Effect of Certain Compounds on *in vitro* Degradation of Serotonin by Ceruloplasmin.* (26176)

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It was recently demonstrated in this laboratory that *in vitro* degradation of serotonin by ceruloplasmin is strongly inhibited by both isoniazid and iproniazid(1). This finding may be of importance in connection with the psychopharmacologic and other clinical effects of these compounds. Moreover, it may have significance with regard to a concept which postulates that fibrosis may result from an imbalance between serotonin and serotonin degradation mechanisms at the tissue level(1,2,3). In view of these and other considerations, it became of interest to determine the *in vitro* effect of a wide range of other compounds on this pathway of serotonin oxidation.

Accordingly, tests were performed with selected reducing, sulfhydryl containing, and metal binding compounds; with hydrazine derivatives and amine oxidase inhibitors; and with a number of antihypertension and psychopharmacologic agents. The findings form the basis of this report.

Materials and methods. Initial titration tests with purified preparations of ceruloplasmin confirmed the activity of this enzyme over a wide pH range. Although optimal activity was at pH 6.0, it was elected to perform these *in vitro* tests at a more closely physiological pH, that is, 7.4. Accordingly, serial 2-fold dilutions of ceruloplasmin were made in physiological saline solution. The enzyme concentration which was found to metabolize between 65% and 80% of the substrate (300 μg serotonin-creatinine sulfate) was used in the final test system. This degree of enzyme activity was found to be present in a 1:8 dilution of the human ceruloplasmin employed. Dilutions of 3 different preparations of ceruloplasmin were used. These were designated DWZ 37, DWZ 42, and DWZ 44, and had been prepared by the Kabi Co., Stockholm, Sweden†

Procedure. To a 20 ml beaker were added 1.0 ml of ceruloplasmin solution; 1.0 ml of 0.5 M phosphate buffer, pH 7.4; and 0.5 ml of the solution containing the substance to be tested for inhibition of the reaction. The beakers were pre-incubated for 15 minutes on a Dubnoff metabolic shaker. Solutions of the test substances were freshly prepared and placed in the test system within 10 minutes of preparation. Final concentration in the incubation mixture was 4.9×10^{-4} M for serotonin. Incubation was continued for 1 hour, and determination for residual serotonin was made by the colorimetric method of Udenfriend, *et al.*(4). Controls included tests with boiled ceruloplasmin, and, for color interference, recovery of serotonin after incubation with the respective test substance in absence of enzyme.

The following substances were tested for reaction inhibition at a final concentration of 1×10^{-3} M: sodium metabisulfite, ascorbic acid, glutathione, British anti-lewisite (BAL), cysteine, methionine, D, L penicillamine, sodium cyanide, and ethylene diamine tetracetic acid (EDTA). Among the hydrazine derivatives and among oxidase inhibitors tested at the same concentration were: amphetamine, procaine amide, iproniazid,‡ isoniazid,‡ isocarboxazid‡ (Marplan),® hydralazine§ (Apresoline),® Compound Ba-20523§ (an analog of hydralazine), thiocarbohydrazide, semicarbazine, and aminoguanidine. Hydrazine hydrate, phenylhydrazine, 2-4 dinitrophenylhydrazine, 1-acetyl-2-phenylhydrazine, and diphenylthiocarbazone were employed in a final dilution of 1×10^{-5} M be-

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cause of color interference at greater concentrations.

The antihypertensive and psychopharmacologic substances tested at a final concentration of 1 x 10^{-4} were Su-7057§ (a thiazide diuretic), Su-5879§ (Esidrix,® hydrochlorothiazide), Su-6835§ (an analog of hydrochlorothiazide), reserpine, Su-3118§ (syrosingopine, Singoserp,® a synthetic analog of natural Rauwolfia alkaloids), Su-5171§ (an isomer of syrosingopine), chlorisodamine chloride§ (Esidrix),® hexamethonium bromide, tetraethylammonium chloride, methylphenidate hydrochloride§ (Ritalin),® and Ro-50690‡ (Librium).® Su-5864§ (guanethidine, an antihypertensive agent), and Su-6187§ (a thiazide diuretic) were employed in a final concentration of 0.0285 mg/ml.

All of the reagents were chemically pure substances except for BAL which was prepared from a solution containing peanut oil and benzyl benzoate.

Results. The majority of test findings are presented in Fig. 1-3. At pH 7.4, an inhibitory effect on *in vitro* degradation of serotonin was exhibited by sodium metabisulfite, ascorbic acid, glutathione, BAL, isoniazid, iproniazid, isocarboxazid, hydralazine, Ba-20523, thiocarbohydrazide, and hydrazine hydrate. An effect about equal to the latter was also noted with compounds Su-3118 (Singoserp) and its isomer, Su-5171. Lesser changes in activity were found in presence of reserpine, tetraethyl ammonium chloride, and Su-7057, as well as with phenylhydrazine, 2-4-dinitrophenylhydrazine, and 1-acetyl-2-phenylhydrazine.

