unhandled rats did differ significantly from the controls, however, ($p<.025$). Sixty minutes after stress, the only groups which differed from one another were stressed unhandled rats and non-stressed handled rats. However, 80 minutes after stress, stressed unhandled subjects differed significantly ($p<.025$) from all other groups, which did not differ from one another. Therefore, the hypothesis that rats stimulated in infancy recover from stress more rapidly than non-stimulated rats is supported.

The other results of particular interest are the significant Sex difference, and the Sex $\times$ Infantile Experience interaction. The higher glucose levels found in males, as compared with females are consistent with the sex differences found with other measures frequently assumed to reflect emotional reactivity(2). Differential reactions of the sexes to infantile stimulation are not usually reported. Levine(4), however, found that stimulated rats survived leukemia longer than non-stimulated rats. This effect was due to the difference between stimulated and non-stimulated males. The results of the present experiment, which suggest that the handling effect may be attributable to males rather than females, are consistent with these findings.

1. Bell, R. W., Reisner, G., Linn, T., Science, 1961, v122, 1428.
2. Broadhurst, P. L., Brit. J. Psychol., 1957, v8, 1.
3. Hoffman, W. S., J. Biol. Chem., 1937, v51, 120.
4. Levine, S., in Roots of Behavior, ed., E. L. Bliss, Harper & Brothers, New York, 1962, p246.

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Radiosensitivity and Biological Properties of Two Tumor Types Indigenous to the Same Host. IX. Swelling Properties of Mitochondria Isolated from Mouse Tumor and Liver Tissue.* (30293)

LEO A. MILLER† AND ANNA GOLDFEDER
Cancer and Radiobiological Research Laboratory, Department of Hospitals, City of New York, and Department of Biology, New York University, New York City

During investigation of the biological properties of tumor types, we investigated the swelling phenomenon of their mitochondria under various conditions of isolation, resuspension in various media, and incubation. Previous electron microscopic studies revealed a difference in morphological structure of mitochondria of the 2 tumor types(1). Differences in oxidative phosphorylation of the 2 tumor types were also ascertained(2), and some enzymic and metabolic differences between neoplastic and normal tissue mitochondria have been discussed by Kit and Griffin (3).

Mitochondrial swelling can be induced by various agents such as phosphate and thyroxin. The action of these agents is thought to be mediated by their effect upon the electron transport system of this organelle(4,5). Other compounds, such as ascorbate(6) and glutathione(7,8), are believed to exert their effect directly upon the mitochondrial membrane. Spontaneous swelling of mitochondrial preparations has also been reported(9,10).

Evidence is herein presented showing dependence of the rate and extent of swelling on the quality of the isolated mitochondrial fraction of 2 mouse tumor types and of mouse liver.

Materials and methods. Two tumor types, an epithelial cell, slow growing, and a spindle cell, fast growing, served as basic test material. Both tumors had originated in the mammary glands of female mice of the inbred DBA/212 strain and are being carried in isologous parent hosts by serial transplants. Liver mitochondria from normal mice of the same strain were assayed as controls for comparative purposes.

