Histochemical Demonstration of Aryl Sulfatase[1]

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In a previous search for a suitable substrate to demonstrate sulfatase activity histochemically, it became apparent that enzymatic activity was sufficiently low in mammalian tissues to require hours of incubation, that the optimum pH was too low for rapid coupling with diazonium salts, and that the enzyme itself was readily diffusible. By the selection of a colorless substrate, made soluble by the sulfate group, that would yield a naphthol sufficiently insoluble in water to remain at the site of enzymatic hydrolysis, coupling to a blue azo dye was made possible at higher pH afterward (*1*). The problem was also made easier by the discovery that aryl sulfatase activity in rodents withstood fixation of tissue in cold 10% neutral formalin (*1, 2*). This was not the case with monkey and human tissues. Whereas diffusion of the enzyme produced artifacts after incubation of fresh frozen sections for more than 2 hr, similar diffusion artifacts usually did not appear until after 3 hr of incubation of formalin-fixed tissue.

Following exploration of several substituted naphthols, 6-bromo-2-naphthol provided the most satisfactory sulfate ester (*1*). However, incubation for 12–24 hr was necessary in order to produce tissue sections with enough stain (*1*). Following the synthesis of 6-benzoyl-2-naphthol (*3*), it was found that its sulfuric acid ester was hydrolyzed enzymatically only slightly faster than the bromo analog. However, the color density of the azo dye produced with tetrazotized diorthoanisidine was about twice as great per mole in the case of the benzoyl naphthol as with the bromonaphthol. This enabled shortening the incubation period to 2–4 hr with tissues of high enzymatic activity and 6–12 hr with others. With formalin-fixed tissue, diffusion of the enzyme was usually not significant in 3 hr. With tissues requiring longer incubation and with all unfixed tissues, enzyme diffusion was prevented only by increasing the salt content of the medium. This procedure has been necessary in the case of other diffusible enzymes: esterase (*4, 5*), acid phosphatase (*6*), and β-D-galactosidase (*7*). In the case of β-D-glucosidase, all attempts to control enzyme diffusion by increasing the salt content of the medium failed because of inhibition of enzymatic activity (*8*).

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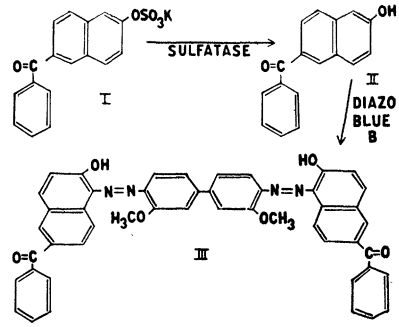


FIG. 1.

Sulfatase activity in tissue sections was slightly inhibited by 3% sodium chloride, whereas fresh tissue homogenates were not affected.

The substrate,[4] potassium 6-benzoyl-2-naphthyl sulfate (Fig. 1, I), was prepared from 6-benzoyl-2-naphthol (II) (*3*) as follows: Chlorosulfonic acid (7.5 ml) was added cautiously in small portions to anhydrous pyridine (50 ml) in an Erlenmyer flask surrounded by an ice bath. In the course of a vigorous reaction a white crystalline mass of sulfur trioxide formed. A solution of 6-benzoyl-2-naphthol (25 g) in anhydrous pyridine (50 ml) was added all at once. The mixture was stirred and heated to the boiling point, where it was held for 2–3 min. The solution was cooled and transferred to a 3-liter beaker with absolute methanol (100 ml). A saturated solution of methanolic potassium hydroxide was added in small portions until the solution was alkaline. A darkening of the solution occurred on passing the neutral point. A large excess of alkali was avoided. A white heavy precipitate separated during this procedure. Dry ether (2 liters) was added, and the salts were collected with suction, washed with

