Although these experiments are far from complete, it appears possible from the data obtained that histaminase might play an important part at the onset and during labour. It may be suggested that histaminase, which is very effective in the placenta during pregnancy, might become inhibited at the end of pregnancy by the action of some compounds, possibly the sex hormones, which are known to play an important role at the onset of labour. It may be recalled that in 1937 (Kapeller-Adler, 1937) it was shown that the gonadotropic hormones inhibit the activity of the histidase.

SUMMARY

1. The activity of the histaminase in the sera and placentae of pregnant women has been studied with two different substrates, cadaverine and histamine, in parallel tests.

2. The histaminase reaction is negative in the serum of non-pregnant women.

3. In normal pregnancy serum the histaminase test was found to be positive without exception, irrespective of the substrate used.

4. The activity of the histaminase is diminished in mild cases of pre-eclamptic toxæmia and only a trace of activity or a negative enzymic reaction was obtained in cases of *hyperemesis gravidarum*, in severe cases of pre-eclamptic toxæmia and in eclampsia.

5. The results obtained in investigations on 45 placentae suggest a possible correlation of the amount of the histaminase in the placenta with the character of the labour, the activity of the histaminase in the placenta having been found to be inversely proportional to the uterine efficiency.

6. The results obtained render doubtful Zeller's claim that the histaminase and diaminoxidase are identical.

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A Study of the Determination of Glucuronic Acid by the Naphthoresorcinol Reaction, with the Photoelectric Absorptiometer

By S. W. F. HANSON, G. T. MILLS AND R. T. WILLIAMS, *Department of Biochemistry, University of Liverpool*

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Methods for the determination of glucuronic acid in biological materials have appeared intermittently in the literature for the last 50 years. That none of these methods is entirely satisfactory is not surprising, since the reactions upon which they are based are not specific. The problem of the accurate determination of glucuronic acid in biological materials is therefore a difficult one.

Glucuronic acid may be estimated by the following methods: (1) those based upon measurement

of its reducing properties, e.g. by Shaffer-Hartman and Bertrand's reagents (Goebel & Babers, 1933); by Benedict's reagent (Quick, 1924, 1925); by the ferricyanide reduction method of Miller & Van Slyke (Fishman, 1938); (2) those depending upon the formation of furfural by the action of hydrochloric acid on glucuronic acid (e.g. C. Tollens, 1909; Haendel, 1929; Tanabe, 1938); (3) those depending upon the carbon dioxide set free by the decarboxylation of glucuronic acid with mineral acids and other

reagents (B. Tollens & Lefèvre, 1892, 1907; Buston, 1932; Voss & Pfirschke, 1937; Freudenberg, Gudjons & Dumpert, 1941); (4) those depending on the violet colour produced when glucuronic acid is heated with naphthoresorcinol (1:3-dihydroxynaphthalene) and hydrochloric acid (B. Tollens & Rorive, 1908; B. Tollens, 1908). This reaction is given by other uronic acids, and pigments are also formed by sugars such as glucose, fructose and pentoses (cf. Neuberg & Kobel, 1931; Green & White, 1932).

Our purpose was to estimate the glucuronic acid output in urine after feeding 2:4:6-trinitrotoluene (α -T.N.T.) had been fed. Some of the results on T.N.T. have already been published (Channon, Mills & Williams, 1944). For pure solutions of glucuronic acid and conjugated glucuronic acids we found Fishman's method (1938) to be accurate and convenient. But for urine a ferricyanide method was useless, unless the glucuronide could be selectively extracted by a solvent, as in the case of menthylglucuronide. The method which seemed to offer the greatest possibilities was the one based on the Tollens naphthoresorcinol reaction (1908). An early attempt to put this colour reaction on a quantitative basis was made by C. Tollens (1909); and later the method was studied by Maughan, Evelyn & Brown (1938), by Kapp (1940), by Mozolowski (1940) and by Deichmann (1943) among others.

