fragments was able to form the typical "crescent" which is a degenerating form of normal mitochondria (*6*). Those observed in 0.6*M* sucrose retained normal morphology for a longer period. The time for the complete process varied from 15 to 35 minutes. The actual division took about 8 minutes.

In general it was noted that there was a gradation of response to the unnatural *in vitro* conditions which appeared to be correlated with the size of the mitochondria. The larger mitochondria formed crescents of a large size in extremely short intervals, whereas the smallest freshly isolated ones took somewhere between 5 and 20 hours to form typical but diminutive crescents. It would appear therefore that this group of small mitochondria were newly formed by unequal division of larger mitochondria and had not yet elaborated intramitochondrial protein.

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References and Notes

1. O. Lindberg and L. Ernster, *Protoplasma* III, A4, 114 (1954).
2. B. Ephrussi, *Nucleo-cytoplasmic Relationships in Micro-organisms.* (Clarendon, Oxford, 1953).
3. E. Faure-Fremiet, *Anat. Anz.* 36, 186 (1910).
4. This work was carried out under grant No. C-2303 from the U.S. Public Health Service.
5. P. E. Kennedy and A. L. Lehnenger, *J. Biol. Chem.* 179, 957 (1949).
6. J. W. Harrnan *et al.*, *Exptl. Cell Research* 3, 509 (1952).

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Dual Requirement of Walker Carcinosarcoma 256 in vitro for Asparagine and Glutamine

A routine procedure for the cultivation of the Walker carcinosarcoma 256 in tissue culture recently was developed in this laboratory (*1*). The technique involved the use of cell suspensions, which were prepared from the freshly excised tumor, as the initial inoculum. The medium consisted of 5-percent rat or horse serum, an amino acid and vitamin mixture, and 0.05-percent Bacto yeast extract with Earle's balanced salt solution (*2*) as the diluting fluid. Both serum and yeast extract were essential for satisfactory growth. The cells adhered to the surface of T-15 flasks and increased several fold within 72 to 96 hours.

Although the medium was adequate for good growth of the Walker tumor, the available amino acids and other factors in the serum and yeast extract precluded the determination of specific nutritive requirements of the Walker tumor *in vitro*. Thus, further simplification of the medium was desirable. The present report describes a simplified medium that permits the study of essential components for the Walker tumor. Furthermore, a dual requirement of the tumor for asparagine and glutamine is demonstrated.

Initial studies consisted of observing the growth-promoting activity of yeast extract after it had undergone treatments or fractionation. The activity was lost upon autoclaving in 2*N* HCl for 1 hour at 15 lb pressure but was retained upon autoclaving in a simple aqueous solution. Decolorizing a solution of yeast extract with Nuchar would not remove the activity, but the active portion was adsorbed on columns of IRA 400 (hydroxyl form) or on Dowex 50 (hydrogen form). Thus, the growth factors appeared to be amphoteric in nature and labile under acid conditions of hydrolysis.

An active fraction was isolated from the Dowex-50 column by elution with 1.5*N* HCl in a chromatographic procedure similar to that of Stein and Moore (*3*). This fraction was found to contain asparagine, by chromatography on paper buffered at *p*H 6.2 according to the procedure of McFarren (*4*). Upon replacement of yeast extract with 0.05–10m*M* L-asparagine (5), the cells proliferated as rapidly in 72-hour test periods as they had previously on whole-yeast extract. Without L-asparagine or yeast extract, the cells did not survive. D-Asparagine (*5*), was inactive, although it did not inhibit growth appreciably when it was added at 20m*M* in the presence of 1.5m*M* L-asparagine.

Following this work, the use of dialyzed serum was studied. Human serum was dialyzed for 24 hours at 4°C in a slow-moving shaker against 100 vol of Earle's solution. This preparation adequately replaced the requirement of whole serum, whether rat, horse, or human. The vitamin complement of the medium was altered to insure fortification for prolonged growth. The composition of this new simplified medium (herein referred to as medium 2) is shown in Table 1. Medium 2 was found to support subcultures indefinitely.

The biological and chemical similarity of glutamine to asparagine, as well as the essentiality of glutamine to other mammalian cells in tissue culture (*6, 7*), prompted the investigation of the glutamine in addition to the asparagine requirement of the Walker-256 cells. The cultures were established as was previously described (*1*) and grown for 48 hours in 2 ml of medium, which was then replaced by 3 ml of fresh medium. Growth was determined by whole-cell counts in a hemocytometer with an overall accuracy of ± 10 percent.

