interaction may be simply the result of dispersion, although we are not entirely satisfied with this explanation.

We believe that much higher Ni^{2+} concentrations, and thus higher gains, are possible with modified crystal-growing methods. Such a development would permit easy determination of all the magnetoelastic coupling constants and could greatly facilitate ultrasonic research at higher frequencies and with lossy materials.

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7. Prior to optical polishing of the end faces, the rod was exposed to a hydrogen atmosphere at 1200°C for several hours. This procedure reduced Ni^{3+} to Ni^{2+}, approximately doubling the concentration of the latter. The final Ni^{2+} content as determined by calibrated electron paramagnetic resonance measurements was approximately 5 parts per million.
8. Although the pump cavity was designed to operate in one of the $TE_{0,2,n}$ modes, there were in fact several other closely spaced modes in the neighborhood of 53 Ghz, which is a result of the large ratio of length to diameter of the cavity. Consequently, it was impossible to identify the particular mode being excited, so that in practice a cavity mode was chosen solely on the basis of strong coupling to the V-band pump.
9. The pulse-echo technique is described by E. B. Tucker, in *Physical Acoustics*, W. P. Mason, Ed. (Academic Press, New York, 1966), vol. 4, pt. A, pp. 77–78.
10. Work supported by NSF grant GP 6448. We thank Mr. Charles Sahagian of Air Force Cambridge Research Laboratories for providing the sapphire boule, Dr. John de Klerk of the Westinghouse Research Laboratories for graciously evaporating the CdS transducing films, and Dr. Theodore Castner of the University of Rochester for his active interest and many helpful discussions.

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Nucleoside Triphosphate Termini from RNA Synthesized in vivo by Escherichia coli

Abstract. *Alkaline hydrolyzates of RNA made in vivo by* Escherichia coli *contain ribonucleoside-3′-monophosphate-5′-triphosphates. These probably arise by hydrolysis of the initial nucleoside triphosphate from the 5′ terminus of the nascent RNA chains. Logarithmically growing cultures, labeled for 45 seconds with ^{32}P-labeled phosphate, yield about 2000 molecules of labeled tetraphosphate per cell, this yield increasing only slightly with continued labeling. Only the tetraphosphates of adenosine and guanosine have been found in* Escherichia coli, *and these two are present in approximately equal amounts.*

Studies in vitro of the DNA-primed RNA polymerase reaction have shown that RNA synthesis proceeds in the 5′ to the 3′ direction and that a triphosphate moiety is retained at the 5′ end of product RNA molecules (*1, 2*). In these cell-free experiments, RNA was synthesized with RNA polymerase and γ-labeled ^{32}P-nucleoside triphosphates (*pppX) (*3*) in the presence of a variety of DNA primers. Alkaline hydrolysis of the resulting RNA produced unlabeled nucleoside monophosphates (Xp) from the internal nucleotides of the molecules, a nucleoside (X) from the 3′-hydroxyl ends, and a ^{32}P-labeled nucleoside tetraphosphate from the 5′-triphosphate ends of the molecules (*pppXp). These studies have been performed with RNA polymerase from *Escherichia coli* and *Azotobacter vinlandii*. In both these systems, the RNA made was preferentially initiated by a purine ribonucleoside triphosphate (adenine or guanine), while the ratio of adenine to guanine termini was dependent on the source and physical state of the DNA used as primer.

Experiments in vivo have shown that the synthesis of RNA in *E. coli* is in the 5′ to 3′ direction, as in the in vitro systems (*4*). However, in *E. coli*, purine nucleotide monophosphates have been found at the 5′ termini of 23*S* ribosomal RNA (pGpX. . .), 16*S* ribosomal RNA (pApX. . .), and transfer RNA (pGpX. . .) (*5*). Terminal triphosphates have been found on the RNA of some RNA containing bacteriophage (*6*).

