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Histochemical enzymatic survey of 25 "normal" human carotid bodies removed at autopsy from patients three days to 83 years of age revealed a variety of oxidative and hydrolytic enzymes in the lobular and interlobular cells. The intensity of the reactions was greater in the lobular cells. Acid phosphatase was the only enzyme that was limited to the cells comprising the lobules.

Despite many anatomic and physiologic studies on the carotid body there still remains a considerable void in our knowledge of its physiology. Histochemical enzyme stains have been employed on human[13] and animal[1,9,11] carotid bodies but generally have been limited to one or several enzymes. This study is an attempt to explain the metabolic pathways available in the human carotid body by the application of a battery of oxidative and hydrolytic enzyme technics.

Materials

The carotid bodies from 25 unselected patients — 11 women and 14 men three days to 83 years of age (Table I)—were removed 1 to 40 hours after death, frozen in a mixture of dry ice and isopentane and stored for one day to 7 months (average 2 weeks) at -90°C prior to serial sectioning at -20°C. The sections were stored at -90°C for 1 to 22 days prior to staining. The first and last sections from each block were stained with 0.5% thionin and in 20% alcohol to verify the presence of the carotid body. Material was insufficient to perform all enzyme reactions in every case.

Methods

Oxidative enzymes. We used 6μ unfixed sections except for dopa and cytochrome oxidase and monamine oxidase for which 16 and 26μ sections, respectively, were employed. We also employed the block method for dopa oxidase in three cases. Dehydrogenases were studied utilizing incubating medium containing 3- (4,5 - dimethylthiazolyl -2) -2, 5 - diphenyltetrazolium bromide-cobalt-polyvinyl pyrrolidine (MTT-Co-PVP) (.9 cc), 2 mg of DPN or TPN* and 0.1 cc 1 molar substrate (except succinate and barium phosphogluconate substrates which were 0.75 and 0.01 molar respectively). MTT-Co-PVP (.9cc) and 2 mg DPNH or TPNH† were used for the respective diaphorases. The final concentration of the MTT was 0.22 mg/cc. The pH

*Department of Pathology.
†DPN, TPN, DPNH, TPNH & MTT were purchased from Nutritional Biochemical Corp., 21010 Miles Avenue, Cleveland 28, Ohio.
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TABLE I

AGE, SEX AND POSTMORTEM INTERVAL OF CASES STUDIED

No. Cases	Hrs. Postmortem	Age (Years)	Sex	
			M	F
4	7 & 18 H.	< .5	2	2
3	1, 3 & 6 H.	1-10	2	1
2	3 & 6 H.	11-20	2	
0	-	21-30		-
2	4 H.	31-40	1	1
4	2, 3 & 6 H.	41-50	2	2
5	5 - 40 H.	51-60	3	2
2	2 & 13 H.	61-70	-	2
3	1, 3 & 10 H.	71-80	2	1

of the incubating solution was determined with the Beckman 76001 pH meter and adjusted to 7.1 with tris buffer when necessary. The incubating solution was placed on the sections in a moist chamber at 37°C for 30 minutes after which they were fixed 10 minutes in formol saline and mounted in water soluble Paragon medium. The remaining oxidative enzymes were performed by the technic given in Table II. Rat liver and kidney were used as positive controls and were exposed with the cases under investigation to solutions of MTT-Co-PVP and TPN, MTT-Co-PVP and DPN, MTT-Co-PVP and water for 30 minutes at 37°C in addition to the MTT-Co-PVP, coenzyme, substrate solutions. In one case 0.9 cc of nitro-blue terazolium** in O.1M phosphate buffer at pH 7.4 (0.4 mg/1 ml) was used in place of the MTT-Co-PVP, and in another the reactions were carried out with both MTT and nitro BT.

Hydrolytic enzymes. We used unfixed frozen sections for this group except the diazo dye method for alkaline and acid phosphatase and esterase. Fifteen minutes postfixation in graded acetone at 4°C was used for the phosphatases and 15 minutes postfixation in formalin vapor at room temperature for esterases. We studied the effect of inhibitors and activators on the alkaline phosphatases in six cases by incorporating .0025M of Na parachlorømercuribenzoate (PCMB) or .0025M cysteine HCl in the incubating medium or by incubation of the sections at room temperature for 4 hours in 1% EDTA (pH_4) or 0.1$MgCl_2$ in 0.2M acetate buffer (pH_4) prior to exposure to the substrate solution.[5,6,10] The first two chemicals were used in sequence.

