Identification and Localization of Catecholamines in the Nervous System of *Limulus polyphemus*

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SUMMARY

The concentrations of various catecholamines in the nervous system of the horseshoe crab *Limulus polyphemus* have been determined by high-performance liquid chromatography with electrochemical detection. Dopamine, norepinephrine, epinephrine, and their precursor L-Dopa were present in appreciable quantities in discrete regions of the central nervous system and cardiac ganglion. The catecholamines were localized more precisely by use of the glyoxylic-acid-histofluorescence technique of de la Torre and Surgeon (1976). Catecholamine fluorescence appeared in protocerebral and tritocerebral neuropile, including regions of the central body and optic medulla. Posterior to these brain areas, tracts extended through the circumesophageal ganglionic ring and laterally out each of the pedal ganglia. Small clusters of large fluorescent somata were present in the protocerebrum. No fluorescence was observed in the corpora pedunculata.

INTRODUCTION

The catecholamines dopamine (DA), norepinephrine (NE), and, to a lesser extent, epinephrine (E) are present in all Metazoa (Florey, 1967; Sakharov, 1970; Welsh, 1972; Kerkut, 1973; Gerschenfeld, 1973; Klemm, 1976; Walker and Kerkut, 1978). For invertebrates, however, information about the quantities, distribution, and pharmacology of these important compounds is fragmentary.

In arthropods DA and NE are the predominant catecholamines, with DA usually present in greater concentrations (for reviews see Gerschenfeld, 1973; Kerkut, 1973; Klemm, 1976; Walker and Kerkut, 1978; Evans, 1980). Epinephrine has not been detected in any arthropod nervous system examined thus far (Gerschenfeld, 1973; Kerkut, 1973; Klemm, 1976; Walker and Kerkut, 1978), although it is present in the venom sacs of social wasps, where it is thought to play a defensive role through its action on the nervous tissue of rival arthropods (Ishay et al., 1974). Histofluorescence studies reveal that in insect central nervous systems (CNS) the highest concentrations of catecholamines appear to be in the protocerebral neuropile (Frontali, 1968; Elofsson et al., 1966; Elofssen,

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Nassel, and Myhreberg, 1977; Klemm and Björklund, 1971; Klemm and Schneider, 1975) although the distribution varies considerably from species to species (Klemm, 1976). Complementary quantitative and histological data are available for only a few insect species (*Periplaneta americana:* Frontali and Norberg, 1966; Frontali, 1968; Frontali and Häggendal, 1969; *Anabolia nervosa:* Björklund, Falck, and Klemm, 1970; Klemm and Björklund, 1971; *Schistocerca gregaria:* Klemm and Axelsson, 1973; Hiripi and S.-Rozsa, 1973; Klemm and Schneider, 1975). This paucity of information results in part from the lack of simple, inexpensive, sensitive neurochemical techniques which are readily applicable to invertebrate nervous tissue. The two methods used in this study offer many advantages in this respect.

This investigation of catecholamines in *Limulus* was motivated by indications that in the *Limulus* CNS and cardiac ganglion, the role of catecholamines can be analyzed at the cellular level. In this article are reported the concentrations and distributions of catecholamines in the nervous system of *Limulus*. The following article (Augustine, Fetterer, and Watson, 1982) describes the effects of catecholamines on the *Limulus* cardiac ganglion.

