Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

Luigi Musajo, Carlo Alberto Benassi, Antonio De Antoni, Graziella Allegri, Carlo Costa

Substances influencing pyridoxal phosphate-dependent enzymes regulating tryptophan degradation extracted from human urine

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **51** (1971), n.5, p. 381–386.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1971_8_51_5_381_0>

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Accademia Nazionale dei Lincei, 1971.

Chimica biologica. — *Substances influencing pyridoxal phosphate–dependent enzymes regulating tryptophan degradation extracted from human urine* (*). Nota di LUIGI MUSAJO, CARLO ALBERTO BENASSI, ANTONIO DE ANTONI, GRAZIELLA ALLEGRI e CARLO COSTA, presentata (**) dal Corrisp. L. MUSAJO.

RIASSUNTO. — Continuando gli studi sulla presenza nelle urine umane di sostanze attivanti e inibenti la chinureninasi e la chinurenin transaminasi, enzimi piridossal fosfato–dipendenti che regolano il metabolismo del triptofano, sono stati isolati e identificati tre agenti attivanti.

Le urine di uomini sani di 20–24 anni tenuti a dieta normale dopo acidificazione hanno fornito estratti eterei ed esanici che sono stati sottoposti a ripetute cromatografie con vari adsorbenti ed eluenti. Si sono ottenuti in forma pura due indigoidi dall'estratto esanico, indigotina ed indirubina, ed uno dall'estratto etereo, una ossiindirubina non nota.

Questi indigoidi hanno mostrato, a determinate concentrazioni, effetti attivanti sulla chinureninasi e chinurenin transaminasi. L'indirubina inoltre saggiata sulla tirosin decarbossilasi, anch'essa piridossal fosfato–dipendente, ha mostrato pure proprietà attivanti.

Sono ora oggetto di studio i precursori di queste sostanze.

INTRODUCTION

For many years we have studied the urinary excretion of tryptophan metabolites " via kynurenine " in man [1]. These investigations have led us to consider the presence, in human urine, of substances interfering with pyridoxal phosphate-dependent enzymes which, as is known, regulate the tryptophan degradation.

Our preliminary studies [2] showed, in fact, the existence in human urine of some agents that inhibit and activate kynureninase (L–kynurenine hydrolase, EC 3.7.1.3) and kynurenine transaminase (L–kynurenine: 2-oxoglutarate aminotransferase, EC 2.6.1.7), pyridoxal phosphate–dependent enzymes. We have also considered another enzyme, i.e. tyrosine decarboxylase (L–tyrosine carboxy–lyase, EC 4.1.1.25), which requires the same coenzyme.

The action mechanism of the inhibitory substances has been studied *in vitro* using both crude and partially purified urinary extracts. The nature of the inhibitory effects involved a competition for the assayed apoenzymes between the pyridoxal phosphate and the agents present in human urine [2].

The urines of about one hundred subjects have been investigated [3]. The excretion of these substances presented a large variability in relation to sex, age, pregnancy and pathological conditions.

(*) Lavoro eseguito nell'Istituto di Chimica Farmaceutica dell'Università – Centro di studio per la Chimica del Farmaco e dei prodotti biologicamente attivi del Consiglio Nazionale delle Ricerche, Padova.

(**) Nella seduta del 13 novembre 1971.

In order to try to isolate the active agents we preferred, so as to have basal information not influenced by non-normal situations, to select the pooled urine of young men aged 20–24 years fed on a normal diet excluding drugs and other substances unrelated to food.

Filtered urine, after acidification, was extracted at first with hexane and then with ethyl ether.

Crude urinary extract and the isolated and purified substances have been assayed on kynureninase, kynurenine transaminase and tyrosine decarboxylase.

The object of the present work is the isolation and identification of activating agents. We found them to be indirubin, indigotin and an unknown hydroxyindirubin. They are present in examined urine in small amounts (about 40–60 μg each per litre were extractable).

The biological activity of indirubin was assayed on the three above-mentioned enzymes, and that of the other two substances on kynureninase and kynurenine transaminase.

Methods

After a long series of preliminary trials the extractive conditions were fixed in this way: filtered urine, acidified to pH 1 with hydrochloric acid, heated at 95° for 30 min., was extracted with hexane in a liquid-liquid apparatus for 72 hours. After adjusting the pH to 3.9–4.0 with alkali the extraction was continued for 72 hours with ethyl ether. The solvent was then removed from the extracts *in vacuo* under nitrogen.

Enzyme assays.

1) Rat kidney kynurenine apotransaminase was partially purified and assayed at 37° according to the method of Mason [4], in a total volume of 1.5 ml, containing 0.8 ml of enzyme preparation (1 mg protein/ml), 3 μmoles of α–ketoglutarate, 0.06 μmoles of pyridoxal phosphate, 1.5 μmoles of L–kynurenine, 75 μmoles of potassium phosphate buffer at pH 7.4. Aliquots of crude or purified residues were dissolved in the same buffer adjusting the pH to 7.4 with 0.1 N NaOH.