TABLE 1

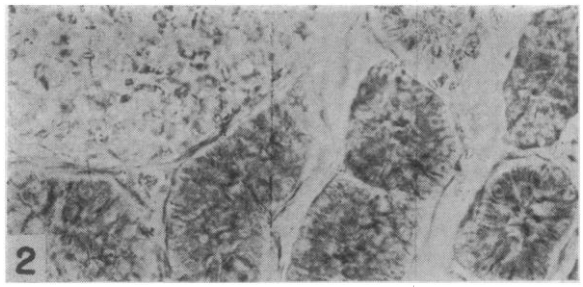
ARYL SULFATASE ACTIVITY OF ORGANS FROM 4 RATS MEASURED BY INCUBATING AQUEOUS EXTRACTS OF HOMOGENATES (10 MG) WITH POTASSIUM 6-BENZOYL-2-NAPHTHYL SULFATE FOR 4 HR AT PH 6.1 AND 37° C

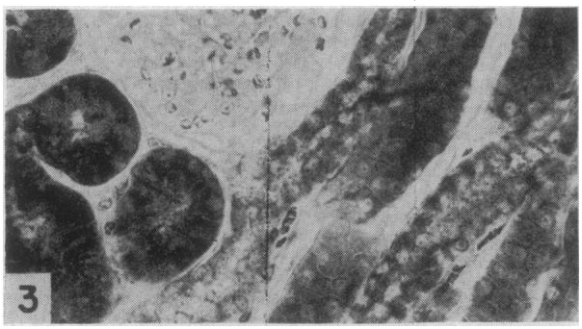
Organ	Micrograms 6-benzoyl-2-naphthol formed in 4 hr	
	Range	Average
Liver	36–64	45
Kidney	22–32	28
Pancreas	18–40	26
Adrenal	16–18	18
Testis	6–10	7
Small intestine	6–8	7
Lung	6–8	7
Large intestine	1–9	6
Brain	4–7	6
Spleen	2–10	5
Heart	4–7	5
Skeletal muscle	4	4
Salivary gland	2–6	4
Stomach	2–4	3

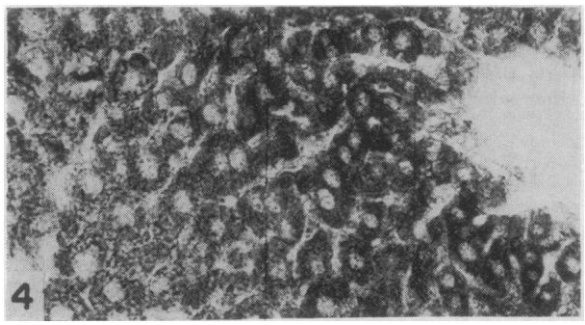
[4] The substrate and reagents may be purchased from Dajac Laboratories, Monomer-Polymer, Inc., 511 Lancaster St., Leominster, Mass.

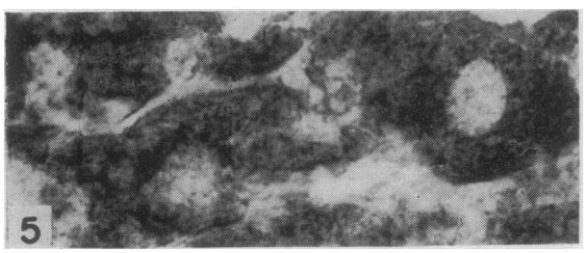
ether, and dried. The white powder was suspended in 100 ml of cold distilled water for 30 min in order to remove potassium chloride and potassium sulfate (inhibits aryl sulfatase). The poorly soluble potassium 6-benzoyl-2-naphthyl sulfate was collected with suction, washed with a little cold water, acetone, and ether, and dried; yield 26 g (74%).

