SUMMARY

1. The 3:4-dihydroxyphenylalanine (DOPA) decarboxylase activities of extracts of rats' livers and kidneys, and the cysteic acid decarboxylase activities of extracts of rats' livers, have been measured.

2. The DOPA decarboxylase activity of extracts of rat liver is comparatively high, being of the same order as that previously recorded for extracts of guinea pig liver. The rat is unusual in that the DOPA decarboxylase activity of an extract of its kidney

is much lower than that of its liver; the ratio is about 1/10.

3. The mean cysteic acid decarboxylase activity of extracts of the livers of male rats is about twice that of females. This difference can be abolished by the spaying of the females, but not by the castration of the males; it is partly restored by the injection of oestrone into the spayed females.

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Methods for the Determination of *N*-Methyl-2-pyridone-5-carboxylamide and of *N*-Methyl-2-pyridone-3-carboxylamide in Human Urine

By W. I. M. HOLMAN AND D. J. DE LANGE
South African Institute for Medical Research, Johannesburg

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Knox & Grossman (1946, 1947) showed that *N*-methylnicotinamide can be oxidized by an enzyme in rabbit liver to *N*-methyl-2-pyridone-5-carboxylamide and isolated the latter compound from human urine after the ingestion of doses of 0.6–0.9 g. of nicotinamide per day. The conversion of part of the *N*-methylnicotinamide formed in the body into the pyridone may explain why, in the past, it has been possible to account for only a small proportion of a dose of nicotinamide in the urine in the form of then known metabolites of nicotinic acid, and why the urinary excretion of *N*-methylnicotinamide shows little correlation with the dietary intake of nicotinic acid (Perlzweig & Huff, 1945; Knox & Grossman, 1946); but before the full significance of this new metabolite of nicotinic acid can be elucidated, an accurate method for its determination in urine is required.

Holman & Wiegand (1948) synthesized *N*-methyl-2-pyridone-5-carboxylamide by methylation and oxidation of nicotinic acid, and conversion of the

resulting *N*-methyl-2-pyridone-5-carboxylic acid to the corresponding amide. They found that the methylation and oxidation of nicotinamide gave *N*-methyl-2-pyridone-3-carboxylamide. The work of Knox & Grossman gave no evidence that the latter compound is excreted in human urine after administration of nicotinamide, but further information on this point is desirable.

Since pure specimens of both the isomeric amides were available, a study of their properties was made, in an effort to find reactions which could be used for their determination in urine. It was observed that the 2:3-amide could be nitrated at room temperature in the presence of concentrated sulphuric acid, and that the resulting nitro compound gave an intense and reasonably stable colour when made alkaline with sodium hydroxide. The 2:5-amide also could be nitrated, but only by the application of heat; its nitration product gave a yellow colour with weak alkalis as well as with sodium hydroxide. The colour faded rapidly in the presence of sodium hydroxide,

but was reasonably stable in the presence of weak alkalis. The compounds could be estimated colorimetrically by these reactions.

Before the methods could be applied to urine it was necessary to remove other substances which are capable either of being nitrated to derivatives which are coloured in alkaline solution (e.g. derivatives of phenol, imidazole, purine and pyrimidine), or of substances interfering in other ways, e.g. by charring in concentrated sulphuric acid. It was found possible to separate pyridones either present in, or added to, urine from almost all extraneous matter by making use of their slight solubility in chloroform and of their capacity to be adsorbed by Lloyd's reagent. By the application of these principles, procedures were worked out for the determination of both amides in human urine.

METHODS

Reagents

5N-H₂SO₄; 0.1N-H₂SO₄; 0.4N-NaOH in methanol (diluted from saturated aqueous solution of NaOH with A.R. methanol); methanol (A.R.); N-NaOH; CHCl₃ (grade for anaesthesia); nitrating mixture (1 g. pure KNO₃ dissolved by warming in 6 ml. pure conc. H₂SO₄; freshly prepared and cooled before use); 95% (v/v) ethanol; 15% (w/v) Na₂CO₃; standard solutions of *N*-methyl-2-pyridone-3-carboxylamide and *N*-methyl-2-pyridone-5-carboxylamide (0.1 mg./ml.; the solutions were stable for at least a month when kept in a refrigerator).