EXPERIMENTAL

In the methods of Maughan *et al.* (1938), of Kapp (1940) and of Deichmann (1943) ether was used to extract the pigment produced in the Tollens reaction. The intensity of the colour of the ethereal solution was then measured by means of a photoelectric colorimeter (Maughan *et al.*, and Deichmann) or a Stüfen photometer (Kapp). In the present work, colour intensity was measured with a Spekker photoelectric absorptiometer, but we found ether an inconveniently volatile solvent. Amyl ether was suitable but its purification is hazardous and it was therefore discarded. Of a number of alcohols tried, amyl alcohol proved satisfactory. Extraction of the pigment was rapid and complete, and the resulting solution had a blue colour with a sharp maximum absorption at 615–620 μ (see Fig. 1). The filter employed (Ilford no. 607 orange, one of the set of spectrum filters H. 558 issued by Adam Hilger, Ltd. for use with the absorptiometer) cuts off all light of wave-length less than 570 μ , thus removing the interfering absorption in the region of 510 μ when normal urine is used and at 480–505 μ when urine from T.N.T.-fed rabbits is used. The amyl alcohol solution of the pigment produced from both normal and T.N.T. urines showed a sharp maximum at 615–620 μ which is characteristic of that shown by the

pigment from *l*-menthylglucuronide or glucurone (the curves from these compounds are almost identical (Fig. 1)).

Fig. 1. Absorption curves of amyl alcohol extract of pigment from I, *l*-menthylglucuronide; II, T.N.T. urine (rabbit); III, normal urine (human); IV, *l*-menthylglucuronide in presence of an equimolecular amount of fructose; V, absorption curve of Ilford spectrum filter no. 607 (orange) reproduced from Ilford's catalogue (Ilford Colour Filters, p. 39).

Optimum conditions for colour production

Time of heating. In agreement with Kapp (1940) we found that 4 hr. in a boiling water-bath was necessary for

Fig. 2. Effect of acid concentration on colour production. 2 ml. solution plus 2 ml. 0.25% naphthoresorcinol plus variable quantity of conc. HCl.

maximum colour production. Over 90% of the colour had developed in 2 hr., and for economy of time this period of heating was adopted.

Concentration of HCl. Maximum colour production is obtained when 3 vol. conc. HCl in a total of 7 vol. solution are used (see Fig. 2). Kapp (1940) found optimum colour production when the above ratio was 3 to 10.

Concentration of naphthoresorcinol. There is no sharp maximum for colour production on varying the naphthoresorcinol concentration. The colour intensity increased rapidly with rising concentrations to 0.25% solution and then only slowly for concentrations above this. Above 0.25% naphthoresorcinol, however, the blank value was significant and consequently a concentration of 0.25% was chosen.

Method of estimation

Reagents. Naphthoresorcinol (British Drug Houses, Ltd.), 0.25 g. dissolved in 100 ml. distilled water by keeping at 37° for 1 hr. The solution is filtered and stored at 0° in a dark bottle. It is stable for about 1 week. Hydrochloric acid, concentrated ('pure', J. W. Towers & Co. Ltd.). Amyl alcohol (Analar, British Drug Houses, Ltd.). Ethanol (absolute).

Procedure. 2 ml. of solution containing 10–80 μ g. glucuronic acid, 2 ml. 0.25% naphthoresorcinol solution and 3 ml. conc. HCl in 6 $\times$ $\frac{1}{8}$ in. pyrex tubes, are mixed by rotation and placed in a boiling-water-bath for 2 hr. The tubes are cooled in ice for 10 min., 5 ml. amyl alcohol added, and the tubes stoppered and shaken vigorously for 15 sec. The contents are poured into 50 ml. separating

Fig. 3. Calibration curve for the estimation of glucuronic acid.

funnels, and washed out with a further 5 ml. amyl alcohol added to the separating funnels, the tubes being allowed to drain for 1 min. The aqueous layer is separated, the amyl alcohol solution run into graduated tubes, and the funnels allowed to drain for 1 min. The solution is made up to 11 ml. with ethanol (to dissolve any fine droplets of water suspended in the amyl alcohol), the contents of the tubes mixed and the colour estimated. A calibration curve was constructed for the Spekker photoelectric absorptiometer (10 mm. cells, Ilford 607 orange filters) with *l*-menthylglucuronide and glucuronic acid. The curves for these two compounds were identical and the common curve is illustrated in Fig. 3.