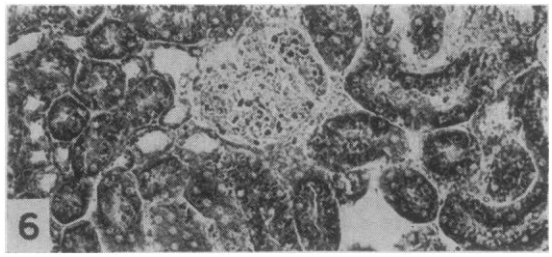
By means of a calibration curve prepared with 6-benzoyl-2-naphthol and tetrazotized diorthoanisidine (Diazo Blue B) in the presence of tissue protein, aryl sulfatase activity was determined colorimetrically in extracts of fresh tissue homogenates. Although details of the procedure will be published elsewhere, enzymatic activity of several organs of the rat is given in

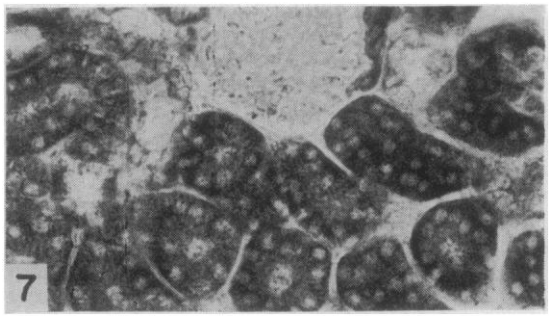


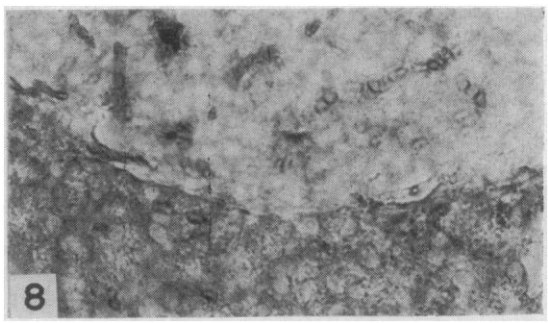


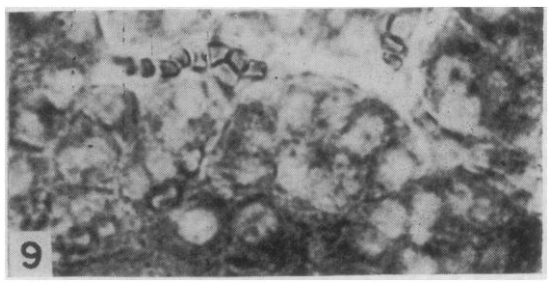












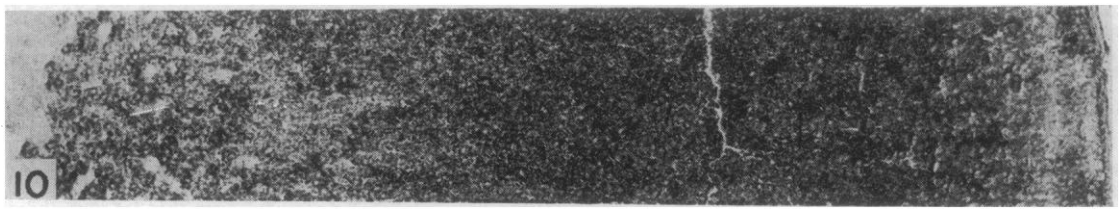


Table 1. The highest activity was found in liver, kidney, pancreas, and adrenal gland.

Phenol sulfatase demonstrated colorimetrically with potassium *p*-nitro phenyl sulfate has been reported highest in liver, adrenal gland, and kidney of the rat (*9*). Phenol sulfatase demonstrated gravimetrically with potassium phenyl sulfate was found in liver, kidney, muscle, and brain of rabbits (*10, 11*); kidney, brain, liver, duodenum, spleen, lung, muscle, small intestine, and pancreas of man (*12*); and kidney and brain of pig, calf, and rabbit (*13*).