**Nitro BT was purchased from Dajac Laboratory, Philadelphia, Pa.

Two sections from each case were first exposed to the PCMB-adenosine triphosphate (ATP) for 15 minutes, and then one of the pair was placed in the cysteine ATP substrate for 45 minutes.

Results

It was often difficult to determine the identity of the cells in which the enzymatic activity occurred. It was impossible to distinguish the lobular and perilobular cells

TABLE II

ENZYMATIC REACTIONS OF HUMAN CAROTID BODY

	Lobular Cells	Interlobular Cells
DPNH[a]	4+ (12)	4+ (12)
TPNH	4+ (12)	4+ (12)
Succinic Dehydrogenase	0-3+ (7/10)	0-1+ (7/10)
Dehydrogenases		
Lactate DPN	3-4+ (12)	2-4+ (12)
TPN	0-3+ (7/11)	0-2+ (7/11)
Malate DPN	1-4+ (12)	1-2+ (12)
TPN	0-3+ (5/11)	0-1+ (5/11)
Isocitrate DPN	0-2+ (6/11)	0-1+ (5/11)
TPN	0-1+ (3/12)	0-1+ (3/12)
Glutamate DPN	0-2+ (6/13)	0-1+ (6/13)
TPN	0 (12)	0 (12)
α Glycerophosphate DPN	0-2+ (11/13)	0-1+ (11/13)
TPN	0-2+ (2/12)	0-1+ (2/12)
Alcohol DPN	0-3+ (8/11)	0-1+ (8/11)
TPN	0 (13)	0 (13)
β Hydroxybutyrate DPN	0-3+ (6/13)	0-2+ (6/13)
TPN	0 (13)	0 (13)
6 Phosphogluconate DPN	0-3+ (6/13)	0-1+ (6/13)
TPN	0 (13)	0 (13)
Glucose - 6 - Phosphate DPN	0-2+ (6/13)	0-1+ (6/13)
TPN	0-3+ (8/13)	0-1+ (8/13)
MTT - DPN	0	0
MTT - H_20	0	0
MTT - TPN	0	0
Oxidases		
Cytochrome 12 (p. 901, Burstone)	1-3+ (10)	0-1+ (5/10)
Monamine 12 (p. 906)	0-1+ (6/11)	0 (11)
Dopa 12 (p. 905)	0 (15)	0 (15)
Peroxidase 2 (Washburn)	0 (5)	0 (5)

TABLE II (CONT'D)

Hydrolytic	Lobular Cells		Interlobular Cells	
Acid Phosphatase (azo dye pH 5.2) [12 (p. 882)]	1-2+	(10)	0	(10)
Alkaline Phosphatase (azo dye pH 8.3) [12 (p. 874)]	0	(9)	0	(9)
Alkaline Phosphatase (Cobalt, pH 9.2 & 7.5) [12 (p. 868)]	0	(16)	0	(16)
Esterase [12 (pp. 886, 888 α naphthyl)]	4+	(8)	4+	(8)
Leucine aminopeptidase [12 (p. 913)]	0-2+	(8/10)	0-1+	(8/10)
ATPase [12 (p. 877 Padykula - Herman)] (pH 9.2)	3-4+	(21)	0-1+	(21)
(pH 7.5)	1-2	(21)	0-1+	(21)
Glucose - 6 - Phosphatase [12 (p. 880)]	1-2+	(2/4)	0	(4)
5-Nucleotidase [12 (p. 874)] (pH 9.2)	3-4+	(21)	0-1+	(21)
(pH 7.5)	1-2+	(21)	0-1+	(21)
Aryl sulfatase [12 (p. 914) c]	4+	(8)	4+	(8)
Glutaminase [8] I $\bar{c}$ substrate	3-4+	(5)	2-3+	(5)
$\bar{s}$ substrate	0	(5)	0	(5)

a. DPN, TPN, DPNH, TPNH, di- and triphosphopyridine nucleotides and their reduced forms; MTT, 3 - (4, 5 - dimethylthiazolyl - 2) - 2, 5 diphenyltetrazolium bromide; dopa, 3 - 4 - dihydroxyphenylalanine; ATPase, adenosine triphosphatase.

b. Parentheses indicate total cases studied (denominator) and positive cases (numerator).

c. 6 - Bromo - 2 - naphthyl sulfate used as a substrate.