MATERIALS AND METHODS

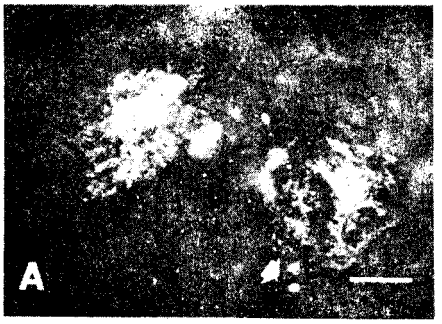
Male and female *Limulus polphemus* were obtained from the Marine Biological Laboratory, Woods Hole, MA, and maintained in a saltwater aquarium. Small animals measuring 5–10 cm across the carapace were used in the histofluorescence studies and larger animals measuring 12–24 cm were used for analytical procedures except where noted. For tissue histofluorescence studies, the brain, circumesophageal ganglia, and ventral nerve cord were rapidly dissected out of each animal and immediately frozen on the quick-freeze stage of a cryostat (−25 to −30°C). The tissue was then sectioned at 8 μm or 10 μm and the sections melted onto microscope slides. The mounted sections were processed according to the method of de la Torre and Surgeon (1976), except that the reacting solution contained 3 mL of 50% glyoxylic acid (Fisher reagent), 10.2 g of sucrose, and 4.8 g of KH_2PO_4 in 150 mL of water, pH 7.4. The slides were dipped into this solution for 3 s, air-dried for a few minutes, incubated at 80°C for 5 min, and coverslipped with Nujol. The sections were viewed with a Reichart fluorescence microscope using incident epiillumination with a BG 12 exciter filter and a Wratten 4 barrier filter. Sections serving as controls were treated similarly except that they were not exposed to the glyoxylic acid.

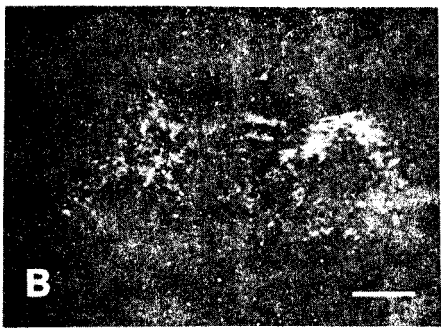
Limulus tissues were analyzed for catecholamines by means of high-performance liquid chromatography coupled with electrochemical detection (HPLC–EC; see Adams, 1976; Kissinger, Bruntlett and Shoup, 1981). Catecholamines were extracted according to the method of Shellenberger and Gordon (1971). Selected tissues were dissected out of the animals, weighed, and immediately placed in ice-cold 0.4*N* perchloric acid containing 1 g/L sodium bisulfite and 0.5 g/L disodium EDTA. The tissues were homogenized and centrifuged for 10 min at 3.6×10^4 rpm. The supernatant was adjusted to pH 8.6 with Tricine buffer (17.9 g Tricine and 25.0 g disodium EDTA/L 0.525*N* NaOH). The catecholamines were extracted from the supernatant with alumina prepared

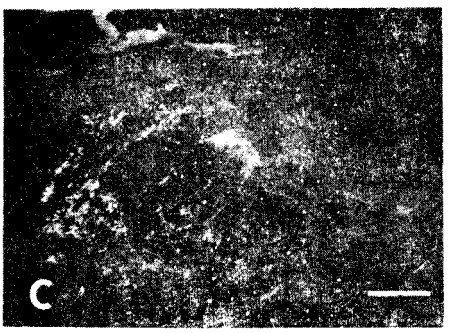
TABLE 1
Recoveries of the Catecholamines as a function of Alumina Extraction and of Presence of Tissue[a]

	NE	E	DA
A. Efficiency of alumina extraction			
Extracted sample	38.2 ± 4.9	31.6 ± 1.9	21.8 ± 1.3
Unextracted sample	50.3 ± 1.0	40.7 ± 3.0	28.3 ± 1.0
Percent recovery	75.9 ± 4.9	77.6 ± 5.7	77.0 ± 1.3
B. Effect of tissue on recovery			
	NE	E	DA
Extracted with tissue	28.0 ± 5.4	19.4 ± 5.4	10.3 ± 14.5
Extracted without tissue	33.5 ± 1.7	22.5 ± 2.0	11.8 ± 1.0
Percent recovery	83.5 ± 11.8	86.1 ± 11.8	87.9 ± 14.6

[a] Values given are in mean peak response (nA) ± SD. $N = 10$ in all cases.







