ACTA CHEMICA SCANDINAVICA 14 (1960) 2081–2088

Oxidation of 3-Hydroxy-Anthranilic Acid by Human Serum

GÖSTA EHRENSVÄRD*, JAN LILJEKVIST**, and ROBERT G. HEATH***

Institute of Biochemistry, University of Lund, Lund, Sweden, and Department of Psychiatry and Neurology, Tulane University School of Medicine, New Orleans, Louisiana, U.S.A.

The oxidation of 3-hydroxy-anthranilic acid by human serum has been investigated by fluorimetric techniques. The influence of some activators and inhibitors has been studied. By examination of approximately one hundred samples of sera from various origins, it has been found that the partial inhibition by cyanide is subject to a considerable variation. In sera from schizophrenic subjects the cyanide inhibition in per cent of total oxidation ranges from 0 to 6 %; in some cases there is a slight activation. In normal controls the corresponding range is from 6 to 20 %. The overlapping between the groups is about 15 %.

In the plethora of reactions involved in tryptophan metabolism 3-hydroxy-anthranilic acid (HOA) keeps a position as an intermediary metabolite. Its formation through enzymatic cleavage of 3-hydroxy-kynurenine has been demonstrated in a variety of organisms [1–4]. In man the main route for disposal of 3-hydroxykynurenine seems to involve its transformation *via* oxidative desamination and ringclosure to xanthurenic acid and derivatives of this compound [5]. Since, however, a minor fraction of tryptophan metabolized in man is providing part of his nicotinic acid supply [6,7], and this reaction in mammals has been demonstrated to involve an oxidative splitting and rearrangement of HOA [3–10] the existence of the latter compound in human organism is indirectly indicated. The demonstration by Wiss [3] of the presence of kynureninase in pig liver, catalyzing the conversion of 3-hydroxy-kynurenine to HOA, gives additional, although indirect, support to the assumption of HOA partaking in human metabolism as an intermediate. Another indication is the recent finding of conjugated derivatives of HOA in human urine [11,12].

* Chairman, Institute of Biochemistry, University of Lund; Visiting Professor to the Department of Psychiatry and Neurology and the Department of Biochemistry, Tulane University School of Medicine.

** Assistant, Institute of Biochemistry, University of Lund; Visiting Research Associate to the Department of Psychiatry and Neurology, Tulane University School of Medicine.

*** Professor and Chairman, Department of Psychiatry and Neurology, Tulane University School of Medicine.

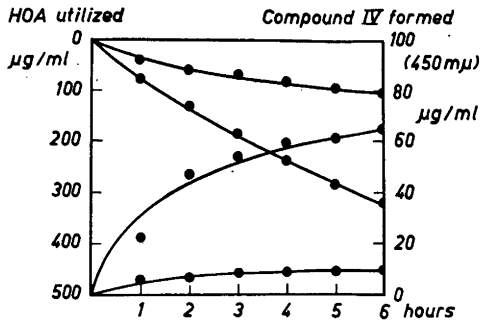

Fig. 1. Diagram of HOA-oxidation by human serum and the corresponding formation of compounds absorbing at 450 mµ. Figures in µg per ml of reaction mixture.

In view of the marked tendency of HOA to form a variety of oxidation products under various conditions, as shown by Butenandt *et al.*[13], it should be expected that a small fraction of HOA in circulation should give rise to similar products, related to phenyl-quinoneimides and phenoxazines. An indication of this is a finding that rat liver homogenate forms from HOA a red pigment, possibly related to the phenoxazines of Butenandt [14]. The present work is an investigation of the oxidation of HOA by human serum under different conditions.

As a preliminary experiment 2 ml of serum were incubated at 30°C with 2 ml of a 0.1 % solution of HOA in 0.02 M tris-buffer. At intervals 25 µl samples were taken and analyzed for HOA and compounds absorbing at 450 mµ; see Fig. 1. The result showed a rapid disappearance of HOA and a steady, but not stoichiometric rise of compounds absorbing at 450 mµ. The similar situation, but at a slower rate appears in a parallel run without serum; *i.e.* the result of pure autoxidation. Examination by paper chromatography of the formed reaction products showed the same distribution of spots, coloured and fluorescent in UV, for both runs, the solvent being butanol-propanol-water (1:2:1). The R_F-values and the colours of some of the spots indicate identity with Butenandt's "compounds III and IV" [14].

