of which might conceivably be due to interaction with the zinc prosthetic group of the enzyme. Perhaps some interference with zinc transfer in insulin synthesis may account for the action of alloxan on the β -cells of the pancreatic islets.

SUMMARY

1. Four diabetogenic ureides were examined for their power to inhibit alkaline phosphatase and were all highly active. Of ten related compounds examined, two were poor inhibitors of phosphatase

although not diabetogenic and non-inhibiting for phosphatase. The anti-phosphatase activity seems to reside in a free 5-hydroxyl group.

2. This action on phosphatase has been demonstrated in the presence of plasma and blood and in tissue sections. It is reversible, non-competitive and unaffected by magnesium.

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The Estimation of Histidine In Urine and its Excretion by Normal and Pregnant Women

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The urinary excretion of histidine has been a matter of interest since Voge (1929) demonstrated an increase in the excretion during normal pregnancy. This finding has been confirmed (Kapeller-Adler, 1934; Niendorf, 1940; Racker, 1941; Langley, 1941; O'Brien & Quelch, 1943; Dawson, 1944), together with the fact that the excretion drops in pre-eclamptic toxæmia. These results have been obtained by examination of a few urine samples from a considerable number of individuals, but in studying the metabolism of histidine it is of interest to examine the excretion for the duration of the pregnancy in order to ascertain whether any definite trends in histidine excretion are observed. The results obtained in the course of two pregnancies are presented in this paper together with some observations on the excretion of histidine by normal women at different times during the menstrual cycle. The latter work was undertaken as a result of a suggestion by Boxer & Kapeller-Adler (1937) that the gonadotrophic hormones inhibited the activity

of liver histidase thus giving rise to the increased excretion of histidine during pregnancy. This conception would be strengthened if it could be demonstrated that normal women showed a rise in histidine excretion at the time of greatest gonadotrophic hormonal activity in the menstrual cycle, although Boxer & Kapeller-Adler found the rise in excretion occurred approximately at the time of the first occurrence of a positive Ascheim-Zondek test which suggested that a minimal level of circulating gonadotrophic hormone was essential to affect the histidine excretion.

The method of estimation of urinary histidine is based on a colour reaction described by Knoop (1908) and consists of a bromination of the histidine, removal of excess bromine and heating the product at 100° when a characteristic colour develops. The method as applied by Armstrong & Walker (1932) and Kapeller-Adler (1934) has been criticized on the grounds of interference by the urea, urates, phosphates and pigments of the urine (Racker, 1940;

Inhibition of phosphatase in kidney sections by alloxan.

A. S. V. BURGÉN AND J. I. LORCH—THE ACTION OF ALLOXAN
AND RELATED COMPOUNDS ON ALKALINE PHOSPHATASE

Langley, 1941). It has been found that two distinct pigments can be produced according to the pH at which the heating is carried out and a technique has been devised in which the pH is controlled and errors due to other causes have been overcome.

METHODS

The estimation of histidine in urine

Reagents. (a) 10% (v/v) H_2SO_4 , (b) N - and 0.1 N - $KMnO_4$, (c) bromine reagent: bromine (1 ml.) is added to 100 ml. glacial acetic acid and the volume made up with distilled water to 200 ml. This reagent is kept in the dark and renewed every 3 days. (d) 0.5% phenol and (e) a fresh standard solution of histidine hydrochloride containing 1 mg./ml.

Procedure. To a series of four reaction tubes graduated at 10 ml., varying amounts of the standard histidine hydrochloride solution are added and the same volume of the urine added to each tube, this volume being such that the final amounts of histidine present lie within the range 0.8–1.6 mg. (the volume required is determined by a previous rough estimation). Sulphuric acid, 0.5 ml. of 10% (v/v), is added and the contents diluted to approximately 5 ml. with distilled water. Potassium permanganate is then added drop by drop to each tube until a pink colour, permanent for 30 sec., is obtained. With concentrated urines, N - $KMnO_4$ must be added initially until the pink colour is permanent for approximately 20 sec. and then the treatment completed with 0.1 N - $KMnO_4$; with weaker urines only the latter is necessary. Bromine reagent (0.5 ml.) is then added to each tube and the contents allowed to stand for 5 min. For the majority of urines this amount has been found sufficient, but the tube containing the greatest concentration of histidine may be examined at 2 min. intervals for the presence of excess bromine by means of starch-iodide paper, and if none is present a further 0.5 ml. bromine reagent should be added to all tubes. Excess bromine is removed at the end of the 5 min. period by addition of 0.5 ml. of 0.5% phenol. The volumes are then adjusted to 10 ml. and the tubes heated in the dark in a boiling water bath for 5 min., cooled and the volumes again adjusted to 10 ml. The histidine value represented by the colour developed is determined either from a standard curve determined at the same time under identical conditions with the Hilger 'Spekker' photoelectric absorptiometer using Ilford spectrum blue filter or by direct comparison with the colour developed from known amounts of histidine with a Klett Bio-colorimeter. When the Spekker photoelectric absorptiometer is used, a urine blank is included which receives identical treatment except that 50% acetic acid is substituted for the bromine reagent, and the relationship between the optical density of the reaction mixture and histidine concentration was found to be represented by a straight line between the limits of 0.8–1.6 mg. histidine/10 ml.