as Costero described them using silver stains. The perilobular cells in the following description are not to be taken therefore as representing Costero's perilobular cells.[3,4] Enzymatic staining, which was graded 0-4+, was of greater intensity and more diffuse in the lobular cells (Zellballen) than in the elongated cells in the loose interlobular tissue (Fig. 1). All enzymes studied gave reactions in perilobular and/or lobular cells (Table II) with the exception of alkaline phosphatase, peroxidase and dopa oxidase. The greatest variation in activity was noted among the dehydrogenases;

the diaphorases and lactate and malate dehydrogenases gave the strongest and most consistent reactions (Fig. 2). The coenzyme dependency of the dehydrogenases was similar to that previously described except for the 6-phosphogluconate and glucose-6 phosphate dehydrogenases which gave reactions with coenzymes other than those generally considered best for their demonstration; 12(p573) reactions were more often positive with DPN than with TPN. The results using nitro-BT varied little from those with MTT. In one case where both tetrazolium salts were employed, there was slightly greater activity with nitro BT except for the glucose-6 phosphate, 6 phosphogluconate and alpha glycerophosphate dehydrogenases. However, a weak reaction was noted with the controls—nitro BT-DPN and nitro BT-TPN solutions devoid of substrate—which was not observed with similar MTT controls.

Among the oxidases examined, only cytochrome and monamine oxidase was detected. The former was consistently present and located principally in the lobular cells (Fig. 3), while the latter was less frequently encountered producing a weak reaction that was difficult to localize but appeared to be in the lobular cells and some nerves. Peroxidase and dopa oxidase reactions occurred only in the occasional neutrophils present in some cases. The block method for dopa oxidase was also negative.

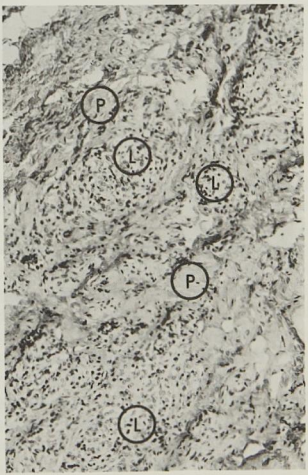

Figure 1

Human carotid body demonstrating well-defined cellular lobules with capillaries (L) and the intervening spindle cell vascularized fibrous tissue with varying size nerves (P). H & E x 115.

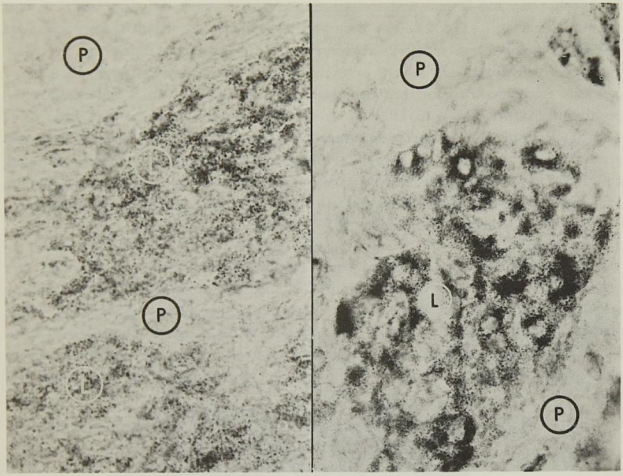

Figure 2

Succinic (A) and alcohol (B) dehydrogenase activity is present in the lobular cells (L) and negative or weak in the perilobular cells (P). Coenzyme DPN and tetrazolium salt MTT x 720

Hydrolytic enzyme reactions in the lobular and interlobular cells also varied in their intensity, being strongest in the lobular cells. Esterase, arylsulfatase and glutaminase I were the most intense in both cell types while the reaction of leucine aminopeptidase and the phosphate splitting enzymes were less intense and unevenly distributed. They were most notable in the lobular cells. The acid phosphatase and glucose-6 phosphate reaction was confined to the lobular cells except for a rare isolated acid phosphatase-positive cell of uncertain identity located in the perilobular tissue.

