Observations on the Oxidation of Homogentisic Acid in Urine

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Homogentisic acid, which is considered to be an intermediate in the metabolism of tyrosine and phenylalanine, is excreted in a number of different conditions. The most widely studied of these, which is caused by a genetic inability to oxidize homogentisic acid, was termed alcaptonuria, owing to the characteristic darkening of the urine on standing. It has also been shown (Sealock & Silberstein, 1940; Rogers & Gardner, 1949) that, normally, ascorbic acid plays some part in the metabolism of homogentisic acid, since the latter has been claimed to be present in the urine of scorbutic subjects and of premature infants (Levine, 1949). In both these latter cases, but not in congenital alcaptonuria, the administration of ascorbic acid stops the excretion of homogentisic acid. Dietary amino-acid imbalance may also interfere with the normal metabolism of tyrosine and phenylalanine and result in excretion of homogentisic acid (Glynn, Himsworth & Neuberger, 1945; Neuberger & Webster, 1947). Homogentisic acid is the reduced form of 1:4-benzoquinone-2-acetic acid, both together constituting an oxidation-reduction system. The high positive redox potential of benzoquinone-acetic acid, however, makes it appear extremely unlikely that it could exist for any appreciable length of time in a biological medium. It is therefore surprising that Fishberg (1948) reported its presence in urines from a case of enterogenous cyanosis, from scorbutic animals and from cases of rheumatic fever. The last observation was of special interest since it suggested a possible relationship of rheumatic fever to a vitamin C disturbance, a relationship for which some evidence already existed (Rinehart, 1938). Since the methods for detecting the presence of benzoquinoneacetic acid depend largely on its oxidizing properties, special care must be taken to avoid the development of oxidizing substances in urine after its collection. This could arise, for instance, as a result of the growth of nitrite-forming bacteria. The nitrite so produced would not only give several of the tests used by Fishberg (1948) to demonstrate benzoquinoneacetic acid, but might oxidize any homogentisic acid present to the quinone and thus account for her unexpected findings. It was therefore decided to investigate the action of oxidants on homogentisic acid in urine; and secondly, in the light of

these results, to re-investigate Fishberg's claims to have found benzoquinoneacetic acid in urines from rheumatic fever cases.

EXPERIMENTAL

Paper chromatography was used to demonstrate the presence of homogentisic acid in urines. The property of ready oxidation to the quinone has been used to provide a delicate and specific test. Homogentisic acid was separated from interfering substances in urine on paper chromatograms, and estimated quantitatively, after elution, by treatment with phosphomolybdate and measurement of ultraviolet absorption at 355 m μ . (Consdén & Stanier, 1951).

Reactions of homogentisic acid in urine towards oxidizing conditions were followed by means of ultraviolet absorption spectrophotometry. Homogentisic acid and benzoquinoneacetic acid have characteristic absorption spectra, sufficiently different from each other to enable one to be detected in the presence of the other.

Materials. Homogentisic acid was a pure sample kindly supplied by Dr A. Neuberger, F.R.S. 1:4-Benzoquinone-2-acetic acid was prepared from this by oxidation with chromic acid (Mörner, 1912). It liberated 96% of the theoretical quantity of I₂ from acidified KI, and by titration with alkali had an equivalent of 162 (theoretical, 166).

Paper chromatography. In *n*-butanol-acetic acid-water (4:1:5 by vol.), homogentisic acid had an *R_F* value of 0.80. A satisfactory reagent was ammoniacal AgNO₃, which produced an immediate black or brown spot on the chromatogram; 1 μ g. could be clearly detected in this way. In this solvent system, benzoquinoneacetic acid was almost entirely converted to homogentisic acid. On chromatograms developed with *n*-butanol in an atmosphere of NH₃, oxidation of homogentisic acid quickly occurred, producing a brown spot at the origin and the presence of the quinone in this spot could be demonstrated by spraying with acidified KI-starch solution (1% KI and 1% starch made acid with a few drops 6*N*-HCl) which produced a slowly developing blue colour (*R_F*, 0). Owing to the instability of the quinone at high pH, the KI reaction gives much less colour on chromatograms developed for long periods such as 17 hr. than when developed after only 2-4 hr. Benzoquinoneacetic behaved exactly as homogentisic acid in *n*-butanol-NH₃ (1 μ g. being detectable), but, in the acetic acid system, was converted to homogentisic acid, since no brown spot positive to KI was present, but instead, a spot with *R_F* of