We have analyzed hydrolyzates of RNA from growing cultures of *E. coli* for the presence of pppXp terminal groups. Because of the length of RNA polymers and the probable short life of the terminal groups (they do not appear on the stable RNA species), we sought conditions to label the terminal phosphates selectively. With this in mind we administered ^{32}P-phosphate to cultures for short periods only. This procedure is based on the observation by Bolton and Roberts (*7*) that, after addition of ^{32}P-phosphate to a culture of *E. coli*, ^{32}P appears first in the β- and γ- phosphates of ATP and only after a lag in the α position. The *E. coli* strain ML 30 was grown at 37°C (*8*) and ^{32}P-phosphate was administered for periods between 45 and 120 seconds. The cells were then collected in 5 percent TCA, washed, and hydrolyzed with KOH. The protein, DNA, and K^+ were subsequently removed by addition of perchloric acid, and the remaining RNA nucleotides were applied to a DEAE-cellulose column and eluted with buffers containing 7*M* urea (*9*). Adenosine-5′-tetraphosphate (ppppA), which contained some ADP and ATP as well, or a ribonuclease digest of yeast RNA, was added to the samples to provide ultraviolet-absorbing markers for the chromatography. This system (*9*) has been useful for separating small oligonucleotides, but it is also convenient for separating mono-, di-, tri-, and tetraphosphomononucleotides (Fig. 1). The pyrimidines are eluted slightly ahead of the purines, the order of elution being cytidine, uridine, adenosine, guanosine, so that a double peak is observed when all four nucleotides are present (Figs. 1 and 2). Separate chromatography of the four nucleoside triphosphates in pairs showed that they are eluted in this order as well (Fig. 2a). In this experiment a pancreatic ribonuclease digest of RNA was added as a marker to facilitate comparison of these data to those of others who studied the elution of pppXp compounds in similar chromatographic systems (*5*).

In the RNA hydrolyzate from cells labeled for 2 minutes, a large amount of ^{32}P-label is present in the monophosphate region and in the di- and triphosphate regions as well (*10*), but there is little label in the eluent containing the ultraviolet-absorbing peak of ppppA. There is a distinct double peak of phosphate radioactivity in the region following the ppppA peaks where pppXp compounds with a net negative charge of 6 (*p*H 7.7) would be expected to be eluted (see below). The first half of this peak is referred to as ppp*I*p, and the latter half as ppp*II*p. In individual experiments there was a slight predominance of one or the other but in these regions from six different chromatograms, an average of 49 percent of the total radioactivity was in the ppp*I*p

peak and 51 percent was in ppp*II*p peak.

Initially the cultures were labeled for 45 seconds to minimize ^{32}P incorporation into the internucleotide phosphates of the RNA, but in light of the wide separation of the pppXp from the remainder of the labeled compounds, a 2-minute labeling time was chosen in an effort to increase the yield of tetraphosphate. The longer labeling time resulted in only a 15 percent increase in the amount of ^{32}P-labeled material in the pppXp region, whereas the amount of label in the monophosphate peak was increased fourfold. This suggests the pppXp terminus may have a relatively short half-life in vivo.

The pppXp peaks are eluted in the same region as the tetranucleotides (pXpXpXpX, Fig. 2), but the lack of increased radioactivity in the pppXp