For the histochemical procedure, tissues were obtained from freshly exsanguinated animals, at operation, or at autopsy on man. It was found that animal tissues could be stored at 4° C for 24–48 hr without serious loss of enyzmatic activity. The tissues of rat, mouse, and hamster withstood fixation in cold, neutral, 10% formalin for as long as 120 days with some loss (10–50%) in enzymatic activity. In the monkey and man, however, sulfatase activity of liver, kidney, and pancreas was nearly completely destroyed by cold formalin within 24 hr. Prior exposure of liver and kidney of the mouse and rat to other fixatives at 4° C for 4 hr resulted in loss of enzymatic activity as follows: acetone or absolute ethanol, 20–40%; ethylene glycol dimethyl ether, 60–80%; and absolute methanol, 90–100%.

After fixation in formalin the blocks of tissue (3–5 mm thick) were washed twice in 0.85% sodium chloride solution for 30 min, and frozen sections (15–20 μ) were cut with a freezing microtome, and 8–10 sections were transferred directly from the knife and floated on 10 ml of the substrate solution. They were then transferred to 20 ml of the substrate solution in a beaker for incubation. Tissues from monkey and man were not fixed, and frozen sections (10–20 μ) were cut according to the Coons' modification of the Linderström-Lange technique (*14*), mounted on glass slides, and air-dried. The slides were then incubated in the substrate solution made hypertonic according to the procedure given below. The enzymatic reaction was somewhat slower in sections which were mounted on glass slides and air-dried.

The substrate solution was prepared by dissolving potassium 6-benzoyl-2-naphthyl sulfate (25 mg) in hot, 0.85% sodium chloride solution (80 ml) and adding 0.5 *M* acetate buffer, pH 6.1 (20 ml). The solution was stable and could be stored at room temperature for at least 15 days. Substrate solution was made hypertonic by the addition of solid sodium chloride (2.6 g) to each 100 ml of substrate solution. To prevent extensive histologic distortion, the sections were immersed briefly (2–5 min) in three solutions of sodium chloride (0.85%, 1%, and 2%) prior to incubation in the hypertonic substrate solution (*7*).

Tissues with greater enzymatic activity were incubated at 37° for 2–3 hr in the substrate solution, and tissues with low activity were incubated for 4–16 hr in the hypertonic substrate solution. All fresh frozen sections were also incubated in the hypertonic substrate solution. Sections from each organ were incubated in separate containers.

Following incubation the sections were washed twice (10–15 min each) in cold saline (fresh tissue) or water (formalin-fixed tissue). Sections that had been incubated in hypertonic substrate solution were returned to 0.85% sodium chloride solution after prior immersion in graded concentrations of the salt. After washing, the sections were transferred to a cold (4° C), freshly prepared solution of tetrazotized diorthoanisidine (1 mg/ml) in 0.05 *M* phosphate buffer, pH 7.6. The sections were gently agitated for 5 min. They were washed 3 times in cold 0.85% sodium chloride solution and water (15 min each) and mounted on a glass slide with glycerine or glycerogel. The cover slip was sealed with fingernail polish.

Coupling of 2 molecules of 6-benzoyl-2-naphthol with the tetrazonium compound yielded a deep blue dye (Fig. 1, III), whereas monocoupling produced a red dye. However, solutions of the blue dye in many organic solvents and in lipoid were red by transmitted light. The intensity of the pigment, as well as the color, was related to the degree of enzymatic activity. Tissues with high sulfatase activity stained blue, and those with low activity stained red or purple. The blue dye was stable in these sections for only about 15 days at 4° C. Thereafter, the blue pigment changed to purple and finally red, and the localization of the dye became fuzzy. This process was accelerated when sections were

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FIGS. 2–10 : FIG. 2. Dog kidney fixed in formalin for 24 hr. Sulfatase activity is absent. Incubation period, 24 hr ; no filter. × 350.

FIG. 3. Dog kidney fixed in formalin for 24 hr (adjacent section to Fig. 2). Incubated 24 hr together with sections of fresh rat kidney. Sulfatase activity is present in the section of dog kidney (*cf.* Fig. 2). Staining is confined to the cytoplasm of tubular epithelium, presumably as a result of diffusion of the enzyme from the sections of rat kidney. Green filter. × 350.

