The average respiration difference is more than several standard deviations. At zero urethan concentration, we observed no significant differences between the mitochondria with and without tocopherol, so the presence of the tocopherol did not stimulate mitochondrial respiration in our experiments.

The tests for malondialdehyde formation as an index of lipid peroxidation in these groups of mitochondria did not show a significant difference.

Injected mitochondria and tocopherol. The mitochondrial oxygen consumption of the mice which were given tocopherol was significantly greater than the urethan-induced controls as shown in the Table.

Discussion. Our results indicate that tocopherol protects mitochondria against the depressing effects of urethan on respiration both in vitro and in vivo. Although free radical scavenging is a frequently invoked mechanism for protective action of α-tocopherol, our failure to detect significant differences in lipid peroxidation suggests that another property of the tocopherol is operating here. In view of the recent emphasis on the catalytic effect of tocopherol in the respiratory chain, we are inclined to think in these directions.

The relationship of these findings to urethan carcinogenesis is interesting. There is evidence that urethan must first be metabolized before it can act as a carcinogen[5]. However, from studies on mitochondrial biogenesis, it is known that anaerobiosis leads to altered cellular morphology including enlarged nuclei, enlarged mitochondria with fewer cristae and other changes similar, to cancerous cells[6]. Therefore we have some evidence that in urethan carcinogenesis, the first steps may be a respiratory impairment.

Our results also suggest that experimentation on urethan carcinogenesis in the presence of tocopherol be undertaken. Large scale experiments, along the lines of the i.p. injections may yield an answer to the problem of whether the dominant mechanism in urethan carcinogenesis is a result of the respiratory impairment. Lysergic acid diethylamide counteracts the anesthetic effect of urethan but does not affect the carcinogenecity[7]. The relation of vitamin E to anesthetic action remains to be investigated. Therefore these experiments would be of practical and theoretical importance[8].

Résumé. La dépression induite par l'uréthane sur la respiration des mitochondries a été mesurée en présence de la vitamine E (α-tocophérol). Ces agents furent utilisés in vitro et aussi injectés intrapéritonellement. Nos résultats montrent que l'apport de vitamine enraye la diminution de la respiration causée par l'uréthane. Ils attestent le rôle que joue l'uréthane dans la carcinogénèse et la necessité de poursuivre ces expériences sur une plus grande échelle.

A. E. SMITH, R. ROY, C. SCOTT and I. WAHEED

Departments of Physics and Biology, Sir George Williams University, Montreal 107 (P.Q., Canada), 5 December 1972.

Mitochondrial respiration (n*M* O_2/min/mg mito protein)

Injection	Run				Average S.D.
	1	2	3	4	
Urethan (0.5 mg/g body wt.)	19	21.8	26	19	21.5 ± 2.86
Urethan (0.5 mg/g body wt.) and α-tocopherol (1 IU/g body wt.)	31	31	31	34	31.8 ± 1.30

[5] K. WILLIAMS, W. KUNZ, K. PETERSEN and B. SCHNEIDERS, Z. Krebsforsch. *76*, 69 (1971).
[6] N. HOWELL, C. A. ZUICHES and K. D. MUNKRES, J. Cell Biol. *50*, 721 (1971).
[7] S. S. MIRVISH, Adv. Cancer Res. *11*, 1 (1968).
[8] This work was partly supported by the National Research Council (Canada).

Effect of Endotoxin and Dietary Phosphorus on the Proliferation of Liver Reticuloendothelial and Bone Marrow Cells[1]

Guinea-pigs fed diets containing high levels of phosphorus are more resistant to salmonellosis than controls[2]. We have recently observed that the RE clearance rate of ^{32}P-labeled, killed *Salmonella typhimurium* organisms is elevated in guinea-pigs 3 days after *Salmonella typhimurium* endotoxin administration[3]. The rate was significantly greater in guinea-pigs fed 1.0% than in those fed 0.4% of dietary phosphorus and clearance was effected primarily by the liver. KELLY et al.[4] also observed that endotoxin induced proliferation of liver reticuloendothelial (RE) cells 3 days after endotoxin challenge. This study was initiated to determine whether or not increased proliferation of liver RE cells accounts for the greater clearance rate observed in animals fed higher levels of phosphorus.

