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6. *Rhithropaneus harrisii* passes through four zoeal stages and one postlarval stage (megalopa) before metamorphosing to the benthic juvenile crab. In our experiments, larvae were added to the vertical column either a few hours prior to the molt to zoeal stage 3 (laboratory-reared) or during stage 3 (field-caught). The record therefore includes data from late stage 2 (laboratory-reared), and stages 3 through the megalopa. Molting from one stage to the next occurred every 2 or 3 days.
7. The column was constructed of transparent Lucite (0.64 cm). The dimensions of the enclosed water column were 8.2 cm by 7.0 cm by 1.9 m, and the volume of water was 10.9 liters.
8. The desired salinity was obtained by diluting natural seawater, collected off the Beaufort Inlet, North Carolina, with distilled water. The salinity used for laboratory-reared larvae was 20 per mil, for field-caught larvae it was 15 per mil.
9. The monitoring system consisted of a television camera (Cohu model 4350) with a silicon intensified target vidicon, a videotape recorder (Panasonic model 30205D), and an elevator system to raise and lower the camera parallel to the vertical column. Light was provided by a pair of 8-foot (2.44-m) General Electric red fluorescent tubes further filtered and diffused by a red plastic screen and red cellophane to give only dim diffuse light of wavelengths greater than 600 nm. Larvae of *R. harrisii* are very insensitive to such a light source [R. B. Forward, Jr., and J. D. Costlow, Jr., *Mar. Biol.* **26**, 27 (1974)]. Since the light was mounted parallel to the vertical column, it presented no vertical orientation cues. A mechanical clock was used to show the time of each monitoring scan.
10. The center of the larval distribution was determined by multiplying the number of larvae in each segment by the depth of the center of that segment and dividing by the total number of larvae counted. This was a good descriptor of the larval depth distribution for two reasons; first, few or no larvae settled to the bottom of the chamber; and, second, the population vertical distribution was generally symmetrical about the center and concentrated near it.
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17. We thank B. Hunnings for help with construction of the column and for design and construction of the elevator system. M. Swift and D. Stearns provided helpful comments on the manuscript. Supported in part by NSF grant OCE 77-26838.

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Subfornical Organ Efferents to Neural Systems for Control of Body Water

Abstract. *The subfornical organ, a circumventricular structure of the central nervous system, has efferent neural projections to sites within the brain known to be involved in drinking behavior and secretion of antidiuretic hormone. By using anterograde tracing techniques, it is shown that the subfornical organ projects to the nucleus medianus of the medial preoptic area, to the organum vasculosum of the lamina terminalis, and to the supraoptic nuclei bilaterally. Its efferent connectivity is confirmed by retrograde transport of horseradish peroxidase. The organum vasculosum of the lamina terminalis, another circumventricular organ and a suspected receptor site for angiotensin II, is involved in the circuitry of the subfornical organ and also has an efferent projection to the supraoptic nuclei.*

The circumventricular organs (CVO's) of the brain are unique structures that lack a blood-brain barrier (*1, 2*). They are highly vascular and lie in the midline of the brain at strategic positions on the surface of the cerebral ventricular system (*3–6*). Without a blood-brain barrier, they are ideally suited as receptor sites for blood-borne biologically active substances normally excluded by the brain (*7, 8*). Initially, their functional role was presumed to be neurosecretory on the basis of morphological evidence (*2, 4, 5, 9*). From the results of earlier experimental work it was speculated that the subformical organ (SFO) played a role in water balance (*10*). This has been supported by recent work showing that lesions of the SFO inhibit the natriuretic response to intracarotid hypertonic saline (*11*) and that extracts of the SFO given intracerebroventricularly affect salt-water balance (*12*). More significantly, Simpson and Routtenberg (*8*) and Simpson *et al.* (*13*) demonstrated that the SFO is an extremely sensitive site in the brain for the induction of drinking behavior by angiotensin II (AII). This was the first direct demonstration of a functional role for the SFO and firmly supported the contention that the SFO is involved in the regulation of water balance. However, it may not be the sole receptor site for AII-mediated drinking behavior. Other work implicates the periventricular tissue of the anteroventral third ventricle, which contains the organum vasculosum of the lamina terminalis (OVLT), another CVO (*14–16*). Both sites are very sensitive. The medial preoptic area is a third possible receptor site; however, it is a less likely candidate since it requires at least three to four orders of magnitude more AII to produce drinking (*17*) than does the SFO (*13*).