Procedure

Specimens of urine were collected every 24 hr. in 25 ml. of a mixture of equal volumes of glacial acetic acid and toluene. If the total volume was <1500 ml. the specimen was diluted with water to 2 l. Specimens were usually filtered immediately after collection and the filtrate analysed without delay. Samples stored in a refrigerator for 5–10 days and then filtered gave slightly lower results, apparently owing to loss by adsorption. In such cases it was necessary to dissolve the sediment as well as possible by warming and mixing, and to omit filtration.

Determination of

N-methyl-2-pyridone-5-carboxylamide

Adsorption on Lloyd's reagent. With normal urines, 25 ml. of the filtered sample were transferred to each of four 50 ml. centrifuge tubes. If the pyridone content was exceptionally high, e.g. after the ingestion of a dose of nicotinamide, it was necessary to dilute the urine to a suitable extent before analysis. Two of the tubes were labelled *U* (unknown). To each of the remaining tubes was added 1 ml. of the standard solution of *N*-methyl-2-pyridone-5-carboxylamide and the tubes were labelled *R* (recovery). From this stage onwards, until the final extraction with CHCl₃ had been completed, all four tubes were treated in exactly the same way. One ml. of 5N-H₂SO₄ was added to each tube, followed by 2 g. Lloyd's reagent. The tube was stoppered, shaken for 5 min., centrifuged and the supernatant fluid discarded. To the Lloyd's reagent in the tube were added 25 ml. of 0.1N-H₂SO₄ and, after shaking and centrifuging, the supernatant

fluid was again discarded. To the Lloyd's reagent in the tube were added 10 ml. 0.4N-NaOH in methanol and, after shaking for 5 min. and centrifuging, the supernatant fluid was transferred to a 150 ml. glass-stoppered extraction flask. The residue in the centrifuge tube was washed with 5 ml. methanol and, after mixing and centrifuging, the washings were added to the contents of the flask. The extract and washings were neutralized with glacial acetic acid (0.15 ml.) and evaporated in a boiling water bath to a volume of 1 ml., and then to dryness with the aid of suction, taking care to avoid loss by sputtering and to obtain uniform drying.

Extraction with chloroform. Exactly 0.5 ml. aqueous N-NaOH and 25 ml. CHCl₃ were added to the dry residue in the flask, which was stoppered (no grease) and shaken for 5 min. The mixture was filtered through a dry paper (Whatman no. 42, 12.5 cm.), taking care to minimize evaporation, into a glass-stoppered 50 ml. measuring cylinder containing 10 ml. of a dilute aqueous solution of acetic acid (2 ml. glacial acetic acid/l.) until the total volume was exactly 30 ml. The contents of the measuring cylinder were shaken for 5 min. and the layers allowed to separate.

Portions (5 ml.) of the aqueous layer were transferred from each measuring cylinder to a 7 × 1 in. Pyrex test tube and the appropriate tubes were marked *U* and *R*. The fluid remaining in the four measuring cylinders was combined, the layers were allowed to separate, and 5 ml. of the aqueous layer transferred to a fifth test tube and marked *B* (blank). The contents of the five tubes were evaporated to dryness in a boiling water bath; the residue was perfectly white in colour.

Nitration. Exactly 0.3 ml. of nitrating mixture was added to the bottom of each tube which was shaken to dissolve the residue. The tubes marked *U* and *R* were heated for 1 hr. in a boiling water bath and the tube marked *B* was left at room temperature for 1 hr.

Development of the colour. This step was carried out under carefully controlled conditions. An apparatus was used consisting of an electrically driven stirrer with a blade which fitted closely into the bottom of a 7 × 1 in. test tube, and three burettes so arranged that they could deliver water, 15% (w/v) Na₂CO₃ and ethanol, respectively, into the tube with continuous stirring. The stirrer was made by flattening the end of a glass rod and shaping to fit the tube. A freezing bath at -6° was prepared by insulating a wide-mouthed vessel of about 1 l. capacity with asbestos and filling with a solution of 100 g. NaCl in 900 ml. water. The temperature was reduced to -6° by the addition of powdered 'dry ice' and maintained by the occasional addition of a further spoonful of 'dry ice'.