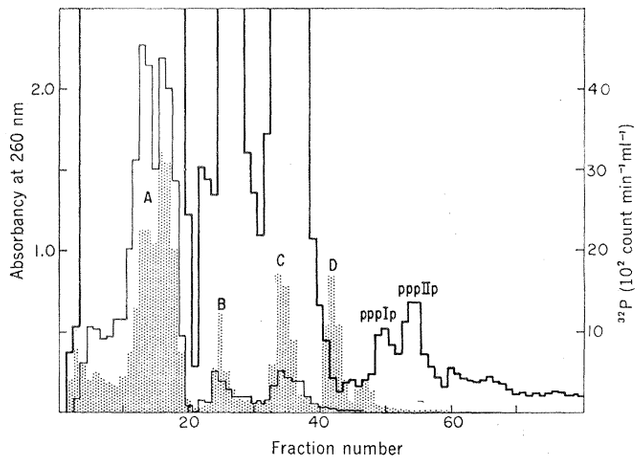

Fig. 1. Chromatography of alkaline hydrolyzate of $^{32}PO_4$-labeled RNA and adenosine phosphate standards. Shaded areas, absorbance of effluent at 260 nm; heavy solid line, ^{32}P (count/min); light solid line, ^{32}P (count/min) on scale reduced 20-fold. Peak A, mononucleotides; peak B, ADP; peak C, ATP; peak D, adenosine-5′-tetraphosphate. A culture of 100 ml of *E. coli* strain ML 30 was grown at 37°C in a tris-buffered salts medium (*8*) containing 0.4 percent glucose and $10^{-3}M$ K_2HPO_4. When the cells reached an optical density of 0.5 at 540 nm, they were harvested by centrifugation at 20°C for 10 minutes at 10,000 rev/min and resuspended in one-third their original volume of warmed, aerated medium, as above, but containing $10^{-5}M$ K_2HPO_4. After growth was resumed (5 minutes), 0.4 mc of carrier-free $H_3{}^{32}PO_4$ was added, and 120 seconds later the cells were precipitated by the addition of 20 percent of the culture weight of ice (from distilled water) and enough 50 percent TCA to give a final concentration of 5 percent. The cells were collected by centrifugation in the cold and washed twice with 5 percent TCA, once with 80 percent ethanol at 37°C for 20 minutes, and once with a mixture of 95 percent ethanol and ether (1:1) at 37°C for 20 minutes. The residue was collected by centrifugation after each wash. The final pellet was suspended in 4 ml of 0.3*N* KOH and incubated for 18 hours at 37°C. This hydrolyzate was then chilled and acidified by addition of 0.4 ml of 50 percent perchloric acid. The precipitate was removed by centrifugation, and the supernatant was adjusted to neutrality by addition of tris to a final concentration of 0.01*M* and then 10*N* KOH as necessary. The precipitate was again removed by centrifugation, and a portion [two-thirds of the supernatant, containing 50 absorbancy (A_{260}) units], was diluted to 10 ml with H_2O. To this was added about 2 mg of adenosine-5′-tetraphosphate (Calbiochem), and the resulting solution was applied to a DEAE-cellulose column (0.9 by 20 cm) at 3°C (*9*). The elution was carried out with a 500 ml linear gradient of NaCl from 0.02 to 0.25*M* in a solution of 0.0025*M* tris-HCl (*p*H 7.7) and 7*M* urea. Fractions of 5 ml were collected at 0.5 ml/min. Portions (0.2 ml) of each sample were dried onto planchets and counted in a gas-flow counter.