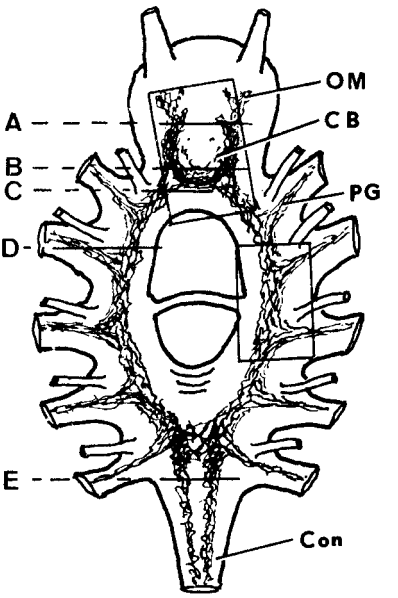


Fig. 1. Catecholamine histofluorescence in the prosomal CNS of *Limulus*. The diagram summarizes the general distribution of catecholamine-fluorescent fibers, and indicates the approximate locations of the cross sections shown in (A)–(E). Rectangles indicate areas shown in Fig. 2. (A) Cross section through protocerebrum, showing paired tracts of catecholamine-containing fibers as well as somata (arrow). Dorsal is up in all cross sections. (B) Section through posterior protocerebrum. The paired catecholamine tracts join across the midline, just ventral to the central body. Arrow indicates individual fibers. (C) Section through left tritocerebral ganglion. (D) Section through circumesophageal ring and pedal ganglion (lower left). (E) Section through posterior end of circumesophageal ring, showing paired catecholamine tracts extending posteriorally toward the opisthosoma. Abbreviations: CB, central body; Con, connective between prosomal and opisthosomal CNS; OM, optic medulla; PG, first pedal ganglion. Scale: 200 μm.

