SUMMARY

1. Serine and alanine have an inhibitory effect upon indole production from tryptophan by *Esch. coli*. This effect is not a simple relation between the tryptophan and serine concentrations, and only holds when the enzyme is saturated with tryptophan.

2. Fildes's (1945) finding that there is no evidence of tryptophan synthesis from indole and serine using cells of *Esch. coli* is confirmed.

3. Using partition chromatographic analysis and retarding the reaction with mepacrine, alanine has been detected as an intermediate; under similar conditions the presence of serine has never been detected.

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Studies on Alcaptonuria

1. THE ESTIMATION OF HOMOGENTISIC ACID

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An accurate and specific method for the estimation of homogentisic acid applicable to small amounts of urine and plasma was required in connexion with experiments which are described in subsequent papers (Neuberger, Rimington & Wilson, 1947; Neuberger & Webster, 1947). Since it was intended to feed substances closely related to homogentisic acid, it was important to define clearly the specificity of the methods used. The present paper deals firstly with methods already described in the literature, and secondly with a new method which, it is believed, is both very specific and sensitive for homogentisic acid.

Baumann (1892), who established the structure of homogentisic acid, also described a method for its estimation in the urine, based on the reduction of silver nitrate in the presence of ammonia. The method is not specific and it is known that normal constituents of urine such as uric acid also reduce silver ions in an alkaline medium (Garrod & Hurtley, 1905). But in spite of this lack of specificity the many important results on human alcaptonuria obtained by the method of Baumann can be considered substantially correct, for the concentration of homogentisic acid in the urine of alcaptonurics is very high compared with that of other substances having similar reducing properties. For our purpose, however, the method was unsuitable and we have not investigated it further.

A more convenient method which makes use of the reduction of phosphomolybdic acid by hydroquinone and its derivatives in acid solution has been described by Briggs (1922). This method, which has been widely used in America, has considerable disadvantages if other substances arising from the metabolism of aromatic amino-acids are present. Thus Medes (1932) found that both *p*-hydroxyphenylpyruvic acid and 3:4-dihydroxyphenylalanine reduce the phosphomolybdate reagent in an acid medium, though the latter only very slowly. A similar experience was reported by Sealock & Silberstein (1940) who tried to differentiate between the two acids by reading the colour 5 min. after the addition of the final reagent, making allowance for the amount of colour produced in that period by the keto acid. Since similar difficulties were anticipated in our work, we have studied the rate of development of the colour with different concentrations of homogentisic acid in some detail, and have also investigated the validity of Beer's law and the specificity of this reaction.

Another method for the estimation of homogentisic acid is based on an observation by Wieland (1910) who found that hydroquinone is quantitatively oxidized to *p*-quinone by iodine in the presence of sodium bicarbonate without any substitution taking place in the benzene ring. On acidification the quinone reacts with the iodide ion, and is itself reduced again to hydroquinone and a corresponding amount of iodine is liberated which can be titrated with

thiosulphate. That homogentisic acid behaves in an identical manner was shown by Metz (1927). Conditions for the estimation of this acid in the urine were worked out (Lieb & Lanyar, 1929; Metz, 1930) and it was shown that homogentisic acid added to normal urine can be recovered to the extent of 97–98%. As might be expected, the consumption of iodine in slightly alkaline solution is not a specific reaction, since many constituents of normal urine are oxidized under such conditions. The liberation of iodine on acidification, however, should be peculiar to substances having oxidation-reduction potentials of the same order of magnitude as homogentisic acid and showing a similar variation with pH. It was shown by Blix (1932) that this reversible reaction of hydroquinone and homogentisic acid with iodine is a consequence of the variability of the oxidation-reduction potential of the hydroquinone-quinone system with pH, whilst the corresponding potential of the iodine-iodide system is independent of acidity changes. Blix has shown that it is necessary to reduce the pH to about 0, in order to ensure that the reaction goes practically to completion, i.e. to about 99.5%.

