vations here are based on measurements of hundreds of individual cells in smears obtained from five patients. Four of these patients had histologically proven epidermoid carcinoma. The fifth patient was a normal premenopausal female whose cells were used for control and comparison purposes.

The smears were photographed at different wavelengths, with apparatus which included a Bausch and Lomb mercury light source and monochromator set to a sufficiently narrow bandwidth to isolate the 2536 Å, 2652 Å, 2803 Å, and 2967 Å mercury lines at the exit slit. A Carl Zeiss ultrafluar 100/1.25 glycerine immersion objective was used, an achromatic 0.85 NA condenser, a quartz 10× eyepiece, and a Polaroid Land camera attachment with a viewing eyepiece. Polaroid type-32 film was used at exposures ranging between 0.1 and 5 seconds.

The absorption profiles of cells were obtained by modifying the photographic equipment. The mechanical stage of the microscope was arranged to drive the slide electrically through the center of the field at the rate of 2 μ per second. An aperture and a 1P28 photomultiplier were substituted for the camera. The effective aperture size at the slide was less than 0.5 μ. The photomultiplier signal was amplified by a d-c amplifier with a bandwidth of 10 cy/sec, and the signal was recorded as a function of time on a Moseley chart recorder. The signals were normalized at each wavelength by manual adjustment of the amplifier gain while an area without cells was being scanned.

The smears were observed under visible light, and specific cells were selected to be photographed and scanned at different wavelengths. A parfocal viewing eyepiece and the achromatism of the optics enabled focus to be maintained at ultraviolet wavelengths. A scan profile line was established by correlating the aperture and the mechanical stage movement with an engraved disk in the viewing eyepiece. The reproducibility of the profiles was verified by scanning at each wavelength in succession and then repeating the measurements at all wavelengths.

Figure 1 shows photographs of typical mature squamous cells, and Fig. 2 shows the scan profile of one of these cells at wavelengths 5460 Å, 2967 Å, and 2652 Å. The nuclear boundary of these cells can be distinctly discerned at all wavelengths because of the small amount of cytoplasmic absorption.

The most striking characteristic of some of the cancer cells shown in Figs. 3, 4, and 5 is the marked absorption of the nucleus and the cytoplasm at 2652 Å due to the great amount of DNA and RNA in these cells. This often results in the disappearance of the nuclear boundary in such cells at this wavelength. The boundary is clearly present in all photographs at wavelengths above 2967 Å. In other cancer cells shown in Figs. 3 to 5, there is increased absorption of the nucleic acids but the delineation of nucleus from cytoplasm is preserved. Differences in the absorption profiles among 2652 Å and 2967 Å and visible light are shown in Figs. 6 to 8 for certain cells indicated in the figures.

Especially in Fig. 3, the absorption due to cytoplasmic RNA in some of the cancer cells is as intense as that of the nucleus, and it is not possible to distinguish the cytoplasm from the nucleus in the photograph taken at 2652 Å. It must be pointed out that in some of the other cancer cells, also present in this field, this absorption phenomenon is not nearly as complete as it is in cells *D* and *E* of Fig. 3. The inference suggested by this observation is that the cytoplasmic absorption may vary inversely with the degree of differentiation of the cancer cells. Cell *A* of Fig. 3 is an example of a well-differentiated cancer cell. In such cells the nuclear boundary is not obliterated at 2652 Å.

We believe that, with an electronic scanning system in which two wavelengths are used, cells such as *D* and *E* of Fig. 3 can be readily identified among other cells. While we have observed these cells in the four cancer cases studied, it remains to be seen by further efforts whether every epidermoid cancer will contain such cells. Special concern will be needed for some of the precancerous states in which the abundance of RNA may not equal that in cells *D* and *E* of Fig. 3, and might produce a pattern approaching that of cell *A*. It further remains to be seen to what degree actively growing or metabolizing benign cells with increased cytoplasmic proteins will exhibit the total absorption phenomenon (*7*).