FIG. 4. Mouse liver fixed in formalin for 24 hr. Incubated 4 hr. Enzymatic activity is highest (blue) in the periportal area (right) and is lower in the surrounding region (red). Hepatic cell nuclei, walls of blood vessels, and erythrocytes are free of sulfatase. Green filter. × 375.

FIG. 5. Rat liver fixed in formalin for 24 hr. Incubated 4 hr. Cytoplasm localization of the enzyme is shown. Green filter. × 950.

FIG. 6. Mouse kidney fixed in formalin for 24 hr. Incubated for 8 hr in hypertonic substrate solution. Cytoplasm of tubular epithelium has sulfatase activity. Cell nuclei, glomeruli, and blood cells in the glomeruli are unstained. Green filter. × 350.

FIG. 7. Rat kidney fixed in formalin for 24 hr. Incubated for 4 hr. Same localization of sulfatase as Fig. 6. Green filter. × 375.

FIG. 8. Rat pancreas fixed in formalin for 48 hr. Incubated in hypertonic substrate for 12 hr. Islet of Langerhans (top) has much less sulfatase activity than acinar cells. Erythrocytes in capillaries of the islet are unstained. Green filter. × 400.

FIG. 9. Unfixed human pancreas (surgical biopsy). Incubated in hypertonic substrate for 4 hr. Cytoplasmic localization of sulfatase activity is shown. Green filter. × 850.

FIG. 10. Rat adrenal gland fixed in formalin for 96 hr. Incubated for 4 hr. All zones of the adrenal cortex are stained. The adrenal medulla (left) and transitional zone (right) show very little sulfatase activity. Erythrocytes are unstained. Green filter. × 150.

stored at room temperature (2 days) or exposed to heat (minutes). The sections were redder with tissues containing much lipoid. The use of higher than recommended concentrations of the tetrazonium compound or coupling above 4° C resulted in the formation of more of the red than of the blue pigment.

Diffusion of the enzyme was carefully checked in every batch of sections by testing for the naphthol in the supernatant incubation medium. In order to do this the pH was elevated to 7.4 to 7.6 with solid sodium bicarbonate, and tetrazotized diorthoanisidine was added. Significant diffusion was shown by production of the azo dye. Under conditions that prevented diffusion of the enzyme, or when the incubation period was sufficiently short, only a slight pink coloration was produced in the supernatant; otherwise the color was deep red to blue. The importance of diffusion of the enzyme was shown in experiments in which frozen sections of fresh or formalin-fixed dog kidney, which normally do not have sulfatase activity (Fig. 2), were incubated in the same flask (10 ml) with an equal number of fresh frozen sections of rat kidney for 4–24 hr. The sections of dog kidney were intensely stained (Fig. 3), and the pattern of distribution was similar to that of rat kidney. When the sections of fresh dog kidney were incubated separately in an aqueous extract of homogenized rat liver or kidney for 4 hr at 37° and rinsed briefly, subsequent incubation in substrate medium for 4 hr gave a similar distribution, but paler and less distinct stain in the dog kidney. Longer washing prior to incubation in the substrate, or the use of formalin-fixed sections of dog kidney, resulted in even less staining. These experiments indicated that diffusion of the enzyme rather than diffusion of naphthol was largely responsible for the phenomenon.