None of the other substances appeared to alter the reaction rate under the conditions of testing.

Discussion. It is evident from the foregoing results that enzymic degradation of serotonin by ceruloplasmin is markedly inhibited at physiologic pH by a number of widely differing compounds. Reducing agents such as sodium metabisulfite, ascorbic acid, glutathione, and BAL are particularly active. In this connection, it may be noted that Holmberg and Laurell demonstrated reduction of ceruloplasmin to a colorless compound by sodium hydrosulfite, ascorbic acid, hydroxylamine, and thioglycolic acid(5), while ascorbic acid was further shown to diminish enzyme activity(6). This property of ascorbic acid has been utilized by R. B. Davis to block the destruction of serotonin present in specimens of clotted blood(7). Holmberg and Laurell also found that potassium cyanide inhibited oxidation of paraphenylene diamine, especially on the acid side of pH 7(6). This is in contrast to the minimal effect of

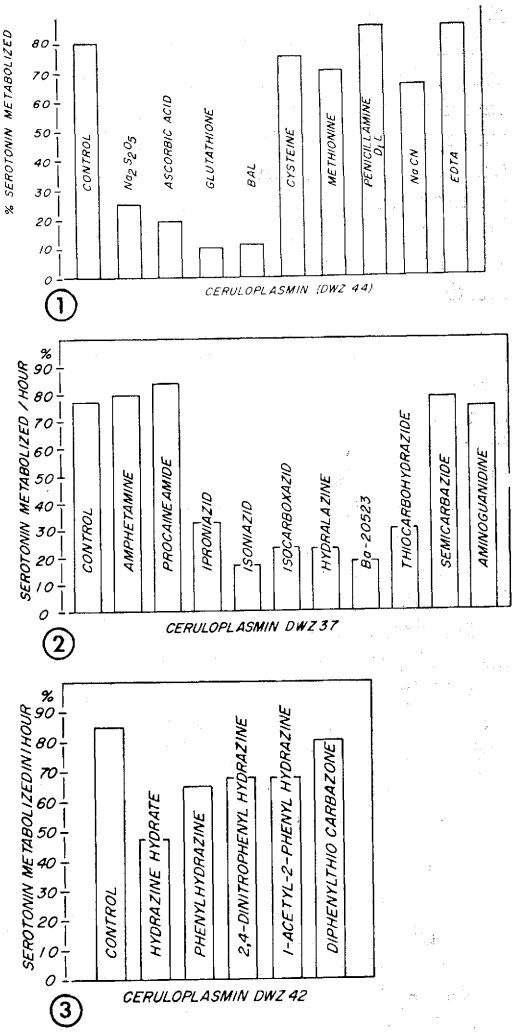

FIG. 1. *In vitro* effect of certain reducing, sulfhydryl containing, and metal binding agents on serotonin degradation by ceruloplasmin.

FIG. 2. Effect of indicated compounds on *in vitro* degradation of serotonin by ceruloplasmin preparation DWZ 37.

FIG. 3. Effect of certain hydrazine derivatives on *in vitro* degradation of serotonin by ceruloplasmin preparation DWZ 42.

NaCN as observed in this study at pH 7.4.

It is of interest that EDTA and penicillamine did not inhibit the reaction. It appears, therefore, that the copper of ceruloplasmin was not chelated. R. P. Davis observed a similar failure to chelate enzyme metal in studies with carbonic anhydrase(8). He suggested that the lack of chelation of carbonic anhydrase zinc might reflect a more internal location of this metal in the carbonic anhydrase molecule than is the case for other zinc enzymes where chelation results in competitive inhibition and where a superficial location is necessary in the substrate binding function of those enzymes. It seems reasonable to consider that a similar relationship probably exists with regard to the copper of ceruloplasmin.

With regard to the hydrazine derivatives, Martin, *et al.*(9) demonstrated that oxidation of 5-hydroxytryptamine by serum from normal and pregnant humans was inhibited by .003 M iproniazid. These workers suggested that the enzyme involved was ceruloplasmin and further noted that this offered a potential degradation pathway for serotonin transformation other than the monoamine oxidase system. The present studies with purified ceruloplasmin revealed not only iproniazid, but also isoniazid, hydralazine, an analog of hydralazine (Ba-20523), isocarboxazid, and thiocarbohydrazide are inhibitory, as is hydrazine hydrate to a lesser extent. On the other hand, semicarbazide, aminoguanidine, and diphenylthiocarbazone were without effect. Thus, the presence of the basic hydrazine nucleus in a compound is not sufficient in itself to produce inhibition of this reaction.