The isolation of mitochondrial fractions

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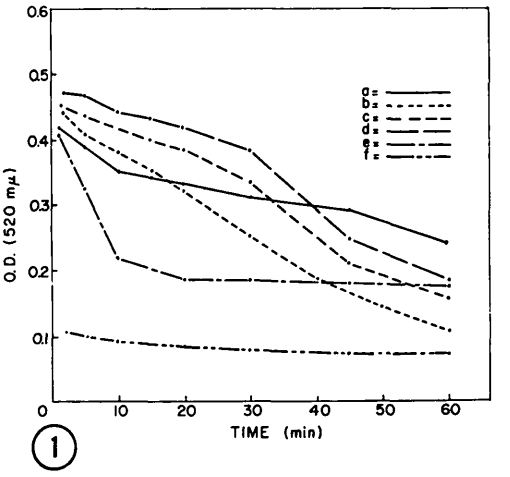
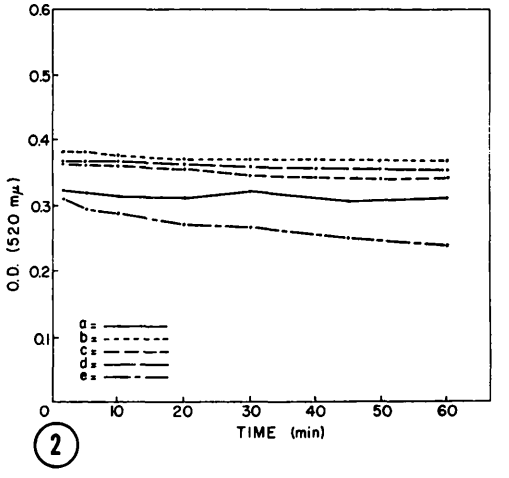
† Present address: Dept. of Pomology, Univ. of Calif., Davis.

from the 3 types of tissue in both 0.25 M sucrose and 0.88 M sucrose was carried out according to a procedure previously described (11).

The isolated mitochondria were resuspended in either 0.25 M or 0.88 M sucrose and used directly for swelling measurements within 30 minutes from the time of isolation by observing a decrease in optical density at 520 mμ at room temperature (23-25°C). The relationship between mitochondrial swelling and optical density was ascertained by Tedeschi and Harris(12) and serves as a basis for the present assay. The total volume in the cuvette was 3.0 ml and included 0.3 M tris buffer at pH 7.5. Appropriate dilution of the mitochondrial suspensions was made so that the initial O.D. readings for liver mitochondria ranged from 0.400 to 0.500 and were approximately 0.350 to 0.450 for tumor mitochondria. Protein determinations were made by the method of Lowry(13). Crystalline bovine plasma albumin from Armour Laboratories was used as standard material.

Results. The data reported in the Tables show that the resuspension medium influences the initial optical density of mitochondrial protein. For example, resuspension in 0.25 M sucrose results in a greater O.D./mg protein value indicating a more contracted state even though the mitochondrial fraction had previously been isolated from 0.25 M or 0.88 M sucrose. This effect is more pronounced for liver than for tumor mitochondria.





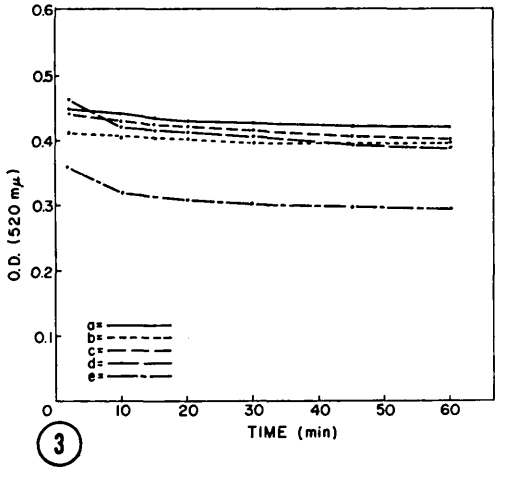


FIG. 1. Liver mitochondrial swelling. a) isolated and resuspended in 0.25 M sucrose; b) isolated in 0.25 M sucrose and resuspended in 0.88 M sucrose; c) isolated in 0.88 M sucrose and resuspended in 0.25 M sucrose; d) isolated and resuspended in 0.88 M sucrose; e) isolated in 0.25 M sucrose and resuspended in 0.15 M KCl; f) isolated and resuspended in 0.88 M sucrose and "aged" 24 hours.

FIG. 2. Spindle cell tumor mitochondrial swelling. a) isolated and resuspended in 0.25 M sucrose; b) isolated in 0.25 M sucrose and resuspended in 0.88 M sucrose; c) isolated in 0.88 M sucrose and resuspended in 0.25 M sucrose; d) isolated and resuspended in 0.88 M sucrose; e) isolated in 0.25 M sucrose and resuspended in 0.15 M KCl.