RESULTS

Typical figures for the recovery of glucuronic acid from pure solutions of various glucuronides are presented in Table 1. This table shows that most glucuronides can be estimated with an error of about $\pm 5\%$. Low results were obtained, however, with *o*-aminophenylglucuronide, *o*-amino-*p*-sulphonamidophenylglucuronide and euxanthic acid. The reason for these low results was investigated in the case of *o*-aminophenylglucuronide. This may be caused by either interference by the aglycone set free during hydrolysis or lack of hydrolysis of the glucuronide under the conditions of the estimation, i.e. heating for 2 hr. The first possibility was tested by estimating *l*-menthylglucuronide in the presence of an equimolecular proportion of *o*-aminophenol. The following typical results were obtained, which showed that this phenol had no appreciable effect on the estimation:

Substances present	Glucuronic acid	
	Calculated (μ g.)	Found (μ g.)
<i>l</i> -Menthylglucuronide alone	56.0	53.5, 54.5
<i>l</i> -Menthylglucuronide + <i>o</i> -aminophenol (33.6 μ g.)	56.0	54.0, 52.5

The second possibility was tested by using longer periods of heating and comparing the rate of increase in colour produced by *l*-menthylglucuronide and

Fig. 4. Effect of increase of time of heating on colour production from *l*-menthylglucuronide (I) and *o*-aminophenylglucuronide (II). III is the theoretical curve for *o*-aminophenylglucuronide if it behaved in the same manner as *l*-menthylglucuronide.

o-aminophenylglucuronide after 2 hr. It was found that the rate of increase in colour between 2 and 4 hr. heating was greater for the latter substance than for the former (see Fig. 4 and Table 1). This result indicates that the low results for the *o*-amino

phenylglucuronides are very probably due to incomplete hydrolysis under the standard conditions outlined above.

Table 1. *Recovery of glucuronic acid from pure solutions of glucuronides*

Glucuronide	Glucuronic acid	
	Calculated (μg.)	Found (μg.)
<i>l</i> -Menthylglucuronide	53.0	57.0, 56.0
<i>d</i> -Isomenthylglucuronide*	40.6	41.0, —
<i>p</i> -Amino- <i>o</i> -sulphonamidophenylglucuronide†	31.0	32.5, 34.0
<i>p</i> -Aminophenylglucuronide‡	44.9	47.5, 49.8
<i>m</i> -Aminophenylglucuronide‡	51.0	53.0, —
<i>m</i> -Aminophenylglucuronide‡	48.0	48.8, —
Sodium pregnandiolglucuronide‡	57.0	55.0, —
<i>o</i> -Aminophenylglucuronide‡	54.0	46.0, 45.0 (51.0) , (56.5)
<i>o</i> -Amino- <i>p</i> -sulphonamidophenylglucuronide‡	31.3	22.5, 22.5
Euxanthic acid§	61.0	52.0, 52.6

* Williams (1938).

† Williams (1943).

‡ Venning (1938).

§ Euxanthone glucuronide, prepared from Indian yellow (Purree).

|| After 3 hr. heating.

|| After 4 hr. heating.

In the absence of interfering substances, added *l*-menthylglucuronide could be almost quantitatively recovered from normal human urine. The urine was diluted 1 in 50 before analysis and each estimation carried out in duplicate. Recoveries of 98–106% were obtained. Similar results were obtained from normal rabbit urine.

Mucic acid and sugars such as glucose, fructose and pentose interfered. The interference was approximately equal for glucose and arabinose and slightly greater for fructose. Glucose, however, does not present a serious problem when present in amounts less than 50 μg. in 2 ml. of test solution, i.e. 2.5 mg./100 ml. The results in Table 2 illustrate this point. When more than about 50 μg. glucose is present the apparent glucuronic acid rises rapidly, even though the actual glucuronic acid remains constant.

Table 2. *Recovery of glucuronic acid in the presence of glucose*

Glucose added (μg.)	Glucuronic acid*	
	Added (μg.)	Found (μg.)
0	53.6	54.0
18	53.6	56.5
36	53.6	57.0
54	53.6	61.0

* Added as *l*-menthylglucuronide.

Since it may often be necessary to estimate glucuronic acid in the presence of glucose, the removal of the latter by fermentation was investigated (cf. Kakinuara, 1939, 1940). In our hands this method did not prove effective. The glucose content of the sample was reduced to 30–40 μg. by incubation with fresh washed brewer's yeast, but the results in Table 3 show that yeast contains water-soluble material, not completely removed by exhaustive washing with water, which produces a pigment when heated with HCl and naphthoresorcinol. This material appears in increasing amounts when the yeast is fermenting glucose. No fructose could be detected in the fermenting medium and the presence of small amounts of ethanol had little effect on colour production.

Table 3. *Effect of the products of fermentation of glucose on glucuronic acid estimation*

(Washed brewer's yeast suspended in 5 vol. tap water. Tubes incubated at 30° for 1 hr. and then centrifuged at 3000 r.p.m. for 5 min. 2 ml. solution used for each estimation in duplicate. Three experiments.)