In order to elucidate some aspects of the serum factors involved in HOA-oxidation, a series of experiments with various inhibitors were performed. The results indicate that only a part of the total oxidation was sensitive to inhibitors, and thus could represent an enzymatic mechanism. In view of the fact that metal ions in combination with serum proteins could bring about a nonspecific oxidation of a variety of substances (for a general review of this subject see Ref.[15]) the influence of some metal ions upon the HOA-oxidation was studied with and without the presence of human serum. As a further development a comparative study of the HOA-oxidation in human serum from various sources was performed, with special reference to subjects with clinical symptoms of schizophrenia, and with normal healthy subjects as controls.

EXPERIMENTAL

For the determination of the rate of HOA-oxidation under various conditions the following procedure was used: To a series of 10 ml Erlenmeyer flasks 2 ml of serum, or 2 ml of a 0.9 % NaCl-solution, was added, followed by additional solutions of various activators and/or inhibitors in 100 μl quantities. (Corrections for these minor additions were made when necessary in order to keep the volumes constant.) When cyanide was used as inhibitor, the solution (0.4 M) was neutralized with HCl against phenol red. Thereafter 2 ml of a 0.1 % HOA-solution in 0.02 M tris-buffer, pH 7.5, were added and the flasks incubated at 30°C under gentle shaking. (Dubnoff Metabolic Shaking Incubator). At intervals, 25 μl samples were withdrawn and diluted with 2 ml of a 0.9 % NaCl-solution. These diluted samples were analyzed in an Aminco-Bowman Spectrophotofluorimeter, for their HOA-content; activation maximum 330 mμ fluorescence wave-length 415 mμ. The instrument was calibrated within the range used by a series of HOA-solutions of known concentrations with the same amount of serum added from a mixture of 2 ml of HOA-solution and 2 ml of serum, 25 μl were taken and diluted as above with 2 ml of saline solution.

The human sera were usually prepared from bloodsamples drawn in the early morning, before eating. They were immediately stored at + 2°C, and, as a rule, analyzed the same day. The schizophrenic patients providing the serum for this study were hospitalized on a special Tulane University research unit at the East Louisiana State Hospital at Jackson. These patients were under intensive observation on the unit for a minimum period of 3 years. Agreement on the diagnosis of schizophrenia had been reached by a minimum of 4 psychiatrists and 2 clinical psychologists. The various clinical subcategories of schizophrenia were represented almost equally, *i.e.*, catatonia, paranoia, hebephrenia, and chronic undifferentiated schizophrenia. All patients selected for these special wards were required to meet exacting clinical and laboratory criteria. They had been hospitalized for a minimum of 3 years without clinical remission. They were under 45 years of age

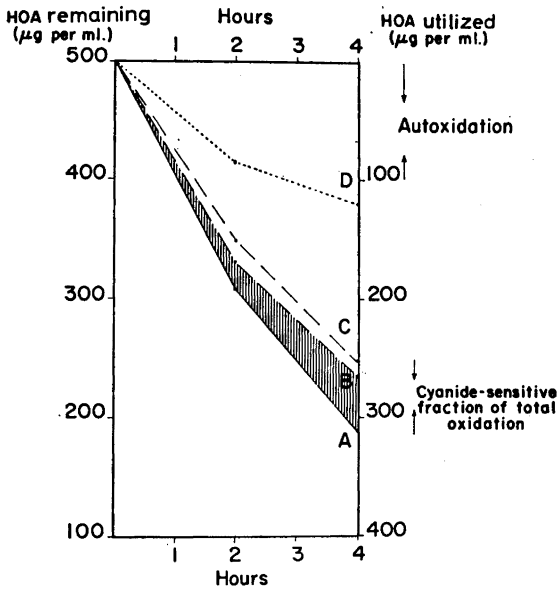

Fig. 2. Diagram of HOA-oxidation by human serum during 4-hour period, curve A. The curves B and C represent the rate of oxidation in the presence of 0.01 M NaCN, and 0.001 M NaN_3. The shaded region between curves A and B represents the cyanide-sensitive fraction of total oxidation. Curve D shows the oxidation rate without serum.