LOUIS A. KAMENTSKY
I.B.M. Watson Research Center, Yorktown Heights, New York
HERBERT DERMAN
City of Kingston Laboratory, Kingston, New York
MYRON R. MELAMED
Memorial Hospital for Cancer and Allied Diseases, New York, New York

References and Notes

1. G. N. Papanicolaou, *Atlas of Exfoliative Cytology* (Harvard Univ. Press, Cambridge, 1954); L. G. Koss, *Diagnostic Cytology and Its Histopathologic Bases* (Lippincott, Philadelphia, 1961).
2. D. A. Boyes, H. K. Fidler, D. R. Lock, *Brit. Med. J.* **1**, 203 (1962).
3. E. G. Diacumakos, E. Day, M. J. Kopac, *Ann. Acad. Sci.* **97**, 498 (1962).
4. R. C. Mellors and R. Silver, *Science* **114**, 356 (1951).
5. T. Caspersson, *Cell Growth and Cell Function, a Cytochemical Study* (Norton, New York, 1950).
6. R. C. Mellors, J. F. Keane, Jr., G. N. Papanicolaou, *Science* **116**, 265 (1952).
7. We thank Leopold G. Koss, of the Memorial Center for Cancer and Allied Diseases, and Robert C. Mellors, of the Hospital for Special Surgery.

5 November 1963

Carbamyl Phosphate Synthesis in the Earthworm *Lumbricus terrestris*

Abstract. *The enzymatic synthesis of citrulline from ammonia, bicarbonate, adenosine triphosphate, and* L-*ornithine takes place in the soluble fraction of gut tissue of the earthworm. The synthesis occurs at low ammonia concentrations, is dependent upon added* N-*acetyl-*L-*glutamate, and results in incorporation of the bicarbonate carbon into the ureido group of the citrulline molecule. Carbamyl phosphate is the intermediate in the reaction and its biosynthesis in the earthworm is mediated by a carbamyl phosphate synthetase system similar to that of ureotelic vertebrates.*

The key role of carbamyl phosphate in the *de novo* biosynthesis of arginine and uridylic acid has previously been discussed and the importance of the comparative biochemistry of this compound has been emphasized (*1–3*). Three distinct enzyme systems differing in mechanism of action are known for the synthesis of carbamyl phosphate. These are the carbamate kinase of bacteria, the carbamyl phosphate synthetase found in the fungus *Agaricus bisporus*, and the carbamyl phosphate synthetase of ureotelic vertebrates. The vertebrate system has been of interest in relation to biochemical evolution because of its role in ammonia detoxification by way of the urea cycle. The aquisition of this detoxification mechanism during evolution has been implicated as an important factor in the exploitation of the land habitat by ancestral vertebrates (*1, 2*). In the animal kingdom, studies on the synthesis and metabolism of carbamyl phosphate and the distribution of the participating enzymes have been mainly restricted to vertebrates (*1*). The utiliza-

tion in vitro of carbamyl phosphate by invertebrate tissue extracts for citrulline synthesis was first shown in the land snail *Otala lactea* (*4*), and it is now known to occur in several other invertebrate species (*5, 6*). The synthesis in vitro of carbamyl phosphate had not been previously attained with invertebrates although indirect evidence suggested that it was synthesized in vivo (*6*).

In this report, we show that the biosynthesis of carbamyl phosphate in gut tissue of the common earthworm is mediated by a carbamyl phosphate synthetase similar to that of ureotelic vertebrates. Thus, animal carbamyl phosphate synthetase is distributed among invertebrates as well as vertebrates. The occurrence of carbamyl phosphate synthetase and other urea-cycle enzymes (*6*) in the gut tissue of the earthworm may represent an early stage in the evolutionary appearance of enzymes of the urea cycle in intestinal cells prior to their association with a hepatic diverticulum and subsequent localization in the liver of ureotelic vertebrates. In mammals, the only extrahepatic tissue showing appreciable synthetase activity is intestinal mucosa (*7*). Because of the ontogenetic origin of the liver from the primitive gut, this intestinal synthetase activity in mammals may thus represent a vestigial condition.