Since the SFO has been shown to be a receptor site for AII in the induction of drinking behavior, identifying its efferent projections offers the chance to trace the neural circuitry mediating a specific behavioral response elicited by a specific hormone at a specific site in the brain. Little is known about the efferent connections of the CVO's. The single exception is a projection from the area postrema to the neighboring nucleus of the solitary tract described in the cat (*18*). Nothing has been described for the SFO or OVLT. In this study we describe efferent connections of the SFO traced by labeling both anterograde and retrograde transport within the axons of SFO neurons.

The SFO is a small (0.4 by 1.0 mm) structure which lies in the anterior and dorsal part of the third ventricle below the ventral surface of the ventral hippocampal commissure and between the interventricular foramen of the lateral ventricles. Suspended from the fornix and separated from immediately adjacent nuclei by a fiber bundle, the SFO is ideally positioned for the application of anterograde autoradiographic tracing techniques (*19*). In this procedure tritiated amino acids in nanoliter volumes are locally injected at specific sites within the brain. They are taken up by neuronal perikarya, incorporated into protein, and carried down axons by axoplasmic transport mechanisms to their terminals, which are identified by autoradiography.

We examined the brains of 11 Sprague-Dawley male albino rats in which tritiated L-leucine ([3,4,5-^{3}H(N)], 79.8 Ci/mmole) in small volumes (10 or 20 nl, 50 or 25 μCi/μl, respectively) was injected through a 33-gauge beveled cannula (*20*).

Four rats had injections involving the SFO. In two of these rats the cannula terminated in the ventral hippocampal commissure just dorsal and anterior to the SFO and minimally involved the triangularis and septofimbrial nuclei of the septum (caudal septal nuclei). In the remaining two rats the cannula terminated more anteriorly and the injection site included the caudal septal nuclei. Of the

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remaining seven rats, one had a control injection into the dorsal third ventricle; another had a sham injection; three rats had control injections into the three nuclei most adjacent to the SFO (the septofimbrial and triangularis nuclei of the septum and the dorsal nucleus medianus) which did not spread to the SFO; and two had injections in the more distant medial septal nucleus.

We report three projections from the SFO. Grain patterns characteristic of terminal fields (*9*) were observed over the nucleus medianus of the medial preoptic area (NM), the OVLT, and the supraoptic nuclei (SON) bilaterally, as shown in Fig. 1, C, D, and E, respectively. Quantitatively, the density of labeling of these terminal fields was correlated with the extent of SFO involvement in the injection site and not with the extent of caudal septal nuclei involvement. In two rats the SFO was labeled by the injection, and the terminal fields over the NM, OVLT, and SON were moderately dense. In a third rat the SFO was labeled less and the resultant labeling of the NM and OVLT was light; labeling was at background levels in the SON. The SFO of the last rat was more heavily involved by the injection than the SFO's of the other rats (see Fig. 1A) and the terminal fields of the NM, OVLT, and SON were more densely labeled. The SON were labeled bilaterally and most heavily at their ventral borders in the middle third of their extent along their anterior-posterior axis. Because of the close proximity of the caudal septal nuclei we could not exclude or support the existence of an SFO projection to them. In addition, we did not exclude the possibility of more distant projections from the SFO; for example, to the brainstem.

Labeling of a projection through the stria medullaris to the habenular nuclei was used as an indication of the involvement of the caudal septal nuclei (*21*) within the spread of the injection, which is of some concern in interpreting the source of the projections to the NM, OVLT, and SON. This concern is assuaged by the following evidence. First, others using the same techniques have not reported projections to these areas from the caudal septal nuclei (*21*). Second, our control injections involving only the caudal septal nuclei (two rats) did not result in labeling of these sites, but did label the stria medullaris and habenular nuclei, confirming the adequacy of the injection (see Fig. 1B) (*21*). The dorsal NM is adjacent to the SFO at its anterior-ventral extent. A control injection here that did not spread into the SFO did not label the ventral NM, OVLT, or SON, but did label the arcuate nucleus. These results from control rats do not confirm a previous finding (*22*), obtained by silver staining of degenerating axons, of afferents from the triangularis nucleus of the septum and the dorsal NM to the SFO. Last, a ventricular control injection resulted in heavy labeling of the ependyma and underlying parenchyma of the dorsal and anterior third ventricle. No characteristic labeling of the NM or SON occurred, which rules out ventricular spread of the isotope as a cause of the pattern of labeling observed in the previous cases. Since the OVLT is a periventricular structure, it was labeled by the ventricular injection, but grain densities were localized superficially.

