DISCUSSION

The method described is based on two observations. The first, made by Jackson (1938), is that small amounts of iron can be quantitatively precipitated as sulphide from ashes of biological material with a high P : Fe ratio. The second, that the ferrous sulphide so precipitated can be redissolved without separation from the interfering ions present, and combined quantitatively and preferentially with $\alpha\alpha'$-dipyridyl, was made while trying to apply Jackson's methods to ashes of protein hydrolysates. These ashes contain an iron complex very resistant to acid hydrolysis. It is therefore unlikely to be iron pyrophosphate, which can be hydrolyzed by acids (Jackson, 1938; Howe, 1944). The nature of the complex is not known, but it may be significant that iron metaphosphates can be formed at 200–300° and are stable up to 800° (Brasseur, 1944). Direct experimental confirmation of the presence of the metaphosphate ion is difficult; its solutions, however, are known to have marked tendencies to sequester multivalent ions. After reduction and precipitation of iron as sulphide, one or more possible interfering ions are left in solution. It seemed that introduction of $\alpha\alpha'$-dipyridyl and re-solution of the iron would be likely to lead to the preferential formation of stable ferrous dipyridyl rather than any of the unwanted ferric complexes. Any tendency for the iron to revert to the ferric state would be checked by the nascent H_2S evolved from the dissolving ferrous sulphide. This appeared to be the case.

SUMMARY

1. A method for determining iron in biological materials with a high P : Fe ratio is described. It is applicable over the range 0·5–10 μg. Fe/ml. All analytical manipulations, except the final centrifuging before colour reading, are performed in the crucible in which the sample has been ashed. Blanks are therefore kept at a minimum.

2. The method has proved satisfactory with protein hydrolysates and cows' milk and may be suitable for other materials where phosphate interference is met.

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The Excretion of $l(-)$-Tyrosine in Urine

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Estimation of the $l(-)$-tyrosine content of urine is of value in the assessment of hepatic disease (Lichtman, 1934) and in the study of $l(-)$-tyrosine metabolism. The methods hitherto available lack the necessary sensitivity and specificity for the measurement of normal and pathological tyrosinuria. Thus the tyrosinase method of Lichtman & Sobotka (1929) fails to detect the tyrosine present in normal urine, and Millon's reaction lacks specificity.

In the present communication a method is described which estimates only $l(-)$-tyrosine and l-3:4-dihydroxyphenylalanine and is sufficiently sensitive to measure the $l(-)$-tyrosine content of normal urine. The material estimated is referred to throughout this paper as $l(-)$-tyrosine because the presence of l-3:4-dihydroxyphenylalanine in normal urine is considered unlikely. The possibility that a mixture of the two substances has been estimated has not however been excluded. Data have been obtained for a small series of normal men and women and a rough assessment made of the $l(-)$-tyrosine output of a few normal domestic animals. The output of $l(-)$-tyrosine has also been measured in pregnancy and in a few pathological conditions.

Evidence for the occurrence of tyrosine in normal urine was obtained by Embden & Reese (1906), although a pure product was not isolated. Pouchet (1880) is quoted by Neubauer & Huppert (1910) as

having detected tyrosine in normal urine, but the original publication is not accessible. Rosenbloom & Gardner (1914) reported the occurrence of tyrosine crystals in the urine of a normal pregnant woman.

METHODS

$l(-)$-Tyrosine was estimated by submitting 2·0 ml. of urine, or urine concentrate, to the action of a specific preparation of $l(-)$-tyrosine decarboxylase (Epps, 1944) and measuring the CO_2 evolved in a Warburg manometer as in the analysis of protein hydrolysates described by Gale (1945). This enzyme decarboxylates $l(-)$-tyrosine and l-3:4-dihydroxyphenylalanine specifically (Epps, 1944; Gale, 1940).