Several of the test substances are classified by some as "monoamine oxidase inhibitors," while others are termed "diamine oxidase inhibitors." Among the former are amphetamine, procaine amide, iproniazid, and isocarboxazid. It can be seen from the results that some inhibited ceruloplasmin activity whereas others did not. Similarly, thiocarbohydrazide, a "diamine oxidase inhibitor," markedly depressed ceruloplasmin oxidation, while semicarbazide and aminoguanidine were ineffective.

Hydralazine, and its analog, Ba-20523, are both potent diamine oxidase inhibitors. Hydralazine also strongly inhibits monoamine oxidase, but its analog is about 30 times less active in this regard(10). Both substances were markedly inhibitory to ceruloplasmin activity. The findings, therefore, fail to show correlation with either monoamine or diamine oxidase inhibition.

Numerous reports have dealt with the relationship of serotonin and other amines, such as norepinephrine, to central nervous system activity. The psychopharmacologic effects of monoamine oxidase inhibitors, for example, are believed to be mediated through inhibition of this pathway of destruction of brain serotonin and/or norepinephrine(11). Recent observations by Sulser and Brodie(12) underscore the complexity of interaction of these mechanisms at the brain tissue level. Their findings suggest that the tranquilizing action of reserpine is not related to brain norepinephrine loss but rather to a change in level of brain serotonin.

Our findings indicate an even more complex relationship is probably involved. Thus, reserpine and 2 of its analogues (syrosingopine and its isomer, Su-5171) were found to have some inhibitory effect on degradation of serotonin by ceruloplasmin. Degree of inhibition was not as striking as that obtained with hydralazine, its analog (Ba-20523), isoniazid, and isocarboxazid. Nevertheless, it should be apparent that modifying effects on pathways of serotonin degradation other than monoamine oxidase must also be taken into account when interpreting the clinical actions of various compounds.

Of the remaining substances tested in this study, only TEAC and Su-7057 exhibited some inhibitory effect on the reaction test-system. That TEAC produced inhibition despite the presence of the chloride ion is of interest since Holmberg and Laurell(13) showed that certain monovalent anions, including Cl^- and Br^-, had an accelerating effect on this enzymic process. Hexamethonium bromide, however, was without evident effect. Su-7057, which showed slight inhibition, was the only thiazide derivative tested to do so. The reason for this finding is not known.

Summary. In an *in vitro* test system at pH 7.4 it was found that degradation of serotonin by ceruloplasmin was markedly inhibited by sodium metabisulfite, ascorbic acid, glutathione, BAL, isoniazid, iproniazid, hydralazine, an analog of hydralazine (Ba-20523), isocarboxazid, thiocarbohydrazide, hydrazine hydrate, syrosingopine (Su-3118) and its isomer, Su-5171. Lesser effects were produced by reserpine, tetraethylammonium chloride, and a thiazide diuretic (Su-7075). A number of other sulfhydryl containing, chelating, anti-hypertension, and psychopharmacologic agents were without effect in the test system employed. It was not possible to correlate reaction inhibition with simple presence of sulfhydryl groups, the hydrazine nucleus, or with monoamine oxidase and diamine oxidase inhibition.

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Pyridoxal Phosphate in Plasma and Leukocytes in Patients with Leukemia and Other Diseases.* (26177)

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The concentration of pyridoxal phosphate, the coenzymatically active form of Vit. B_6, can be estimated in leukocytes and plasma using a modification of the manometric method of Umbreit *et al.*(1). Previously, this has been done in the blood of normal (2,3,4) and pregnant subjects(3,4) and in the cord blood of new born babies(3,4). In addition, load tests were performed in normal controls and in pregnant women with measurement of the B_6 content of plasma and leukocytes following oral administration of pyridoxine hydrochloride(4). The present communication deals with estimation of pyridoxal phosphate in the blood and plasma of patients with chronic and acute leukemia and various other diseases.

Method. For simultaneous estimation of pyridoxal phosphate in leukocytes and plasma, 5 ml of venous blood were collected in a tube containing about 0.002 g of sodium versenate. The assay method is based on coenzyme function of pyridoxal phosphate in a tyrosine decarboxylase system according to Boxer *et al.*(2) with minor changes as previously described(4). Streptococcus fecalis was obtained from the Dept. of Bacteriology, Rutgers Univ., New Brunswick, N. J. Leukocytes were isolated using freeze-dried phytohemagglutinin prepared from navy beans. The phytohemagglutinin does not contain any measurable B_6. The samples were usually

* Supported by Vitamin Fn.