0.80 was demonstrated with ammoniacal AgNO_3 . A more specific test for homogentisic acid is carried out as follows: the solution to be tested is applied to Whatman no. 4 paper and duplicate chromatograms developed in *n*-butanol-acetic acid for 2–4 hr. One chromatogram, after drying, is sprayed with the silver reagent. The other is spotted on a suitable area with bromothymol blue indicator and then sprayed with the acidified KI-starch reagent. It is then hung for 1.5–2 min. in a chamber containing NH_3 vapour, produced by a 3% NH_3 solution; during this time the indicator spot becomes blue. The chromatogram is then removed and after a short airing becomes acid, as shown by the change of colour of the indicator spot to yellow. A blue spot now appears in the same position as that occupied by the Ag spot on the control chromatogram. The procedure can be repeated, which usually enhances the blue KI spot. 1 μg . homogentisic acid in 5 μl . urine can readily be detected in this way.

Quantitative estimation of homogentisic acid. Homogentisic acid (25–50 μg .) was run on paper chromatograms for 4–6 hr. in butanol-acetic acid. The areas containing the homogentisic acid, as determined by a 'marker' chromatogram treated with ammoniacal AgNO_3 , were cut out and eluted with water in the apparatus described by Consden, Gordon & Martin (1947), but instead of running the eluate into a capillary tube, the liquid was allowed to flow down a thin strip of filter paper into a 5 ml. graduated flask. An end of the strip was touched against the corner of the cut when it was sufficiently wet, and the strip then remained in position by capillary attraction. After about 0.1 ml. of eluate had been collected, KH_2PO_4 (0.4 ml. of 1% solution) and ammonium molybdate (0.4 ml. of 5% solution in 5N- H_2SO_4) were added and the solution made to volume with water. After allowing to stand for 1 hr. the optical densities at 355 $m\mu$. were read. A portion of the chromatogram not containing homogentisic acid was treated in the same way to provide the control solution. This paper blank had a negligible absorption when measured against the reagents alone. Comparison of the absorptions with the standard (Consden & Stanier, 1951) gave 90–100% recoveries. Several other methods were tried for the estimation of small quantities of homogentisic acid from paper chromatograms. Estimation of the optical densities of eluates at 290 $m\mu$. was not successful owing to comparatively high and variable paper blanks, which could not be eliminated. The KI titration method of Neuberger (1947) was too insensitive for the small amounts handled. The method of Park & Johnston (1949) for the estimation of reducing sugars, consisting of production of Prussian blue from ferri-cyanide, was applied to homogentisic acid, and although it is even more sensitive than the phosphomolybdate method when dealing with 2–10 μg ., it could not be applied to homogentisic acid from chromatograms as the paper blanks were high and variable.

Recovery from urine. 150 μl . urine containing 30 μg . homogentisic acid were applied to paper, with intermediate drying, across a line 4–5 cm. in length. The development of the chromatogram, the elution of the homogentisic acid and treatment with phosphomolybdate were carried out as described above. The blank was obtained from a similar area of the chromatogram, which was free from reducing substances as shown by the Ag 'marker'. The densities at 355 $m\mu$. measured against the blank gave recoveries of 90%. When larger amounts of homogentisic acid in urine were estimated in this way, better recoveries were obtained, e.g. 100% recovery for 60 μg .

Ultraviolet absorption spectra

Solutions were measured in a Unicam spectrophotometer, Model SP 500, using 1 cm. quartz cells. For measurement, homogentisic acid or benzoquinoneacetic acid (0.004% in water) was mixed with an equal volume of M/15 phosphate buffer, pH 6.8. M/30 Phosphate was the control solution. Solutions of homogentisic acid, if kept at 0°, showed no alteration even after several months, whereas benzoquinoneacetic acid required to be freshly prepared before measurement, since marked changes in the absorption curve are liable to occur on standing, similar to those shown in Fig. 3C.

Action of nitrous acid on homogentisic acid. To homogentisic acid (0.1 ml. of 0.1%) was added freshly prepared 1% NaNO_2 solution (0.1 ml.); M/15 phosphate buffer (2 ml.) was added, followed by 0.1N-HCl (0.5 ml.). The solution (acid to methyl orange) was allowed to stand for 5 min. and was then made up to 5 ml. with M/15 phosphate (final pH, 6.7). A control was prepared containing all the constituents except homogentisic acid. The absorption curve is shown in Fig. 2B. No change in the absorption curve was observed without acidification of the nitrite-containing solution.