Carbamyl phosphate synthesis was measured indirectly as either citrulline or carbamyl aspartate formation when earthworm gut tissue extracts were incubated with adenosine triphosphate (ATP), NH_4^+, and HCO_3^- in the presence of excess ornithine transcarbamylase and L-ornithine or aspartate transcarbamylase and L-aspartic acid. The reaction mixture is described in Table 1. Under the conditions of this assay system, citrulline or carbamyl aspartate synthesis was linear for 30 minutes with low concentrations of enzyme. Because of endogenous ammonia in the supplementary enzymes, the actual NH_4^+ concentration was slightly higher than 0.01*M*. Maximum synthesis was obtained, however, at or below 0.015*M* NH_4^+. Omission of ATP, $MnSO_4$, *N*-acetyl-L-glutamate, or L-ornithine and L-aspartic acid greatly diminished or abolished the synthetase activity. When Mg^{+2} was used as the divalent ion, 25 percent of the activity given by Mn^{+2} was obtained; Co^{+2} would not replace Mn^{+2}. Omission of the ATP-generating system decreased the synthesis by 60 percent. Omission of either transcarbamylase decreased, but did not abolish, the synthesis. This effect was greatest in carbamyl aspartate formation. The ratio of endogenous ornithine transcarbamylase activity (*6*) to aspartate transcarbamylase activity is approximately 100 : 1 in the gut tissue of the earthworm. The addition of 2.5 μmole of $ZnSO_4$ to the reaction mixture caused a 75 percent inhibition of the reaction. No stimulation of either citrulline or carbamyl aspartate formation was obtained when L-glutamine was included in the assay. The optimum *p*H for the reaction was around 8.0 in either imidazole or glycylglycine buffers. With this method of assay, the net synthesis of citrulline was from 2.5 to 5.0 μmole/g of tissue per hour, or approximately 0.05 μmole/mg of soluble protein per hour. Equivalent results were found for carbamyl aspartate synthesis.

Because of the low activity, a clearer definition of the requirements for citrulline synthesis could be obtained by studying the incorporation of bicarbonate-C^{14} into the citrulline synthesized. These data are presented in Table 2. By means of the assay system described in Table 2, citrulline or carbamyl aspartate was identified as the product of the reaction as follows. After incubating the complete reaction mixture, four volumes of ethanol were added to terminate the reaction. Ten μmole of carrier citrulline or carbamyl aspartate were then added and the solutions were prepared for chromatography (*8*). The extracts containing the carbamyl compounds were streaked on Whatman No. 3MM paper and developed twice with a mixture of 2-butanol, 88 percent formic acid, and water (75 : 15 : 10, by volume). Radioautographs were made of the chromatograms to detect the radioactive citrulline or carbamyl aspartate. These compounds were positively identified by comparing the exposure on the radioautographs with the color reaction given by *p*-dimethylaminobenzaldehyde on the chromatograms. The compounds were then eluted from the paper, plated on aluminum planchets, and counted in a gas-flow counter. In order to show bicarbonate-C^{14} incorporation into the ureido carbon of the citrulline, the citrulline was eluted from the chromatograms and arsenolyzed (*6*). The ureido carbon, as CO_2, was trapped in NaOH and precipitated; the precipitate

Table 1. Intracellular localization of carbamyl phosphate synthesis in gut tissue of the earthworm. The assay system contained, in μmoles per ml: $NaHCO_3$, 10; $(NH_4)_2SO_4$, 5; $MnSO_4$, 10; ATP, 5; N-acetyl-L-glutamate, 10; 3-phosphoglycerate, 10; NAD, 1; L-aspartic acid, 10; and imidazole-HCl buffer, *p*H 7.9, 50. Supplementary enzymes included 20 units of aspartate transcarbamylase from *Escherichia coli* and 0.3 mg of a fraction from rabbit muscle containing 3-phosphoglycerate kinase as an ATP-generating system. For the measurement of citrulline formation, L-ornithine was used in place of L-aspartic acid and the transcarbamylase was rat-liver ornithine transcarbamylase. The enzyme from the earthworm was then added and the mixture was incubated at 28°C. The reaction was terminated with 1 ml of 1*M* $HClO_4$ and the carbamyl aspartate or citrulline formed was determined colorimetrically (*8*). Controls consisted of a complete reaction mixture deproteinized at the start and a complete system containing boiled enzyme. The tissues were homogenized and fractionated in a solution of 0.4*M* mannitol, 0.1*M* K_2SO_4, and 0.015*M* potassium glycylglycinate, *p*H 7.5, and centrifuged at 0° C for 8 to 10 minutes. The intestine posterior to the gizzard was used. A unit of activity represents μmoles product per hour.

Cell fraction	Total units carbamyl phosphate synthesis as:	
	Carbamyl aspartate	Citrulline
Homogenate (25 percent wt/vol)	4.92	5.08
600*g* residue	0	*
600*g* supernatant	4.90	*
15,000*g* residue	0.02	*
15,000*g* supernatant	3.78	3.78

* Not determined.