Materials and methods. 5-week-old female guinea-pigs were fed diets containing either 0.4% or 1.0% phosphorus[2] for 6 weeks then treated i.p. with *Salmonella typhimurium* endotoxin[3]. Three days after endotoxin treatment a pulse dose of ^{3}H-thymidine (specific activity, 20 Ci/mmole) was given i.p. (25 μCi/100 g body weight), and 24 h later the animals were anesthetized and heparinized. The livers were perfused in situ with a phosphate buffered (pH 7.4) glucose + KCl + NaCl solution[5]. The entire liver was homogenized in water, and the DNA extracted[6] and determined spectrophotometrically[7]. Radioactivity was determined[8] on the DNA extract. Femur bone

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 6468. Supported in part by NIH Postdoctoral Fellowship No. 1F2FR36 for R. L. Doak.
[2] R. L. DOAK, G. B. GARNER, P. F. HUEBNER and B. L. O'DELL, Proc. Soc. exp. Biol. Med., *141*, 953 (1972).
[3] R. L. DOAK, G. B. GARNER and B. L. O'DELL, Proc. Soc. exp. Biol. Med., submitted.
[4] L. S. KELLY, E. L. DOBSON, C. R. FINNEY and J. D. HIRSCH, Am. J. Physiol. *198*, 1134 (1960).
[5] J. S. GARVEY, Nature, Lond. *191*, 972 (1961).
[6] H. N. MUNRO and A. FLECK, in *Methods of Biochemical Analysis* (Ed. D. GLICK; John Wiley and Sons, New York 1966), vol. 14, p. 159.
[7] R. W. WANNEMACHER, W. L. BANKS and W. H. WUNNER, Analyt. Biochem. *11*, 320 (1965).

marrow was removed, homogenized in water, and processed in the same manner.

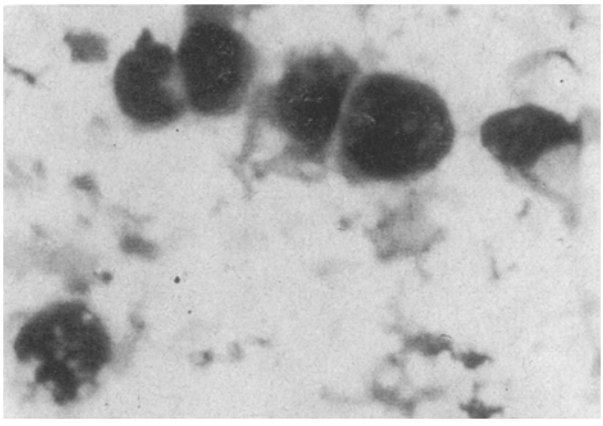
To determine the validity of an increase in specific activity of whole liver DNA as a measure of RE cell proliferation, 2 animals were treated with ^{3}H-thymidine (specific activity, 8 Ci/mmole) as above and the RE cells concentrated from the perfused livers by an enzymic digestion technique[9,10]. Two g of each liver were used to determine the specific activity of whole liver DNA. The remainder was treated[11] with collagenase and pronase to concentrate the RE cells prior to determination of their DNA specific activity.

Results and discussion. As shown in the Table, endotoxin administration significantly ($p < 0.05$) increased the rate of ^{3}H-thymidine incorporation into liver DNA. The specific activity increased approximately 50% regardless of dietary phosphorus level. Thus, endotoxin administration increased the rate of proliferation of RE cells but there was no interaction with the level of dietary phosphorus. Although an increased rate of cell proliferation may be a partial explanation for the elevated RE clearance rate which occurs in guinea-pigs 3 to 4 days after endotoxin challenge, it does not explain the earlier observation[3] that animals fed the higher phosphorus level, have a higher clearance rate.

That the radioactivity determined arose primarily from RE cells is shown by data obtained from the cell isolates. The cells concentrated enzymatically were predominately mononuclear cells as shown in the Figure; parenchymal cells made up only 15 to 20% of the isolate. The specific activity of DNA in this cell population after in vivo labeling with thymidine averaged 15.9 ± 2.6 while that of DNA from the same whole livers was 3.4 ± 0.8, a 5-fold difference. Thus, the increase in specific activity of whole liver DNA is a valid measure of RES proliferation. The radioactivity in mouse liver as evaluated by autoradiography after ^{3}H-thymidine administration also showed high specificity in the labelling of RE cells[12]. Few parenchymal cells were labeled before or after endotoxin administration.

As shown in the Table, endotoxin decreased significantly ($P < 0.05$) the specific activity of bone marrow DNA in the guinea-pigs fed 1.0% phosphorus. Thus, there was an interaction of endotoxin treatment and high phosphorus to depress the rate of incorporation of ^{3}H-thymidine into bone marrow cell DNA. Endotoxin has been shown to induce leukocytosis[13] by increased release of mature cells from bone marrow[14,15] and greater proliferation of bone marrow stem cells[16,17]. The leukocyte count is greater both prior to and after *Salmonella typhimurium* infection in guinea-pigs fed the higher level of phosphorus[2]. Release and/or proliferation of bone marrow cells may be greater in high phosphorus animals. If so, the reduction in specific activity could be explained by a greater release of a highly labeled cell population, or a dilution of the amount of ^{3}H-thymidine available to each cell in the larger stem cell population.