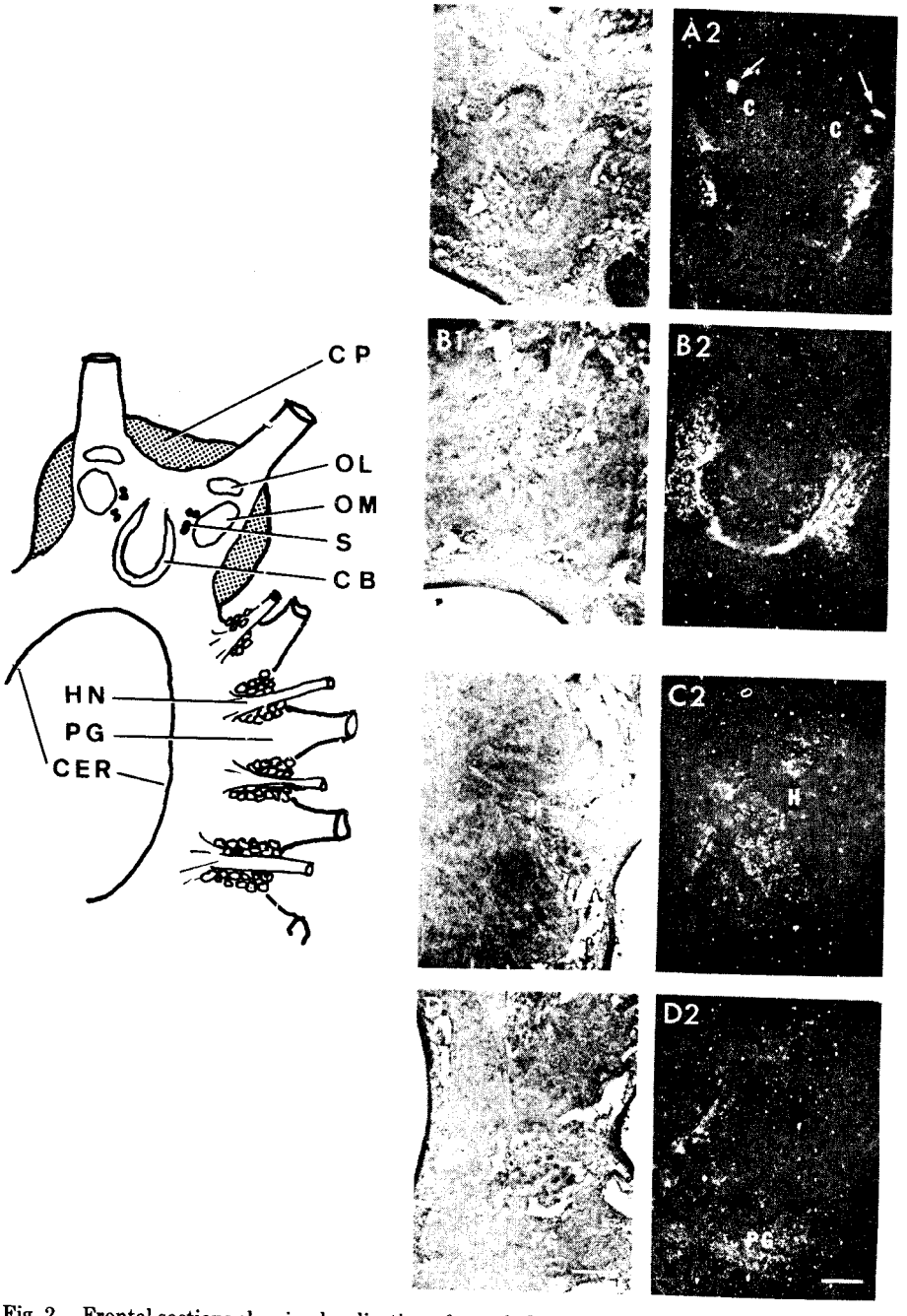


Fig. 2. Frontal sections showing localization of catecholamine fluorescence in prosomal CNS. Diagram represents a dorsal view of brain and right anterior portion of circumesophageal ring (CER) in the same orientation as sections (anterior is up). Abbreviations: CP, corpora pedunculata (which also extend ventral to the other brain structures); OL, optic lamina neuropile; OM, optic medulla neuropile; S, clusters of fluorescent somata; CB, central body neuropile; HN, hemal nerve; PG, pedal ganglion. Sections in (A) and (B) are of the brain region shown in anterior rectangle in Fig. 1; sections in (C) and (D) are of the region of circumesophageal ring shown in posterior rectangle in Fig. 1. Compare sections stained with hematoxylin–eosin [(A1), (B1), (C1), (D1)] with adjacent glyoxylic-acid-treated sections [(A2), (B2), (C2), (D2)]. (A) Dorsal sections of brain in region of central body (C). Arrows in (A2) indicate dorsal paired clusters of CA fluorescent somata. (B) Sections through the same area as (A), but ca. 64 μm more ventral. Anterior tips of central body curve ventrally, and are labeled (C) in (B1). Catecholamine-fluorescent fibers ventrolateral to the central body are shown

by the method of Anton and Sayre (1962) and were then eluted from the alumina with 1*N* acetic acid. This acid eluate was frozen for later analysis or was immediately injected into the column. The catecholamines and the catecholamine precursor L-Dopa were separated on a 50-cm × 0.9-mm i.d. glass column containing a strong cation exchange resin (Vydac, obtained from The Separations Group, Hesperia, CA). The mobile phase was prepared by diluting 1 mL of concentrated H_2SO_4 and 11.4 g of Na_2SO_4 to 2 L with triply distilled water. This mobile phase was pumped through the column at a rate of 0.1 mL/min (300 psi). All separations were carried out at room temperature (25°C). The catecholamines were detected with an electrochemical transducer (Bioanalytical Systems, West Lafayette, IN), the output of which was displayed on a strip-chart recorder equipped with a disc-chart integrator.