But apart from the equilibria involved, there are other factors to be considered. The reactions, both oxidation and reduction, are not instantaneous and their respective rates should vary with the pH. Moreover, the quinone is not very stable in alkaline solution and the possibility of a substitution by iodine has also to be considered. Our first aim was therefore to establish the most favourable conditions for a quantitative recovery of homogentisic acid. It also appeared possible that substances containing amino or thiol groups might react with the quinone and thus interfere with the estimation. Finally we wished to investigate whether this reaction also occurs with 1:2-dihydroxybenzenes, such as dopa and catechol.

During the course of this work it was found that, though the iodometric method was more specific than that of Briggs, several substances which might either occur normally in urine or be formed in our feeding experiments behaved towards iodine exactly like homogentisic acid, whilst others interfered to some extent with the reaction. Attention was therefore directed towards finding an even more specific method of estimation and it was proposed to take advantage of the relative solubilities of homogentisic acid in different solvents and of the fact that it reduces silver ions in acid solution. It was proposed therefore, to extract the homogentisic acid first from water into ether and then back again into water and then estimate it by determining its reducing power at a well-defined pH. If homogentisic acid is added to a silver nitrate solution in the presence of acetate buffers between pH 4–5, colloidal silver is slowly precipitated, but this precipitate cannot be readily estimated by turbidimetry. In the presence of a protective colloid such as gum arabic a silver sol is formed, but it was found that the colour and therefore presumably the particle size varied in an uncontrollable manner. This agrees with the work of Voigt & Heumann (1928) who noted that silver sols rarely obeyed Beer's law. These authors also found that if the reduction of the silver salt is carried out in the presence of colloidal gold of uniform particle size, fairly homogeneous silver sols are obtained. We have used this observation and have added a minute amount of gold sol to the buffered mixture before the addition of homogentisic acid. The most satisfactory results were obtained when the gold sol was prepared by the old method of Faraday (1857).

PART 1. BRIGGS'S METHOD

EXPERIMENTAL

Reagents. The composition of the reagents used was the same as that given by Briggs (1922), viz. a phosphate solution containing 1% KH_2PO_4 (A.R.) and a molybdate solution containing 5% ammonium molybdate (A.R.) in 5N- H_2SO_4 . The standard was a solution of 0.1% homogentisic acid which was made up fresh every second day and kept at 0°. The homogentisic acid used was a synthetic sample thrice recrystallized from an ether-chloroform mixture.

Procedure. A known volume of the solution to be estimated was diluted to about 15 ml. with water. To this was added 2 ml. of molybdate solution and 2 ml. of the phosphate solution. The volume was then made up with water to 25 ml. Readings were taken after exactly 1 hr. unless otherwise stated with a Hilger absorptiometer with a red filter.

RESULTS

Development of the colour. The maximum colour is not produced at once at any concentration tested and the rate of colour development varies with the concentration of homogentisic acid (Fig. 1). At the

Fig. 1. The rate of colour development in the Briggs reaction with different concentrations of homogentisic acid and *p*-hydroxyphenylpyruvic acid.

lower concentration the rate of change after 1 hr. is only small and this period was therefore allowed in all later experiments for the colour to develop. At

the higher concentration the colour increases considerably over several hours and the light absorption observed after 4 hr. is not proportional to the concentration of homogentisic acid. The rate of the development of colour with *p*-hydroxyphenylpyruvic acid was also measured (Fig. 1) and the results obtained were similar to those reported by Medes (1932). The colour development with the keto acid is slower than with homogentisic acid, but the difference is not very great. It appears therefore that a differentiation of the two acids based on separate readings at suitably chosen intervals would be very inaccurate.

Application of Beer's law. At higher concentrations Beer's law is not obeyed, at least if a reading is taken within 5 hr. after mixing the reagents (Fig. 1). However, with concentrations of homogentisic acid varying between 100 and 600 $\mu\text{g./25 ml.}$ the colour produced is linearly proportionate to the concentration. It is advisable therefore to dilute the sample sufficiently so that the final concentration of homogentisic acid should not exceed the value of 600 $\mu\text{g./25 ml.}$

Recovery of homogentisic acid added to normal urine. Some normal urines give a very slight colour with the Briggs reagent, but the error introduced by the reduction of the molybdate reagent through substances other than homogentisic acid in the urine of an alcaptonuric can be safely neglected. Added homogentisic acid is satisfactorily recovered, though there is a slight deflexion, especially at higher concentrations.