Mechanisms other than RES proliferation must be invoked to explain the larger clearance rate in guinea-pigs fed 1.0% phosphorus. For example, endotoxin is known to increase RES clearance rates in mice by the activation of the preexisting liver RE cells[12] and to increase the adsorption activity in preexisting mouse peritoneal macrophages[18]. Endotoxin also enhances serum opsonin levels as reflected by the increased intravascular clearance of ^{32}P-labeled *Salmonella typhimurium* and carbon pretreated with serum from endotoxin-treated animals[19].



Photomicrograph of 6 mononuclear cells typically observed in RE cell isolates prepared enzymatically from guinea-pig livers. Nuclei were dark purple, and some were irregular in shape. Cytoplasm was abundant in some and scant in other cells. Vacuoles in the cytoplasm were fairly common. The amorphous stained background material is guinea-pig plasma used to preserve morphology. Wright-Giemsa stain. × 800.

Incorporation of ^{3}H-thymidine into liver and bone marrow DNA after endotoxin treatment[a]

Dietary phosphorus (%)	Endotoxin treatment	Specific activity of DNA Liver (dpm/μg)	Bone marrow (dpm/μg)
0.4	− (10)[b]	17.0 ± 1.9	215.4 ± 20.3
	+ (13)	25.4[c] ± 4.0	222.6 ± 15.7
1.0	− (9)	16.1 ± 1.2	239.3 ± 9.6
	+ (12)	24.9[c] ± 4.0	171.4[d] ± 18.8

[a] 25 μCi ^{3}H-T/100 g body weight injected i.p. 24 h before sacrifice and 3 days after endotoxin treatment. [b] Number of animals. [c] Two-way analysis of variance of the liver data shows a significant difference ($p < 0.05$) due to endotoxin irrespective of the dietary phosphorus level. [d] There was a significant ($p < 0.05$) interaction between phosphorus level and endotoxin treatment, and a significantly ($p < 0.05$) lower S.A. of DNA in bone marrow of endotoxin-treated animals fed high phosphorus.

[8] R. Tye and J. D. Engel, Analyt. Chem. *37*, 1225 (1965).

[9] J. C. Pisano, J. P. Filkins and N. R. DiLuzio, Proc. Soc. exp. Biol. Med. *128*, 917 (1968).

[10] B. Roser, J. reticuloendothel. Soc. *5*, 455 (1968).

[11] Collangease, Type I and Pronase, Type VI were purchased from Sigma Chemical Co. St. Louis, Mo. Collagenase was prepared from *Clostridium histolyticum* and contained some protease and peptidase activity. Pronase is a proprietary name for a non-specific protease isolated from *Streptomyces griseus*.

[12] L. S. Kelly and E. L. Dobson, Br. J. exp. Path. *52*, 88 (1971).

[13] P. A. Chervenick, D. R. Boggs, J. C. March, G. E. Cartwright and M. M. Wintrobe, Proc. Soc. exp. Biol. Med. *126*, 891 (1967).

[14] J. W. Athens, O. P. Haab, S. O. Raab, A. M. Mauer, H. Ashenbrucker, G. E. Cartwright and M. M. Wintrobe, J. clin. Invest. *40*, 989 (1961).

[15] G. J. Fruhman, Life Sci. *3*, 737 (1964).

[16] P. A. Chervenick and D. R. Boggs, J. reticuloendothelial Soc. *9*, 288 (1971).

[17] G. E. Hanks and E. J. Ainsworth, Radiat. Res. *32*, 367 (1964).

[18] I. R. Tizard, Infec. Immunity *3*, 472 (1971).

[19] C. R. Jenkin and D. Rowley, J. exp. Med. *114*, 363 (1961).

Resumen. El grado mayor de proliferación de las RE células después del tratamiento de endotoxina no fué afectado por la cantidad de fósforo del régimen de alimentación pero 1.0% del fósforo abatió el thymidine incorporado dentro del DNA de la médula órea.

R. L. DOAK[20] and B. L. O'DELL

Department of Agricultural Chemistry, University of Missouri, Columbia Missouri 65201, (USA), 11 October 1973.

[20] Department of Veterinary Microbiology and Pathology, Purdue University, Lafayette (Indiana 47907, USA).

