Isolation from Mammalian Brain of Coproporphyrin III and a Uro-Type Porphyrin.* (20162)

T. Paul Blanshard. (Introduced by C. J. Watson.)

From the Department of Medicine, University of Minnesota Hospital, Minneapolis.

Klüver(1,2), using ultraviolet light and a direct vision spectroscope, was the first to describe the occurrence of porphyrin in the normal central nervous system of warm blooded animals. He found a sharp band with a maximum at about 625 mμ in the fluorescence spectrum and on this basis identified the substance as a coproporphyrin. He also confirmed this finding chemically but, being unable to obtain a melting point owing to an insufficiency of material, was not able to state whether the coproporphyrin was present as the type I or type III isomer. Later he tentatively identified it as a type III isomer(3). Since then the presence of coproporphyrin has been confirmed in this laboratory(4) and again the isomer was tentatively identified as coproporphyrin III, by means of the florescence quenching technic.

The finding of an increased excretion of coproporphyrin III in the urine of patients with poliomyelitis(5) raised the problem of the origin of this excess of porphyrin and its possible relation to the coproporphyrin of the central nervous system. Because of this and other more general considerations it was believed desirable to identify this porphyrin with certainty. The present report is concerned with the isolation of crystalline porphyrin from mammalian brains.

Methods. Fresh beef brains were used and extracted by the method of Schwartz and Wikoff for red blood cells(6). The brains were stripped of membrane, washed as clean of blood as possible, homogenized in a Waring blender and extracted in a ball-mill using a 4:1 ethyl acetate-glacial acetic acid mixture. In all 9.665 kg of brain were processed. The ethyl acetate-acetic acid extract was washed with 1% sodium acetate in a separatory funnel and the porphyrins were extracted with 3 N HCl. This was washed with ether to remove some of the liquid material which was invariably present and which interfered considerably with purification at all stages of the procedure. The 3 N HCl was then neutralized with saturated sodium acetate and, after the addition of a few ml of glacial acetic acid, shaken with ethyl acetate. This was washed with water and extracted repeatedly with 0.3 N HCl to remove the coproporphyrin. The latter solution was then diluted to 0.1 N and washed with chloroform to remove any traces of chloroform-soluble porphyrins. The 0.1 N HCl solution, after addition of sodium acetate and glacial acetic acid, was shaken with ether and the latter in turn extracted with 1.5 N HCl. The content of total coproporphyrin in the 1.5 N HCl, assayed fluorimetrically, was approximately 193 μg or 2 μg per 100 grams of brain, which is in rough agreement with previously published figures of the average content of human brain(7). The total coproporphyrin was concentrated in 3 N HCl and esterified by the addition of 10 volumes of a mixture of methyl alcohol-sulphuric acid (20:1) The porphyrin ester was purified by the method of Grinstein, Schwartz and Watson(8). Chloroform-petroleum ether was used to dissolve the residue and develop the chromatogram. Some lipid material appeared to be present throughout and caused a cloudiness in the chloroform-methyl alcohol used for attempted crystallization. This was successfully removed by suspending the porphyrin in petroleum ether and filtering. The porphyrin was then redissolved from the filter paper in chloroform and crystallized from methyl alcohol in the usual way(8). Coproporphyrin III methyl ester was obtained crystallizing slowly in the typical rosettes of straight needle-like prisms. On determining the melting point initially, shrinkage of the crystals was observed from 148°C onward with generalized melting at 174°C. Recrystallizations gave melting points of 146-149° and 146°C.

Results. During the initial phase of extraction the sodium acetate washings of the

* Aided in part under contract with the Atomic Energy Commission.

ethyl acetate were concentrated on aluminum oxide, eluted with 1.5 N HCl and examined for uroporphyrin. None was found. However, it was observed that the neutralized 0.1 N HCl after extraction with ether continued to fluoresce red in ultraviolet light. This fluorescence was not removed by repeated extraction with ether or ethyl acetate at the prevailing pH, but on adjusting the pH to about 3.5 with HCl, it was found that the porphyrin passed into ethyl acetate. From this it was taken into 1.5 N HCl. The amount of uro-type porphyrin in the aqueous solution was found to represent from 0.15-0.25 μg per 100 grams of brain. Due to various losses in the course of purification the total amount of crystalline material finally obtained was not more than 12 μg. It was esterified and crystallized in the same manner as the coproporphyrin. On account of apparent lipid impurities it was rechromatographed and recrystallized. The crystals were composed of imperfect rosettes of curved hairlike needles. Successive recrystallizations yielded melting points of 272°, 272-3° and 273-4°. The absorption spectrum of the methyl ester in chloroform, using a Zeiss grating comparison spectrometer gave a band in the red, maximal at 626.1 mμ. This agrees well with a uroporphyrin(8). The absorption curve of this porphyrin showed a Soret band maximal at 404.5 mμ for uroporphyrin I. Unfortunately there was insufficient material for decarboxylation and analysis of the isomer type.