Table 1. *The reaction of various substances with phosphomolybdic acid in acid solution*

(Strongly positive indicates that the intensity of the colour is of the same order as that found with homogentisic acid and that the colour develops within the first 10 min. Slightly positive means that the colour is less than 5% of that of homogentisic acid on a molecular basis and that the appearance is delayed.)

Strongly positive	Slightly positive	Negative
Hydroquinone	Catechol	2:5-Dihydroxyphenylpyruvic acid lactone
Gentisic acid	3:4-Dihydroxyphenylalanine	Cysteine
2:5-Dihydroxyphenylalanine	Uric acid	Glutathione
Ascorbic acid	—	Phenylpyruvic acid
<i>p</i> -Hydroxyphenylpyruvic acid	—	—

Specificity of the reaction. All substances which have two hydroxyl groups in the benzene ring in *p*-position to each other resemble homogentisic acid in its reduction of the molybdate reagent (Table 1).

Thus hydroquinone, gentisic acid and 2:5-dihydroxyphenylalanine would be estimated as homogentisic acid. Ascorbic acid, which is also a normal constituent of urine, gives a strongly positive reaction and might account for the slight colour produced with many normal urines. The behaviour of *p*-hydroxyphenylpyruvic acid which has already been discussed above, contrasts with that of phenylpyruvic acid showing that the keto acid group as such does not reduce Briggs's reagent. The lactone of 2:5-dihydroxyphenylpyruvic acid does not reduce, indicating that the lactone ring does not open under the conditions of the test.

If the hydroxyl groups in the benzene are in *o*-position to each other, only a very slow reaction with the Briggs reagent is observed. Thus catechol and dopa will not interfere with this estimation.

PART 2. THE IODOMETRIC METHOD

EXPERIMENTAL

Reagents. The reagents used were those described by Lieb & Lanyar (1929) and Metz (1930).

Procedure. A. *Macromethod.* To 10 vol. of freshly collected urine 1 vol. of approximately 3N-HCl was added and the sample was stored at 0°. To 10 ml. of acidified urine were added 40 ml. of water, 5 ml. of the 5% KI solution, 5 ml. of the starch solution (1% in 20% NaCl) and 0.55 g. of NaHCO₃. If the urine was strongly pigmented, the amount of water was increased. The addition of bicarbonate to the acidified urine produces a buffer system and the pH obtained varied between 7.5 and 7.8. At this slight alkalinity both the destruction of the quinone-acetic acid and substitution by iodine appear to be minimal and the oxidation is sufficiently fast for the purpose.

The solution is then titrated with 0.05N-iodine solution until a definitely blue colour which persists for 45 sec. is obtained. The rate of titration is adjusted in such a way that a large excess of iodine is avoided. The solution is then acidified with 25 ml. of 7N-H₂SO₄ and left to stand for 3 min. before titration of the liberated iodine with thiosulphate. If the volume of the solution before the acidification exceeds 70 ml. the amount of acid added has to be increased accordingly. The amount of 0.05N-thiosulphate used up in ml. multiplied by 4.2 gives the amount of homogentisic acid in the acidified sample in mg.

B. *Micromethod.* The micromethod was used for rat urine and for deproteinized plasma filtrates. The rat urines were acidified in the same proportions as described above for human urines. For the estimation of homogentisic acid in blood, 2.5 ml. of 10% trichloroacetic acid were added to 5 ml. of heparinized plasma and the mixture was centrifuged. The clear supernatant was stored at 0° in a dark bottle and a measured sample used for titrations.

The samples were diluted when necessary, to a volume of 10–12 ml.; 1 ml. each of the KI and starch solution were added and an amount of NaHCO₃ to bring the pH to about 7.6. The titration with iodine was done as described above. The solution was then acidified with 5 ml. of 7N-H₂SO₄ and the liberated iodine was titrated, after standing for 3 min., with 0.01N-thiosulphate. The colourless solution was then

back-titrated with iodine until a faint blue colour was obtained. The amount of thiosulphate used up minus the amount of iodine consumed in the back-titration in ml. multiplied by 0.84 gives the amount of homogentisic acid in the sample in mg. Microburettes were used for these titrations.