Action of NH_3 on homogentisic acid. 0.1N- NH_3 (1 ml.) was added to a solution consisting of 1% homogentisic acid (0.2 ml.) and M/15 phosphate buffer (1 ml.). The solution darkened and liberated I_2 from acidified KI solution. After allowing to stand for 10–15 min., the solution was made to 10 ml. with phosphate buffer. The absorption curve measured against M/15 phosphate buffer is shown in Fig. 3A. The absorption curve of this solution measured after 17 hr. is also shown in Fig. 3C.

Effect of ascorbic acid on homogentisic acid treated with nitrous acid, or with ammonia. After treatment as described above with HNO_2 or NH_3 , ascorbic acid (0.15 mg.) was added to the final buffered solution, and the absorption then measured (Fig. 2C).

Effect of HNO_2 on homogentisic acid treated with NH_3 . N- NH_3 was added to a solution consisting of 1% homogentisic acid (0.5 ml.) and M/15 phosphate (2 ml.) until just alkaline to thymol blue. The solution was made up to 5 ml. with water and allowed to stand for 15 min. To 2 ml. was added NaNO_2 (1 ml. 0.1%) and N-HCl (0.2 ml.) until acid to methyl orange. After 15 min. the solution was diluted to 10 ml. with M/15 phosphate. The absorption curve measured against a control solution containing all the above constituents except homogentisic acid is shown in Fig. 3B.

Spectrophotometry of urine containing added homogentisic acid. The absorption curve of urine from a healthy individual is shown in Fig. 4A. The urine was diluted 200 times with water and phosphate buffer (final concentration M/30), and the absorption was measured against M/30 phosphate buffer. The same urine containing 0.4% added homogentisic acid and diluted similarly gave the curve shown in Fig. 4B. To demonstrate the effects of HNO_2 and NH_3 , the urines containing homogentisic acid were treated with these reagents as described above in the case of homogentisic acid solutions and absorption spectra after dilution were measured against the homogentisate-free urine treated with the same reagents. Similar experiments were carried out on urines containing 0.02% added homogentisic acid, but for spectrophotometry the final dilutions were 40 instead of 200 times.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 1. Ultraviolet absorption spectra of homogentisic acid (*A*) and benzoquinoneacetic acid (*B*) in *M*/30 phosphate buffer, pH 6.8. (All spectra shown in Figs. 1–6 were derived from solutions made up with *M*/30 phosphate buffer pH, 6.6–6.8.)

Fig. 2. Ultraviolet absorption spectra of a mixture of homogentisic acid and benzoquinoneacetic acid (*A*), homogentisic acid after treatment with nitrous acid (*B*) and homogentisic acid treated with nitrous acid or ammonia, then re-treated with ascorbic acid (*C*). Final concentrations of all solutions correspond to 0.002% homogentisic acid or benzoquinoneacetic acid.

Fig. 3. Ultraviolet absorption spectra of homogentisic acid after treatment with ammonia (*A*), treatment of this solution with nitrous acid (*B*), and solution treated with ammonia measured after 17 hr. (*C*). Final concentrations of all solutions correspond to 0.002% homogentisic acid.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 4. Ultraviolet absorption spectra of normal urine (*A*) the same urine containing 0.4% added homogentisic acid (*B*). Urines diluted 200 times.

Fig. 5. Ultraviolet absorption spectra of urine containing homogentisic acid (0.4%) after treatment with nitrous acid (*A*), and ammonia (*B*). Both solutions were diluted 200 times, and absorptions were measured against the homogentisate-free urine treated and diluted similarly.

Fig. 6. Ultraviolet absorption spectra of urine containing homogentisic acid (0.02%) after treatment with nitrous acid (*A* and *B*) and ammonia (*C*). Control solutions for curves *B* and *C* were homogentisate-free urines treated similarly and for curve *A* was untreated homogentisate-urine. Final dilution of urines, 40 times.

RESULTS AND DISCUSSION

The absorption curves of homogentisic acid ($\epsilon = 3430$ at $\lambda_{\max}$ 290 m μ .) and benzoquinoneacetic acid ($\epsilon = 10790$ at $\lambda_{\max}$ 250 m μ .) are shown in Figs. 1A and B, respectively. The absorption curve for a mixture of these two substances is shown in Fig. 2A. If these curves are compared with those which Fishberg (1948) claimed to be the curves of benzoquinoneacetic acid, it will be seen that the latter correspond much more closely, as regards their maxima and minima, with homogentisic acid.