FIG. 3. Epithelial cell tumor mitochondrial swelling. a) isolated and resuspended in 0.25 M sucrose; b) isolated in 0.25 M sucrose and resuspended in 0.88 M sucrose; c) isolated in 0.88 M sucrose and resuspended in 0.25 M sucrose; d) isolated and resuspended in 0.88 M sucrose; e) isolated in 0.25 M sucrose and resuspended in 0.15 M KCl.

TABLE I. Effect of Sucrose, Thyroxin and Phosphate on Mitochondrial Swelling.

Tissue type	Isolation medium (M sucrose)	Resuspension medium* (M sucrose)	Swelling,† mg protein	Swelling		
				Control	PO_4	Thyroxin‡
Liver	.25	.25	1.33	.180	.150	.120 (67)
	.25	.88	.92	.339	.290	.272 (80)
	.88	.25	1.32	.286	.280	.150 (52)
	.88	.88	.86	.294	.316	.243 (82)
Epithelial tumor	.25	.25	.47	.021	.028	.028
	.25	.88	.37	.014	.016	.016
	.88	.25	.49	.043	.043	.045
	.88	.88	.37	.080	.090	.100
Spindle cell tumor	.25	.25	.74	.013	.023	.020
	.25	.88	.45	.018	.021	.018
	.88	.25	.64	.022	.032	.036
	.88	.88	.51	.018	.016	.009

* The unwashed mitochondrial pellets were kept in an ice bath homogeneously resuspended in the appropriate medium and used for swelling determination within 30 min from time of isolation. Dilutions of suspensions were made so that the initial O.D. readings for liver mitochondria ranged from 0.400 to 0.500 and that for tumor mitochondria, approximately 0.350 to 0.450.

† Swelling is calculated as ΔO.D. per hr. Each assay cuvette also contained 0.03 M Tris, pH 7.5, sucrose to a final concentration 0.25 M or 0.88 M and 0.01 M KPO_4, pH 7.5 or 1×10^{-6} M L-thyroxin when used.

‡ Values in parentheses represent % of control values.

Further, the extent of swelling, which is indicated by O.D./hr occurs spontaneously in liver mitochondria and is not influenced by addition of phosphate. Thyroxin is actually inhibitory to the spontaneous swelling of the liver mitochondria and is most effective in this respect when the mitochondria have been resuspended in 0.25 M sucrose rather than 0.88 M sucrose.

Curve I shows the effect of combinations of sucrose isolation and resuspension media on rate of swelling of liver mitochondria. Note the large initial swelling which is caused by addition of liver mitochondria to a medium containing 0.15 M KCl. The relatively low initial O.D. of a liver suspension which has been "aged" 24 hours at 5°C, compared with the original fresh mitochondrial suspension which was isolated and resuspended in 0.88 M sucrose, is also of interest. The "aged" mitochondria shows very little additional swelling over a period of one hour.

Curves II and III show that both tumor types are similar and contrast with liver in that they show very little spontaneous swelling when isolated or resuspended in either 0.25 M or 0.88 sucrose. Table I indicates that phosphate or thyroxin also had no effect. Curves II and III, however, do indicate an increase in swelling for tumor mitochondria when added to a medium containing 0.15 M KCl.

As depicted in Table II, various combinations of additives and pre-treatments of the spindle cell tumor mitochondria resulted in about 50% mitochondrial swelling of the spindle cell tumor as compared to that of the liver mitochondria. However, this difference was significantly smaller where the epithelial mitochondria were concerned. The most effective combination appeared to be pre-incubation of the mitochondria at 38°C for 20 minutes and addition of 0.15 M KCl, phosphate, and thyroxin. Cyanide was also able to increase mitochondrial swelling.