Table 2. The formation of C^{14}-citrulline from $NaHC^{14}O_3$ by gut tissue extracts of the earthworm. The complete assay system contained, in micromoles per 5 ml: $NaHC^{14}O_3$ (~100,000 count/min per μmole), 30; $(NH_4)_2SO_4$, 20; $MnSO_4$, 50; imidazole-HCl buffer, *p*H 7.9, 500; L-ornithine, 70; *N*-acetyl-L-glutamate, 50; ATP, 25; 3-phosphoglycerate, 30; NAD, 5; K_2HPO_4, 0.5. Supplementary enzymes were 0.9 mg of rabbit muscle extract and 250 units of rat liver ornithine transcarbamylase. The earthworm enzyme source was 1 ml of a 15,000*g* supernatant from a 25 percent tissue homogenate sedimenting at 15,000*g* in a mixture of 0.4*M* mannitol, 0.1*M* K_2SO_4, and 0.015*M* potassium glycylglycinate, *p*H 7.5. Citrulline was isolated from the reaction mixture by paper chromatography; it was eluted from the chromatogram, plated, and counted in a gas-flow counter.

Assay modifications	Incubation time (min)	Total radioactivity in citrulline (count/min)
None	0	0
None	15	18,250
None	30	38,200
None (boiled enzyme)	30	97
Minus ATP	30	108
Minus *N*-acetyl-L-glutamate	30	795
Minus $MnSO_4$	30	1265
Minus $MnSO_4$, plus $MgSO_4$	30	2330

was counted for radioactivity as $BaCO_3$ by standard methods. In experiments in which this arsenolysis method was used, results equivalent to those presented in Table 2 were obtained.

The limitation of both citrulline and carbamyl aspartate synthesis by the same reaction and the incorporation of bicarbonate carbon into the ureido carbon of the citrulline molecule strongly suggests that carbamyl phosphate is the intermediate in carbamylation reactions in the earthworm (*1*). The requirement of *N*-acetyl-L-glutamate and the operation of the reaction at low ammonia concentrations are characteristic of the carbamyl phosphate synthetase system found in mammals and amphibians. The failure of L-glutamine to stimulate citrulline synthesis distinguishes this system from that in certain fungi (*9*). Thus carbamyl phosphate synthesis in the earthworm is mediated by an enzyme system more closely related to that in ureotelic vertebrates than to that in either bacteria or fungi.

The earthworm carbamyl phosphate synthetase is only detectable in the soluble fraction of the cell in contrast to the synthetase in the vertebrates which is found mainly in the mitochondrial fraction. The intracellular localization in the earthworm gut tissue is presented in Table 1. The only variation encountered in this localization was in two of five fractionations where as much as 24 percent of the total units was found in the residue sedimenting at 600*g*. Of 14 different solutions tested, the combination of 0.4*M* mannitol, 0.1*M* K_2SO_4, and 0.015*M* potassium glycylglycinate, *p*H 7.5 was found to be most effective in protecting the quite labile activity during fractionation. The supernatant from tissue homegenates prepared in this solution and centrifuged at 15,000*g* was routinely used as the enzyme source.

A high "carbamyl phosphate phosphatase" activity is present in mitochondrial preparations of the earthworm as well as of other invertebrates. This may account for previous failures to demonstrate carbamyl phosphate synthetase activity with such preparations. We found a marked inhibition of carbamyl phosphate synthesis by water-lysed liver mitochondria from an amphibian (*Rana pipiens* or *Bufo valliceps*) when they were mixed with water-lysed mitochondrial preparations from three species of invertebrates (the earthworm, the land snail *Otala lactea*, and the flatworm *Hymenolepis diminuta*).

The adenosine triphosphatase activity of both the invertebrate and amphibian mitochondria was of the same order of magnitude when measured under the conditions of the synthetase assay. Under conditions where the reaction proceeded to completion on the basis of the ATP present, an increase in the amount of amphibian mitochondria did not result in inhibition of carbamyl phosphate synthesis. An increase in the amount of ATP-generating system did not overcome the inhibition given by the invertebrate mitochondria. The inhibition was not due to the metabolism of *N*-acetyl-L-glutamate since the effectiveness of this cofactor in the amphibian system was not changed after its incubation for up to 3 hours with the invertebrate mitochondria.