After the nitration each tube was treated, in turn, as follows. The tube was clamped so that the stirrer fitted closely into its base. After switching on and ensuring that the 0.3 ml. of nitrating mixture was being mixed thoroughly and that the stirrer speed was about 300 r.p.m., the freezing bath was raised until the tube was well immersed. After stirring for 0.5 min., 1 ml. distilled water was added, drop by drop, over a period of approx. 1 min., followed by 5 ml. 15% (w/v) Na₂CO₃ drop by drop, over a period of 4–5 min. The freezing bath was lowered until the tube no longer dipped into it and 20 ml. ethanol were run in during 1 min. The stirrer was switched off, the tube removed, and the contents transferred to a centrifuge tube and centrifuged for 10 min. at 3000 r.p.m. to remove precipitated Na₂CO₃; 20 ml. of the supernatant fluid were pipetted into a flask

and diluted with 5 ml. distilled water (to prevent the appearance of turbidity on standing).

The colour intensity of each solution was measured against the blank in a photometer at 450 m μ ., using a 3 cm. cell. The blank was colourless.

Calculation of results. *N*-methyl-2-pyridone-5-carboxylamide (mg. excreted/24 hr.) = $U/(R - U) \times 0.1 \times V/v$, where U and R are the average optical densities of unknown and recovery respectively; V is total vol. (ml.) of 24 hr. sample of urine after all dilutions have been made; and v is vol. (ml.) of urine taken for analysis (25 ml.).

Determination of *N*-methyl-2-pyridone-3-carboxylamide

The procedure was very similar to that described above. Except that 1 ml. of the standard solution of the 2:3-amide was used, the technique for the adsorption on Lloyd's reagent and for the extraction with CHCl₃ was identical in the two methods.

After extracting with CHCl₃, 5 ml. of the aqueous layer were transferred from each measuring cylinder to a test tube and evaporated to dryness. A separate blank was not required. To each tube was added 0.3 ml. nitrating mixture and the residue dissolved by rotating the tube. After standing for several minutes at room temperature, the contents were diluted and neutralized at -6°, as described above. After adding 20 ml. ethanol and centrifuging off the precipitated Na₂CO₃, 20 ml. of the supernatant fluid were diluted with 5 ml. distilled water. The optical density of the solution was read against distilled water at 420 m μ ., using a 3 cm. cell, before and after the addition of 0.05 ml. saturated aqueous KOH. The 24 hr. excretion in mg. was calculated by the formula given for the 2:5-amide, taking U as the average difference between the optical density of the unknown before and after the addition of KOH, and R as the corresponding difference obtained with the recovery.

RESULTS AND DISCUSSION

Accuracy of the methods

Under the conditions of nitration and colour development described above, the relationship between optical density and concentration of pyridone is linear (Fig. 1). To obtain constant colour development with the 2:5-amide, care must be taken with the dilution and neutralization, since the colour intensity is greatly influenced by the conditions of temperature and pH. Sodium carbonate gave the best results of a number of weak alkalis tested. The effectiveness of the above technique probably lies in the fact that the carbon dioxide evolved during the reaction between the carbonate and the diluted acid mixture keeps the pH at the optimum value. In the case of the 2:3-amide these conditions are not so important.

With both isomers losses occur in the adsorption on Lloyd's reagent as well as in the extraction with chloroform, but the proportion of different added amounts of either the 2:5- or the 2:3-amide which is recovered by the present technique (about 30%) is

constant for any particular sample of urine, provided that the analyses are carried out side by side in the same way, and that the amounts of added pyridone do not vary over too wide a range. There is a slight tendency for the percentage recovery to decrease as the amount of added pyridone is increased, but the effect is insignificant if the amount does not vary more than about threefold.

Fig. 1. Typical standard curves for *N*-methyl-2-pyridone-5-carboxylamide (A), and *N*-methyl-2-pyridone-3-carboxylamide (B). A Lumetron colorimeter was used, with 1 cm. cells.

The degree of precision of the method for the 2:5-amide, which has been examined in more detail than that for the 2:3-amide, should be adequate for most purposes. The average deviation between the optical densities of pairs of unknown solutions or recoveries has been found to be 2.5%, and that between results for the pyridone content of urine calculated on the basis of one unknown and one recovery to be 10%. Results calculated on average values for the optical density of two unknown solutions and two recoveries show a considerably lower degree of variability, of the order of 5%.