That diffusion of the naphthol from sections was not significant was shown in other experiments in which 8–10 sections containing the naphthol released by enzymatic hydrolysis intracellularly were suspended in 20 ml of wash fluid and gently agitated for 30–40 min. Naphthol could not be demonstrated in the wash fluid. Furthermore, it was shown that movement of naphthol from the supernatant fluid into the section was not likely to produce serious artifacts, since formalin-fixed sections, incubated for 4 hr at 37° C in a saturated aqueous solution of 6-benzoyl-2-naphthol, and subsequently washed and coupled with the tetrazonium compound, showed only a diffuse, indistinct, pale-purple stain in the cytoplasm of epithelial cells. Longer incubation or incubation in 15% ethanol solution containing more of the naphthol (40 μg per ml) produced more intensely, but diffusely and irregularly stained, sections. Fresh frozen sections were stained more intensely than formalin-fixed sections. The naphthol could be extensively removed by washing them prior to coupling, but it was less readily removed if it had been liberated intracellularly by enzymatic hydrolysis. This suggests that the naphthol in the diffusion experiments may be attached to different portions of the cell; hence it was necessary to take the precautions described above in order to avoid artifacts caused by diffusion of enzyme and naphthol.

The four organs (rat) found to have high enzymatic activity by colorimetric determination (Table 1) were studied histochemically in the mouse, rat, hamster, guinea pig, rabbit, dog, rhesus monkey, and man. The intensity of staining of each organ varied among individuals of each species and particularly from one species to the next, but the pattern of distribution for each organ was similar in all species. In general, enzymatic activity was present in the cytoplasm of epithelial cells and was absent from cell nuclei, smooth muscle, and connective tissue. Although elastic tissue of some blood vessels (human pancreas) was well stained, this presumably was not due to enzymatic hydrolysis, because other naphthols, particularly those with electron-attracting groups which enhance the acidity of the hydroxyl group, have shown an affinity for elastic tissue (*15*).

The kidney and liver of the mouse, rat, monkey, and man, and the kidney, but not the liver, of the hamster stained intensely. These organs showed very little activity in the guinea pig, rabbit, and dog. A high order of activity was noted in the pancreas of the rat, hamster, monkey, and man, but not in the mouse, guinea pig, rabbit, and dog. The adrenal gland of the rat and hamster stained less intensely than the corresponding kidney and pancreas. Mouse adrenals showed even less activity than in the rat and hamster, and very little activity was seen in the adrenals of guinea pig, rabbit, dog, monkey, and man. Organs with a very low order of sulfatase activity showed a diffuse, light-purple stain after long incubation (16–24 hr). Localization of the enzyme was unsatisfactory in these sections because of enzyme diffusion and lipoid solubility of the azo dye.

The cytoplasm of epithelial cells of the liver (Fig. 5) was stained a diffuse blue to purple color. More activity was noted in the hepatic cells of the periportal (Fig. 4) and pericentral regions of the liver lobule. In the intermediate areas the cells were red and purple.

In the kidney cortex (Figs. 6, 7) the cytoplasm of the epithelial cells of all parts of the nephron except the glomerulus was stained intensely blue or purple. The glomerulus and blood cells in the capillaries of the glomerulus were unstained. The proximal and distal convoluted tubules were darker than other parts of the nephron. Collecting tubules and other elements of the medulla were not stained.

In the pancreas (Figs. 8, 9) the cytoplasm of acinar cells was stained purple, and the pigment was concentrated in the perinuclear reigon. In a few acini, staining was most marked in the basilar portion of the cells. The islets of Langerhans stood out from the surrounding acinar cells because of their much paler stain (Fig. 8). Epithelial cells of the ducts were also less intensely colored than the acinar cells, and the pigment was confined to the apical portion of these cells.

Sulfatase activity was present in all parts of the adrenal cortex (Fig. 10). Color was most intense in the zona fasciculata and least in the transitional zone.

cytoplasmic staining was homogeneous except in the zona reticularis, where red and purple droplets stood out against the red cytoplasmic background, perhaps because of solution of the dye in fat droplets. Cell nuclei and the capsule of the gland were unstained, and cells of the adrenal medulla stained very lightly.

Sulfatase activity was not demonstrable in rat and mouse collagen, cartilage, bone, and blood cells.