To confirm the existence of SON terminals originating from neurons in the SFO, we applied the horseradish peroxidase (HRP) retrograde labeling technique (*23*). The HRP is taken up by axon terminals and not by undamaged fibers of passage. The material is retrogradely transported to the soma of the neuron, where it can be observed with appropriate histochemical staining. A group of six Sprague-Dawley male rats were given 100 to 150 nl of HRP solution (250 μg/μl; Sigma type VI, lot 26C-9680) by injection either unilaterally or bilaterally through a 33-gauge cannula stereotaxi-

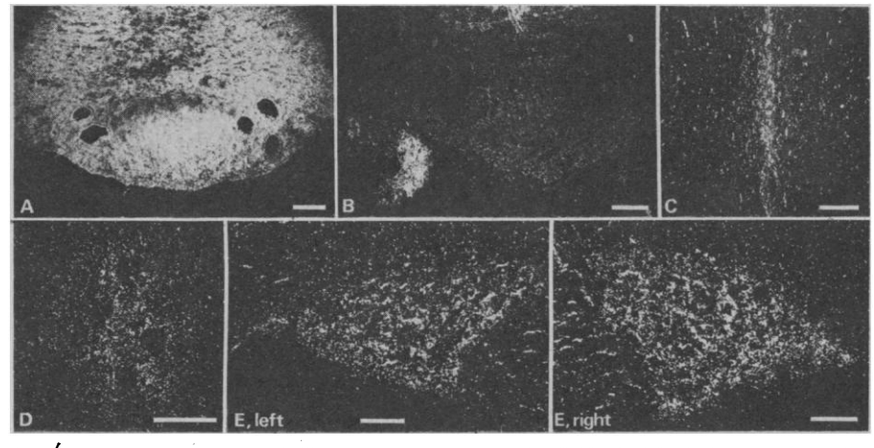

Fig. 1. Anterograde tracing of efferent projections from the SFO: darkfield photomicrographs. (A) Coronal section through the SFO heavily labeled with tritiated leucine. The injector terminated in the ventral hippocampal commissure above the SFO. (B) Horizontal section through the SFO from a rat used as a control. The injection was made into the nucleus triangularis septi (TS); there was no spread of the isotope into the SFO. The boundary of the spread can be seen dorsally in the ventral hippocampal commissure. In the lower right the stria medullaris is heavily labeled, indicating that the TS was hit by the injection since it is known to project by way of the stria medullaris to the habenular nuclei (*21*). (C) Coronal section through the NM. The vertically aligned grain density overlies the NM. (D) Coronal section through the labeled dorsal OVLT. (E, left, right) Coronal sections through the SON. There is an increased density of grains overlying these nuclei, particularly along the ventral boundary. Scale bar, 100 μm.

