A New Compound Excreted in Urine and Developing Colour Reaction with *p*-Dimethylaminobenzaldehyde.

First Report.

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In urine are already known various compounds which react with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. Urobilinogen, Freudenberg's anti-sheep hemolysin (a glucosamine-containing polysaccharide), Jorpes' anti-isoagglutinin (a glycidamin) and urinomuroid (Kobayasi) give the reaction immediately without any preliminary treatment and kynurenine after oxidation with H_2O_2 . During the course of our investigations on those group-specific substances, we became aware of the presence of another compound, which is similar in behaviour toward the reagent, in the filtrate of the basic-lead precipitate of the anti-sheep hemolysin or of corresponding carbohydrates of B and O urine. Separation of it to establish its nature has been tried over and over again, all but with failure and regretfully no decisive claim can be put forward at present. However, its several properties examined will be worthy of description for a further investigation:—

1) It was ultrafiltrable (collodion membrane) as well as dialyzable (animal membrane).

2) Soluble not only in water but also in absolute alcohol and butanol¹⁾ and partially extractible into acetone and pyridine from its solid mixtures with other urine components. Insoluble in acetic ester and ether.

3) As dissolved in water it was scarcely adsorbed to activated charcoal and kaolin.

4) When heated with hydrochloric acid in advance, it formed on treatment with alkaline acetylacetone solution a structure, which gives a red colour with the Ehrlich reagent. The spectrum of this colour³⁾ exhibited an absorption band from λ 5700 to 5200 Å that divided in a short while (15') into two bands, one lying at 5700 Å and the other ranging from 5300 to 5200 Å.

It also gave a light red coloration by the indirect process of Osaki and Turumi³⁾ but not by the direct.

5) It was readily oxidized by mercuric oxide at neutral and alkaline reactions in the cold and rendered incapable of showing the colour reaction in 4).

6) Its output was larger when the urine was more acid. 9 normal individuals excreted 29 (pH of the urine, 6.8)—334 mgm. (pH of the urine, 5.6) in a day (Table III). In normal rabbit urine, which is alkaline, it appeared scarcely, but in urine at pH of some 6 of those rabbits to which menthol or β -naphthol had been injected noticeable amounts of it were found (Table II).

An example of fractionation trial of urine to separate the substance will be given in the experimental part.

EXPERIMENTAL.

Determination Procedure.

The procedures of Yositate⁴⁾ and of Hamasato and Akakura⁵⁾ for hexosamine determination were combined as follows.

First method.

20 cc. of urine (acidulated with acetic acid when the urine is alkaline) are precipitated with 4 cc. of 37.9% lead acetate solution. The filtrate is removed from lead with the aid of H_2S and after aeration off of H_2S , ultrafiltered through a collodion membrane (Zsigmondy apparatus). The ultrafiltrate is freed from acetic acid by passing ether in a continuous extractor for 1.5 hours and 6 cc of it heated together with 4 cc. of 2 n. HCl in a 25 cc.-containing ampoule (hard glass) at $150^\circ C$ (in an oil bath) for 75 minutes. Cooled. The digestate is transferred perfectly into a beaker with the aid of two 5 cc. portions of 25.7% NaCl solution. And 250 mgm. of stannous chloride are dissolved and subsequently sodium carbonate powder added until the solution becomes to pH of 5.6. The solution is made up to 25 cc. in a measuring flask with water and filtered and this filtrate subjected to colorimetric analysis according to Hamasato and Akakura, after addition, when necessary, of a smallest requisite quantity of glucosamine hydrochloride to cause sufficient colour development.

By 75 minutes digestion mentioned of the ultrafiltrate is the maximum value obtained. In urine of an individual, for instance, the colour appeared in depths corresponding to 0.050, 0.083 and 0.05 mgm. of glucosamine, when 1 cc. of the digestates after heating for 60, 75 and 90 minutes was adapted to colorimetric analysis (Colorimetry was carried out after addition of 0.5 mgm. glucosamine hydrochloride per cc. of the digestates and the amount of glucosamine added was subtracted from the values

found). This duration of heating appears to sufficiently decompose urea and other urine components, which interferes with the colour reaction, and longer heating to gradually break up the substance in question, when considered from the results obtained on ovomucoid added to urine (Table I). Ovomucoid was chosen here for reasoning by analogy because the unknown substance may be of complex nature giving the colour reaction only after hydrolysis as are mucoids. That this mucoid is hydrolyzed completely

TABLE I.

Recovery of Glucosamine Added in the Form of Ovomucoid.

300 mgm. of ovomucoid (glucosamine content, 14.5%) were added to 20 cc. of human urine. Glucosamine in 1 cc. of the heat digestate, that was subjected to colorimetric analysis, amounted to 0.52 mgm.