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The Destruction of Epinephrine by the DOPA-Oxidase System of Ocular Tissue[1]

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The principal enzyme systems responsible for the inactivation of epinephrine in the body are generally considered to be monoamine oxidase and cytochrome oxidase (*1–3*); in addition, epinephrine is conjugated in the liver. In seeking a possible enzymatic basis for the observation that mydriatics are more effective in light than in dark eyes (*4*), assays were conducted on homogenates of the irides and ciliary bodies of pigmented and albino rabbits for enzymes which might be involved in the destruction of the cholinergic or adrenergic mediators. No significant differences were found between the two groups in respect to monoamine oxidase (*5*), cytochrome oxidase, succinic dehydrogenase (*6*), cholinesterase (*7*), or reducing substances (*8*), employing the specific substrates called for by the methods listed. Dihydroxyphenylalanine (DOPA) was oxidized by the pigmented but not by the albino homogenates; DOPA-oxidase has previously been demonstrated in the pigment granules of steer irides (*9*). It appeared that this enzyme was responsible for

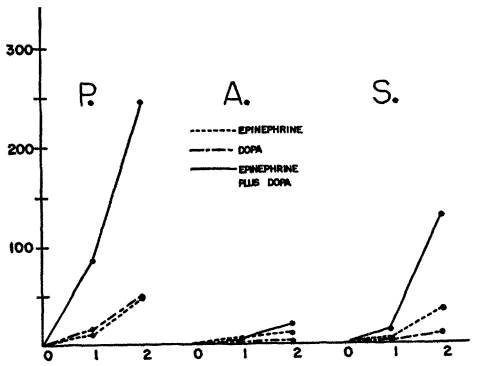

FIG. 1. Rates of oxygen uptake of homogenates of pigmented (*P.*) and albino (*A.*) rabbit iris-ciliary bodies, and spontaneous oxygen uptake (*S.*), in the presence of epinephrine (2.75 mg), D-L-DOPA (2.0 mg), and epinephrine plus DOPA. Each flask contained 60–75 mg wet wt tissue in 0.133 *M* phosphate buffer (pH 7.0), 38° C, total fluid volume 3.0 ml. Points represent averages of two to four determinations. Ordinates: μl O_2/100 mg wet wt tissue; abscissae: time in hours.

the small but consistent difference in O_2-uptake observed when epinephrine was used as substrate, since preparations of melanomas have been reported to oxidize epinephrine, although at rates considerably below those for DOPA (*10, 11*). When a mixture of epinephrine plus DOPA was used as substrate, the O_2-uptake by the pigmented homogenates was considerably greater than the sum of the values with the two substances alone (Fig. 1). When the concentrations of the two substrates were varied, the rate was found to be dependent upon the concentration of each. Bio-assays of trichloracetic acid filtrates of the reaction mixtures by the dog's blood pressure method showed that significant amounts of epinephrine were inactivated during the reaction. A lower degree of augmentation of O_2-uptake was recorded with the vessels in which autoxidation was followed, but none occurred with the albino homogenates. Recent evidence has indicated that mammalian "DOPA-oxidase" and "tyrosinase" are identical (*12*). The addition of 0.001 *M* α-naphthothiourea (ANTU), a relatively selective inhibitor of plant tyrosinase (*13*), reduced the O_2-uptake by 50% during the first hour in vessels containing the combined substrate and pigmented homogenate, but did not affect the rate of autoxidation. No significant degree of deamination or decarboxylation occurred during the enzymatic or spontaneous oxidation. When mixtures of DOPA and norepinephrine were used as substrate, the augmentation of O_2-uptake was absent.

The development of a reddish-orange color prior to a fine black precipitation in the autoxidation vessels and in the supernatant solutions with pigmented homogenates, and the absence of deamination or decarboxylation, indicate that both substrates followed the same path of oxidation to and beyond indole-quinones as they have been shown to follow with plant tyrosinase (*14, 15*). The basis of the greatly augmented O_2-uptake with the combined substrates remains to be determined. The quinone and indole-quinone forms of epinephrine and DOPA constitute redox systems; the

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