Preparation of urine. If concentration of the urine is not required, the acidity of the urine is adjusted to pH 5·5 with acetic acid. In the case of normal urine the $l(-)$-tyrosine content is too low for direct estimation, and the following procedure for concentration of the urine is used. Urine (200 ml.) and crystalline oxalic acid (6 g.) are concentrated by vacuum distillation almost to dryness. The oxalic acid maintains a pH low enough to retain $l(-)$-tyrosine in solution and prevent hydantoin formation (Dakin, 1910), and precipitates much urea as urea oxalate. The concentrate is washed into a weighed flask; its volume and the washings should not exceed about 30 ml. The preparation is then filtered by suction and a sample of the total fluid component, determined gravimetrically, brought to pH 5·5 with a measured volume of 10N-NaOH. The dilution involved in this neutralization is noted and allowed for in the calculation of the $l(-)$-tyrosine content.

Manometric estimation. The concentrate (2·0 ml.) at pH 5·5 is measured into the main chamber of the Warburg manometer cup and 0·2 ml. of 2·2M-citrate buffer of pH 5·5 added. Into the side arm is measured 0·8 ml. of a 2·5% (w/v) suspension of enzyme preparation in 20% (w/v) Na_2SO_4 buffered to pH 5·5 with 0·2M-citrate. After temperature equilibration, the enzyme is tipped into the main chamber and readings taken until a constant value is obtained. A control unit is set up in which the enzyme suspension is replaced by buffered Na_2SO_4, and its readings are deducted from the test readings. The weight in mg. of $l(-)$-tyrosine in the manometer cup is $0·00844 \times (\mu l. $ of CO_2 evolved). This factor allows for the finding of Gale (1945) that the manometric output of CO_2 is 96% of the theoretical value. From this finding the $l(-)$-tyrosine content of the urine can be readily calculated. When unconcentrated urine, or $l(-)$-tyrosine standards are analyzed the enzyme is suspended in 0·2M-citrate buffer instead of buffered Na_2SO_4. The 20% (w/v) Na_2SO_4 and the 2·2M-citrate buffer are supersaturated solutions at room temperature and should be warmed sufficiently to secure complete solution immediately before use.

RESULTS

Control readings in absence of $l(-)$-tyrosine. The tipping of the buffered enzyme preparation into the 0·2M-citrate buffer used in these experiments causes no manometric change. When urine concentrate is used in the main chamber a large positive blank reading is obtained on tipping in 0·8 ml. of 0·2M-buffer. This blank can be shown not to be due to

liberation of CO_2 by arranging for CO_2 absorption in the centre cup, when the blank reading is unchanged. If the buffer in the side arm contains 20% (w/v) of Na_2SO_4, a very small blank reading is obtained with most urine concentrates, and this concentration of Na_2SO_4 was therefore used in the enzyme suspension when analyzing samples of concentrated urine.

Effect of urea and of Na_2SO_4 upon decarboxylase activity. When $l(-)$-tyrosine equivalent to 74 $\mu l.$ of CO_2 was treated with decarboxylase in the presence of 20% (w/v) of urea, 84% inhibition was observed. A similar experiment in the presence of 20% (w/v) Na_2SO_4, instead of the urea, showed no inhibition, as would be expected from the absence of salt effect up to 33% saturation with $(NH_4)_2SO_4$ found by Epps (1944). Thus 20% Na_2SO_4 can be safely used to suspend the enzyme in the side arm.

Recovery of $l(-)$-tyrosine added to urine. Solutions of $l(-)$-tyrosine in normal urine were prepared over a range of concentration that might be encountered in clinical work (Lichtman, 1934). These solutions gave on analysis the recoveries shown in Table 1.