The reaction was stopped at zero time for the blanks and after 30 minutes by means of 7.5 ml of 95% ethanol containing 1% boric acid.

Enzyme activity was measured spectrophotometrically by the change in absorbence at 333 nm resulting from the formation of kynurenic acid during 30 minute incubation period.

2) Kynureninase was prepared according to Wiss [5] and the measurements performed with a modification of Saran's method [6].

The frozen rat liver was homogenized with a cold 0.05 M phosphate buffer, pH 8, preparing a mixture with 20% liver. After freezing for a day at —20° it was thawed and centrifuged for 35 minutes at 9000 rev./min. Each assay was performed as follows: 1.0 ml of supernatant fraction (2 mg protein/ml);

0.3 ml of 40 μM pyridoxal phosphate in a 0.05 M phosphate buffer, pH 8, and a 1.6 ml of a 0.05 M phosphate buffer, pH 8. Samples of the examined substances were dissolved in the same buffer, adjusting the pH to 8 with 0.1 N NaOH when necessary. After a 15 minute pre-incubation period at 37° 0.1 ml of 0.01 M L–kynurenine in a 0.05 M phosphate buffer, pH 8, was added.

Duplicate blanks of 1.2 ml at zero time and test samples after 30 minutes were taken from the incubation mixture.

The reaction was stopped with 0.2 ml of 15% trichloroacetic acid and the precipitate removed by centrifugation at 5000 rev./min. for 10 minutes.

0.1 ml supernatant was brought to 4 ml with a 0.1 M phosphate buffer, pH 8. The anthranilic acid formed was determined spectrofluorometrically with an Aminco-Bowman apparatus at 410 nm (excitation 310 nm).

3) Tyrosine apodecarboxylase was obtained from cells of " Streptococcus faecalis R 8043 " according to Umbreit *et al.* [7].

The assay was performed as follows: each vessel of the Warburg apparatus contained; in the side arm, 0.5 ml of a suspension of 0.03 M L–tyrosine in a 0.5 M acetate buffer pH 5.5; and, in the main compartment, 1.5 ml of the same acetate buffer, 0.5 ml of a suspension of cells (8 mg cells/ml water), 0.1 ml of 2.5×10^{-7} M pyridoxal phosphate; aliquots of examined substances dissolved in the acetate buffer pH 5.5, and water to give a final volume of 3 ml. The CO_2 evolution was measured, after a 10 min preincubation, every 5 min over a 30 min period at 28°.

Purification of the urinary extracts.

A preliminary purification of both hexane and ethyl ether extracts obtained from 12 l urine at a time was carried out by silica gel column chromatography.

Elution was performed with chloroform, chloroform at increasing concentrations of acetone, acetone, and methanol. The fractions collected were assayed for the enzyme preparations.

RESULTS

Some hectolitres of pooled urine were worked on.

A) *Fractioning of the activating agents from the hexane extract.*

Silica gel column chromatography of the hexane extract by elution with chloroform yielded various fractions, only two of which, one blue and one red-violet always emerging very closely, showed activating effects. From the blue fraction indigotin was separated, from the red-violet one indirubin.

B) *Isolation of the activating substance from the ethereal extract.*

Silica gel column chromatography of the ether urinary extract by elution with chloroform-acetone (90 : 10, *v/v*) yielded a violet fraction that showed activating effects. Continuing the elution of the column different fractions

were obtained. Two of these showed inhibitory activity regarding the enzymes under consideration [2].

From the violet fraction it was possible to obtain a violet crystalline substance.

The U.V. and I.R. spectra showed many analogies with those of indirubin. However this substance appeared more soluble in polar organic solvents; it was crystallized from chloroform. It begins to decompose at 320°.

Found :	C 68.93 ;	H 3.74 ;	N 9.85 ;
Calc. for $C_{16}H_{10}O_3N_2$:	69.06 ;	3.62 ;	10.07 % .

This substance is a hydroxyindirubin.

By repeated methylation in an ether solution with diazomethane a violet substance was obtained, that was crystallized by methanol, m.p. 241°.

Found :	$-OCH_3$	10.18 ;
Calc. for $C_{17}H_{12}O_3N_2$:		10.61 % .

The determination of the hydroxyl group position was not successful because of the small amount of the isolated substance. NMR investigations were not conclusive.

C) *Enzymatic determinations.*

For the enzymatic assays we were able to work only with low concentrations of the activating agents because the pigments were slightly soluble. Table I reports the effect of these agents on the enzymatic activities considered at significant concentrations of indigoid.

TABLE I.

Effect of indirubin, indigotin and hydroxyindirubin on enzymatic activities (*).

ENZYME (*)	Indirubin concentration M	Increase of activity %	Indigotin concentration M	Increase of activity %	Hydroxy-indirubin concentration M	Increase of activity %
Kynurenine transaminase	3.3×10^{-7}	27	1.5×10^{-7}	18	5.5×10^{-8}	30
	6.6×10^{-7}	43	2.0×10^{-7}	24	9.2×10^{-8}	25
Kynureninase	6.6×10^{-8}	30	8.7×10^{-8}	20	2.2×10^{-7}	26
	1.3×10^{-7}	26	1.7×10^{-7}	16	4.4×10^{-7}	30
	1.0×10^{-6}	—24				
Tyrosine decarboboxylase	3.8×10^{-6}	66				
	7.6×10^{-6}	110				

This effect resulted constant in many repeated trials.

