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Presence of a Hitherto Unrecognized Nicotinic Acid Derivative in Human Urine.*

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In the course of some studies on the excretion of thiamin in urine by means of the thiochrome method, using the procedure of Hennessy and Cerecedo,[1] it was noted that treatment of the KCl eluate of urine with alkali, even in the absence of ferricyanide, yielded a small amount of a substance soluble in butyl alcohol which gave a bluish fluorescence with ultraviolet light. This fluorescence could be distinguished from that given by thiochrome even with the naked eye, being a whitish blue without any tinge of violet. Specimens of urine from a large series of normal individuals of various ages were found to exhibit such fluorescence in slight degree. A patient receiving nicotinic acid therapy, however, was found to excrete it in large amount, suggesting that nicotinic acid was the precursor of this substance. Following this observation the effect of taking nicotinic acid was studied in normal individuals, and it was found that a dose of 50 mg of nicotinic acid, given to an adult, produced a prompt increase in the excretion of the unknown material. An increase in its concentration in the urine could be detected within an hour, and persisted for 4 to 6 hours.

We have attempted to identify the unknown urinary constituent by studies of the fluorescence of 27 different pyridine derivatives.† These compounds were dissolved in 25% KCl solution, both with and without treatment with alkali; the aqueous solution was then extracted with butyl alcohol, the alcoholic extract being tested for fluorescence with ultraviolet light. The following substances were tested:

nicotinic acid
nicotinic acid amide

* This study was aided by a grant from Mead Johnson and Co., Evansville, Ind.

1 Hennessy and Cerecedo, *J. Am. Chem. Soc.*, 1939, **61**, 179.

† Most of these compounds were furnished us through the courtesy of Dr. Charles E. Bills. Dr. William A. Perlzweig was kind enough to supply us with samples of trigonelline and nicotinuric acid, and Dr. Eric G. Ball with the sample of diphosphopyridine nucleotide; the vitamin B_6 hydrochloride was furnished by Merck and Company.

nicotinuric acid
diphosphopyridine nucleotide
vitamin B_6 hydrochloride
dinicotinic acid
alpha picoline
alpha picoline methiodide
beta picoline
5,6-dichlor-nicotinic acid
trigonelline
5-amino-nicotinic acid
3,5-diamino-2,6-dimethyl pyridine
2,6-dimethyl pyridine
2,6-dimethyl pyridine hydrochloride
2,6-dimethyl dinicotinic acid (K salt)
diurethyl lutidine (3,5-diurethyl-2,6-dimethyl pyridine)
pyridine 2,3,5,6-tetracarboxylic acid
2,4,6-trimethyl dinicotinic acid (K salt)
1,4-dihydro-, 3,5-dicarbethoxy-, 2,4,6-trimethyl pyridine
1,4-dihydro-, 3,5-dicarbethoxy-, 2,6-dimethyl pyridine
3,5-dicarbethoxy-, 2,6-dimethyl pyridine
3,5-dicarbethoxy-, 2,4,6-trimethyl pyridine
quinoxaline 2-3-dicarboxylic acid
pyrazine monocarboxylic acid
pyrazine 2,3-dicarboxylic acid
2-methyl, 3-hydroxy-quinoxaline

Most of these compounds gave negative results. Both compounds containing a 5-amino substitution in the pyridine ring gave a fluorescence with an indigo blue tint resembling that of thiochrome, rather than that of the unknown; alkali was not needed to bring this out. Both compounds containing the 1,4 dihydro-, 3,5 dicarbethoxy substitutions gave a strong fluorescence, the color in both instances showing distinct differences from the unknown—the dimethyl compound giving a deep blue fluorescence and the trimethyl a violet emission; fluorescence appeared without the addition of alkali. The 3,5 dicarbethoxy dimethyl and trimethyl pyridines gave, also without alkali addition, a bluish fluorescence somewhat resembling the unknown, but the spectra given by these solutions were not identical with that of the fluorescent urinary extracts, from which it would appear that these compounds were not identical with the unknown. Diphosphopyridine nucleotide showed a blue fluorescence similar to that of the unknown, which likewise developed only after the addition of alkali. The fluorescent spectrum, however, showed marked differences from the unknown. Since the fluorescent spectrum of diphosphopyridine nucleotide has not, so far as we are aware, been studied, it is reproduced herewith. (Fig. 1.)

None of the compounds tested could, therefore, be identified with

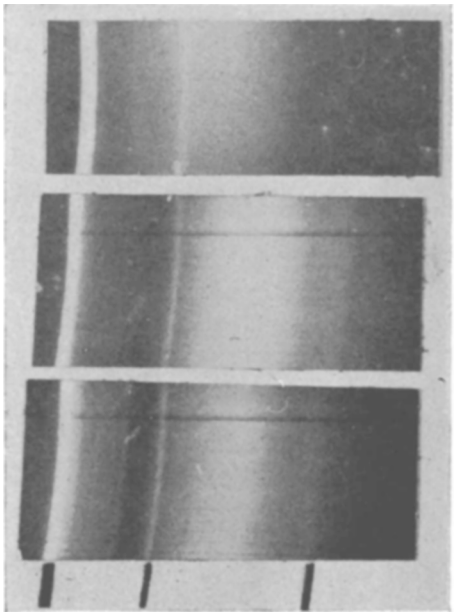

FIG. 1.
Photographs of Fluorescent Spectra.