Treatment of homogentisic acid with nitrous acid or ammonia results in almost complete conversion to the quinone (Figs. 2B and 3A). This reaction is largely reversed by excess of ascorbic acid (Fig. 2C). With ammonia, presumably atmospheric oxidation takes place. Retreatment of the ammonia-treated homogentisic acid with nitrous acid appears to remove substances absorbing at short wavelengths (Fig. 3B). Homogentisic acid in urine can be easily detected spectrophotometrically, if present in sufficiently large amounts (Fig. 4B). Treatment of such urine with nitrous acid or ammonia results in the formation of benzoquinoneacetic acid, though, as would be expected, owing to the presence of reducing substances, the conversion may not be so complete as with homogentisic acid alone. This is illustrated in Fig. 5B, where atmospheric oxidation in the presence of ammonia gives a curve similar to that of a mixture of homogentisic acid and benzoquinoneacetic acid (Fig. 2A). On the other hand, nitrous acid results in more complete oxidation (Fig. 5A) in spite of the competing urinary substances, e.g. urea. This effect of nitrous acid could also be reproduced in urine containing nitrite-forming bacteria. Urine containing 0.2% homogentisic acid was inoculated with nitrite-producing bacteria obtained from a contaminated sample of urine which gave reactions positive for nitrite. After incubation, the inoculated urine, on acidification, gave the absorption curve for benzoquinoneacetic acid.

The effects of nitrous acid and ammonia could be demonstrated on urines containing much smaller amounts of homogentisic acid, owing to the smaller dilutions which had to be employed in spite of the interfering absorption of other urinary constituents which is relatively high. Urine containing 0.02% homogentisic acid, when treated with nitrous acid, gave the curve shown in Fig. 6A when measured against the untreated homogentisic acid containing urine, and the curve shown in Fig. 6B when measured against the homogentisate-free urine treated with nitrous acid. A similar curve (Fig. 6C) was obtained after treatment with ammonia and measuring against the homogentisate-free urine also treated with ammonia.

Solutions of homogentisic acid, when made alkaline with ammonia and allowed to stand for a short time, liberated iodine from acidified potassium iodide solution. This reaction was observed even in urine containing only 0.02% homogentisic acid. The presence of homogentisic acid in urine may therefore be demonstrated by three tests described above: (a) by paper chromatography; (b) by the development of oxidizing properties on treatment with ammonia; and (c) by the disappearance of the peak at 290 m μ . in the ultraviolet absorption curve after treatment with ammonia or nitrous acid, and the corresponding appearance of a peak or inflexion at 250 m μ .

Examination of rheumatic fever urines

Urices from febrile rheumatic fever children gave absorption spectra similar to urine containing added homogentisic acid (Fig. 4B). However, this substance was absent as shown by paper chromatography. Treatment of the urine with ammonia failed to modify the absorption curve, and it is concluded that the peak at 290 m μ . is due to excess of substance other than homogentisic acid. This is probably uric acid, since the intensity of this spot on the paper chromatogram was greater than that of a spot obtained under similar conditions from normal urine, and preliminary experiments showed that the absorption curve of uric acid was unaffected after treatment with ammonia. Some of the rheumatic fever urines after standing for 24 hr. liberated iodine from potassium iodide, and gave positive tests with most of the reagents used by Fishberg (1948) for demonstrating benzoquinoneacetic acid. However, these tests are not specific, as they are also given by nitrite. Nitrites were demonstrated in these urines by the red colour produced after addition of sulphanilic acid, acidification and addition of alkaline β -naphthol. This reaction is negative with homogentisic acid and benzoquinoneacetic acid.

Examination of scorbutic guinea pig urines

Urices from scorbutic guinea pigs showed no evidence in their ultraviolet absorption curves of any excess of substance having a maximum at 290 m μ . This was found also for ethyl acetate extracts of the acidified urines. The absence of homogentisic acid was confirmed by paper chromatography.