A typical urine estimation is reproduced in Table 1. Where the recovery of added histidine differs markedly from the theoretical the urine value is obtained by calculation but in the majority of instances the recovery is almost theoretical and the value can be obtained from the graph.

Table 1. *Typical estimation of histidine in normal urine using photoelectric colorimeter*
(vol. of urine taken 1.5 ml./tube)

Tube	Amount of histidine HCl added (mg.)	Optical density (1 cm. cell)	<i>l</i> -Histidine HCl	
			(mg./tube) from standard curve	(mg./100 ml.) original urine
1	—	0.300	1.015	67.7
2	0.2	0.375	1.205	71.2
3	0.4	0.466	1.410	68.4
4	0.6	0.556	1.610	68.2

The effect of pH on the production of colour

Hunter (1922) observed a variation in the colour produced in the above reaction according to the pH of the solution, recording a dull yellow-brown in acid solution and a pink colour in alkaline solution, using NaOH for pH adjustment and a purple colour when using NH_4OH . Kapeller-Adler (1934) recommended the use of ammonia and an alkaline reaction while Langley (1941) preferred an acid reaction.

Fig. 1. Titration curves of pigments derived from histidine. Curve I. Purple pigment produced at pH 9. Curve II. Same materials as I after acidification to pH 3. Curve III. Brown pigment produced at pH 3.

Hunter's findings have been confirmed and it has been further shown that the purple pigment is formed at pH values greater than 5 and is converted to the reddish brown material by lowering the pH beyond this value. At pH values below 5 only the reddish brown pigment is produced but this is not converted to the purple substance on raising the pH. The existence of two distinct substances has been confirmed by means of titration curves determined on coloured products prepared at various pH values. For the preparation of the curves 12.3 mg. histidine HCl were dissolved in 5 ml. distilled water and then treated with excess bromine, allowed to stand for 5 min., excess bromine removed by a stream of nitrogen, the pH adjusted to the required value, the volume made to 10 ml. and the mixture heated in a boiling water bath for 5 min., cooled and diluted to 100 ml. The titration curve was determined by means of a glass electrode and pH meter. Fig. 1 shows a series of

typical curves. There is a point of inflexion in curve I at approximately pH 5.5 indicating the presence of a weak basic group, which is absent from curves II and III.

In employing this colour reaction in the routine estimation of histidine it is essential that the conditions used should be certain to yield one or other of these pigments and not a mixture of them both. Although the purple pigment is preferable for colorimetric work, the use of the brown pigment is recommended as interference by urine pigments and dissolved phosphate is considerably reduced at an acid pH. The conditions described above for the reaction yield a pH of approximately 2 and this has been found to be most satisfactory.

RESULTS

Histidine excretion by normal women

The subjects were 38 women medical students. The procedure adopted was to examine a 24 hr. urine sample at intervals of 7 days for 5 weeks in order to cover a complete menstrual cycle. The period of maximum activity of the gonadotrophic hormones is about 14 days after the onset of the period and that of minimum activity during the last 8 days before the onset of the next period (Frank, 1935). Consequently a maximum excretion of histidine would be expected 10–20 days after the onset of the period and the minimum excretion during the last 8 days of the cycle, if the suggestion of Boxer & Kapeller-Adler (1937) is correct.

The individual excretions all fell within what is recognized as the normal range, i.e. 0–400 mg./day, with two exceptions where all the samples examined had a histidine content of 400–600 mg./day. Variation in histidine content from one sample to another for the same individual was considerable and only 7 subjects showed an excretion which was constant within 100 mg. over the whole period. The maximum and minimum points of the excretions for the 38 subjects were spread evenly over the whole menstrual period, so that no correlation was apparent between histidine excretion and gonadotrophic hormone activity.