Indirubin was assayed for the three enzymes showing activating effect on kynurenine transaminase and tyrosine decarboxylase. For the assay with kynureninase, the activating effect occurred only at lower concentrations. When the concentration was raised the effect decreased until an inhibition of the activity occurred with a concentration of 1.0×10^{-6} M.

Hydroxyindirubin and indigotin were assayed for kynurenine transaminase and kynureninase showing activating properties. However, indigotin showed lower activating effects.

DISCUSSION

The presence of indigoid pigments in human and animal urine has already been noted by various Authors.

Rosin [8] isolated indirubin from horse urine and observed the formation of indirubin and indigotin in the urine of patients with intestinal disorders and also in apparently normal subjects.

Dorner [9] found indigotin in the urine of a patient with kidney stones.

Indirubin was also isolated by chromatography of the benzene extract of the urine of pregnant mares [10].

Musajo [11] isolated indirubin with small amounts of indigotin from the urine of rats and rabbits fed with a hyperproteinic diet.

Rimington [12] described the presence of indirubin and indigotin in a ratio of 2 : 1 in the urine of patients with sprue.

Friedman *et al.* [13, 14] isolated indirubin from urine of leucaemic patients 3 times superior to that of the urine of normal subjects.

Indirubin and indigotin were also found in the urine of patients with amyloid nephrosis [15] and in cancerous patients [16, 17].

One should not exclude the question that the formation of indigoids can derive, during the extractive operations, from simpler substances, i.e. indoxyl, which, as is known, is present in urine in a conjugated form. In fact various researchers [8, 18–21] have demonstrated that indigotin and indirubin can form in urine from indoxyl and isatin, substances that are derived from tryptophan metabolism.

In another work we shall report the results obtained also studying the activities of the precursors of isolated indigoids or of correlated ones.

Furthermore this research will of course be continued on highly purified enzymatic preparations. The reported data should be considered, from an enzymatic point of view, as an introduction to a development of the problem.

REFERENCES

[1] L. MUSAJO and C. A. BENASSI, « Advan. Clin. Chem. », 7, 63 (1964).

[2] L. MUSAJO, C. A. BENASSI, E. LONGO and G. ALLEGRI, Symp. Chem. and Biol. Aspects Pyridoxal Catalysis, Rome 1962, Pergamon Press, New York 1963, p. 333.

[3] L. MUSAJO, C. A. BENASSI, G. ALLEGRI, A. DE ANTONI and E. LEVORATO, Symp. on Pyridoxal Enzymes, Nagoya 1967, Maruzen Co., Tokyo 1968, p. 209.

[4] M. MASON, « J. Biol. Chem. », *227*, 61 (1957).
[5] O. WISS, « Z. Physiol. Chem. », *303*, 232 (1956).
[6] A. SARAN, « Biochem. J. », *70*, 182 (1958).
[7] W. W. UMBREIT, W. D. BELLAMY and I. C. GUNSALUS, « Arch. Biochem. », *7*, 185 (1945).
[8] H. ROSIN, « Arch. path. », *123*, 159 (1891); « Biochem. J. », *40*, 669 (1946).
[9] G. DORNER, « Münch. med. Wschr. », *69*, 661 (1922).
[10] R. DUSCHINSKY and E. LEDERER, « Bull. Soc. Chim. Biol. », *17*, 1534 (1935).
[11] L. MUSAJO, « Gazz. Chim. Ital. », *67*, 235 (1937).
[12] C. RIMINGTON, « Biochem. J. », *40*, 669 (1946).
[13] E. FRIEDMAN and W. JACOBSON, « Nature », *157*, 337 (1946).
[14] E. FRIEDMAN, D. H. MARRIAN and M. F. PERUTZ, « Biochim. Biophys. Acta », *5*, 45 (1960).
[15] M. FERNANDEZ–CRIADO, « Rev. Clin. espan. », *50*, 39 (1953).
[16] J. MAKY, « Nippon Ya Kurigatu Zasshi », *55*, 1358 (1959).
[17] S. MATSUMOTO, « Kumamoto Med. J. », *13*, 247 (1960).
[18] L. MAILLARD, « Chem. Ztg. », *25*, 415 (1901).
[19] A. ELLINGER, « Hoppe–Seyl. Z. », *39*, 44 (1903).
[20] A. ELLINGER, Neubover and Huppert's « Analyse des Harns », Wiesbaden: Kreidel, 1913; « Biochem. J. », *40*, 669 (1946).
[21] L. MAILLARD, *Dictionnaire de physiologie*, cited by Fearon et Thompson, Paris, 1913; « Biochem. J. », *40*, 669 (1946).