Table 1. *Recovery of $l(-)$-tyrosine added to urine*

Nature of urine	$l(-)$-Tyrosine added (mg./100 ml.)	Recovery of added $l(-)$-tyrosine	
		Observations (%)	Mean (%)
Normal (not concentrated)	57·1	97, 99	98
Normal (not concentrated)	40·0	100, 103	102
Normal (not concentrated)	19·0	99, 100	100
Normal (not concentrated	5·0	105	
Normal (not concentrated	3·0	104	
Normal (not concentrated)	2·0	120, 161	141
Urine containing bile pigment	30·0	98	
Urine preserved with toluene	30·0	99	
Normal urine (concentrated)	3·0	95, 101	98
Normal urine (concentrated)	2·5	97, 99	98

Although good recovery was obtained without concentration of the urine with additions of $l(-)$-tyrosine as low as 3·0 mg./100 ml., yet it is advisable to concentrate the urine whenever the $l(-)$-tyrosine content falls below 20 mg./100 ml.

The excretion of $l(-)$-tyrosine in urine. Urines (24 hr. collections) were obtained from six normal men and six normal women, seven normal pregnant women, four cases of toxaemia of pregnancy, three cases of liver disease and one case of nephritis.

Table 2. *Excretion of $l(-)$-tyrosine in urine*

Source of sample	$l(-)$-Tyrosine content (mg./100 ml.)		24-hr. excretion of $l(-)$-tyrosine (mg.)		Difference between duplicate analyses (% of mean)	
	Range	Mean	Range	Mean	Range	Mean
		(a) Normal subjects				
6 normal men	0·9–1·9	1·4	16–39	25	2–10	5·5
6 normal women	1·0–1·7	1·3	11–28	19	2–9	4·9
7 normal pregnant women	2·2–6·2	3·2	19–82	52	2–6	5·1
		(b) Individual abnormal urines				
Toxaemia of pregnancy	2·7		35		2	
Toxaemia of pregnancy	2·1		17		6	
Toxaemia of pregnancy	0·9		17		8	
Cirrhosis of liver. Case 'C': 24. iv. 46	3·6		42		1	
9. v. 46	3·9		68		Single estimation	
Case 'N'	3·8		14		9	
Acute yellow atrophy of liver	115		—		1	
Chronic nephritis	1·8		22		9	

Toluene was added as preservative, and duplicate analyses carried out on the day after the completion of the collection. Loss of $l(-)$-tyrosine was found if appreciable bacterial decomposition of the urine was allowed to occur. The results of these analyses are given in Table 2. $l(-)$-Tyrosine was also found in single samples of urine from normal pigs ($\male$ and $\female$), sheep and dog in concentrations of 0·5, 0·5, 1·6 and 0·7 mg./100 ml. respectively, but analysis of these urines presents difficulties due to deep pigmentation and precipitation of much solid matter on concentration, and the technique used for human urine is not completely satisfactory for these samples.

DISCUSSION

The chief difficulty in estimating $l(-)$-tyrosine in urine concentrates by means of the specific decarboxylase is the inhibitory effect of high concentrations of urea. A urea concentration of 6% (w/v) or less does not seriously interfere with the enzyme action, and the urea content of the concentrate can be kept down to this level by evaporating the urine in the presence of excess oxalic acid. Urea oxalate has a solubility at 20° of approximately 9·4% (w/v) in 0·2M-citrate buffer at pH 5·5. This corresponds to 5·3% (w/v) urea, which is slightly below the values generally found in concentrates. If the copious precipitate of urea oxalate and other solids is well washed with successive small volumes of cold water good recovery of $l(-)$-tyrosine is obtained (Table 1). Concentration is carried out by vacuum distillation in order to avoid excessive darkening, which would interfere with colorimetric adjustment of pH, and possible destruction of $l(-)$-tyrosine.

No difference is apparent between the $l(-)$-tyrosine concentration in the urine of normal men and normal women. The concentration is less variable than the daily output. There is increased concentration of $l(-)$-tyrosine in the urine in normal pregnancy of about 6 months' duration. It is of interest to note that the concentration of histidine is also raised in the urine during normal pregnancy (Kapeller-Adler, 1933). It may be that further work with sensitive and specific methods will show that the excretion of other amino-acids is similarly influenced by pregnancy. As was to be expected, the cases of liver disease showed increased concentration of $l(-)$-tyrosine in the urine. The increase was slight in two cases of cirrhosis of the liver without very severe symptoms, but in the case of acute yellow atrophy of the liver, which rapidly terminated fatally, a concentration of 115 mg. of $l(-)$-tyrosine/100 ml. of urine was observed. Tyrosine crystals were not found on microscopical examination of this urine.