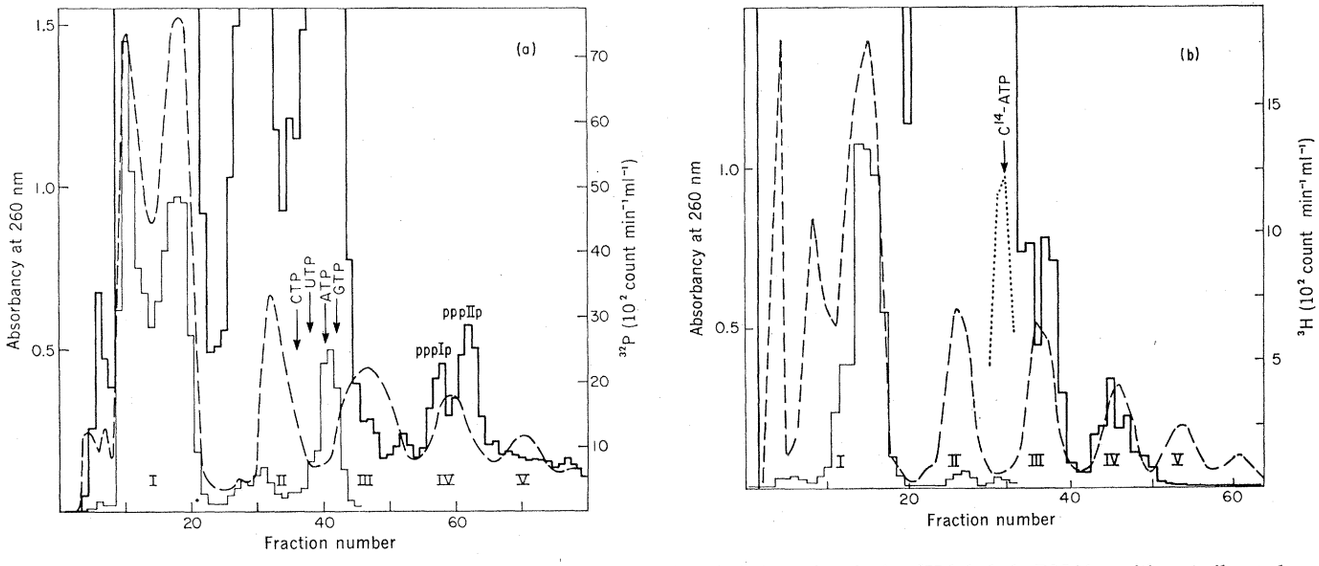

Fig. 2. Chromatography of alkaline hydrolyzates of phosphate-^{32}P-labeled and adenine-^{3}H-labeled RNA's with a ribonuclease hydrolyzate of yeast RNA. Broken line, absorbance at 260 nm; (a) heavy solid line, ^{32}P (count/min); light solid line, ^{32}P (count/min) on scale reduced 20-fold. (b) Heavy solid line, ^{3}H (count/min); light solid line, ^{3}H (count/min) on scale reduced 200-fold. Peak I, mononucleotides; peak II, dinucleotides; peak III, trinucleotides. The procedures of growing cells, labeling, preparing extracts, chromatography were as described for Fig. 1. An ultraviolet-absorbing marker was provided by adding 75 A_{260} units of a pancreatic ribonuclease digest to each sample. Ribonuclease (1 mg, Worthington) and 3 mg of yeast RNA were incubated in 5 ml of 0.1*M* tris-HCl (*p*H 7.8) at 37°C for 5 hours. An ATP marker was provided by adding ^{3}H- or ^{14}C-labeled ATP to the samples. Radioactivity was determined by drying portions of each fraction on glass filter paper disks and counting in a standard toluene-based counting fluid in the liquid-scintillation spectrometer. (a) A culture, as for Fig. 1, was given 1.4 mc of H_3PO_4-^{32}P for 1 minute and harvested. The elution positions of the four nucleoside triphosphates were determined on six separate columns with radioactive nucleotides in various combinations. (b) A 100-ml culture growing in medium supplemented with 2 mg/ml of vitamin-free casamino acids (Difco) was used at an optical density at 540 nm of 0.54, without concentration. One millicurie of adenine-8-^{3}H (14 mc/μmole) was given, and the cells were harvested after 3 minutes.

peaks after the longer labeling time indicated that the ^{32}P recovered is not due to the presence of any tetranucleotides which might be resistant to alkaline hydrolysis. The radioactivity in such a compound would increase in proportion to that in the monophosphate moieties.

In order to verify the location of 3′5′-pyrimidine and purine tetraphosphates in the effluent from these DEAE-columns, *pppAp, *pppGp, and *pppUp were synthesized with *E. coli* RNA polymerase (*11*); denatured DNA from salmon sperm was used as primer and either ATP-^{32}P, GTP-^{32}P, or UTP-^{32}P (*12*) as substrate (*1*). These preparations were eluted from the columns in the expected region and in the order expected from the study of nucleoside triphosphates. The elution of pppGp coincided very well with peak ppp*II*p, while the elution of pppUp fell into the ppp*I*p region. The elution of pppAp in two preparations was different; in one instance it eluted in the ppp*I*p region and in the second just prior to the ppp*II*p region. As judged by the mobilities of ATP relative to GTP, this latter position is the expected one; however our further experiments identifying ppp*I*p as the adenosine tetraphosphate would indicate that the earlier elution is correct.

To pursue further the identification of the unknown nucleoside tetraphosphates, the ^{32}P-labeled compounds in each of the pppXp peaks were removed from the 7*M* urea and concentrated by adsorption to and elution from charcoal (*7*), or by rechromatography on DEAE-cellulose and elution with ammonium carbonate buffer (*13*). The latter procedure resulted in the recovery of 85 to 90 percent of the ^{32}P in each peak. Approximately 85 percent of the ^{32}P in the concentrated peaks was adsorbable to charcoal when tested. After incubation of the concentrated material with alkaline phosphatase (*14*) for 30 minutes at 37°C, 97 percent of the ^{32}P could no longer be adsorbed on charcoal. These data support the conclusion that the ^{32}P in these peaks is externally bound to a nucleotide.

The ppp*I*p and ppp*II*p peaks, concentrated by DEAE-cellulose chromatography (*13*), were subjected to high-voltage paper electrophoresis in 0.06*M* sodium citrate at *p*H 4.1 (*8*). The pppAp, pppGp, and pppUp prepared in vitro were also subjected to electrophoresis under the same conditions, and it was found that they moved about 8 to 10 cm ahead of their respective triphosphates (Fig. 3a). Electrophoresis of the ppp*I*p material resulted in a single sharp peak of ^{32}P, which moved about halfway between GTP and UTP and 8 to 10 cm ahead of CTP and ATP, which have the same mobility at this *p*H (Fig. 3a). Occasionally some ^{32}P was present in the region beyond UTP, but always a small fraction of the total ^{32}P applied. When examined separately, there was no difference in the electrophoretic mobility of the early and late portions of the ppp*I*p peak, suggesting that there is only one major component present. Further analysis of ppp*I*p was made by electrophoresis in 0.02*M* potassium phosphate at *p*H 7.6, containing 0.003*M* ethylenediaminetetraacetic acid, for 4 hours at 4000 volts. This showed the ppp*I*p running 10 to 12 cm ahead of ATP and nearly coincident with CTP. This separation, consistent with the unknown being pppAp, was similar to the separation of ppp*II*p and GTP (13 to 15 cm) in the same electrophoresis run.