Examination of urines from rats with methaemoglobinaemia

Methaemoglobinaemia was produced in a group of rats by feeding nitrite. The urines were pooled, acidified, extracted with ethyl acetate, and the concentrated extracts were chromatographed on paper in both butanol-acetic and butanol-ammonia. With

ammoniacal silver nitrate no spot corresponding to homogentisic acid was observed in the butanol-acetic acid chromatogram. In butanol-ammonia, at least six reducing spots were present, with R_f values of 0.1–0.8. A duplicate chromatogram was sprayed with alkaline diazotized sulphanilic acid. Ten spots appeared of various shades of yellow and pink. Six of these appeared to be in positions identical with those of the six reducing spots. Another duplicate chromatogram was sprayed with methyl orange to demonstrate acidic substances. The upper five spots positive to the sulphanilic acid reagent appeared to be acidic. In the above three groups of urines, since 20 μ l. failed to give a positive paper chromatographic test, it is concluded that homogentisic acid was present, if at all, in a concentration of less than 0.005 %.

From the results obtained, it is apparent that great precautions are necessary before the presence of benzoquinoneacetic acid in the urine can be assumed to indicate that this substance is actually being excreted. Bacterial contamination of the urine readily leads to production of ammonia, and somewhat less frequently to nitrite formation. Both of these conditions (the latter, after acidification) will cause almost immediate oxidation of homogentisic acid to benzoquinoneacetic acid. It is therefore only permissible to conclude that any benzoquinoneacetic acid detected was excreted in this form, if bacterial contamination of the urine has been strictly excluded. Moreover, since several of the tests used for benzoquinoneacetic acid depend upon its power as an oxidizing agent, nitrites may themselves be responsible for these reactions in the absence of either benzoquinoneacetic acid or homogentisic acid.

There is no indication in the paper of Fishberg (1948) that the necessary safeguards were taken against bacterial contamination. Our own failure to obtain any evidence of benzoquinoneacetic acid excretion by patients with rheumatic fever strongly

suggests that the positive results of Fishberg should be attributed to nitrite formation in the urine after its passage. The isolation of benzoquinoneacetic acid from her case of autotoxic enterogenous cyanosis appears to rest upon much stronger evidence. But here again it would appear more probable that what was actually excreted was homogentisic acid and that its subsequent oxidation was due to either urinary ammonia, or to nitrite. The presence of nitrite in the blood and urine of patients with autotoxic enterogenous cyanosis is well authenticated (Evans, Enzer, Eder & Finch, 1950), and it is therefore possible that, in such cases, benzoquinoneacetic acid would be formed on acidification of the urine, even in the absence of bacterial contamination, provided of course that homogentisic acid were present. This would imply that the patient was suffering from both methaemoglobinaemia and alcaptonuria.

SUMMARY

1. Paper chromatography of homogentisic acid and benzoquinoneacetic acid is described, and a method is given for the determination of homogentisic acid in urine, after separation on paper chromatograms.

2. The ultraviolet absorption spectra of these two substances have been determined.

3. The behaviour of homogentisic acid in urines under oxidizing conditions has been followed by means of ultraviolet spectrophotometry. Conversion to the quinone takes place in urine, if nitrous acid is present or when the urine is made alkaline.

4. By paper chromatography and by spectrophotometry, homogentisic acid and benzoquinoneacetic acid were found to be absent from the urines of rheumatic fever cases, scorbutic guinea pigs and rats with experimental methaemoglobinaemia.

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REFERENCES

- Consdén, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Consdén, R. & Stanier, W. M. (1951). *Nature, Lond.*, **168**, 298.
Evans, A. S., Enzer, N., Eder, A. J. & Finch, C. A. (1950). *Arch. intern. Med.* **86**, 22.
Fishberg, E. H. (1948). *J. biol. Chem.* **172**, 155.
Glynn, L. E., Himsworth, H. P. & Neuberger, A. (1945). *Brit. J. exp. Path.* **26**, 326.
Levine, S. Z. (1949). *Harvey Lect.* **4**, 303.
Mörner, C. T. (1912). *Hoppe-Seyl. Z.* **78**, 306.
Neuberger, A. (1947). *Biochem. J.* **41**, 431.
Neuberger, A. & Webster, T. A. (1947). *Biochem. J.* **41**, 449.
Park, J. T. & Johnston, M. J. (1949). *J. biol. Chem.* **181**, 149.
Rinehart, J. F. (1938). *Arch. intern. Med.* **61**, 552.
Rogers, W. F. & Gardner, F. H. (1949). *J. Lab. clin. Med.* **34**, 1491.
Sealock, R. R. & Silberstein, H. E. (1940). *J. biol. Chem.* **135**, 240.