-2}$ | 1. | .72† | 72 |
| | $10^{-3}$ | 1. | .85† | 85 |
| | $10^{-4}$ | 1. | .93† | 93 |
| | $10^{-5}$ | 1. | 1. | 100 |
| Azide | $10^{-3}$ | .91 | 1. | 110 |
| | $10^{-4}$ | 1. | 1. | 100 |
| | $10^{-5}$ | 1. | 1. | 100 |
| Arsenite | $10^{-4}$ | 1. | .77† | 77 |
| | $10^{-5}$ | 1. | .77† | 77 |
| | $10^{-6}$ | 1. | 1. | 100 |
| | $10^{-7}$ | .99 | 1. | 101 |
| Urethan | $10^{-1}$ | 1. | .97* | 97 |
| | $10^{-2}$ | 1. | 1. | 100 |
| | $10^{-3}$ | 1. | .92† | 92 |
| | $10^{-4}$ | 1. | .99 | 99 |

* Significant difference (P equals less than 5%).
† Highly significant difference (P equals less than 1%).

‡ "Glucostat" reagent was obtained from Worthington Biochemical Corp., Freehold, N. J.

TABLE III. Effect of Sodium Fluoride, Sodium Azide, Sodium Arsenite and Urethan on Protein Utilization in Earle's Strain L Cells. Avg initial protein concentration, 29 mg/ml. Each figure for protein utilization is an avg obtained for 10 cultures.

| Molar conc. of inhibitor | | Protein utilization, mg/ml Control | Test | % of control |
|---|---|---|---|---|
| Fluoride | $10^{-2}$ | 3.6 | 3.1 | 86 |
| | $10^{-3}$ | 8.6 | 5.2* | 61 |
| | $10^{-4}$ | 5.0 | 4.7 | 94 |
| | $10^{-5}$ | 3.3 | 3.9 | 118 |
| Azide | $10^{-3}$ | 1.3 | .1 | 8 |
| | $10^{-4}$ | 5.6 | 7.5 | 134 |
| | $10^{-5}$ | 1.5 | +1.4 | -93 |
| Arsenite | $10^{-4}$ | 3.1 | 4.8 | 155 |
| | $10^{-5}$ | 4.6 | 8.6* | 187 |
| | $10^{-6}$ | 3.1 | 6.5 | 210 |
| | $10^{-7}$ | 3.5 | 4.0 | 114 |
| Urethan | $10^{-1}$ | 6.6 | 8.5 | 129 |
| | $10^{-2}$ | 4.9 | 6.8* | 139 |
| | $10^{-3}$ | 1.9 | 3.1 | 163 |
| | $10^{-4}$ | 3.9 | 5.0 | 128 |

* Significant difference (P equals less than 5%).

cept azide ($10^{-4}$ M), and zero for those concentrations that inhibited growth.

The cells were also examined to ascertain number of nuclei in each. In control cultures 4% of cells were multinucleate. When exposed to $10^{-4}$ M azide there was a 17% increase in multinucleate cells. Cultures exposed to urethan ($10^{-2}$ M) and fluoride ($10^{-3}$ M) possessed very few multinucleate cells—2%.

Giant cells were frequently observed in both control and test cultures. They were of 2 types, those having a single very large nucleus and those with several smaller nuclei. In cells exposed to $10^{-2}$ M fluoride no giant single nucleate cells were found. The greatest number of giant cells was noted in azide ($10^{-4}$ M). In large multinucleate cells there were many with variable numbers of nuclei and many with nuclei of different sizes, but no mitotic figures were seen.

*Discussion.* Our results indicate that $10^{-4}$ M azide has little effect on glucose utilization, but inhibits cell growth by 63% which conforms with findings of Cailleau *et al.*(2) that azide ($5 \times 10^{-4}$ M) inhibits growth in strain L cells by 89%. Of 4 inhibitors used, arsenite ($10^{-5}$ M) exerts the greatest inhibition, 81%; fluoride ($10^{-3}$ M) decreases growth by 24%. Urethan exerts no significant inhibition.

The failure of azide to affect glucose utilization could be due to its "uncoupling" action allowing oxidation but inhibiting oxidative phosphorylation (Loomis and Lipmann(5); Mehler(6)). Another possible explanation might be in the findings of Stannard(13), that azide acts on one of 2 parallel respiratory systems.

Only preliminary studies have been made on protein utilization of tissue cells (Pasieka *et al.*,(8)). Results of our study show increase in protein utilization when arsenite or urethan is present in certain concentrations; fluoride and azide have an inhibiting effect. Merchant and Kahn(7) found that suspension cultures of strain L cells produce a collagen-like fibrous protein which is released into the medium. Although the work presented here was done differently than that of Merchant and Kahn, we did find considerable variation in protein utilization in control cultures (8.6 to 1.3 mg/ml). This might be explained by assuming that the cells at times utilize protein from the medium and at other times release soluble proteins into the medium, but in stationary cultures the protein remains in solution and does not coalesce into fibers.