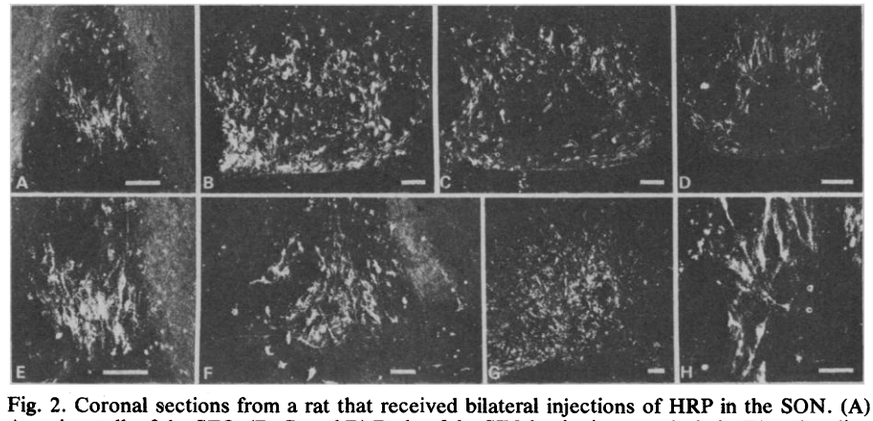

Fig. 2. Coronal sections from a rat that received bilateral injections of HRP in the SON. (A) Anterior stalk of the SFO. (B, C, and D) Body of the SFO beginning anteriorly in (B) and ending caudally in (D). (E) Higher magnification of a segment shown in (A). (F) Dorsal portion of the NM just above the anterior commissure. (G) Coronal section through the OVLT. (H) Higher magnification of the left border of the SFO shown in (D). See text for explanation. Scale bar, 50 μm.

cally aimed for the ventral SON midway along its anterior-posterior extent or for an adjacent control site. The rats were killed 1 or 2 days later and their brains were removed and processed histochemically by the diaminobenzidine procedure (*23*).

The results from one rat injected bilaterally with HRP will be discussed. However, labeling of SFO neurons with HRP was replicated in two other rats; these rats were injected unilaterally and the labeling of SFO neurons was sparser. In the first rat the injections were bilaterally symmetrical and were centered on the ventromedial part of the SON and in the middle third along its anterior-posterior extent. The HRP extended throughout the nucleus and into the immediately surrounding lateral hypothalamus. We observed HRP-positive neurons in the SFO (see Fig. 2). These neurons were labeled with discrete, uniform-sized granules throughout the soma and basal dendrites. The locus of HRP-positive neurons within the SFO shifted depending on the rostrocaudal position in the SFO. The anterior stalk of the SFO has a moderate, uniform distribution of HRP-positive neurons (Fig. 2, A and E), which are predominantly vertically oriented, spindle-shaped, and 10 to 14 μm in width. The distribution of positive neurons disappears from the core of the body (Fig. 2B) and then from its ventral, ventricular boundary (Fig. 2, C and D). The neurons remain along the dorsal border and just medial and adjacent to the large, laterally lying blood vessels of the SFO. These more caudally positioned SFO neurons are also elongated, vertically oriented bipolar types (10 to 18 μm in width) (see Fig. 2H). No HRP-positive neurons were observed in the posterior stalk of the SFO. The dorsal (Fig. 2F) and ventral NM were lightly labeled with HRP-positive neurons (*24*). The OVLT had similar but smaller HRP-positive neurons (see Fig. 2G).

It is unlikely that the labeling of SFO neurons resulted from uptake by terminals outside the SON. First, in the anterograde study no significant labeling above background levels occurred in the area surrounding the SON at the level of the HRP injection. This is suggestive of a lack of SFO terminals just outside the SON. Second, no HRP-positive neurons were observed in the SFO, NM, or OVLT in three control rats in which HRP was injected into the lateral hypothalamic region immediately dorsomedial, dorsal, and lateral to the SON. Other nuclei were labeled in the control rats, indicating the adequacy of the injections as controls. For example, certain septal and anterior thalamic nuclei contained HRP-positive neurons (*25*). This evidence supports the validity of the projections from the SFO, NM, and OVLT.

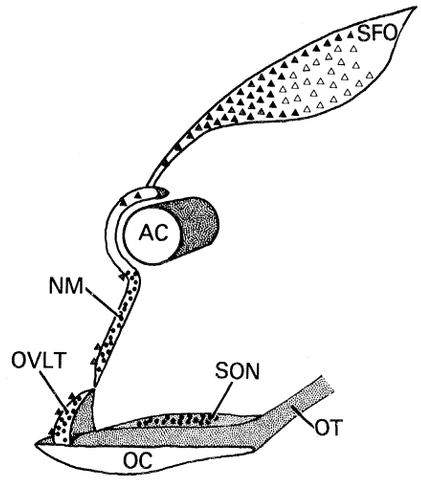

Fig. 3. Schematic (midsagittal view) summarizing the results of the anterograde and retrograde studies. (●) Terminal fields resulting from injections of tritiated leucine into the SFO. (▲) A portion of the HRP-positive neurons resulting from HRP injections in the SON. (△) Labeled neurons out of the plane of the section but in the SFO at its lateral boundary. Abbreviations: *OVLT*, organum vasculosum of the lamina terminalis; *SON*, supraoptic nucleus; *NM*, nucleus medianus; *AC*, anterior commissure; *OC*, optic chiasma; and *OT*, optic tract.