Standard solutions of catecholamines, L-Dopa, tyrosine, and octopamine were used throughout the analysis to estimate sample extraction recovery, to determine retention times, and to calibrate the electrochemical detector response. Standard stock solutions were made up in 1*N* acetic acid and frozen in 5-mL aliquots. Working solutions were prepared from stock solutions as needed. The concentrations of free catecholamines in the stock and final working solution were 80 ng/μL and 80 pg/μL, respectively. Single standards and mixtures of standards were injected directly or were first run through the extraction procedure. In either case each compound had a single peak, clearly separable from the other compounds. The catecholamine nature of the detector response was substantiated further by use of a second electrochemical detector, so that oxidation and reduction currents at specific potentials could be measured simultaneously (Fenn, 1977).

The retention times for the catecholamines and for L-Dopa were as follows: L-Dopa, 5.5 min; NE, 9 min; E, 10.5 min; DA, 14 min. Octopamine, tyramine, and tyrosine could not be detected with our protocol, because octopamine and tyramine are not extracted by alumina (Anton and Sayre, 1962), and tyrosine is not electroactive at the detector potential used (+0.76 V versus Ag/AgCl).

The efficiency of the alumina extraction procedure was estimated by comparing measures of alumina-extracted and unextracted samples of known quantities of catecholamines. As shown in Table 1A, the extraction procedure recovered 75–80% of the catecholamines. Table 1B shows the effect of tissue on catecholamine recovery. Known amounts of catecholamines were added to tubes containing mouse brain tissue which previously had been alumina extracted and to tubes without tissue. These were then alumina extracted and the recoveries compared. Considering both the effect of tissue and of alumina extraction, estimates of overall catecholamine recovery were 63–68%.

Estimates of the tissues levels of the catecholamines were calculated by the following formula:

$$\text{concentration of sample} = \frac{\text{disc integration sample}}{\text{disc integration standard}} \times \text{concentration of standard} \times \frac{1}{\text{percent recovery}}$$

RESULTS

Histofluorescence

With the filter arrangement described, the sections treated with glyoxylic acid were characterized by a brilliant blue–green fluorescence typical of the catecholamine fluorophore (Lindvall et al., 1973; Lindvall and Björklund, 1974a; de la Torre and Surgeon, 1976). Control sections displayed only a yellow and orange autofluorescence. The catecholamine fluorescence generally appeared as discrete fibers and punctate profiles less than 15 μm in diameter. Some of the fibers appeared beaded, with varicosities similar to those for vertebrate catecholaminergic neurons (Lindvall and Björklund, 1974b). The punctate profiles appeared

in (B2). (C) Sections of circumesophageal ring in region of segments 3 and 4 (second and third legs). (C2) shows catecholamine fibers in ganglionic neuropile, and absence of catecholamine-induced fluorescence in (noncardioregulatory) hemal nerve No. 4 (H). (D) More ventral section of the same region as (C). (D2) shows CA fluorescence in the pedal ganglia (PG). Scale: 200 μm for all sections.

to be fibers cut in cross section, and were found only in neuropile regions of the CNS.

The distribution of catecholamine fluorescence in the prosomal (cephalothoracic) CNS is shown in Figures 1 and 2. Two pairs of clusters of large fluorescent somata were located in the brain, lateral to the anterior ends of the horseshoe-shaped central body. Figure 2(A) shows the more dorsal pair of cell-body clusters; the other pair was located in the same area but more ventrally. The dorsal clusters were within the medial region of the ganglion cell layer of the optic medulla (Chamberlain, 1978; and personal communication). The ventral clusters were near the indistinct boundary between the ventromedial ganglion cell layer of the medulla and adjacent unidentified protocerebral cells.