Time of heating min.	Colour development as hexosamine by 1 cc. of the digestate		
	Found mgm.	Recovery of ovomucoid-glucosamine	
		mgm.	In per cent.
30	0.490		
60	0.555	0.505	97
75	0.600	0.517	99
90	0.556	0.506	97
120	0.400		

in 75 minutes was proved by heating its watery solution with hydrochloric acid under similar conditions. The urine used was the one noted on above.

Second method.

In later determinations the precipitation of urine with lead acetate and subsequent ultrafiltration were substituted with single operation of treatment with lead subacetate, namely:—

10 cc. of urine is alkalized with 5% ammonia water to pH 9.0–10.0 and followingly just an enough quantity of the saturated lead subacetate added to throw down all the precipitable substances. The precipitates are filtered off on a Büchner funnel and washed with two 2 cc. portions of water. The filtrate and washings are combined and bubbled with H_2S gas and lead sulfide separated on a filter with suction and washed with two 2.5 cc. portions of water. The filtrate and washings here are acidified together to pH 1–2 with drops of 10% H_2SO_4 and ether passed through. They are then neutralized with 10% NaOH (a few drops) and evaporated under reduced pressure to dryness. The remainder is dissolved in 10 cc. of water and subjected to heating with HCl and following operations described in the former method.

Trials to Increase the Excretion of the Substance in Rabbit Urine.

To start with, menthol, β -naphthol and phenol were given to rabbits. Menthol was administered (2/3–1 gm. per kg. bodyweight) per os as dissolved in lukewarm water by means of a Nalaton's catheter, and phenol (0.25 gm. per kg. bodyweight) as 1% solution in cold water. β -Naphthol was given by forced ingestion (1 gm. of its powder per kg. bodyweight) with a small amount of crushed carrot.

The analysis was made on the whole-day urine. When menthol was given, the pH of the urine was lowered to round 6.0 in about 1 week after the beginning, and the output of the substance increased simultaneously. This raised excretion continued as long as the urine remained at or below this pH, even after the administration was stopped. An example is illustrated in Table II.

TABLE II.

Excretion of the Substance in Question in the Whole-day Urine, When
Menthol was Administered per os to a Grown-up Rabbit (♀).

Date	Bodyweight kg.	Menthol administered gm.	Volume of urine cc.	pH of urine	Output as hexosamine mgm.
Jan. 15	3.09	2	835	8.0	almost nil
16	2.87	"	690	7.8	
17	3.11	"	775	7.8	
18	3.01	"	575	6.5	9.8
19	2.86	3	728	6.5	
20	2.87	"	585	6.4	9.0
21	3.04	"	590	6.4	112.1
22	3.02	"	600	6.2	
23	3.05	"	400	6.2	152.0
24	3.05	"	515	6.2	
25	2.86	"	125	6.0	68.8
26	2.70	"	60	5.4	60.0
27	2.70	0	50	5.2	180.0
28	2.70	0	50	5.2	
29	2.70	0	50	5.2	

During a long application of β -naphthol only temporary (a few days) increase of the output took place with acidification of urine. Phenol was ineffective at all in both of the respects.

In normal human urine, which is acid, were found comparatively large quantities of the substance varying in parallel with the strength of acidity, as is demonstrated in Table III.

From these findings it might be plausibly assumed that the excretion of the substance is promoted by acidosis, and so, to afford a further evidence, subcutaneous injection of neutral inorganic salts (CaCl_2 , NH_4Cl , NaCl , Na_2SO_4 and Na_2HPO_4) and peroral application of HCl (0.1 and 0.2%) were continued day after day on rabbits, but the urine

did not change acid.

TABLE III.

Excretion of the Substance in Question in the Whole-day Human Urine.

Name, sex and age (year)	Body-weight kg.	Volume of urine cc.	pH of urine	Amount of the substance as expressed as hexosamine (mgm.)	
				In 1 cc of urine	In whole-day urine
K.H., ♂, 39	54.0	1,450	5.6	0.23	333.5
K.T., ♂, 34	49.5	1,670	6.0	0.08	136.6
E.I., ♂, 40	54.0	1,700	6.0	0.10	170.0
S.T., ♂, 23	60.6	1,300	6.2	0.069	89.7
M.U., ♂, 31	54.2	1,620	6.2	0.058	94.0
Z.Y., ♂, 27	60.4	1,800	6.4	0.029	52.2
K.O., ♂, 34	52.5	1,600	6.4	0.029	46.4
Y.H., ♂, 33	51.2	1,700	6.4	0.029	49.3
N.H., ♂, 33	49.1	1,630	6.8	0.017	29.4

Fractionation of Urine.