The specificity of the two methods has been studied in considerable detail. Of a number of compounds which were nitrated in amounts of 0.5 mg., the following gave no colour when nitrated at room temperature or at 100°, and when made alkaline either with sodium carbonate or with potassium hydroxide: phenol, histamine, caffeine, xanthine, adenine, uric acid, guanine and thymine. Histidine, tyrosine and tyramine gave yellow colours but they were produced after nitration either at room temperature or 100°, and in the presence of either sodium carbonate or potassium hydroxide. Since

the blanks in the methods for both isomers are colourless, it is obvious that these compounds, if present in normal urine, are removed by the adsorption and extraction processes and do not interfere.

In the method for the 2:3-amide a trace of brown colour of unknown origin develops on the addition of potassium hydroxide and gives apparent values of 0.1–1.5 mg./day for the urinary excretion of this isomer. Since the colour differs widely in spectral absorption properties from the yellow colour given by the 2:5-amide, the true amounts excreted must be less than these apparent values. The fact that no significant increase in the apparent values occurred after the ingestion of a large dose of nicotinamide (Table 1), strongly suggests that the 2:3-amide is not excreted in human urine.

Although the 2:5-amide gives no colour in the method for the 2:3-amide, the latter compound, if present in urine, would interfere in the method for the 2:5-amide, since, when nitrated at 100°, it behaves in a similar manner to the 2:5-amide, giving a yellow colour with sodium carbonate. Evidence has been provided above, however, that the 2:3-amide is not present in urine. Confirmatory evidence that the method for the 2:5-amide is specific for the latter compound is afforded by the fact that the yellow colours given by a large number of samples of urine, when read against the corresponding blanks, invariably showed the same spectral absorption properties as the yellow colour derived from pure *N*-methyl-2-pyridone-5-carboxylamide.

Although both the corresponding acids, namely, *N*-methyl-2-pyridone-5-carboxylic acid and *N*-methyl-2-pyridone-3-carboxylic acid can be nitrated and give the same respective colour reactions as the amides, they are not extracted from alkaline aqueous solution by chloroform, and therefore, if present in urine, would not be included in the result. A method for the determination of the 2:5-acid in urine is being

worked out and will be reported in a later communication. *N*-ethyl-2-pyridone-3-carboxylamide, *N*-benzyl-2-pyridone-3-carboxylamide, and *N*-benzyl-2-pyridone-5-carboxylic acid give similar colour reactions after nitration, but there is no reason to suspect the presence of these compounds in urine. *N*-methylnicotinamide gives no colour reaction. The specificity of the methods has been tested only with normal urines and with urines excreted by normal adults after administration of nicotinic acid and related compounds.

Application of the methods

In Table 1 are shown the results obtained by the present methods for the excretion of the 2:5- and the 2:3-amides by two healthy adult European subjects (no. 1, a male of 27 years; no. 2, a female of 36 years) on successive days before and after the oral administration of 500 mg. of nicotinamide. The corresponding values for the urinary excretion of the total acid-hydrolysable derivatives of nicotinic acid and of *N*-methylnicotinamide are also included in the table. The results show that the 2:3-amide is of no significance in nicotinic acid metabolism, but that the 2:5-amide is the most important metabolite of nicotinic acid which has been recognized up to the present time. Of the dose of 500 mg. of nicotinamide, 47–57 % was excreted as the 2:5-amide, while only 23–26 % was accounted for in the form of all other known metabolites together.

The results suggest that the sum of the daily excretions of the 2:5-amide and of *N*-methylnicotinamide may possibly afford a much better index of nicotinic acid status than any criterion which has been suggested up to the present time. Further results on the excretion of the 2:5-amide and of other metabolites of nicotinic acid after the ingestion of various derivatives of nicotinic acid will be reported in a later communication.