This study firmly establishes the existence of efferent neural connectivity of the SFO and specifies at least three terminal fields: the NM, the OVLT, and the SON (see Fig. 3). This is contrary to negative findings recently reported (*17*), which were most likely due to an injection technique that disrupted the integrity of the SFO (*26*). An important implication of this finding is that the SFO has a capacity for influencing brain function through neural connectivity as well as by local neurosecretion.

The efferent connectivity of the SFO described here is probably incomplete, and there is no evidence that SFO neurons labeled in this study actually serve as a neurological substrate for the circuitry of water balance. However, the sites of termination of SFO projections and the function ascribed to these terminal areas in a variety of studies compel this suggestion and add more evidence that the SFO is within a neural network that mediates water balance both physiologically and behaviorally. The following facts support this contention. The SFO is extremely sensitive to AII for the production of drinking (*8, 13*). Lesioning the SFO eliminates the drinking response to physiologically meaningful doses of AII given intravenously (*8, 13*). The SFO projects to the SON, which produces antidiuretic hormone, the major determinant of water reabsorption by the kidney. Interestingly, AII is excluded by the blood-brain barrier (*27*), but can stimulate the release of antidiuretic hormone by a central action on the brain (*28*). The SFO or OVLT is in a position to act as a receptor for substances excluded by the blood-brain barrier and affect the SON through the neural projections demonstrated here. The SFO also projects to the NM and OVLT, which are two major structures of the anteroventral third ventricular area of the forebrain. Lesion studies show that brain damage to the anteroventral third ventricular area disrupt drinking and SON function. There are transient adipsia and permanent deficits in drinking responses to cellular dehydration and AII, as well as hyperosmolemia and hypernatremia (*15*). Last, the SFO has neurons that are specifically activated by iontophoretically applied AII (*29*).

In conclusion, the SFO and OVLT, the two anterior circumventricular organs of the brain, have efferent neural projections. The connectivity of the SFO is appropriate for its role in mediating AII-induced drinking since the areas of the brain it projects to are involved in the neuronal circuitry of thirst and of the physiological control of body fluid balance (*30*).

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20. Injections intended for the SFO were stereotaxically aimed for the middle anterior dorsal border of the SFO with the beveled surface of the injector facing caudally toward the SFO. This prevented neuronal damage to the SFO and probable disconnection from terminal projection sites, but permitted diffusion of the isotope directly into the SFO from the cannula tip in the ventral hippocampal commissure. The coordinates were: anterior to the ear bars, 8.4 to 8.5 mm; lateral to the sagittal sinus, 0.0 mm; down from the surface of the cortex, 4.5 mm, which was adjusted to lie in the horizontal plane. The solution was infused over a period of 25 minutes, using a Hamilton 1-μl syringe in a Harvard infusion pump. The rats were killed 2 days later, perfused with 10 percent formalin, and their brains were removed and prepared for standard paraffin embedding. Brains were sectioned coronally, parasagittally, or horizontally at 12 μm and the sections were mounted on glass slides and coated with Kodak NTB-3 nuclear emulsion. Sections were exposed for 27 to 30 days and developed. They were counterstained with cresyl violet or thionine and systematically examined by using dark- and lightfield microscopy.
21. L. W. Swanson and W. M. Cowan [*Adv. Behav. Biol.* **20**, 37 (1976)] demonstrated that the caudal septal nuclei (septofimbrial and triangularis nuclei) project through the stria medullaris to the habenular nuclei.
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24. This confirms a report [L. W. Swanson, *J. Comp. Neurol.* **167**, 227 (1976)] in which a projection was observed from the NM to the SON by using anterograde tracing techniques.
25. Sparse HRP-positive neurons were observed in the medial and triangularis nuclei of the septum, the nucleus of the diagonal band, and the thalamic periventricular and parataenial nuclei. Neurons in these areas were labeled in rats with HRP injections into the SON and in control rats with HRP injections into the surrounding hypothalamus, indicating that the terminals could be in the SON or the surrounding hypothalamus, or both. The HRP-positive neurons were also observed in the paraventricular nucleus (PVN) and nucleus circularis of the hypothalamus in SON-injected rats but not in controls. This confirms a projection from the PVN to the SON observed by anterograde tracing [L. C. A. Conrad and D. W. Pfaff, *J. Comp. Neurol.* **169**, 221 (1976)]. A complete description by R. E. Shapiro, R. R. Miselis, and P. J. Hand is in preparation.
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31. We thank R. Berger, B. Gorin, and J. Woolsey for their aid and A. N. Epstein for reading the manuscript. Supported in part by NIH grants RR 54616, RR 07083, and NS 06716 and by a grant from the American Philosophical Society. R.R.M. is an Alfred Sloan Fellow for basic research in the neurosciences.
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3-Methoxy-4-Hydroxyphenethyleneglycol Production by Human Brain in vivo