Table 1. Influence of Cu^{2+} and Mn^{2+} in a concentration of 0.0001 M upon the oxidation of HOA in the presence of human serum and without, 4 hours incubation at 30°C. Figures denote μg HOA consumed per ml of incubation mixture. Starting concentration 500 μg per ml.

	Without serum			With serum		
	0	Cu^{2+}	Mn^{2+}	0	Cu^{2+}	Mn^{2+}
+ 0.01 M NaCN	50	145	405	280	310	437
0	30	195	170	315	328	472
Difference:	+ 20	−50	+ 235	−35	−18	−35

completely negative on physical and neurological examinations and on a large number of routine laboratory tests, including a battery of liver tests. The minimal educational level for the group was 9th grade. A special diet was provided for the patients on these wards which included extra rations of meat and daily vitamin supplements.

The normal control group was composed of staff and faculty of the Tulane Department of Psychiatry and Neurology and carefully selected medical students. Each student was screened by a minimum of 2 members of the team of research psychiatrists after having been observed for a minimum of 3 weeks while on the psychiatric teaching block of the curriculum. All normal controls were required to have a negative family history for nervous and mental disease.

RESULTS

A comparison between the rate of HOA-oxidation by human serum and plain autoxidation gives an approximate ratio of 2:1 for a 2-hour period, and 2.5:1 for a 4-hour period; see Fig. 2. The influence of inhibitors is shown in curve B and C, depicting the oxidation rate in the presence of 0.01 M NaCN and 0.001 M NaN_3. As seen the inhibition is not very great, 15 % at the most for a 4-hour run. However, this percentage represents most likely the part of the total oxidation, which is connected with an enzymatic mechanism; the rest being a non-specific oxidation brought about by metal-protein complexes. For simplicity in the following, we will use the category type I- and type II-oxidations, of which type II represents the cyanide-azide-sensitive fraction.

In order to evaluate the influence of metal ions upon HOA-oxidation of type I and type II, experiments were carried out under the same standard conditions, with and without the presence of various metal ions at a total concentration of 0.0001 M. The result is shown in Table 1.

This experiment demonstrates the relative activating influence of copper and manganese ions upon the HOA-oxidation, whereby manganese exerts a stronger influence than copper. The activating effect of the manganese is especially marked in the presence of cyanide, without serum. In the presence of serum, the activating effect of manganese is not further enhanced by cyanide. Probably the effect of Mn^{2+} in the concentration used, both upon cyanide, 0.01 M, and serum, has a common denominator, namely a tendency to formation of a complex which would function as a HOA-"pseudo"-oxidase.

Table 2. Influence of Cu^{2+} and Mn^{2+} in a concentration of 0.0001 M upon the oxidation of HOA in the presence of coeruloplasmin, 300 μg per ml, 3 hours incubation at 30°C. Figures denote μg HOA consumed per ml of incubation mixture. Starting value 500 μg per ml.

	Without Coeruloplasmin		With Coeruloplasmin		
	Cu^{2+}	Mn^{2+}	0	Cu^{2+}	Mn^{2+}
+ 0.01 M NaCN	75	340	66	120	315
0	130	90	45	330	210
Difference:	−55	+ 250	+ 21	−210	+105

As both Cu^{2+} and Mn^{2+} are normal serum constituents, the former to some extent or possibly totally, protein bond as coeruloplasmin [16], it was appropriate to investigate the oxidation of HOA by this metal-protein alone, and in the presence of cyanide, with and without the addition of free Cu^{2+} and Mn^{2+} ions. For comparison, a similar set of experiments was carried out with the copper-containing enzyme ascorbic acid oxidase (AAO), isolated by Dawson [17]. The results of these two experiments are shown in Tables 2 and 3.*