Discussion. Evidence is herein presented that the rate and extent of mitochondrial swelling may characterize the structural and functional integrity of the mitochondria. This is indicated by the high initial O.D. per mg protein and by the greater rate of swelling for liver mitochondria as compared to the tumor mitochondria. Other investigators have also noted a swelling difference between normal and aged mitochondria(9). Previous electron microscopic studies showed that the spindle cell tumor mitochondria are of inferior quality, containing fewer cristae and more fragile, as compared to those of the epithelial tumor mitochondria and to those

TABLE II. Effect of Various Additives and Treatments upon Tumor Mitochondrial Swelling.

Exp #	Addendum†	Mitochondrial swelling (ΔO.D./hr)* Epithelial cell	% of control	Spindle cell	% of control
1	None	0.034	—	0.067	—
	Succinate	0.026	76	0.061	91
	DPN	0.032	94	0.072	107
	ATP	0.030	88	0.071	106
	Succinate and DPN + ATP	0.030	88	0.068	101
2	None	0.036	—	0.070	—
	KCN	0.050	139	0.088	126
	KCN + KPO_4 + thyroxin	0.037	103	0.080	14
3	None	0.037	—	0.055	—
	38°C; 20 min	0.064	173	0.094	171
	38°C; 20 min + KCN	0.076	205	0.106	193
4	None	0.034	—	0.065	—
	38°C; 20 min	0.055	162	0.118	182
	38°C; 20 min + KPO_4 + thyroxin	0.072	212	0.133	205
	38°C; 20 min + DNP	0.043	126	0.071	109

* The mitochondrial fraction was isolated and resuspended in 0.25 M sucrose. The assay medium contained 0.15 M KCl, 0.03 M Tris buffer at pH 7.5 and 0.5 ml of mitochondrial suspension in a total volume of 3.0 ml.

† The final concentration of the additives are as follows: NaCN, 1.0×10^{-3} M; DPN, 6.7×10^{-4} M; KPO_4, 1.0×10^{-2} M; L-thyroxin, 1.0×10^{-5} M; Na succinate, 1.0×10^{-3} M; ATP, 3.3×10^{-4} M; and DNP, 1.0×10^{-3} M.

of liver(1). The inferior quality of tumor mitochondria as compared to those of liver was thoroughly discussed by Kit and Griffin (3).

When liver mitochondria are "aged" and are therefore in a relatively degraded state, they not only have a much lower initial O.D. per mg protein compared to the fresh preparations, but their rate of swelling is also very low. Since the tumor mitochondrial suspensions also show significantly lower initial O.D. per mg protein, and the rate of swelling is also comparatively low, the tumor mitochondria, in this respect, may resemble those of aged liver mitochondria.

The observations (Table II) that various agents and resuspension media can cause further tumor mitochondrial swelling would indicate that these mitochondria are at some intermediate level with respect to morphological integrity relative to liver mitochondria.

Summary. Isolated mitochondria from 2 tumor types indigenous to DBA/212 inbred strain (an epithelial cell, slower growing, and a spindle cell, faster growing) have relatively low initial O.D. (520 mμ) per mg protein and a very low rate of mitochondrial swelling. On the other hand, mitochondria from liver from the same strain of mice exhibit a higher initial O.D. per mg protein and have a high rate of swelling *in vitro*. "Aged" liver mitochondria have a much lower initial specific O.D. and also swell at a very low rate. Thus, it is proposed that the initial O.D. of isolated mitochondria and its rate of swelling under similar conditions of isolation, resuspension, and incubation is indicative of the inherent quality and integrity of the mitochondrial fraction. Certain conditions of pre-treatment and some specific additives increased mitochondrial swelling of both tumor types, indicating that the mitochondrial integrity of the tumors is at some intermediate state with respect to that of liver mitochondria.

1. Goldfeder, A., Miller, L. A., Int. J. Rad. Biol., 1963, v6, 575.

2. Goldfeder, A., Acta Union Inter. Con. Can., 1960, v16, 1060.

3. Kit, S., Griffin, A. C., Cancer Research, 1958, v18, 621.

4. Lehninger, A. L., Ray, B. L., Schneider, M., J. Biophys. Biochem. Cytol., 1959, v5, 97.

5. Lipsett, M. M., Corwin, L. M., J. Biol. Chem., 1959, v234, 2448.

6. Hunter, F. E., Jr., Scott, A., Hoffstein, P. E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B., Fink, J., Ford, L., Smith E., ibid., 1964, v239, 604.