The response of the Walker-tumor cells to aspartic acid and glutamic acid and their amides is shown in Table 2. One can observe the nonessential nature of L-aspartic acid and L-glutamic acid to the Walker cells. L-Aspartic acid in higher concentrations did not appear to be exceptionally toxic. However, L-glutamic acid exhibited a striking toxicity to the cells in concentrations above 2m*M*. This is in contrast with the nontoxicity of L-glutamic acid reported for other mammalian cells under tissue-culture conditions (*7*).

Evidently, the Walker-256 cells have a dual requirement for asparagine and glutamine, since the cells failed to become established and died rapidly upon omission of either compound from the medium. It was also interesting to note that the requirement for glutamine was several times as great as the requirement

Table 1. Composition of medium 2.

Component	Concentration of final medium (μg/ml)	Percent
DL-Tryptophan	6.1	
DL-Phenylalanine	24.8	
L-Tyrosine	8.2	
L-Arginine · HCl	15.8	
L-Histidine · HCl · H_2O	6.3	
L-Lysine · HCl	27.4	
L-Cysteine · HCl · H_2O	7.9	
DL-Methionine	13.4	
L-Isoleucine	18.8	
L-Leucine	18.8	
DL-Valine	35.2	
DL-Threonine	35.7	
Glycine	11.3	
DL-Alanine	27.7	
L-Proline	16.8	
Hydroxy-L-proline	18.1	
DL-Serine	31.8	
L-Aspartic acid	20.0	
L-Glutamic acid	22.1	
L-Glutamine	219.2	
L-Asparagine · H_2O	30.0	
Thiamine · HCl	0.2	
Riboflavin	0.2	
Pyridoxine · HCl	0.5	
Pyridoxal · HCl	0.5	
Nicotinic acid	0.5	
Nicotinamide	0.5	
Ca pantethenate	0.2	
Biotin	0.2	
Folic acid	0.2	
Choline chloride	5.0	
Inositol	1.0	
PABA	1.0	
Ascorbic acid	0.5	
Glutathione	0.5	
B_{12}	7.5×10^{-5}	
Na penicillin G	50.0	
Streptomycin sulfate	50.0	
Phenol red	2.5	
NaCl		0.65
KCl		0.04
$MgSO_4 \cdot 7H_2O$		0.02
$NaH_2PO_4 \cdot H_2O$		0.014
$NaHCO_3$		0.22
Glucose		0.30
Dialyzed human serum		5.0

Table 2. The response of Walker carcinosarcoma 256 to aspartic acid, glutamic acid, and their amides.*

Concentration in final medium	Response (cells × 10^{-3}/ml)†			
	L-Aspartic acid	L-Glutamic acid	L-Asparagine	L-Glutamine
(m*M*)				
0	626	538	25	18
0.01			56	
0.02			241	
0.05	572	518	996	
0.1	696	522	1013	80
0.2	476	480	1032	212
0.5			1038	632
1.0	603	396	990	976
1.5				830
2	664	351	879	905
3				659
5	559	128	828	504
10	667	53	779	376
20	464	20	638	312

* Cells were grown 72 hours in basal medium described containing the single amino acid or amide in the concentration indicated. Medium was renewed at 48 hours. The initial inoculum was 200,000 cells per milliliter.
† Corrected to compare with initial volume.

for asparagine. The optimal concentration of L-glutamine appeared to be approximately 1.5m*M*, while the optimal concentration of L-asparagine was approximately 0.2 m*M*. These observations were confirmed using cells that had become established in the complete medium and were then exposed to a deficient medium. When the concentration of L-glutamine or L-asparagine was in excess of 10m*M*, a moderate inhibiting effect was observed.

In attempts to replace the asparagine and glutamine requirement, neither L-aspartic acid nor L-glutamic acid, with or without ammonium chloride and ATP, were able to replace the requirement for its corresponding amide. Massive quantities of glutamine were incapable of replacing the requirement for asparagine, nor could asparagine replace the requirement for glutamine. The specific requirement for both asparagine and glutamine by the Walker tumor is particularly interesting, since we have been unable to find evidence of a living system, mammalian or microbiological, which requires both of these amides and exhibits no requirement for the corresponding amino acids (*8*).

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References and Notes

1. T. A. McCoy and R. E. Neuman, *J. Natl. Cancer Inst.* 16, 1221 (1956).
2. R. C. Parker, *Methods in Tissue Culture* (Hoeber, New York, ed. 2, 1950), p. 80.
3. W. H. Stein and S. Moore, *Cold Spring Harbor Symposia Quant. Biol.* 14, 179 (1949).
4. E. F. McFarren, *Anal. Chem.* 23, 168 (1951).
5. H. M. Chemical Co., Santa Monica, Calif.
6. H. Eagle, *Science* 122, 501 (1955).
7. ——— *et al., J. Biol. Chem.* 218, 607 (1956).
8. We gratefully acknowledge the technical assistance of Jane Avery, Merle D. Maxwell, and Eugene Conway.