RESULTS

Recovery. The values obtained for solutions of homogentisic acid in water were 98.5–99.5 of the theoretical and the iodine uptake in alkaline solution was found to be equivalent to the amount of iodine liberated on acidification. If homogentisic acid was added to urine the iodine uptake was greater than the theoretical due to the oxidation of other substances, but the amount of iodine liberated on addition of acid was again about 99 % of the theoretical. In the many samples of normal human or rat urine tested without addition of homogentisic acid there was either only a slight liberation of iodine in acid solution or in most cases none at all.

Recovery from plasma, however, was not quantitative. If amounts of homogentisic acid corresponding to concentrations of 5–25 mg./100 ml. were added to plasma, recovery varied between 85 and 95 %, the higher values being obtained with higher concentrations. With metaphosphoric acid as de-proteinizing agent, recoveries were slightly worse. It is believed that this loss is due to absorption by the protein precipitate. Moreover most samples of normal human plasma showed a blank corresponding to an apparent homogentisic acid content of 0.5–1.0 mg./100 ml.

Possible interference by compounds containing amino or thiol groups. It appeared possible that the quinone-acetic acid would react with substances containing amino or thiol groups to form *N*- or *S*-substituted hydroquinones (Snell & Weissberger, 1939) which might be further oxidized and certainly not be reduced on acidification. Homogentisic acid was therefore titrated in the presence of varying quantities of urea, glycine and cysteine. Table 2 shows that, though the iodine uptake in the case of cysteine is considerably increased, the consumption of thiosulphate in acid solution is not affected. It is concluded that the compounds do not interfere with the estimation.

Specificity of the reaction (Table 3). Other compounds having hydroxyl groups in 1:4-positions in the benzene ring, such as gentisic acid and hydroquinone behave exactly like homogentisic acid and cannot be differentiated from each other by this method. But 2:5-dihydroxyphenylalanine behaves in an atypical fashion. Its iodine uptake in alkaline solution exceeds considerably the expected value of two equivalents and there is only a slight, but variable, liberation of iodine in acid solution. It is likely that when an amino and a quinolic structure exist in the same molecule, internal condensation of the type discussed above, occurs leading to further oxidation and/or substitution. The lactone of 2:5-dihydroxyphenylpyruvic acid is apparently oxidized in alkaline solution by iodine, but again this reaction cannot be reversed by acidification; the amount of thiosulphate used up is only a fraction of the theoretical value.

Table 2. *The titration of homogentisic acid with iodine in the presence of glycine, cysteine and urea*

(In all three experiments 200 μ g. of homogentisic acid were added to solutions containing the stated amount of amino-acid or urea.)

Nature and amount of substance added	Theoretical iodine uptake and liberation at acid or neutral pH	Iodine uptake at pH 7.4 (ml. of 0.01 N-I ₂)		Iodine liberation at pH 0
500 μ g. glycine	0.238	0.274		0.240
500 μ g. cysteine hydrochloride	0.238	0.602		0.236
500 μ g. urea	0.238	0.263		0.240

Table 3. *The titration of several derivatives of hydroquinone and catechol with iodine*

(All titrations were carried out by the micromethod using 0.01 N-iodine and thiosulphate solutions.)

Substance	Amount taken (μ g.)	Theoretical iodine uptake and liberation	Iodine uptake at pH 7.4 (ml.)		Iodine liberation at pH 0
Homogentisic acid	300	0.357	0.358		0.360
Gentisic acid	300	0.390	0.392		0.399
Hydroquinone	300	0.545	0.540		0.543
Catechol	300	0.545	0.980		0.265
2:5-Dihydroxyphenylpyruvic acid lactone	200	0.224	0.440		0.065
2:5-Dihydroxyphenylalanine hydrate	300	0.279	0.870		0.115
3:4-Dihydroxyphenylalanine	300	0.300	0.770		0.210

It was pointed out above that the reaction of compounds possessing a hydroquinone structure, with iodine in acid and neutral solution respectively, is correlated with the magnitudes of the oxidation-reduction potentials involved and their change with pH. The E_h values of *o*-quinones in the benzene series are higher by about 0.1 V. than those of *p*-quinones. Catechol and its derivatives should therefore, like hydroquinone, consume 2 atomic equivalents of iodine at pH 7.4 and liberate the same amount at pH 0. Catechol at pH 7.0–8.0 has an iodine uptake greater than the theoretical and there is only a slight liberation of iodine on acidification (Table 3). It is known that *o*-quinones are much less stable than *p*-quinones and this in addition to a substitution probably accounts for the results obtained. 3:4-Dihydroxyphenylalanine shows a similar behaviour.