Abstract. *A direct method has been employed to estimate the rate of production by human brain of 3-methoxy-4-hydroxyphenethyleneglycol, the major metabolite of brain norepinephrine, a brain neurotransmitter. Venous specimens were obtained from the internal jugular vein from ten awake human subjects at a puncture site above the common facial vein, the first major source of extracranial inflow. Arterial specimens were simultaneously obtained from the radial artery. Plasma samples were assayed and a highly significant difference was found in the concentration of the metabolite in plasma coming out of the brain (venous blood) as compared to plasma entering the brain (arterial blood). This venous-arterial difference was calculated to be 0.7 ± 0.1 nanogram per milliliter of blood. Assuming an adult brain weight of 1400 grams and normal cerebral blood flow, it is estimated that the rate of production of 3-methoxy-4-hydroxyphenethyleneglycol by the awake human brain is approximately 597 nanograms per minute or 35.8 micrograms per hour. Urine specimens were also collected from six of these subjects during a period of 1 to 3.5 hours, which bracketed the time the blood samples were obtained. For these six subjects the output of 3-methyoxy-4-hydroxyphenethyleneglycol by whole brain was estimated to be 40.9 micrograms per hour, whereas the rate of its excretion into urine was 64.5 micrograms per hour.*

The functioning of brain noradrenergic systems has been related to a variety of pathological states such as obesity, hypertension, depression, and mania (*1*). For these reasons clinical investigators have been particularly interested in the problem of determining the degree to which levels of plasma or urinary 3-methoxy-4-hydroxyphenethyleneglycol (MHPG), the major metabolite of brain norepinephrine (NE) (*2–4*), may reflect the functioning of noradrenergic neurons within the central nervous system (CNS). Previous approaches to this problem have utilized indirect methods; while these studies, in general, suggest that measures of urinary MHPG in larger mammals may provide an index of NE metabolism, there are points of disagreement and several assumptions and inferences are required to reach this conclusion (*3*, *5*). Interest in this problem led to recent studies with monkeys (*Macaca arctoides*), which have shown that there are consistently higher levels of MHPG and other neurotransmitter metabolites, such as homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), in venous blood of the internal jugular bulb than in aortic blood. When these venous-arterial (V-A) differences are multiplied by the rate of cerebral blood flow (CBF) a measure of the rate of production of a particular neurotransmitter metabolite per 100 g of monkey brain per minute can be obtained (*6*). It therefore seemed that one might also be able to measure a difference in neurotransmitter metabolites between internal jugular vein and arterial blood in humans. This proved to be the case, and the initial results of experiments dealing with this question for MHPG are reported here. To our knowledge this is the first demonstration that it is possible to measure in the awake human brain a V-A difference for a neurotransmitter metabolite. The data obtained were also used to estimate the relationship between the rate of production of MHPG by human brain and the quantity of MHPG found in urine.

In this study venous blood samples (10 ml) were obtained from the internal jugular vein of six volunteers at a puncture site 3 to 4 cm above the common facial vein and at the same time samples (14 ml) were taken anaerobically from the radial artery. Similar samples were obtained in four patients in the course of inserting balloon-tipped flotation-direct-

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