As seen, the pattern of activation and inhibition of coeruloplasmin compared to AAO differs so far as AAO is a quite effective oxidase for HOA, whereas coeruloplasmin (CP) is a very sluggish enzyme against this substrate. With regard to inhibition AAO is almost completely inhibited by cyanide, in contrast to CP. The two Cu^{2+} enzymes differ also with regard to the activation. CP is strongly activated by Cu^{2+}, and to a less extent by Mn^{2+}, whereas the activity of AAO on the whole is not enhanced by Cu^{2+}, but is activated to some extent by Mn^{2+}. The intricate interplay between inhibition and activation is illustrated for instance in the case of AAO, where cyanide apparently does not

Table 3. Influence of Cu^{2+} and Mn^{2+} in a concentration of 0.0001 M upon the oxidation of HOA in the presence of ascorbic acid oxidase (AAO), 5 units per ml, 3 hours incubation at 30°C. Figures denote μg HOA consumed per ml of incubation mixture. Starting value 500 μg per ml.

	Without AAO		With AAO		
	$+Cu^{2+}$	Mn^{2+}	0	$+Cu^{2+}$	Mn^{2+}
+ 0.01 NaCN	100	332	30	85	330
0	150	80	225	230	320
Difference:	−50	+252	−195	−145	+10

* For highly purified sample of coeruloplasmin we wish to express our gratitude to KABI AB, Stockholm. Likewise we are greatly indebted to professor C. R. Dawson, Columbia University for a generous gift of crystalline ascorbic acid oxidase.

Table 4. Typical 4-hour run with serum from 4 normal (Nos. 1, 4, 7, 8) and 5 schizophrenic (Nos. 2, 3, 5, 6, 9) subjects. Figures denote μg HOA consumed per ml of incubation mixture. Starting concentration 500 μg HOA per ml.

	1	2	3	4	5	6	7	8	9
+ 0.01 M NaCN	299	305	296	292	322	335	309	287	310
0	311	336	311	327	334	316	330	322	300
Difference:	12	31	15	35	12	−19	21	35	−10
Δ %	3.9	9.2	4.9	10.7	3.5	−5.2	6.4	10.9	−3.3

inhibit the Mn^{2+}-activated enzyme. Yet the explanation could be that the cyanide inhibition of AAO (difference −225) is in this case counteracted by a pure cyanide-activation of Mn^{2+} as such (difference + 272). With regard to CP, it is evident that Cu^{2+} and, to a less extent Mn^{2+}, evoke a HOA-oxidase activity in this copper-protein complex, which may have a bearing to the HOA-oxidase activity in serum, of the cyanide sensitive type II.

DISCUSSION

As a tentative hypothesis we suggest that in human serum the cyanide sensitive part of HOA-oxidation — type II — is brought about by some combination of metal ions and some special proteins. Cu^{2+} and Mn^{2+} have been shown to exert an activating influence upon coeruloplasmin, in itself a copper-protein complex, still it is possible that another complex of similar kind, not implicating coeruloplasmin, could be involved in the HOA-oxidation, type II.

With regard to the absolute rate of HOA-oxidation in sera from various human subjects, it has been found that this rate varies from case to case. Examining the material from approximately one hundred HOA-oxidation tests, we found that on the whole the rate of oxidation is lower for sera from subjects with chronic schizophrenia as compared to sera from the groups of normal, healthy subjects. As seen from Table 4, the differences in oxidation rate between schizophrenics and normals is partly eliminated when cyanide or azide is added to the reaction.

Expressing the cyanide effect, Δ %, as $\frac{a-b}{a} \times 100$, where a is the total HOA consumed during a 4-hour period, and b is the corresponding amount of HOA consumed in the presence of cyanide, we find that the value for schizophrenics is encountered in the lower range −2→6, and the normal controls in the higher range 6→20; see Fig. 3.

These findings indicate that in serum from schizophrenics, the type II-oxidation of HOA constitutes a smaller fraction of the total HOA-oxidation, as compared with serum from the control group. In about 20 % of the schizophrenics type II-oxidation is virtually absent.