Keto acids, like phenylpyruvic and *p*-hydroxyphenylpyruvic acids consume iodine at slightly alkaline reaction, but do not liberate it on acidification. They do not therefore interfere with the estimation.

PART 3. THE SILVER REDUCTION METHOD

EXPERIMENTAL

Reagents. Gold sol. The following modification of the method of Faraday (1857) was used: to 250 ml. of water were added 6 ml. of a 0.5% solution of gold chloride and 5 ml. of 0.25N- K_2CO_3 solution. This was followed by the addition of 1 ml. of an ethereal solution of yellow phosphorus prepared by diluting a saturated solution five times. The solution was then allowed to stand for 30 min. and boiled under reflux for 15 min. The gold sol thus produced was red and contained about 70 μ g. of gold/ml. It was stored in the dark at 0°. For the estimation of homogentisic acid this solution was diluted ten times.

0.2N-Acetic acid.

0.2N-Sodium acetate.

Gum arabic solution 5%. This was prepared by dissolving gum arabic in hot water and filtering the solution through filtercel.

Silver nitrate solution (A.R.) 1%.

Procedure. To a carefully cleaned test tube or measuring cylinder was added 1 ml. of the acetic acid, 0.5 ml. of the sodium acetate solution, 2 ml. of 5% gum arabic, 1 ml. of 1% $AgNO_3$ and 0.5 ml. of the dilute gold sol. The tube was shaken to ensure complete mixture and 1 ml. of the solution to be tested, which should not contain more than 200 μ g. of homogentisic acid, was then added. The solution was then made up to 10 ml. with water and well mixed.

The solutions containing homogentisic acid develop a gold-brown colour and this is read, usually after 2 hr. on a Hilger absorptiometer with a neutral filter. A solution containing all the reagents, but no homogentisic acid, gives a very slight absorption. This is allowed for either by deducting this absorption from the experimental values or by placing the control solution in the compensating cup of

the absorptiometer. Concentrations of between 20 and 200 μ g./10 ml. of final solution can be estimated under these conditions.

RESULTS

Application of Beer's law. Over the range of 20–200 μ g. there is an approximately linear correlation between concentration and absorption coefficient, if the reading is made after 2 hr. (Fig. 2). With much

Fig. 2. The relationship between absorption coefficient and concentration of homogentisic acid in the silver reduction method. The two curves were obtained with reagents prepared in a similar manner.

higher concentrations of homogentisic acid the colour of the silver sol is darker, the Tyndall effect becomes more marked and deviations from Beer's law are observed. The two curves in Fig. 2 were obtained with different sets of reagents and though the curves are very similar, they are not identical. It is advisable therefore to prepare a fresh set of standards of homogentisic acid for each series of tests.

Time factor. James (1939) has shown that the reaction between hydroquinone and silver ions in the presence of gum arabic is an autocatalytic reaction. The same appears to apply to the reaction between homogentisic acid and silver ions. Fig. 3 shows the rate of colour development with two different concentrations of homogentisic acid. With the higher concentration there is little further change after 20 min., but with smaller amounts of homogentisic acid the development of the colour is much slower. It was found that with concentrations above 20 μ g./10 ml. the rate of change after 2 hr. is so slow that readings can be safely taken after that time. There is however, a further, very gradual, change;

thus the colour observed after 24 hr. is darker and the absorption coefficient as measured with a neutral filter is further increased. It is likely that this effect is due to a coagulation of the primary particles by the ions present in the solution.

Fig. 3. The rate of colour development with two different concentrations in the silver reduction method.

Application to urine analysis. The presence of large amounts of chlorides makes it impossible to apply the method directly to urine and it was therefore necessary to extract the homogentisic acid into an organic solvent. Homogentisic acid is about six times more soluble in water than in ether and the relative proportions of ether and water had to be chosen accordingly.