A trial of isolating the substance, though fruitless, will be given for example. 1 l. of acid rabbit urine (pH, 5.2), involving 900 mgm. as hexosamine of the unidentified compound, was collected from four rabbits to which menthol had been given. The urine was alkalinized with ammonia water and the saturated basic lead acetate solution added as in analysis (Second method). The filtrate from the precipitates was removed from lead with H_2S . After eliminating acetic acid with ether, it (pH, 5.6) was condensed under reduced pressure to a syrup and extracted with absolute alcohol repeatedly by boiling under reflux. Into the extracts, amounting to 150 cc. in total and condensed to 100 cc., 70 cc. of 20% alcoholic solution of oxalic acid were poured to precipitate urea, and the centrifuged supernatant liquid boiled under a return condensor with $BaCO_3$ powder, added in portions, until pH of the solution was elevated to 4.8. When cold, barium oxalate and barium carbonate were separated off on a funnel and the filtrate decolorized with activated charcoal (1 spoonful) (This solution will be referred to as A. E.) and evaporated at a low temperature to a syrup. The residue was dissolved with two 50 cc. portions of water. Analysis showed that the substance had been reduced to 495 mgm. The solution reacted neither with naphthoresorcinol (test for hexuronic acid) nor with aniline acetate (test for pentose) but gave the positive Molisch test. Colorimetry with orcinol and indole and measurement of iodine reduction gave figures which coincided with one another as calculated as glucose.

The watery solution was again evaporated and the remaining syrup boiled with three 50 cc. portions of acetone (boiled each time for 1 hour), then the extracts united (323 mgm. of the substance came over) and after distilling off the solvent, the rest was taken up with changes

of cold water (60 cc. altogether). The combined solution was concentrated to 10 cc. and analyzed. 290 mgm. of the substance were found in it. The colour developed thereby showed a spectrum having a band, which divided later into two bands already noted. When the determination procedure was followed by replacing Reagent A of Hamasato and Akakura with this reagent less acetylacetone (the salt solution), the colour reaction did not happen. The residue unextracted by acetone above gave the positive reaction of the indirect Osaki-Turumi test. In it hydrolysable sulfur and sugar (Molisch test) were detected. But Ehrlich diazo reaction of urochromogen and Ehrlich-Bauer aldehyde reaction of urobilinogen resulted in negative, although tested immediately as well as after heating with HCl.⁶⁾

The whole remaining (8 cc.) of the watery solution of the acetone-soluble fraction was condensed to a syrup and fractioned with pyridine (Two 10 cc. portions of this solvent were used and boiled each time for 30 minutes), but again the substance did not accumulate in one fraction (about half of it dissolved).

Crystalline products were obtained during condensation of the extracts at various stages above, but all of them gave no colour reaction by the determination procedure.

An attempt of isolating the substance as an acetyl derivative was made by taking an alcohol-soluble fraction of another urine corresponding to A. E. in the above but was also useless.

Exclusion of the Possibility of the Substance to be Kynurenine.

10 cc. of an A. E., which had been prepared as described and contained 40 mgm. as hexosamine of the compound searched for, were evaporated, the residue taken up in 2.5 cc. of water and kynurenine was precipitated with mercuric sulfate and washed with 6% mercuric sulfate solution according to Kotake and Kawase.⁷⁾ The precipitate was then suspended in 10 cc. of water, kynurenine liberated by means of H_2S and H_2S aerated off. The solution obtained gave no colour with the Ehrlich reagent both by the Osaki and Turumi indirect process and the analytical procedure (the heating with HCl and condensation with acetylacetone included), while it gave the positive reaction of Otani and Honda.⁸⁾ On the other hand, the supernatant liquid after centrifugation of the kynurenine precipitate was removed from mercury in a like way and from H_2SO_4 with $\text{Ba}(\text{OH})_2$. This liquid gave the colour reaction by the Osaki and Turumi indirect process and analysis indicated it contained 35 mgm. of the unknown substance as hexosamine.

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Bibliography and Notes.

- 1) Extracted from water in Dakin's apparatus.
 - 2) The spectrum of the corresponding dye of glucosamine shows a band from λ 5340 to 4750Å.
 - 3) Osaki and Turumi, Tohoku J. Exp. Med., 1947, **49**, 11.
 - 4) Yositate, J. Bioch., 1939, **30**, 199.
 - 5) Hamasato and Akakura, J. Bioch., 1941, **34**, 159.
 - 6) The digestate smelled of menthol distinctly.
 - 7) Kotake and Kawase, Zeits. physiol. Chem., 1933, **214**, 6.
 - 8) Otani and Honda, Osaka Igakkaishi, 1938, **37**, 1.
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