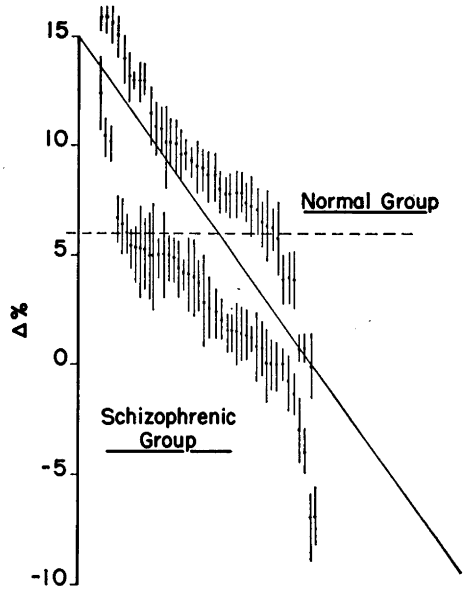

Fig. 3. Diagrammatic representation of Δ %-values for the cyanide effect on HOA-oxidation by serum from schizophrenic subjects as compared with the corresponding values for normal controls. Standard variations of individual test-runs are shown as verticals lines. Overlapping of the two groups less than 15 %. The division line at value 6 is arbitrarily taken as a boundary between the two groups.

Being aware of the fact that the rate of oxidation of various substrates by human serum could be influenced by various external factors, as well as internal, we have, whenever possible, tried to sample the sera examined from subjects on the same diet. With regard to different levels of ascorbic acid in sera, which previously has been shown to influence the rate of other oxidation reactions [18], we find that addition of 50 μg per ml serum before running a HOA test did not influence either the reaction rate or the cyanide effect. With regard to the general concept of diurnal variations under fasting and non-fasting conditions the results of a test series from two schizophrenics show slight variations in the Δ % values, the range being for one subject 1.4→1.6, for the other —4.8 → 2.3 (samples taken at 07.30 and 19.30). As comparison, two normal controls were tested in the same way, one showing the range 7.9→4.9, the other 15.0→3.8, thus considerable variation during the day. It should, however, be emphasized that the delta values recorded in Fig. 3 are all derived from morning samples of sera in order to eliminate diurnal variations. These data demonstrate a convincing difference between the two groups, the overlapping being less than 15 %. The question of the diurnal variations in normals, as well as the general problems of the relationship of the HOA oxidation to the clinical syndrome of schizophrenia, will be subject of continuing investigation.

REFERENCES

1. Jacoby, W. and Bonner, D. M. *J. Biol. Chem.* **205** (1953) 699, 709.
2. Miller, I. L. and Adelberg, E. A. *J. Biol. Chem.* **205** (1953) 691.
3. Wiss, O. *Helv. Chim. Acta* **32** (1949) 1694.
4. Wiss, O. *Z. physiol. Chem. Hoppe-Seyler's* **293** (1953) 106.

5. Price, J. M. and Dodge, L. W. *J. Biol. Chem.* **223** (1953) 699.
6. Dalgliesh, C. E. *Intern. Congr. Biochem. 4th Meeting Symposium* **11**.
7. Horwitt, M. K. *Am. J. Clin. Nutrition* **3** (1955) 244.
8. Bokman, A. H. and Schweigert, D. S. *Arch. Biochem. Biophys.* **33** (1951) 270.
9. Wiss, O. *Z. Naturforsch.* **9b** (1954) 740; **11b** (1956) 54.
10. Wiss, O. *Z. physiol. Chem. Hoppe-Seyler's* **304** (1956) 237.
11. Boyland, E. *Nature* **177** (1956) 837.
12. Boyland, E. *Biochem. J.* **64** (1956) 578.
13. Butenandt, A., Keck, J. and Neubert, G. *Ann.* **602** (1957) 61.
14. Viollier, G. and Süllmann, H. *Helv. Chim. Acta* **33** (1950) 776.
15. Massart, L. and Vercauteren, R. *Annual. Rev. Biochem.* **28** (1959) 527.
16. Holmberg, C. G. and Laurell, C. B. *Acta Chem. Scand.* **2** (1948) 550.
17. Dawson, C. R. and Tarpley, W. B. in Sumner, J. B. and Myrbäck, K. (eds) *The Enzymes*, vol. 2, p. 454. (Academic Press Inc. N.Y. 1951).
18. Åkerfeldt, S. *Science* **125** (1957) 117.

Received June 2, 1960.