Strong reactions were present in the endothelial cells using naphthol AS-MX phosphate (pH 8.3), beta glycerophosphate, ATP or a 5 adenylic acid substrate at pH 9.2 (Fig. 4). At pH 7.5, however, only a weak reaction was observed — consistently with ATP and infrequently with 5 adenylic acid. Both lobular and interlobular cells were negative with naphthol AS-MX phosphate (pH 8.3) and beta glycerophosphate (pH 9.2) except for an occasional unidentified isolated cell in the interlobular tissue. Cytoplasmic and nuclear staining was observed only with ATP and 5 adenylic acid, being strong at pH 9.2 and weak to moderate at pH 7.5 (Figs. 5 and 6). The lobular cells were intensely stained in contrast to the infrequent single interlobular cells which

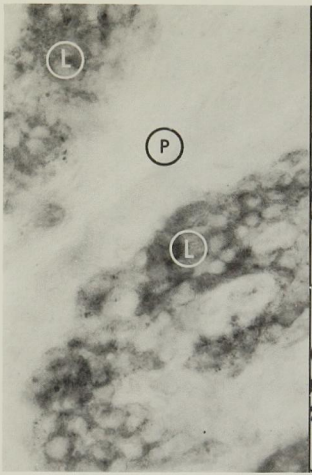

Figure 3

Cytochrome oxidase activity is strongest in the lobular cells (L). Perilobular cells (P) are negative or weakly reactive x 560

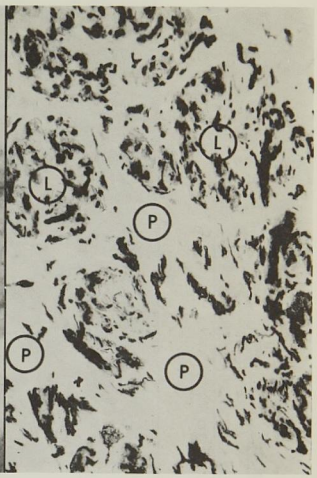

Figure 4

Alkaline phosphatase activity (pH 9.2) is confined to the vascular endothelial cells in the lobular (L) and perilobular tissue (P) x 70

sometimes had long positive staining cytoplasmic prolongations. Small intralobular nerve fibers, particularly at the periphery of the lobules, were more intensely stained using ATP than 5 adenylic acid while the larger nerves were positive only with ATP; the reactions being stronger at pH 9.2 than pH 7.5. The perineural fibrous tissue showed a consistent strong 5-nucleotidase reaction at pH 9.2 which was negative or weak at pH 7.5, and with other substrates.

Inhibition of the alkaline phosphatases varied with the inhibitor used (Table III). Endothelial cell staining at pH 9.2 was abolished by EDTA or $MgCl_2$ acetate buffer using 5 adenylic acid or beta glycerophosphate and was markedly diminished using ATP. Staining of the axons and lobular cells at pH 9.2 and 7.5 was somewhat inhibited by EDTA or $MgCl_2$ acetate buffer, using the 5 adenylic acid substrate, and markedly inhibited by PCMB or $MgCl_2$ acetate buffer using the ATP substrate (Fig. 7).

Cysteine, used in the second incubation following PCMB-ATP, reactivated the ATPAse that had been inhibited by PCMB; 5-nucleotidase and nonspecific alkaline phosphatase activity were not affected by PCMB.

TABLE III

EFFECT OF INHIBITORS ON ALKALINE PHOSPHATASES OF HUMAN CAROTID BODY

Substrate		Reaction without Inhibitor		Reaction with Inhibitor					
				PCMB		EDTA		MgC12 Acetate Buffer	
		pH 9.2	pH 7.5	pH 9.2	pH 7.5	pH 9.2	pH 7.5	pH 9.2	pH 7.5
ATP	Endothelial cells	4+	0-1+	4+	0-1+	1+	0-1+	1+	0-1+
	Small axons*	0-4+	0-2+	0-1+	±	0-4+	0-2+	0-1+	±
	Lobular cells+	2-4+	1-2+	0-1+	±	2-4+	1-2+	0-1+	±
5 Adenylic acid.	Endothelial cells	4+	N	4+	N	N	N	N	N
	Small axons	0-3+	0-1+	0-3+	0-1+	N	N	N	N
	Lobular cells+	2-4+	1-2+	2-4+	1-2+	N	N	N	N
β glycerophosphate	Endothelial cells	4+	N	4+	N	N	N	N	N
	Small axons	N	N	N	N	N	N	N	N
	Lobular cells	N	N	N	N	N	N	N	N

* Axons of large nerves were + only with ATP and their staining was affected similarly by the inhibitors.

\+ The few perilobular cells which stained were less intensely stained but affected similarly by the inhibitors.

N = negative reaction.