SUMMARY

1. The determination of $l(-)$-tyrosine in urine by means of $l(-)$-tyrosine decarboxylase is described.

2. The concentration of $l(-)$-tyrosine in urine from 12 normal adults varied from 0·9 to 1·8 mg./100 ml. (mean 1·3 mg./100 ml.), and the total daily excretion varied from 11 to 37 mg. (mean 23 mg.).

3. An increased concentration of $l(-)$-tyrosine was found in urine from seven normal pregnant women and three cases of liver disease.

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A Note on the Calibration of Barcroft Differential Manometers

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Manometers used for tissue respiration experiments may be calibrated: (i) by introducing known volumes of gas from a pipette (Munzer & Neumann, 1917), (ii) by filling the apparatus with mercury, determining the volume of the several parts, and calculating the constant from the appropriate formula, (iii) by carrying out a chemical reaction in the apparatus which results in the absorption or evolution of a known amount of gas.

The number of reactions suitable for the chemical calibration of manometric apparatus is small, and, so far as the author is aware, no direct comparisons between the results of chemical and physical calibration have been made on an extensive scale. Of the reactions available for chemical calibration, the absorption of oxygen by ferrous hydroxide in alkaline solution, proposed by Harrison (1933), seems particularly well suited to the purpose, since the reagents, unlike those required for some of the methods, are in common use in the laboratory and the iron solutions may readily be standardized with permanganate or dichromate. It has the further advantage of reproducing the conditions under which the manometers are to be used, since the gas exchange measured is an uptake of oxygen.

Gibson (1943) used the method of Harrison (1933) for calibrating Barcroft manometers at 17° with good agreement between the chemical and physical methods, and when it became necessary to calibrate a number of similar manometers for use at 37° the method was again employed. It was now found that there was a systematic difference between the results given by the chemical method and the physical method of Dixon (1943). In the present paper it is shown that changes in vapour pressure on mixing the solutions used were responsible for this difference and that such changes in vapour pressure may be a source of appreciable error in accurate

manometric work, for example in calibration or in the liberation of CO_2 by the addition of strong acid in an R.Q. determination.

METHODS

Apparatus. The manometers used were of the standard Barcroft type with flasks of about 35 ml. capacity. They were shaken at a rate of 120 complete oscillations/min. through a stroke of 2 in. The specific gravity of the manometric fluid was 0·8. All apparatus used was calibrated under working conditions.

Reagents. The iron solutions were prepared by dissolving 4·6–4·8 g. $FeSO_4.7H_2O$ in 50 ml. $0·2N-H_2SO_4$ and diluting to 500 ml. with water. The solutions were standardized at the time of use by titrating portions of 50 ml. with $0·1N-KMnO_4$. The permanganate was filtered through a sintered glass funnel and standardized against dried and powdered sodium oxalate (A.R.). The standardization of the iron and of the permanganate was repeated daily in triplicate.

Method. 2 ml. iron solution were delivered into the main chamber of the manometric flasks, using a microburette, and 0·2 ml. 2·5N-NaOH (approx.) measured into Keilin tubes which were suspended from the centre well of the manometric flasks. The flasks were then attached to the manometers and worked firmly on to the ground joints. Thirty minutes at 37° was allowed for temperature equilibration. The taps were closed and an initial reading taken. The Keilin tubes were then displaced and the manometers shaken for a further period of 30 min. At the end of this time the final reading was taken.

RESULTS

Control values. In each experiment two manometers were set up with water in place of the iron solution. It was found that these control manometers showed an apparent absorption of some 10–12 μl. of gas on mixing the alkali with the water. It seemed that this effect might be due to lowering of the vapour pressure of the solution in the main