Protoporphyrin considerably in excess of coproporphyrin was present in the brain. This in agreement with other observations both in human and animal brain.

It was theoretically possible but unlikely that the porphyrins obtained were derived from the blood contained in the intracerebral blood vessels. Accordingly 50 ml. of beef blood were extracted for coproporphyrin and protoporphyrin(6). A total for the former of 0.72 μg per 100 ml of cells and serum was obtained while for protoporphyrin the value was 16.3 μg per 100 ml. It was clearly impossible that sufficient blood could have been present in the washed brain to account for the amounts of porphyrins obtained.

The isolation and identification of coproporphyrin III confirms the previous tentative results(3,4). The question of significance has been discussed elsewhere(9). The solubility characteristics and melting point of the second compound clearly identify it as a uro-type porphyrin. The melting point of 273-4° C is significantly lower than that of uroporphyrin I methyl ester, 284-6°C(8) yet certain samples of the latter have been noted to melt either lower or higher than this, for reasons that are not clear(8). The slight but significant differences in the Soret bands together with the lower melting point suggest that this may be a Waldenström type porphyrin such as encountered in cases of porphyria, especially in the "cutanea tarda" or "mixed" type of hepatic porphyria(10). This porphyrin incorporates a major portion of uroporphyrin I with a minor fraction of a type III porphyrin having less than 8 COOH groups. The nature of the present porphyrin cannot be determined until larger amounts are available to permit decarboxylation and other studies. Nor is any speculation warranted at the present time as to its biological significance.

Summary. The isolation from beef brain of crystalline coproporphyrin III and of a uro-type porphyrin is described. The ester melting point of the latter is 273-4°C, significantly lower than that of uroporphyrin I methyl ester. The Soret band was also found to differ significantly.

1. Klüver, H., *J. Psychol.*, 1944, v17, 209.
2. ———, *Science*, 1944, v99, 482.
3. ———, *J. Psychol.*, 1948, v25, 331.
4. Chu, E. J. H., and Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 509.
5. Watson, C. J., Schulze, W., Hawkinson, V. E., and Baker, A. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 73.
6. Schwartz, S., and Wikoff, H. M., *J. Biol. Chem.*, 1952, v194, 563.
7. Watson, C. J., and Larson, E. A., *Physiol. Rev.*, 1947, v27, 478.
8. Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, 1945, v157, 323.
9. Watson, C. J., *Duncan's Diseases of Metabolism*, 1952, W. B. Saunders Co., Phila., Pa.
10. ———, *Trans. Assn. Am. Phys.*, 1950, v63, 219.

Received February 5, 1953. P.S.E.B.M., 1953, v82.

Phenylpyruvic Oligophrenia Deficiency of Phenylalanine-Oxidizing System. (20163)

GEORGE A. JERVIS.

From the Research Department, N. Y. State Department of Mental Hygiene, Letchworth Village, Thiells, N. Y.

The biochemical nature of phenylpyruvic oligophrenia (phenylketonuria), a disease characterized by mental deficiency and urinary excretion of phenylpyruvic acid, is still uncertain(1). Some indirect evidence was presented in a previous publication(2) which suggests that the metabolic error consists of the inability of phenylketonuric patients to oxidize phenylalanine to tyrosine. Following the recent report of Udenfriend and Cooper (3) demonstrating that an oxidizing system is present in the liver which is capable of catalyzing the conversion of phenylalanine to tyrosine, it became of interest to investigate whether this enzymatic system is defective in phenylketonuria.

Materials and methods. As a source of the enzyme, fragments of liver obtained at the autopsy of 2 phenylketonurics and 3 normal controls were used. The first patient, V.N., a male 25 years of age, died of pulmonary tuberculosis. The liver showed histologically a moderate amount of fatty infiltration. The second patient, W.J., a male 5 years of age, died of acute bronchopneumonia. The liver was histologically normal. In both patients abnormally high amounts of phenylpyruvic acid and phenylalanine were repeatedly found in the urine and the blood. Control 1 was a male, 52 years of age, who died of gastric hemorrhage originating from carcinomatous ulcer; the liver showed a moderate amount of fatty infiltration. Control 2 was a woman, 60 years of age, who died of mitral stenosis. Control 3 was a girl, 8 years of age, whose cause of death was hydrocephalus. The bodies were placed in the ice box (0°-4°C) within a half hour after death. Autopsy was performed 4 hours after death in patient V.N., 5 hours in patient W.J., and 5, 10, and 15 hours respectively in the 3 controls. Fragments of liver were removed, immediately homogenized in chilled Waring blendor with twice their weight of ice cold isotonic potassium chloride and placed in the deep freeze. Two days later the thawed material, packed in ice, was centrifuged for one hour at 8000 r.p.m. and the supernatant fluid, after removal of fatty material, was immediately used as crude liver extract in the test system of Udenfriend and Cooper(3). This consisted of .5 ml of liver extract, 2 μM L-phenylalanine in .20 ml of water, .5 μM of tridiphosphopyridine nucleotide in .20 ml of water, 5 μM of nicotinamide in .20 ml of water and .20 ml of phosphate buffer (pH 6.7). All preparations were commercial products and were not tested for purity. The mixture was shaken in air at 25°C and at the end of one hour 2.70 ml of water was added followed by 1.0 ml of 30% solution of trichloracetic acid. After centrifugation and filtration, tyrosine was determined on 2 ml of filtrate using Udenfriend and Cooper's method(4). The color was read in a Coleman junior spectrophotometer at 450 mμ against a blank consisting of .5 ml of the liver extract treated exactly in the same way but omitting the phenylalanine substrate and the pyridine nucleotide. All tests were run in duplicate.