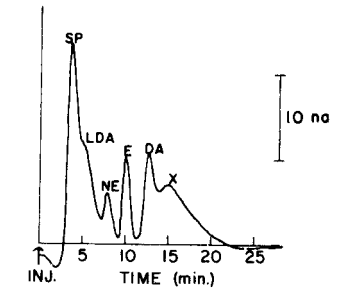
Fluorescent fibers were more prominent in the CNS than were somata. These fibers appeared in discrete tracts extending through the entire prosomal CNS (Fig. 1). The most prominent fiber fluorescence was in prosomal neuropile just ventrolateral to the central body [Figs. 1 (A) and 1 (B); Figs. 2 (A2) and 2 (B2)]. Fibers also extended anterolaterally from the central body region into the optic medulla (Fahrenbach, 1975; Chamberlain, 1978), and posterolaterally into the tritocerebral cheliceral ganglion [Fig. 1(C)] and circumesophageal ring of fused ganglia. This posterior tract of fluorescent fibers extended the length of the dorsal side of the circumesophageal ring, with projections ventrolaterally into each pedal ganglion [Figs. 1(D); Figs. 2(C) and 2(D)]; fiber tracts continued through the prosomal–opisthosomal connectives [Fig. 1(E)]. Fluorescent fibers were observed in the opisthosomal (abdominal) ventral nerve cord and in the cardiac ganglion, but they have not yet been mapped. No catecholamine fluorescence was observed in hemal nerve fibers in the circumesophageal ring [Fig. 2(C)], or in the corpora pedunculata (mushroom bodies) that comprise 80% of the volume of the brain (Fahrenbach, 1975).

Chromatographic analysis

Biochemical analysis of *Limulus* tissues by HPLC–EC demonstrated the presence of appreciable quantities of dopamine (DA) and norepinephrine (NE). Most tissues examined also contained epinephrine (E) and the catecholamine precursor L-Dopa. Figures 3(A) and 3(B) show typical chromatograms obtained from a cardiac ganglion and from a whole prosomal CNS, and demonstrate the degree of resolution obtained and the relative concentrations in each tissue.

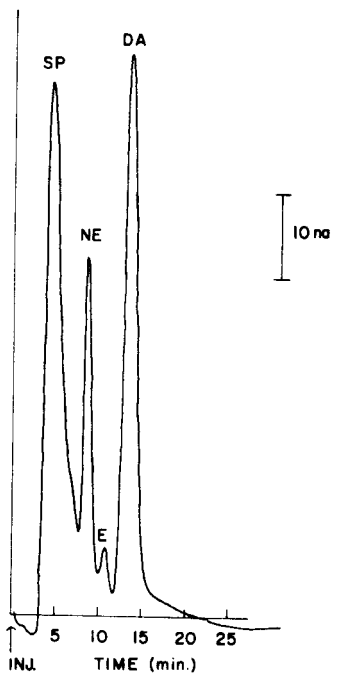
Table 2 summarizes the catecholamine concentrations in the tissues examined in adult *Limulus* (15–18-cm carapace width). In most tissues DA concentrations were greater than or equal to NE concentrations, and both were considerably higher than levels of L-Dopa. High levels of NE and DA were found in all parts of the CNS (brain, circumesophageal ganglionic ring, opisthosomal nerve cord) and in the cardiac ganglion. Appreciable amounts of DA and NE were also found in lateral eyes, optic nerve, and cardioregulatory nerve. Leg nerve and hemolymph contained low-to-undetectable levels of catecholamines. Interestingly, quantifiable levels of epinephrine were found in cardioregulatory nerve No. 9 (25 ng/g) and cardiac ganglia (6 ng/g). Epinephrine was also present in the CNS, but could not be quantified due to incomplete separation from the large adjacent peaks of NE and DA (Fig. 3).

The catecholamine concentrations of specific tissues varied considerably, as indicated by the standard deviations in Table 2. This variability was probably



CARDIAC GANGLION

(a)



PROSOMAL CNS

(b)

Fig. 3. Chromatograms from the analysis of extracts of cardiac ganglion (A) and prosomal CNS (B). SP, solvent peak; LDA, L-Dopa; NE, norepinephrine; DA, dopamine; E, epinephrine; X, unknown (not a catecholamine). Levels of L-Dopa were not quantifiable from the peaks shown, due to overlap with the solvent peak. Other chromatograms provided quantifiable peaks for L-Dopa. Scale: 10 nA.

not a result of the methods employed, since these were monitored with a variety of controls, such as internal standards and comparison of right and left halves of the same brain. These controls indicate that the extraction and chromatographic procedures were very consistent. Some of the variability may represent catecholamine loss during dissection as well as fluctuations in wet weight of tissues, but some appears to result from real differences in catecholamine levels. These variations may reflect differences in the sex and age of the samples and time of day, and time of year at which the samples were collected.