Table 1. *Urinary excretion of various metabolites of nicotinic acid before and after oral administration of 500 mg. of nicotinamide*

Subject no.	Metabolite of nicotinic acid	Urinary excretion (mg./day)					Percentage of dose excreted in the urine
		Before dose		After dose			
		Day 1	Day 2	Day 1	Day 2	Day 3	
1	Total acid-hydrolysable derivatives*	—	2.9	19.1	2.7	2.6	3
	<i>N</i> -methyl-nicotinamide†	—	5.7	127	9.2	11.5	23
	<i>N</i> -methyl-2-pyridone-5-carboxylamide	—	7.5	256	36.6	19.9	47
	<i>N</i> -methyl-2-pyridone-3-carboxylamide	—	<0.9	<1.6	<0.7	<0.6	0
					Total	73	
2	Total acid-hydrolysable derivatives*	0.7	0.6	13.4	0.4	0.6	3
	<i>N</i> -methyl-nicotinamide†	1.8	2.3	110	5.5	4.2	20
	<i>N</i> -methyl-2-pyridone-5-carboxylamide	6.7	6.5	295	61.3	15.6	57
	<i>N</i> -methyl-2-pyridone-3-carboxylamide	<0.1	<0.7	<0.9	<0.7	<0.5	0
					Total	80	

* Determined by the method of Perlzweig, Levy & Sarett (1940).

† Determined by a modification of the method of Najjar & Wood (1940).

While this work was in progress an outline of a fluorimetric method for the determination of *N*-methyl-2-pyridone-5-carboxylamide in urine was reported by Rosen, Perlzweig & Handler (1948). The authors considered that their values for normal urines (5.0–13.2 mg./day) may have been somewhat low, but it is obvious that they are of the same order as those obtained by the present colorimetric method (see Table 1, and also Dean & Holman, 1949).

SUMMARY

1. Methods are described for the determination of *N*-methyl-2-pyridone-5-carboxylamide and *N*-methyl-2-pyridone-3-carboxylamide in human urine. The methods are based on the nitration of the compounds, after isolation from urine by adsorption on Lloyd's reagent followed by extraction with chloroform, and on their colorimetric determination by

means of the yellow colours given by the nitro derivatives in alkaline solution.

2. The accuracy and specificity of the methods is discussed.

3. Results are shown which indicate that the 2:3-amide is of no significance in nicotinic acid metabolism, but that the 2:5-amide is an important metabolite. The 2:5-amide was excreted by two normal adults in amounts of the order of 7 mg./24 hr. After the ingestion of 500 mg. of nicotinamide, the urinary excretion of the 2:5-amide increased greatly, 47–57 % of the dose being excreted in 72 hr. as the latter compound.

4. It was possible to account for 73–80 % of an orally administered dose of 500 mg. of nicotinamide in the urine of two adult subjects as *N*-methyl-2-pyridone-5-carboxylamide, *N*-methylnicotinamide and the total acid-hydrolysable derivatives of nicotinic acid.

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The Terminal Peptides of Insulin

By F. SANGER (Beit Memorial Fellow)
Biochemical Laboratory, University of Cambridge

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One of the outstanding problems of protein chemistry is the elucidation of the relative positions occupied by the amino-acid residues in the protein molecule. A method for the identification of those terminal amino-acids in which the α -amino group is free has already been applied to several proteins (Sanger, 1945; Porter & Sanger, 1948). The protein is treated with 1:2:4-fluorodinitrobenzene (FDNB) and submitted to complete hydrolysis. Thereafter the *N*-2:4-dinitrophenyl (DNP) derivatives of the amino-acids are separated from each other and quantitatively estimated. By this means insulin has been shown to contain glycine and phenylalanine in the terminal position, each present to the extent of two molecules per insulin molecule of molecular weight 12,000.

The method can be adapted in determining the sequence of amino-acids which occupy positions near to the terminal amino-acid residues. For this pur-

pose the protein is submitted to partial hydrolysis leading to the liberation of a series of *N*-2:4-dinitrophenyl (DNP) peptides. These differ from the other products of partial hydrolysis in that they are acids which can be extracted from acid solution by organic solvents and can thus be obtained relatively free from other unsubstituted peptides. The peptide mixture so produced is relatively simple and can be fractionated by chromatography on silica gel. Determination of the structure of the individual *N*-2:4-dinitrophenyl peptides then reveals the order of the amino-acid residues in proximity to the free α -amino groups. The present paper describes the results obtained by applying the method to insulin. The nature and order of the amino-acid residues contiguous to the lysyl residues have also been determined.

Since insulin contains four free α -amino groups, it was concluded that the molecule is built up of four