Results. In Table I the results are presented. It may be seen that tyrosine was formed under the conditions of the experiment in all controls but no formation of tyrosine was observed when liver extracts of phenylketonuric patients were used.

Comment. One may object to the use of autopsy material in the present experiments

TABLE I. Conversion of Phenylalanine to Tyrosine in 2 Phenylketonurics and 3 Controls (2 M L phenylalanine added to each).

		Tyrosine formed, μM
Patient	VN	0
	WJ	0
Control	I	.033
	II	.027
	III	.020

because of the instability of the phenylalanine-oxidizing liver enzyme(3). It should be noted, however, that the controls gave positive results, although the livers were removed somewhat later than in the phenylketonuric patients. Moreover, the amount of tyrosine formed in Control 1 was comparable to that reported by Udenfriend and Cooper(3) in an experiment with rat liver slices in which the liver was immediately removed and all operations carried out in a cold room. In order to avoid further deterioration of the enzymatic activity, no attempt was made at dialyzing the liver preparation and only the crude extract was used.

Although there is a remote possibility that the observed failure to find tyrosine in the phenylketonuric preparations might be the result of a very rapid deamination of phenylalanine to phenylpyruvic acid, the most likely explanation is that the specific enzymatic system described by Udenfriend and Cooper(3) is absent in phenylketonuria. This absence is apparently the essential metabolic characteristic of the disease; in fact, as previously noted(5), the main biochemical findings may be explained on the assumption of a failure in the normally occurring conversion of phenylalanine to tyrosine. Furthermore, the demonstration of the absence of a specific enzyme clarifies the observation that phenylketonuria is determined by a single recessive gene(1), since several instances are on record of specific enzymatic reactions being controlled by single genes.

Summary. Liver extracts of two phenylketonuric patients failed to catalyze the conversion of phenylalanine to tyrosine in the presence of oxygen, phosphopyridine nucleotide and nicotinamide.

1. Jervis, G. A., Mental Deficiency and Aberrant Metabolism, in *The Biology of Mental Health and Disease*, p. 422, Hoeber, Inc., New York, 1952.

2. ———, *J. Biol. Chem.*, 1947, v169, 651.

3. Undenfriend, S., and Cooper, J. R., *J. Biol. Chem.*, 1952, v194, 503.

4. ———, *J. Biol. Chem.*, 1952, v196, 227.

5. Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 715.

Received February 10, 1953. P.S.E.B.M., 1953, v82.

Cross-Dialysis: Description of a Possible Method of Temporary Kidney Substitution. (20164)

MILTON J. KRAININ. (Introduced by Max Michael, Jr.)
(With the assistance of Mary Z. Shumpert.)

From the Medical Research Laboratories, Veterans Administration Hospital, Atlanta, Ga.

The several types of artificial kidneys and their clinical applications have been adequately described(1). Basically they consist of two compartments separated by a dialyzing membrane. Blood of the patient is circulated through one compartment and a bath solution is circulated through the other. The dialyzable substances tend to pass from the compartment in which their concentrations are higher into that compartment in which their concentrations are lower. The rate of transfer depends to a great extent on the physical characteristics of the dialyzable substances; the type, thickness and area of the dialyzing membrane; and the concentration gradient present.

The purpose of this study was to design a system of cross-dialysis whereby the blood of a uremic subject would be cross-dialyzed with that of a subject with normal kidneys. There would be no mixing of blood between the two circulations. In such a system it is anticipated that the normal subject would remove through dialysis "toxic products" from the blood of the uremic subject and then utilize its own kidneys to excrete them. This system would not be an artificial kidney in the true sense as it would use a normal pair of kidneys to regulate certain functions which the patient with diseased kidneys would be unable to do. The following results might be expected: a) Re-