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Induced Biosynthesis of Uricase in Yeast

Uric acid is assimilated by *Torulopsis utilis* (*1*), and the presence of uricase in adenine-adapted yeast has been noted (*2*). This report (*3*) deals with the uptake of uric acid by *T. utilis* cells and the subsequent increase in the uricase activity of cellular extracts.

Torulopsis utilis (ATCC 9950) was grown with aeration in the medium described previously (*2*); the cells were collected by centrifugation, washed with distilled water, and stored at 4°C until used. To increase the uricase content of the yeast, 1 g (wet wt.) of the yeast was aerated in 100 ml of medium of the same composition except that the nitrogen source was 0.1 mg/ml of uric acid. The decreasing uric acid content of the medium was followed by measurement of the optical density of a 1/10 dilution of the medium at 293 mμ after removal of the cells by centrifugation. To estimate the intracellular uric acid content, the sedimented yeast cells were suspended in 10 ml of distilled water and heated in a boiling-water bath for 10 minutes; the optical density of the extract that was obtained upon centrifugation was measured at 293 mμ. Uricase activities were measured by the method of Kalckar (*4*) on yeast extracts in *p*H 9.5 borate buffer obtained with the aid of a Hughes press (*5*) operated at dry-ice temperature. Protein was determined by the method of Lowry *et al.* (*6*) with bovine plasma albumin as the standard.

Results of typical experiments are illustrated in Fig. 1. There is a time lag of about 1 hour before the uric acid starts to disappear from the medium at a high rate (curve *A*). The disappearance of the uric acid from the medium is accompanied by the intracellular accumulation of uric acid (curve *B*); analyses of the cellular extracts with uricase show that the absorbing compound is uric acid. An estimate based on the cell count, cell volume, optical density of the medium, and optical density of the cellular extract indicated that upon completion of the removal of uric acid from the medium, the concentration of uric acid within the yeast cell is about 600 times the original concentration in the medium. The specific activity of the yeast uricase increases only after the rapid accumulation of uric acid by the yeast cell starts (curve *C*). The lag period prior to the rapid uptake of uric acid is abolished by aeration of the yeast in the medium for 2 hours prior to the addition of uric acid, the nitrogen source (curve *A'*). There is no uptake of uric acid in the absence of glucose, and anaerobic conditions do not prevent the uptake with glucose in the medium.

The effect of various inhibitors on the uptake of uric acid by the yeast cell was investigated. Potassium cyanide (0.038*M*), an inhibitor of uricase (*7*), completely stopped the uptake under all conditions. Sodium arsenate ($10^{-3}M$) inhibited the uptake if it was present during a 2-hour aeration period prior to the addition of uric acid but not if it was added with the uric acid after preaeration; if the yeast cells that have been exposed to arsenate for 2 hours are removed from the medium by centrifugation, washed, and resuspended in fresh medium without the inhibitor, uptake occurs upon the addition of uric acid. The inhibition of uptake by $10^{-3}M$ arsenate is prevented if 0.02*M* phosphate is present with the arsenate during the experiment.

A consideration of these results has led to the following tentative conclusions and working hypotheses. (i) In the system described, when there was a lag period in the induced synthesis of uricase, the lag is dependent on the penetration of the cell wall by the substrate. (ii) The accumulation of uric acid by the cell involves active transport (*8*). (iii) Glucose metabolism, accompanied by the

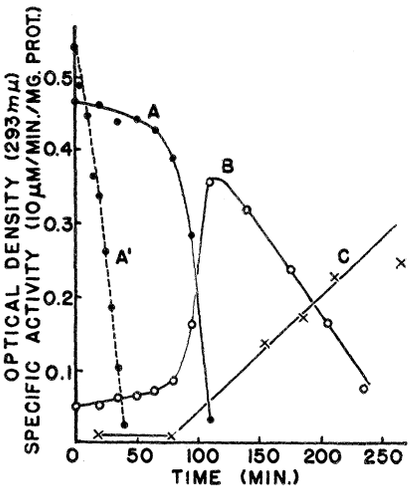

Fig. 1. The intracellular accumulation of uric acid by *T. utilis* and the resulting formation of uricase. (*A*) Optical density of the uric acid medium, yeast added at zero time; (*B*) optical density of the corresponding extracts of boiled yeast; (*C*) specific activity (uricase) of the corresponding extracts of frozen yeast; (*A'*) optical density of the uric acid medium, yeast aerated for 2 hours prior to the addition of uric acid to the medium at zero time.