Tube	<i>l</i> -Menthylglucuronic acid solution (ml.)	Water (ml.)	0.02M-glucose (ml.)	Yeast suspension (ml.)	Glucuronic acid found		
					1 (μg.)	2 (μg.)	3 (μg.)
A	1	2	0	0	45.0	42.0	50.0
B	1	0	1	1	63.7	59.0	85.5
C	1	1	0	1	57.0	54.0	63.5
D	0	1	1	1	33.0	36.0	53.5
Glucuronic acid present in tubes A, B and C					(46.6)	(41.6)	(52.0)

Glucuronic acid content of normal urine

The estimations were carried out as described above, with 2 ml. of urine diluted 1 in 50. The

Table 4. *Glucuronic acid found in normal human urine*

Subject	Urine vol. in 24 hr. (ml.)	Glucuronic acid found	
		(g./day)	(mg./100 ml.)
1	1620	1.053	65.0
1	1070	0.976	91.6
2	1640	1.256	76.6
2	1114	0.929	83.3
2	1190	0.893	75.0
3	1530	1.325	86.6
3	1370	1.210	88.3
3	1295	0.757	58.3
Average	—	1.050	80.0

results showed (see Table 4) that adult laboratory workers on a normal British war-time diet excreted about 1 g. of glucuronic acid/day.

DISCUSSION

Perhaps the earliest attempt at estimating glucuronic acid in normal human urine was that made by Günther, de Chalmot & B. Tollens (1892). They evaporated 200 ml. of urine to dryness, carried out a furfural distillation on the residue, and obtained furfural corresponding to 40–50 mg. of glucuronic acid. If the daily excretion of urine is 1500 ml. their result corresponds to 300–375 mg. of glucuronic acid/day. Similar figures were obtained by C. Tollens & Stern (1910) both by the furfural distillation method and the colorimetric naphthoresorcinol reaction. Lower figures were obtained by Sauer (1930) (220–290 mg./day) and by Boku & Kin (1931) (13 mg./100 ml.). More recently values varying from 350–800 mg./day have been obtained by Maughan *et al.* (1938), by Wagreich, Kamin & Harrow (1940) and by Wagreich, Abrams & Harrow (1940), whilst Deichmann (1943) found the lower figures of 65–239 mg./day. All these recent values have been obtained by the Tollens colorimetric technique with the more modern photoelectric methods for measuring the colour, and it has been tacitly assumed that what is estimated is glucuronic acid, although there is no unequivocal proof of this.

It is claimed that the method developed in the present paper will estimate glucuronic acid added to normal urine with an accuracy of 5% in the absence of certain limiting proportions of sugars. On applying the method to the estimation of the natural glucuronic acid of urine we obtained figures higher than former workers (see Table 4). The material estimated in this way may or may not be entirely glucuronic acid. Eagle (1927) states that glucose is not normally excreted in urine and that what has been considered as glucose, a substance fermentable by yeast, is in reality a group of substances which only gradually decompose under the conditions of fermentation that remove glucose in a short time. However, if the average daily output of glucose in normal urine is 500 mg. and the daily urinary volume is 1500 ml., then 2 ml. of urine diluted 50 times will contain about 13 μ g. of glucose. According to Table 2 this amount of glucose in 2 ml. solution does not interfere with the glucuronic acid estimation.

In the case of rabbits we have carried out a large number of estimations (see Channon *et al.* 1944). One group received a dry diet which produced a daily urine volume of c. 50 ml. In this group the range of glucuronic acid output was 52–218 mg./day with an average of 148 mg. Another group received a wet diet which gave a daily urine volume of c. 250 ml.; here the range was 62–196 mg./day and the average 140 mg.

It is known that a large number of substances excreted in normal urine are conjugated with glucuronic acid; these include phenol, *p*-cresol, indoxyl, sex hormones, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, catechol, to mention only a few. In addition to these there are present complex glucuronic acid-containing compounds such as urinary mucoid, urochrome and possibly mucopolysaccharides and unknown glucuronogenic substances of the diet. If the quantities of these were known it might then be possible to account for the colorimetric glucuronic acid values of normal urine.

SUMMARY

1. A study of the estimation of glucuronic acid by the Tollens naphthoresorcinol reaction is described. The relation of time of heating and of concentration of hydrochloric acid and of naphthoresorcinol to colour production has been studied, the intensity of colour in amyl alcohol being measured in the Spekker photoelectric absorptiometer with an orange filter.