During the inhibition of the reaction, there was a liberation of inorganic phosphate which was directly proportional to the amount of inhibition of carbamyl phosphate (as citrulline) synthesis resulting from the breakdown of carbamyl phosphate by these mitochondria. This phosphatase activity was assayed in a system containing, in micromoles per milliliter, dilithium carbamyl phosphate, 10; tris-HCl buffer, *p*H 7.4, 50; and $MgCl_2$, 5. Controls consisted of the complete system containing boiled enzyme. The activities expressed in micromoles of inorganic phosphate (corrected for control values) liberated per hour per mitochondria from 1 g of tissue at 28°C were as follows: *Lumbricus*, 27.6; *Hymenolepis*, 21.6; and *Otala*, 20.4. The specificity of the phosphatase activity is unknown (*10*). Although the phosphatase activity is in excess of the synthetase activity in the earthworm, it appears to be an important factor only under conditions of tissue homogenization which result in mitochondrial disruption. This activity did not interfere with the detection of carbamyl phosphate synthesis in crude homogenates containing intact mitochondria as is shown in Table 1.

STEPHEN H. BISHOP
JAMES W. CAMPBELL
Department of Biology,
Rice University, Houston, Texas

References and Notes

1. P. P. Cohen and G. W. Brown, Jr., in *Comparative Biochemistry*, M. Florkin and H. S. Mason, Eds. (Academic Press, New York, 1960), p. 161.
2. S. S. Cohen, *Science* **139**, 1017 (1963).
3. M. E. Jones, *ibid.* **140**, 1373 (1963).
4. S. N. Linton and J. W. Campbell, *Arch. Biochem. Biophys.* **97**, 360 (1962).
5. Z. Porembska and J. Heller, *Acta Biochim. Polon.* **9**, 385 (1962); J. W. Campbell, *Comp. Biochem. Physiol.* **8**, 13 (1963); J. W. Campbell and T. W. Lee, *ibid.* **8**, 27 (1963).
6. J. W. Campbell and S. H. Bishop, *Biochim. Biophys. Acta* **77**, 149 (1963).
7. L. M. Hall, R. C. Johnson, P. P. Cohen, *ibid.* **37**, 144 (1960).
8. Rat liver ornithine transcarbamylase was purified according to J. Caravaca and S. Grisolia, *J. Biol. Chem.* **235**, 684 (1960). Aspartate transcarbamylase from an *Escherichia coli* mutant, supplied by A. B. Pardee, was purified according to the procedure of J. C. Gerhart and A. B. Pardee [*J. Biol. Chem.* **237**, 891 (1962)] through the second ammonium sulfate fractionation. The rabbit muscle fraction was that described by E. Racker, *J. Biol. Chem.* **167**, 843 (1947). Citrulline was determined colorimetrically by the method of R. M. Archibald, *J. Biol. Chem.* **156**, 121 (1944) and carbamyl aspartate by a modification of the method of S. B. Koritz and P. P. Cohen, *ibid.* **209**, 145 (1954). Inorganic phosphate was determined as described by W. W. Kielley [in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1955), vol. 2, p. 593], and protein was determined according to O. H. Lowry *et al.*, *J. Biol. Chem.* **193**, 265 (1951). Chromatographic and radioautographic procedures were as described by J. W. Campbell, *Biochem. J.* **77**, 105 (1960).
9. B. Levenberg, *J. Biol. Chem.* **237**, 2590 (1962).
10. S. Grisolia, J. Caravaca, B. K. Joyce, *Biochim. Biophys. Acta* **29**, 432 (1958).
11. Supported by grants from the U.S. Public Health Service (AI 05006) and the National Science Foundation (13406).

1 October 1963

Tree Rat, *Thamnomys surdaster surdaster*, in Laboratory Research

Abstract. *The breeding of successive generations of* Thamnomys *in the laboratory made possible a study of its bionomics and an evaluation of its importance in parasitological and malarial research.*

The tree rat, *Thamnomys surdaster surdaster,* is the natural mammalian host and reservoir of *Plasmodium berghei* (*1*), the malarial parasite most commonly used in laboratory research because it is easily transmitted by inoculation of infected blood into animals such as white mice, albino rats, hamsters and voles. However, until recently, the difficulty of obtaining live specimens of *Thamnomys* from Katanga, and the failure, as reported, of these animals to mate and reproduce in captivity (*2*) prevented their use in malaria and other parasitology research. In the words of the late J. Rodhain, the tree rat has remained *"un obscure representant parmi le tres nombreux rongeurs de l'Afrique Centrale."*

On 12 April 1962, we received a number of *Thamnomys* caught in the wild (*3*). The animals have been mated successfully, and five successive generations of laboratory-bred *Thamnomys,*