7. Lehninger, A. L., ibid., 1962, v237, 946.

8. Hunter, F. E., Jr., Scott, A., Hoffstein, P. E., Gebicki, J. M., Weinstein, J., Schneider, A., ibid., 1964, v239, 614.
9. Emmelot, P., Bos, C. J., Brombacker, P. J., Peyers, I. H. M., Nature, 1960, v186, 556.
10. Emmelot, P., ibid., 1960, v188, 1197.
11. Miller, L. A., Proc. Soc. Exp. Biol. and Med., 1964, v115, 25.
12. Tedeschi, H., Harris, D. L., Arch. Biochem. Biophys., 1955, v58, 52.
13. Lowry, P. H., Rosebrough, N. J., Farr, L. A., Randall, R. J., J. Biol. Chem., 1951, v50, 182.

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Stability of Glutamine *in vitro*.* (30294)

ROBERT ROSENBLUM (Introduced by L. Leiter)
Cardiovascular Metabolic Research Unit, Medical Division, Montefiore Hospital and Medical Center, New York City

Glutamine is considered an unstable compound with two pathways of breakdown: 1) Formation of ammonia and glutamic acid; 2) Formation of ammonia and a cyclical compound, pyrrolidone carboxylic acid(1,2). In modifying the resin chromatographic methods of Hirs, Stein and Moore(3) for quantitative separation of the dicarboxylic amino acids, the impression was obtained that glutamine was stable. The early peaks that emerged from the resin column remained unchanged regardless of age of the specimens. The following experiments were performed to evaluate this observation.

Materials and methods. A modified resin chromatography method was employed to analyze quantitatively glutamic and aspartic acids(4). Glutamine could readily be separated from glutamic acid with this weakly basic anionic resin (Fig. 1). Initially the amino acid† analyses were performed by collecting 100 one ml effluent fractions on a Technicon drop-counter fraction collector and analyzing the samples with ninhydrin(5,6). However, recently the method has been adapted to the Technicon AutoAnalyzer, employing the same Rohn and Haas 100-200 mesh CG-4B anionic resin and 0.2 N sodium acetate buffer, pH 4.5(4,7).

The analyses of ammonia in glutamine standards were also performed by the ion exchange method of Hutchinson and Labby(8), but the colorimetric analysis was carried out by the technique outlined by Chaney(9).

Experiments. 1) One milliliter of 1 mM aqueous and 0.1 N sodium citrate buffer pH 5 solutions of glutamine were repeatedly analyzed on the resin columns during an 8-week period. The solutions were stored at 4°C. Other specimens were repeatedly analyzed after being left at room temperature for 3 to 4 weeks. 2) Pyrrolidone carboxylic acid emerged after aspartic acid as was shown by analyzing the effluent samples with ninhydrin after alkaline hydrolysis. 3) The effluent fractions obtained on chromatography of the glutamine solutions were subjected to alkaline hydrolysis, to search for a peak in the position of emergence previously demonstrated for pyrrolidone carboxylic acid. 4) Fresh solutions of glutamine and glutamine stored at 4°C for several weeks were alkalinized and allowed to distill into the Nessler's reagent *in vacuo* for 30 minutes. After this period the solution was brought back to pH 5 and analyzed with ninhydrin(5,6). 5) When the method for analyzing quantitatively glutamic and aspartic acids was adapted to the AutoAnalyzer, an ammonia peak could be clearly separated from glutamine (Fig. 1). Therefore, chromatograms of fresh and stored glutamine solutions were reanalyzed to observe whether there would be an increase in quantity of ammonia and a decrease in concentration of glutamine with increasing age of the specimens. 6) Two milliliters of glutamine stock solutions were added to a Na K

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† Amino acids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.