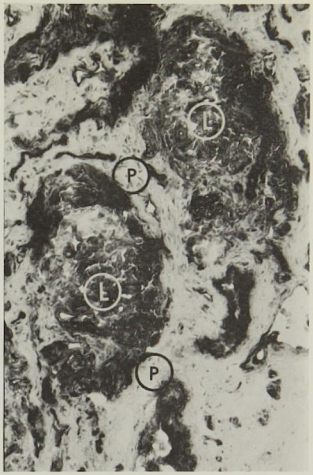

Figure 5

Adenosine triphosphatase activity (pH 9.2) is more diffuse than is the nonspecific alkaline phosphatase (Fig. 4) due to activity which is most marked and frequent in the lobular cells (L) and less intense and sporadic in the perilobular cells (P) and in the small axons which are not seen at this magnification. Endothelial cell staining is similar to that with beta-glycerophosphate substrate x 150

Discussion

Although it was impossible to localize enzymatic activity to the cell types that have been demonstrated by silver technics, it could be established as being lobular or perilobular in most instances. And, although there was variation in the intensity of the dehydrogenase reactions, the presence of a moderate to strong reaction in a number of the cases indicates the carotid body has enzymes necessary for carbohydrate, lipid, and amino acid utilization and those involved in the Krebs' cycle. Failure to demonstrate dopa oxidase activity is not in keeping with the theory of epinephrine and norepinephrine elaboration by the carotid body since that enzyme is necessary in the known pathway for catecholamine production. Its absence, however, does not rule out the formation of these amines by the carotid body since they may be formed via another pathway not requiring dopa oxidase. Also, the enzyme may be present but in insufficient quantity for histochemical detection. Dopa oxidase has been detected in autopsy tissues and it was present in the control sections in the present

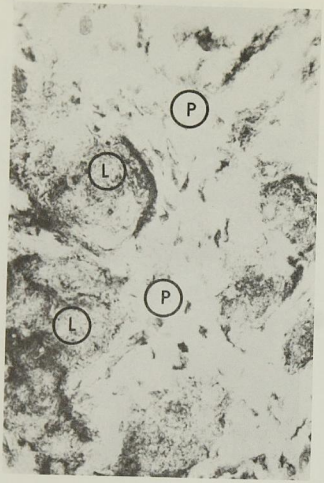

Figure 6

5-nucleotidase activity (pH 7.5) is similar in distribution to ATPase (Fig. 5). Reaction was much stronger at pH 9.2 x 90

study, making it unlikely that faulty technic or postmortem change was responsible for the negative reactions. The presence of minimal monamine oxidase activity is of interest because of its role in the degradation of epinephrine, norepinephrine and serotonin, all of which are believed to be present in the carotid body.[7]

The presence of 5-nucleotidase and ATPase in the carotid body and their distinction from nonspecific alkaline phosphatase activity, which can hydrolize substrates of both the former enzymes, is indicated by the absence of nonspecific alkaline phosphatase activity and by the presence of 5-nucleotidase and ATPase activity at pH 7.5. It is also indicated by the differences in staining reaction with PCMB, cysteine or $MgCl_2$ acetate buffer inhibitors where they are employed with the respective substrates for these enzymes. ATPase is inhibited by PCMB and $MgCl_2$ acetate buffer, activated by cysteine and unaffected by EDTA.[5,6,10] Nonspecific alkaline phosphatase and 5-nucleotidase are inhibited by EDTA and $MgCl_2$ acetate buffer and unaffected by PCMB, while pyrophosphatase activity is not affected by any of these compounds.[5,6]

The positive reaction noted with glucose-6-phosphate may represent nonspecific

Figure 7

Staining of lobular cells and axons has been almost completely blocked by combining PCMB with ATP substrate (pH 9.2). Compare with Fig. 5. Endothelial cell staining of vascular channels is unaffected x 150

acid phosphatase activity rather than glucose-6-phosphatase activity since the reaction site was similar to that of the nonspecific phosphatase.

In evaluating the inconsistent enzymatic reactions, postmortem interval and tissue storage time prior to staining, the patient's age and disease were considered. While the postmortem interval and storage time could contribute to inconsistent results, it appears unlikely since strong reactions were noted in some instances when these times were maximal and weak reactions when they were minimal. Since 45% of the negative results were among patients 3½ weeks to 3½ years of age (representing 20% of the cases studied), carotid body immaturity might be considered as another cause of the inconsistent reactions. If immaturity does play a role, it is probably a partial one since the same positive enzymatic reactions were found in some of this groups as in the older patients. Study of a larger group of patients in the first five years of life might reveal time differences in which the various enzymes make their appearance.

A relationship between the patient's illness and the enzyme reactions could not be established, but such data would be of no significance since the number of patients dying of the same disease was too small for statistical evaluation.

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