To examine whether catecholamine levels in the *Limulus* CNS vary with size

TABLE 2
Catecholamine Contents of Tissues of *Limulus* 15–18 cm in Carapace Width[a]

Tissue	*N*	Tissue weight (mg)	L-DOPA	DA	NE	E
Prosomal CNS[b]	8	497 ± 112	+[c]	767 ± 388	370 ± 138	+
Brain (protocerebrum and tritocerebrum)	2	73 ± 3	124 ± 90	395 ± 120	650 ± 23	+
Circumesophageal ganglia	2	291 ± 10	61 ± 64	290 ± 42	146 ± 22	+
Abdominal nerve cord	12	180 ± 70	+	582 ± 378	396 ± 321	+
Cardiac ganglion	4	10 ± 2	+	830 ± 63	228 ± 68	6 ± 2
Cardioregulatory nerve (hemal nerve No. 9)	Pooled (3)	9	ND[d]	125	96	25
Lateral eye	2	109 ± 18	ND	314 ± 232	108 ± 81	ND
Lateral eye nerve	2	104 ± 58	+	92 ± 60	93 ± 51	ND
Leg nerve	Pooled (3)	85	ND	15	ND	ND
Hemolymph[e]	2	2 ml	2 ± 1	1 ± 0.6	0.7 ± 0.2	0.5 ± 0.1

[a] Values are expressed as ng/g wet weight. Values are means ± SD
[b] Prosomal CNS includes combined brain and circumesophageal ganglia.
[c] +: Compound present at a level too low to measure accurately (0.1 mg).
[d] ND: not detected.
[e] Hemolymph measurements are expressed in mg/mL.

and age, tissues from smaller animals (5–7-cm carapace width) also were analyzed. As shown in Table 3, the smaller, younger animals had higher NE levels in the circumesophageal ganglia and abdominal nerve cord, and lower NE levels in the brain than the larger animals (Table 2). Furthermore, the smaller animals had more NE than DA in the circumesophageal ganglia and abdominal nerve cord (but not in the brain), while in the larger animals only the brain had higher levels of NE than DA. Although the tissues in Table 3 were all disected within a few hours of each other on the same day, the data in Table 3 show as great a variation as those in Table 2. This finding argues that little of the variability in measured catecholamine contents results from diurnal or seasonal differences.

DISCUSSION

Two neurochemical techniques have been utilized here to determine the concentration and distribution of catecholamines in the *Limulus* nervous system. All of the major catecholamines were found in appreciable quantities, at least in the larger animals. Dopamine was present in the highest concentrations in most tissues, but significant quantities of norepinephrine and epinephrine were also present. These results stand in contrast to the few previous studies of

TABLE 3
Catecholamine Contents of Tissues of *Limulus* 5–7 cm in Carapace Width[a]

Tissue	*N*	L-Dopa	DA	NE
Brain (protocerebrum and tritocerebrum)	5	+[b]	346 ± 142	132 ± 46
Circumesophageal ganglia	5	+	478 ± 191	1155 ± 289
Abdominal nerve cord	5	+	222 ± 72	600 ± 150

[a] Values are expressed as ng/g wet weight (mean ± SD). Epinephrine contents were not determined.
[b] +: L-Dopa present but could not be resolved quantitatively from adjacent peaks.

catecholamines in *Limulus*. Townsel, Baker, and Gray (1976), Battelle, Kravitz, and Stieve (1979), and Battelle (1980) have reported the apparent absence of net synthesis of DA and NE from labeled tyrosine in *Limulus* abdominal ganglia, ventral eye nerve, visual system, and brain. Ventral eye nerve has not been analyzed in this work, but the brain, abdominal ganglia, lateral eyes, and lateral eye nerve contain appreciable quantities of catecholamines. Moreover, *Limulus* prosomal CNS and abdominal nerve cords contain both a tyrosine hydroxylase and a Dopa decarboxylase, as determined by HPLC–EC (O'Connor and Tadiri, unpublished).