In this investigation the mitotic index was lowered by all inhibitors except azide. Bullough(1) reported mitotic inhibition in azide and claimed this was due to interference with carbohydrate metabolism. Under the conditions of our experiments, azide did not appear to interfere with carbohydrate metabolism or lower the mitotic index.

Lambert(4) and many others reported that multinucleate giant cells were formed by fusion of many uninucleate cells and that a glass substrate might act as a stimulus for fusion. Our observations indicate that mode of formation may be by division of the nucleus without cytoplasmic cleavage, or in many instances cytoplasmic division occurs followed immediately by fusion of the cells.

*Summary.* 1) Effects of sodium fluoride, sodium azide, sodium arsenite and urethan on growth, glucose utilization, protein utilization and mitosis in strain L cells were studied. 2)

Cell populations in control cultures averaged 3.17 million/ml after 5 days; had utilized an average of 0.99 mg of glucose and 4.0 mg of protein/ml of medium. 3) Fluoride ($10^{-3}$ M), azide ($10^{-4}$ M) and arsenite ($10^{-5}$ M) decreased cell numbers by 24, 63 and 81%, respectively. Although urethan ($10^{-1}$ M) killed the cells, a concentration of $10^{-2}$ M had little effect. 4) Fluoride ($10^{-2}$ M), arsenite ($10^{-4}$ M) and urethan ($10^{-3}$ M) inhibited glucose uptake by 28, 23 and 8%, respectively. Azide ($10^{-3}$ M) produced 10% acceleration of glucose uptake. 5) Azide ($10^{-3}$ M) and fluoride ($10^{-3}$ M) inhibited protein uptake by 92 and 39%, respectively. Arsenite and urethan accelerated protein uptake in all concentrations. 6) The mitotic index of untreated cells was 10.5. All inhibitors except azide produced a lowered index. 7) In control cultures, 4% of cells were multinucleate. Azide ($10^{-4}$ M) increased these forms to 17%.

1. Bullough, W. S., *Biol. Rev.*, 1952, v27, 133.

2. Cailleau, R., Moss, S., Siegel, B. V., *J. Nat. Cancer Inst.*, 1956, v16, 1011.

3. Cornall, A. G., Bardawill, G. J., David, M. M., *J. Biol. Chem.*, 1949, v177, 751.

4. Lambert, R. A., *J. Exp. Med.*, 1912, v15, 510.

5. Loomis, W. F., Lipmann, F., *J. Biol. Chem.*, 1948, v173, 807.

6. Mehler, A. H., *Introduction to Enzymology*. Acad. Press, N. Y., 1957, p385.

7. Merchant, D. J., Kahn, R. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 359.

8. Pasieka, A. E., Morton, H. J., Mcrgan, J. F., *J. Nat. Cancer Inst.*, 1956, v16, 995.

9. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., Shannon, J. E., Jr., *ibid.*, 1951, v11, 773.

10. Simmons, J. S., Gentzkow, C. J., *Laboratory Methods of U. S. Army*. 1944. Lea and Febiger, Philadelphia. 5th ed. p172.

11. Snedecor, G. W., *Statistical Methods*. Iowa State Coll. Press, Ames, 1948, 4th ed. p75.

12. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 69.

13. Stannard, J. N., *Am. J. Physiol.*, 1939, v126, 196.

14. Westfall, B. B., Evans, V. J., Shannon, J. E., Jr., Earle, W. R., *J. Nat. Cancer Inst.*, 1953, v14, 655.

Received April 21, 1960. P.S.E.B.M., 1960, v104.

## Extraction and Concentration of Mammogenic Fractions from Anterior Pituitary Gland.* (25877)

HENRY C. DAMM† AND C. W. TURNER
*Dept. of Dairy Husbandry, University of Missouri, Columbia*

The concept of homogeneity of lactogenic and mammogenic (mammary gland growth promoting) hormones has been disputed for many years. Older studies(1) showing wide differences in relative amount of these hormones have been repeated using improved assay technics for both hormones(2). Since recent evidence(3) indicates that lactogenic hormone discharge from anterior pituitary gland (AP) does not occur until late pregnancy, or until after over 50% of mammary gland growth has taken place, it would appear incongruous with physiological facts to accept the idea that lactogenic hormone is pituitary agent primarily responsible for mammary gland growth during pregnancy. Critics of the mammogen concept have suggested need of extracting a preparation from the pituitary rich in mammogen and free of lactogenic hormone. The present report indicates our progress. It will be shown that mammogenic hormone is poorly extracted by solvents which effectively remove other established hormones (4). Evidence will be presented indicating that after other hormones are removed from AP, a mammogenic factor may be extracted and concentrated from the residue.

*Materials and methods.* To remove gona-

* Contribution from Mo. Agr. Exp. Sta. Journal Series No. 1912. Approved by the Director. This investigation was supported by grants from U.S.P.H.S. and Am. Cancer Soc.

† Public Health Service Research Fellow of Nat. Cancer Inst.