Histidine excretion in pregnancy

Two cases of pregnancy have been examined from about the third month of the pregnancy until delivery. Case I was a normal pregnancy; case II was complicated by anaemia during part of the pregnancy, although the foetus was apparently not affected and delivery was normal. As far as possible early morning urine samples were examined at weekly intervals and in the second case 24 hr. samples at monthly intervals. The early morning samples have varied in volume from 300 to 800 ml. The normal excretion of histidine of both subjects was found to be nil and the excretion of both subjects returned to this value within a few days of delivery. The blood pressure records in both cases were normal throughout the pregnancy.

The records for output of histidine in the early morning samples are shown in Fig. 2 and the 24 hr. output at monthly intervals in case II in Fig. 3.

Fig. 2. Histidine excretion in two normal pregnancies. Case I, $\times - \times - \times$. Case II, $o - o - o$. The arrows indicate time of delivery.

Fig. 3. 24 hr. excretion of histidine at monthly intervals in case II. Arrows indicate period of examination of diet.

There is a striking difference between the two cases; case I shows a histidine output considerably above that of case II with a definite peak at 5 months. The excretion in case II is fairly uniform but at a low level and some of the values recorded have been associated in other patients with pre-eclamptic toxæmia (Dawson, 1944).

In considering the possible causes of the increased histidine excretion in pregnancy, the increased protein intake which frequently occurs may affect the level of histidine excretion and subnormal protein intake may give rise to a lower histidine excretion. To obtain an idea of the protein intake of the two individuals concerned, their diet was weighed for 6 days and its nutritional value calculated from tables (McCance & Widdowson, 1942). The diet of case I was examined during the fifth month and case II during the eighth month of the pregnancy, this period being indicated in Fig. 3 by arrows. The results are shown in Table 2.

Table 2. *Average daily food intake over a selected period during normal pregnancy*

(Values from tables)

	Calories	Protein (g.)	Iron (mg.)
Case I	2723	92	16.4
Case II	2134	66	10.65

The protein and iron intakes are seen to be normal for case I but low for case II for a diet of late pregnancy, but despite this low protein intake there was a retention of 117 g. protein over the 6 days, calculated from urinary total nitrogen and intake.

To examine further the effect of ingested histidine on its excretion, case II ingested 2 g. of *l*-histidine hydrochloride and the 24 hr. excretion values were determined for 5 days before and 4 days after its ingestion. The period chosen was the week during which the diet was recorded and figures for histidine and total nitrogen excretion are shown in Table 3.

Table 3. *Effect of ingestion of histidine on its urinary excretion by a pregnant woman*(2 g. *l*-histidine HCl ingested on day 6)

Day	24 hr. histidine output (mg.)	24 hr. total N output (g.)
1	690	6.6
2	702	8.4
3	685	7.3
4	693	8.2
5	378	6.8
6	784	7.5
7	665	7.5
8	470	8.2
9	314	5.3

There was a considerable drop in histidine output on the fifth control day followed by a rise after ingestion of the histidine with a steady fall over the following 3 days. Following this period the diet was improved, as shown by total nitrogen determinations on 24 hr. urine samples at weekly intervals during the last month of the pregnancy, with a figure of approximately 10.5 g. N/day and the 24 hr. histidine excretion is also seen to rise over this period (Fig. 3).

It is of interest to note that case II showed a haemoglobin level at the third month of 95%, which had dropped to 55% at the time of the dietary determination and had risen again to 92% a week before delivery. Iron salts were administered from 8½ months to delivery.

DISCUSSION

It is apparent from the above results that the histidine excretion of an individual may vary

between wide limits, both in the normal and the pregnant woman. There is some evidence (Kapeller-Adler & Schiller, 1935) that dietary intake of protein which contains histidine may influence output and this may explain variations in excretion of normal individuals, but it will not explain the rise which occurs in pregnancy, although it may influence the degree of the rise. In view of the high histidine content of haemoglobin, this is a possible source of urinary histidine, since considerable quantities will be released on its breakdown and the rise in excretion in pregnancy may be associated with an increased turnover of this material. In this connexion it is interesting to note that case I above showed no abnormalities of haemoglobin level while case II had developed an anaemia by the seventh month and also showed a consistently lower histidine output than case I. In addition, during the last 6 weeks of this pregnancy there was an increased protein intake (as shown by nitrogen excretion figures) and a return of the haemoglobin level to normal accompanied by a rise in histidine output. Even if this suggested connexion between haemoglobin and urinary histidine is correct, this fact sheds no light on the causation of the greatly increased urinary histidine excretion of the normal pregnant woman.