When the material from the ppp*II*p peak was subjected to electrophoresis in citrate buffer, the radioactivity was recovered in a single peak in the region of UTP, which is about 10 cm beyond GTP (Fig. 3b), and corresponds to the position of pppGp. In some instances there was a small second peak with the additional ^{32}P moving slightly more slowly than that in the major peak.

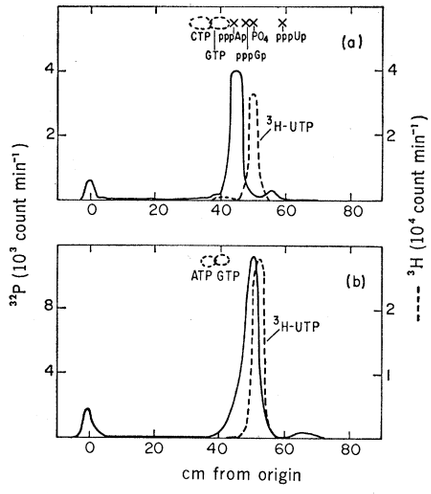

Fig. 3. High-voltage paper electrophoresis of nucleotide triphosphates and 3′5′-tetraphosphates. Solid line, ^{32}P (count/min); broken line, ^{3}H (count/min). (a) ^{32}P-ppp*I*p fractions from a DEAE-cellulose column with UTP-^{3}H. The location of marker compounds indicated by X's were determined on separate runs. The ^{32}P-labeled tetraphosphates of adenosine, guanosine, and uridine were prepared as described. (b) ^{32}P-ppp*II*p fractions from a DEAE-cellulose column with UTP-^{3}H. Electrophoresis was carried out in 0.06*M* sodium citrate buffer (*p*H 4.1) at 3°C for 5.5 hours at 4000 volts (*8*). Nucleoside triphosphates (approximately 0.2 μmole) were added as ultraviolet-absorbing markers. The electropherograms were cut into 2-cm squares which were counted in a standard toluene-based counting fluid in a liquid-scintillation spectrometer.

The material from each of the two pppXp peaks from the DEAE-column was also subjected to hydrolysis by prostatic phosphomonoesterase (*15*). A portion of the ^{32}P-labeled tetraphosphate was incubated with 25 units of the enzyme (1 unit cleaves 1 μmole of *p*-nitrophenylphosphate in 1 minute) in 0.2*M* sodium citrate, *p*H 5.0, containing 0.2 mg of bovine serum albumin per milliliter, for 20 minutes at 30°C. Electrophoresis (citrate buffer) of the treated material showed that 18 percent of the ^{32}P in the ppp*I*p fraction was converted to a compound with the mobility of ATP, and 80 percent of the ^{32}P in the ppp*II*p fraction was converted to a compound with a mobility identical to that of GTP.

The foregoing results were substantiated in experiments in which cells were labeled (3 minutes, 1 mc) with either uracil-^{3}H or adenine-^{3}H. The RNA was hydrolyzed and chromatographed on a DEAE-cellulose column as described. When cells were labeled with uracil-^{3}H, no significant peak of tritium was found in the tetraphosphate region. The results of the labeling with adenine-^{3}H (Fig. 2b) show a distinct peak of a tritium-labeled compound in the ppp*I*p region.

These experiments indicate that ppp*I*p is pppAp and that ppp*II*p is pppGp. Pyrimidine nucleoside tetraphosphates seem absent, in keeping with the results obtained in vitro (*1, 2*).

Initially, however, the clear separation of pyrimidines and purines on the DEAE-cellulose column (Fig. 2) suggested to us that ppp*I*p and ppp*II*p were pyrimidine and purine tetraphosphates, respectively (*17*). We are not yet able to explain why, as tetraphosphates, the two purine nucleotides separate so clearly where comparable separations are not obtained with the purine triphosphates.