The catecholamine content of *Limulus* CNS may be compared with available data for other arthropods. In crustaceans, DA appears to be the predominant catecholamine, with less NE and little or no E reported (von Euler, 1961; Elofsson et al., 1966, 1968; Kerkut, Sedden, and Walker, 1966; Florey, 1967; Cooke and Goldstone, 1970; Elofsson and Klemm, 1972; Sullivan, Friend, and Barker, 1977). In insects, both DA and NE are present in roughly the same concentrations found in *Limulus* (*Periplaneta americana*—2500 ng/g DA, 370 ng/g NE: Frontali and Häggendal, 1969; *Locusta migratoria*—1310 ng/g DA, 240 ng/g NE: Hirpi and S.-Rozsa, 1973; and *Trichoptera*—3500 ng/g DA, 320 ng/g NE: Klemm and Björklund, 1971). The levels in these arthropods are also comparable to those reported in the vertebrate whole brain analyses (DA, 500–800 ng/g; NE, 240–450 ng/g: Refshauge et al., 1974). The presence of epinephrine has not been reported in the CNS of either crustaceans (von Euler, 1961; Elofsson et al., 1966, 1968; Kerkut, Sedden, and Walker 1966; Eloffson and Klemm, 1972), or insects (Frontali and Häggendal, 1969; Björklund, Falck, and Klemm 1970; Klemm and Björklund, 1971; Klemm and Axelsson, 1973; Klemm, 1976). Our detection of epinephrine in the nervous system of *Limulus* might indicate that *Limulus* is unusual in this respect, or alternatively it may reflect the greater sensitivity of HPLC–EC as compared to the techniques used previously. The sensitivity of HPLC–EC in this study was ca. 10 pg, compared to ca. 100 pg for spectrofluorimetry.

The spatial distribution of catecholamines in the *Limulus* brain largely corresponds to that reported in insects (Frontali, 1968; Klemm, 1976), and to a lesser extent in crayfish (Elofsson et al., 1966). The central body region is intensely fluorescent, with rather less intense fluorescence in associated protocerebral and tritocerebral neuropile and in the optic medulla. Battelle (1980) reported that the optic ganglion–central body regions also have high concentrations of octopamine. Catecholamine fluorescence appears to be absent in the Kenyon cells (globuli cells), neuropile, and stalks of the corpora pedunculata. Frontali (1968) reported absence of fluorescence in the corresponding areas in *Periplaneta*, but found considerable fluorescence in the α and β lobes, regions without clear homology in *Limulus*. Fahrenbach (1979) has described two types of afferent endings in the *Limulus* corpora pedunculata that contain dense-cored vesicles, and observes that he has seen induced fluorescence in the neuropile of the corpora pedunculata. This apparent discrepancy with the present results has not yet been resolved.

The presence of catecholamines in the cardiac ganglion and in a cardioregulatory nerve suggests that these compounds may function in cardioregulation. The cardioregulatory nerves arise as branches of prosomal hemal nerves Nos. 7 and 8 and opisthosomal hemal nerves Nos. 9–13 (Patten and Redenbaugh, 1899; Bursey and Pax, 1970). Catecholamines have been demonstrated to excite *Limulus* central neurons (James and Walker, 1979) and eccentric cells in the lateral

eyes (Adolf, 1966; Behrens and Wulff, 1970), and recent evidence indicates that all the catecholamines and octopamine excite the *Limulus* cardiac ganglion (Fetterer and Augustine, 1977; Augustine, Watson, and Fetterer, 1978; Watson, 1978). Pharmacological evidence for the role of biogenic amines in cardioregulation is presented in the following article (Augustine, Fetterer, and Watson 1982).

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