2. Under the standard conditions described, glucuronides can be estimated with an accuracy of $\pm 5\%$ in the absence of certain interfering substances such as glucose and fructose. For certain glucuronides, however, the time of heating must be increased above the standard conditions adopted for routine estimations.

3. The method has been applied to urine and it has been found that the daily output of glucuronic acid is of the order of 1 g. for adult humans and 140–150 mg. for rabbits. The significance of these values is discussed.

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Carotene and Lycopene in Rose Hips and Other Fruits

By F. C. JACOBY AND F. WOKES, *Ovaltine Research Laboratories, King's Langley, Herts*

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Rose hips have been previously reported to contain carotene (Kuhn & Grundmann, 1934; Wokes, Johnson, Organ & Jacoby, 1942) and lycopene (Karrer & Widmer, 1928; Escher, 1928), and they have also been found to possess considerable vitamin A activity (Svensson, 1936). Sufficient care has not always been taken to differentiate between the various carotenoid pigments. Moreover, data are needed on the amounts found in different species.

This paper provides such data by describing a simple method of estimating carotene and lycopene separately in the presence of other pigments, which has also given satisfactory results when applied to tomatoes and *Solanum Dulcamara* berries. The results on dried rose hip extract have been checked by a biological assay confirming the presence of vitamin A activity.

METHODS

Materials. Ripe rose hips of known species collected in the Durham area during October and November 1943 by Prof. Heslop Harrison, and in the Royal Botanic Gardens, Kew, during September 1943 by Dr R. Melville were posted immediately to King's Langley and stored in dry bottles in a refrigerator until examined; tests at intervals during the experimental period showed no loss of carotenoids under the given storage conditions. The samples of dehydrated rose hips were taken from large-scale batches prepared in the Ovaltine Laboratories in 1942-4 from locally grown hips, mainly *Rosa canina* and *R. dumetorum*. Ripe *Solanum Dulcamara* berries were collected in the King's Langley area during October 1943 and examined immediately. Ripe tomatoes grown out of doors in King's Langley in 1943 were preserved in jars by the usual method and when opened for examination 3 months later were in excellent condition.

Extraction of carotenoids. For this we have used an excellent unpublished method for extracting carotene from plant material devised by Dr Vernon Booth, to whom we are greatly indebted for advice and help. It was applied to rose hips as follows:

About 5 g. typical rose hips were selected and weighed. The flesh was dissected from seeds, stalks, etc., divided into two portions and weighed. Tests showed that no significant loss of moisture took place during the dissection, so that results with the flesh could be correlated with those from whole fruits. Each portion of the flesh (c. 1 g.) was ground with quartz powder and a mixture of acetone and petrol ether (2:3 by volume). The supernatant yellow solution was decanted into a separating funnel and the residue ground with more acetone-petrol ether mixture until no more colour was extracted. (With dried extracts and other dry material a few drops of distilled water were added to facilitate extraction.) The combined extracts were then washed with distilled water by a continuous flow apparatus, until the absence of striae as the drops of water fell through indicated that all acetone had been removed. Usually about a litre of water, passing through at the rate of about 2 drops per sec., was required to remove the acetone from 50 to 100 ml. of combined extract. The xanthophylls, which usually formed less than half of the total pigments, were removed by shaking two or three times with one-third volume of diacetone until practically no further colour was removed. The diacetone was then washed out with water.

Chromatographic separation. The adsorbent used in this investigation was Alocol brand colloidal aluminium hydroxide (A. Wander Ltd., King's Langley) activated by heating at 100° for a few minutes immediately before use. Its watery suspension had pH 7.4-7.5 as compared with 6.5-9.5 for other brands of alumina. The average particle size determined by means of haemocytometer was 25-100 μ , which is considerably higher than the 7 μ found by Zechmeister & Cholnoky (1941) for Merck alumina standardized by Brockmann's method. However, comparison with Merck and several other brands of alumina in use in this country at present has shown the Alocol after activation to be as efficient as any for separating carotene and lycopene. Recovery experiments with widely varying proportions of β -carotene and lycopene also gave satisfactory results. Details of this comparison will be published later.

The petrol ether extract, prepared as above, was percolated through a column of alumina (20 $\times$ 2 cm.). The pigments were adsorbed in a zone about 1 cm. deep and about