An estimate of the number of tetraphosphate molecules recovered per cell can be made by dividing the amount of ^{32}P found in the pppXp regions of the columns by the specific activity of the ^{32}P-phosphate in the medium (*18*). This calculation suggests that a total of 2000 molecules of pppAp and pppGp were derived from each cell and indi-

cates that, on the average, 2000 RNA molecules bear triphosphate termini in an *E. coli* cell growing logarithmically at 37°C. The finding that the 5′-terminal nucleotide of RNA made in vivo and in vitro (*1*, *2*) is limited to a purine gives support to the notion that there must exist in the genome specific sites for the attachment of RNA polymerase. The finding of two 5′-terminal nucleotides on RNA suggests there are at least two functional categories of genes, those initiated by cytosine and those by thymidine.

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18. [(2 $\times$ 10^5 dpm in pppXp region) (3 $\times$ 10^{23} molecules/mole of phosphate)]/[(2.2 $\times$ 10^6 dpm/μc) (10^9 μc of ^{32}P per mole of phosphate) (1.5 $\times$ 10^{10} cells)] $\cong$ 2000 molecules pppXp per cell. We assume two of the four phosphates of pppXp are labeled rapidly (*7*).
19. Supported by grants from NIH (GM-15381), NSF (GB-4246), and by Cancer Research Funds from the University of California. L. Buch was a predoctoral fellow of the NIH. We thank B. Hayes for skillful assistance.
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22 November 1968

Complex Synaptic Configurations in Planarian Brain

Abstract. *Complex synaptic configurations which appear to have especial evolutionary and functional significance are shown in the neuropil of the brain of the planarian* Dugesia dorotocephala. *Some of the endings in these synaptic attachments contain dense core vesicles, suggesting that nonadrenaline or serotonin or both are neurotransmitters at a more primitive phyletic level than reported hitherto. The spatial proximity and connectivity of the synapses suggest modes of action permitting greater functional complexity to the planarian brain than previously supposed. Closely adjacent cellular processes which contain polysomal ribosomes, unusual in the neuropil, suggest synaptic transmission–protein synthesis coupling and a possible role in memory.*

Previous studies have elaborated many aspects of the fine structure of the planarian brain (*1*) and shown that these primitive animals engage in complex and plastic patterns of behavior (*2*). Recent experiments have demonstrated that high population densities suppress fissioning in the planarian *Dugesia dorotocephala* and that this effect is mediated via the brain (*3*). It was shown in these earlier electron micrographic studies that all the synaptic junctions between the neurons of the brain occurred in the neuropil (*1*). These synapses were of two types. One involved a terminus of the presynaptic fiber onto the postsynaptic neuron. The other involved the juncture of the lateral surfaces of two more-or-less parallel neural processes. Both kinds of synapses possessed the usual characteristics of an aggregation of small, light core vesicles on the presynaptic side, a heavily staining subsynaptic web, and a sharply demarcated, lightly staining intrasynaptic space of about 150- to 200-Å thickness. These synapses seemed to involve the juncture of only two neurons and only light core synaptic vesicles (although clusters of larger electron dense neurosecretory granules were shown elsewhere in the neuropil).

In this report we describe another kind of synaptic configuration in the planarian brain that is significantly different from those described previously and in which, we believe, the particular ultrastructural morphology has important theoretical and functional implications.

The following methods were used. *Dugesia dorotocephala,* which had been maintained in the laboratory for more than a year in aged tap water, in white enameled dishpans, on a diet of fresh raw beef liver, were decapitated just behind the auricles. The heads were fixed immediately for 1 hour in a 1.0 percent solution of osmic acid buffered to *p*H 7.4 in phosphate buffer. The specimens were then embedded in Epon which was polymerized for 48 hours at 60°C. Sections were cut on an ultramicrotome with a diamond knife (Sorvall "Porter-Blum" M-2) and then stained with lead citrate and uranylacetate according to the method described by Reynolds (*4*). The sections were observed and photographed with a Philips model EM 200 electron microscope. Magnifications of 20,500 and 51,560 times were obtained in the original negatives and further enlargement was achieved photographically.

One feature worth noting is the presence of "dense core" vesicles along with the light core vesicles in the same ending of the presynaptic cell. The outside diameter of these dark core vesicles averages about 600 Å, compared to about 300 Å for the light core vesicles. There is now reasonably convincing evidence that such dense core vesicles are the vesicles of the biogenic amine neurotransmitters, such as serotonin and noradrenaline (*5*). To our knowledge no one has hitherto provided evidence which would suggest that noradrenaline or serotonin is employed as a neurotransmitter by any animal as phyletically primitive as the planarians. These observations would indicate an antiquity for such neurotransmitters in the evolution of the brain that is much greater than previously suspected.

Another curious feature is the com-