Splenic Cellular Responses in vitro After Tumour-Immunization

Some committed small lymphocytes continue their immunological role, with rapid transformation to proliferating cells in the event of re-exposure to the initially sensitizing antigen[1]. Lymphocytes may be stimulated mitotically or transformed by phytohemaggutinin (PHA)[2]. Although the full significance of such stimulation is not known, the reactivity of lymphocytes to PHA has been used as one of the parameters for evaluating the immune status of individuals carrying or immunized to tumours[3-5]. We have described an induction of active anti-tumour immunity to a transplantable methylcholanthrene induced sarcoma with Sendai virus-fused cells in A/Jax mice[6]. In the studies reported here we examined cellular response of spleen lymphocytes from immunized mice with and without addition of PHA in cultures.

Materials and methods. Spleens were removed from mice and physically dissociated to produce a suspension of single cells. Cells were grown in Eagle's minimum essential medium with 5% heat inactivated normal rabbit serum and 1% glutamine. Antibiotics were added to the medium. PHA-P (Difco Laboratories, Detroit, Michigan) was added to some cultures at dilutions of 1:25 and 1:250 from the stock. Triplicate cultures of 2×10^6 cells (2 ml) were prepared for each group in glass tubes with loosely fitted caps. ^{3}H-thymidine was purchased from the New England Nuclear Corporation, Boston, Mass., with a specificity of 6.7 C/n*M* in sterile deionized water. The cells were incubated at 37°C in a humified atmosphere of 5% CO_2 in air.

The incorporation of tritiated thymidine by the cells in culture was estimated according to the method described by DENT[7]. The results were calculated as counts per min (cpm) per culture.

Results and discussion. Splenic cell preparations were studied from 5 immune mice and 5 normal control mice of comparable age. The immune mice had been given 3 separate fused-cell immunizing doses and were resistant to at least one tumour challenge[6]. The results are given in the Table. There was good agreement between the individual radioactivity levels within triplicates, of the order of 5–10%. However, a wide range of variations in the radioactivity was found in individuals within each group, especially among those from immune mice. The data was therefore transformed to logs to stabilize the variance and achieve additivity. As a result of this transformation, the standard deviation varies directly as the mean.

The control spleen cell culture, without adding PHA, showed a low level of radioactivity. By contrast, immune spleen cells exhibited a high rise in radioactivity, this being 23 fold greater than the control (the difference is significant at 1% level). These observations indicate that a high degree of spontaneous DNA synthesis of lymphocytes occurred in the immune mice. This is compatible with an ongoing immune response in the immunized animals in which viable tumour cells were injected 15 days

[1] J. L. GOWANS, Ann. N. Y. Acad. Sci. *99*, 432 (1962).
[2] G. MÖLLER and E. MÖLLER, in *Antibodies to Biologically Active Molecules* (Ed. B. CINADER; Pergamon Press, Toronto 1967), p. 349.
[3] P. C. NOWELL, Cancer Res. *20*, 462 (1960).
[4] D. B. GARRIOCH, R. A. GOOD and R. A. GATTI, Lancet *1*, 618 (1970).
[5] J. STJERNSWARD, P. CLIFFORD and E. SVEDMYR, in *Burkitt's Lymphoma* (Eds. D. BURKITT and D. WRIGHT; Livingston Press, Edinburgh and London 1970), p. 164.
[6] S. K. LIAO and D. H. CARR, Experientia *29*, 597 (1973).
[7] P. B. DENT, J. natn. Cancer Inst. *46*, 763 (1971).

Tritiated thymidine incorporation by normal and immune A/Jax mouse spleen cells with or without PHA stimulation in culture

Experimental group	^{3}H-thymidine incorporation (mean cpm/culture ± S.E.			Ratio of incorporation	
	Unstimulated	PHA stimulated		PHA 1:25 stimulated/ unstimulated	PHA 1:250 stimulated/ unstimulated
		1:25	1:250		
Control	95 ± 17 (2.45 ± 0.22)[a]	4,933 ± 706 (3.74 ± 0.07)	1,932 ± 261 (3.46 ± 0.08)	51.9	20.3
Immune	2,211 ± 1,548 (2.95 ± 0.28)	7,248 ± 1,877 (3.80 ± 0.72)	4,884 ± 896 (3.65 ± 0.10)	3.3	2.2
Ratio of incorporation (immune/control)	23.3	1.3	2.5		
Difference (*t*-value)	3.45[b]	0.95	3.31[c]		

In these experiments, 2×10^6 spleen cells were cultured in 2 ml of medium with or without PHA for 48 h of culture. 2 μc ^{3}H thymidine in 0.2 ml was added for the final 5 h of culture. Results are expressed as the mean counts per min (cpm) of 5 animals in each group having triplicate samples for each spleen in culture. The triplicates in each spleen culture have less than 10% variation in counts. [a] Figures in parenthesis indicate mean cpm/culture ± S.E. after $\log_{10}$ (X) transformation. [b] Significant at 1% level; [c] Significant at 5% level.