SUMMARY

1. A colorimetric method is described for the estimation of histidine in urine.

2. In the reaction used, two pigments may be produced according to the pH at which the reaction is carried out.

3. The 24 hr. urinary histidine excretion of 38 normal women at weekly intervals during a complete menstrual cycle has been examined. No significant trends were observed and no correlation with gonadotrophic hormone activity was apparent.

4. The histidine excretion during the course of two normal pregnancies has been examined. In the first case there was a peak in the excretion at the fifth month of the pregnancy and the excretion in this case was at a generally higher level than in the second.

5. The diets of the two patients were examined; that of the second patient showed a low protein and iron intake. The possible effects of ingestion of histidine-containing protein and haemoglobin formation and breakdown on histidine excretion are discussed.

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Pyruvate Oxidation in Ox Heart

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During the past decade, the *in vitro* oxidation of pyruvic acid by molecular O_2 in the presence of surviving animal tissues has been extensively studied. Published work indicates that, in general, the requirements of these pyruvate oxidation systems for maximal O_2 uptake are (i) inorganic phosphate, (ii) adenine nucleotide, (iii) a C_4 -dicarboxylic acid and possibly (iv) magnesium ions (Banga, Ochoa & Peters, 1939; Smyth, 1940; Ochoa, 1943; Long, 1943, 1946).

Although it is now established that a trace of a C_4 -dicarboxylic acid is needed as a catalyst for maximal O_2 uptake in the pyruvate oxidation system, the effect of the C_4 -acids on the rate of pyruvate disappearance is not yet settled. Thus Annau, Banga, Blazsó, Bruckner, Laki, Straub & Szent-Györgyi (1936), working with rabbit liver, consistently found no increase in the rate of pyruvate utilization when fumarate was added, and Long (1946) obtained the same result with dialyzed pigeon brain suspensions. On the other hand, Krebs & Eggleston (1940), using pigeon breast muscle, reported an increased rate of pyruvate disappearance in one experiment and a decreased rate of disappearance in another.

The present paper summarizes the results of an investigation of the behaviour of a dialyzed ox heart preparation respiring in a medium containing pyruvate. It has been found that maximal activity requires the addition of inorganic phosphate, adenine nucleotide, fumarate and magnesium ions, and that the increased rate of O_2 consumption produced by fumarate addition is accompanied by a corresponding increase in the rate of pyruvate disappearance.

EXPERIMENTAL

Materials

Na pyruvate, Na fumarate and $MgCl_2$ were pure analytical specimens. Ba adenosine triphosphate was prepared from rabbit skeletal muscle by the method of DuBois, Albaum & Potter (1943). When required, the barium was removed as $BaSO_4$ and a stock solution prepared at a concentration of 0.01 M.

Methods

Ox heart was placed on ice as soon as possible after the death of the animal, and on arrival at the laboratory was rapidly washed and freed from fat and connective tissue. The muscle was minced finely in a Latapie mincer, the cutting blade of which was driven by an electric motor. (It has been found that the degree of subdivision of the tissue is a matter of considerable importance: thus if the mincing process is carried too far, the respiration rate of the resulting preparation is low, while if it is insufficient subsequent dialysis will fail to remove substrates and activators.)

A portion of the mince (2.8 g.) was suspended in 25.2 ml. ice-cold 0.9% KCl containing 0.04% $MgCl_2 \cdot 6H_2O$ (tissue diluted 1 in 10), and several 5 ml. portions of the suspension dialyzed in collodion sacs against 4 l. of 0.4% KCl containing 0.002 M- $MgCl_2$ for 3 hr. at 2°. The dialyzer was gently rocked throughout this period so that the movement of an air bubble from one end of the sac to the other kept the tissue suspension in motion.

Portions of the dialyzed suspension, usually 2 ml., were pipetted into the manometric flasks containing substrates, activators, etc. (total volume, 3.4 ml.) and 0.2 ml. 2 N-KOH with filter paper slip was placed in the centre well. Respiration was conducted at 28°, using Barcroft manometers. After 15 min. equilibration, respiration was allowed to continue for a further 2 hr. Pyruvate was estimated by the