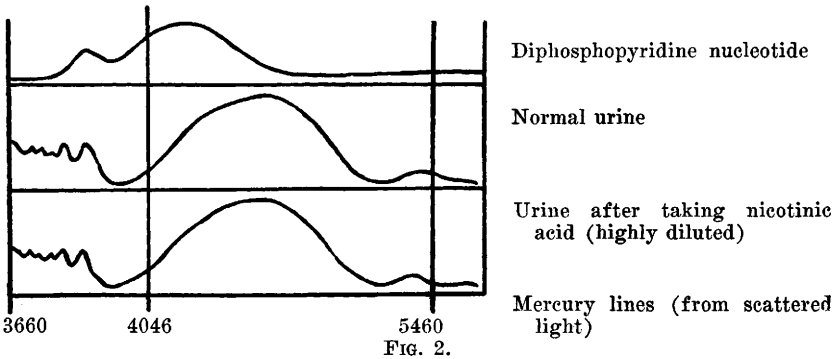

FIG. 2.
Spectrograms.

the unknown. An attempt was made to produce the unknown substance from nicotinic acid and from nicotinic acid amide *in vitro* by incubating normal urine with these compounds for 24 hours at 37° C. This procedure failed to increase the content of the unknown fluorescent material. The possibility that the unknown material might be a porphyrin was considered; however, spectroscopic examination of the KCl eluate failed to confirm this.

Photographs of the fluorescent spectrum were made with eluates

of normal urine and urine after nicotinic acid administration, the eluates being treated as before with alkali and extracted with butyl alcohol. The fluorescent spectra obtained, which are reproduced herewith, indicate that the substance present in small amount in normal urine is identical with that obtained in larger quantity after the ingestion of nicotinic acid. The photographs were taken with a small quartz spectrograph. The mercury lines appear faintly superimposed on the spectrum as the result of Rayleigh scattering of the solvent.

A number of observations were made upon the stability and differential solubility of the unknown substance. Boiling destroys the substance, more rapidly in the presence of alkali. At room temperatures it is destroyed slowly in alkaline solution and slowly by $K_3Fe(CN)_6$. The fluorescence of the butyl alcohol extract is readily destroyed by exposure to sunlight, but not by ultraviolet light passed through a Wood filter. It is readily extracted from aqueous solution by butyl or isobutyl alcohol, but not by amyl alcohol, octyl alcohol, chlorbenzene, benzene or chloroform.

The probability that this substance represents an excretion product of nicotinic acid suggests that its measurement may prove of value in states of nicotinic acid deficiency in man; one might anticipate finding a low value with subnormal excretion following a test dose of nicotinic acid. Up to the present we have had no opportunity of testing patients with nicotinic acid deficiency. Through the courtesy of Dr. C. A. Elvehjem we have tested the urine of a dog with black tongue before and after treatment. We were, however, unable to identify the unknown fluorescent material in the urine of this animal either before or after treatment with nicotinic acid, which suggests that the dog may conjugate this substance by a different mechanism from that which occurs in man.

In the hope that others may have an opportunity to apply this procedure to pellagrins, it is given in detail:

To 20 cc urine are added 10 g permutit ("Decalso" 30 mesh) in a small separatory funnel; this is shaken for 15 minutes gently. The permutit is then washed with 5 portions (30 cc each) of distilled water, the washings being discarded. The permutit is then dried by suction. Ten cc of a 25% KCl solution are then added and the funnel is shaken well for 15 minutes. The fluid is then allowed to drain from the permutit drop by drop the last portion being expelled by air. The permutit is washed with 2 to 4 cc of KCl solution which is added to the eluate previously collected. The eluate is then divided into two equal portions, to one of which one

cc of 15% NaOH is added. Both samples are then shaken immediately with 13 cc butyl alcohol for 3 minutes. The mixture is then centrifuged to separate the butyl alcohol layer and this is treated with anhydrous Na_2SO_4 to remove traces of water, and is allowed to stand in the dark for 20 minutes. Fluorescence is then determined in a Pfaltz and Bauer fluophotometer, the source of light for which is a mercury vapor bulb (General Electric, type H3-85 watts) shielded by a Wood filter (Jena UG-2), the emitted fluorescence being measured after the interposition of a double filter of bright bluish green (Jena BG-14) and bright yellow (Jena GG-3). The difference in fluorescence between the sample treated with alkali and that not so treated represents the fluorescence of the unknown compound. Comparative quantitative measurements can be obtained by comparing the fluorescence with that of a quinine sulfate solution containing 10 to 25 μg % in 0.1 normal H_2SO_4. The daily output of the unknown substance in urine of normal adults gives a fluorescence corresponding roughly to 100 μg of quinine sulfate. After the ingestion of 50 mg of nicotinic acid, the concentration in urine reaches 8 to 10 times its previous value, during the first 4 hours.

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A Simple Method of Preparing Dried Serum Proteins for Therapeutic Use.

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Attempts to preserve serum proteins in a dry form for therapeutic use have been made for a number of years. Flosdorf and Mudd's[1] method of preparing "lyophile" serum is for the most part satisfactory, but requires complicated and expensive special apparatus which was not at our disposal when we began to work with dried serum proteins. We therefore found it necessary to find or devise some method which could be carried out with ordinary laboratory apparatus. After preliminary trials with several other methods, we found the method devised by Hartley[2] for the preparation of dry and lipid-free immune sera to be most suitable,

[1] Flosdorf, E. W., and Mudd, S., *J. Immunol.*, 1935, **29**, 389.

[2] Hartley, P., *Brit. J. Exp. Path.*, 1925, **6**, 181.