Estimation of homogentisic acid in urine. Acidified urine (2.5 ml.) was extracted four times with 20 ml. portions of ether. The ether was freed from peroxides, saturated with water and kept in a dark bottle. For each extraction shaking by hand was carried out for exactly 2 min. at a reasonably constant rate. The combined ethereal solutions amounting to about 120 ml. were dried over anhydrous Na_2SO_4 and filtered into a distilling flask; the Na_2SO_4 was washed carefully and the solution was concentrated to about 15 ml. This solution was then extracted twice with 15 ml. portions and once with a 10 ml. portion of water. Most of the ether was then removed by placing the combined aqueous solutions in a water-bath at 60° for 3–5 min. The volume was then made up to 50 ml. and this solution was used for the estimation of homogentisic acid as described above.

Accuracy of the method. The reproducibility of the colorimetric readings for solutions of homogentisic acid in water was not as good as might be desired. Results obtained with reagent prepared on different days varied within $\pm 5\%$. Many attempts were made to abolish these discrepancies by further purification of the reagents, careful cleaning of the glassware used, elimination of daylight, etc.; however, we were not able to achieve a degree of reproducibility greater than the one stated above.

With urine, recovery was found to be incomplete and averaged 90%, with a standard variation of about 3%. This incomplete recovery is to some extent due to theoretical limitations; thus with

relative proportions of aqueous and ethereal phase used, about 1–1.5% of the homogentisic acid originally present will remain in the aqueous solution. Another source of error is due to unavoidable mechanical losses involved in the extraction, transference of solutions, evaporation, etc. Recovery of added homogentisic acid from plasma varied in different experiments between 78 and 85%. The lower rate of recovery in the case of plasma as compared to urine is probably mostly due to absorption which takes place during the precipitation of the protein.

Specificity of the method. A number of compounds which might be present in urine or plasma reduce silver nitrate under the conditions used in this method, but with most substances tested the reduction is much slower than that observed with homogentisic acid. Thus adrenaline, 2:5-dihydroxyphenylalanine, 3:4-dihydroxyphenylalanine and ascorbic acid produce a yellow colour after some hours. Since these substances, however, are not extracted by ether, they cannot interfere with the method.

The second factor which renders this method so specific is the fact that the rate of the reaction with silver at the pH chosen is rather slow and varies considerably for different compounds. Fig. 4 shows that

Fig. 4. The rate of colour development with gentisic acid, homogentisic acid, hydroquinone and catechol in the silver reduction method.

the reaction with homogentisic acid is faster than with other hydroquinone derivatives tested. Thus with hydroquinone itself there is hardly any colour developed after 2 hr. and even gentisic acid gives a time curve which is very different from that found with the next higher homologue. The reduction with catechol is very slow indeed. By reading the colour of the test solution after 1 and 2 hr. and comparing the ratio with that given by an authentic specimen of homogentisic acid, an identification of the unknown material is possible. Phenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and the lactone of 2:5-dihydroxyphenylpyruvic acid do not react at all with the silver reagent.

DISCUSSION

All the methods discussed in this paper depend essentially on the reducing properties of homogentisic acid. The reduction of phosphomolybdic acid at a very acid pH, which is the basis of Briggs's method, can be brought about in varying degree by a number of other substances which may occur in urine, and the method can therefore only be applied if such compounds are not present in significant amounts relative to that of homogentisic acid. Thus in congenital human alcaptonuria, other aromatic acids metabolically related to tyrosine and phenylalanine are absent (Neuberger *et al.* 1947) and Briggs's method may be expected to give accurate results if the limits of concentration discussed above are observed. However, if the patient receives large doses of salicylates which may give rise to gentisic acid or if the darkening of urine is prevented by medication of massive doses of ascorbic acid, misleading results may be obtained by the phosphomolybdate method. In experimental alcaptonuria in animals and in premature infants the urine may contain appreciable amounts of *p*-hydroxyphenylpyruvic acid; in these cases the Briggs method will yield inaccurate results and it is advisable to use one of the other methods.

The iodometric technique is both convenient and accurate and its specificity is much greater than that of the phosphomolybdate method. It is clear however that any substance having an oxidation-potential smaller than that of the iodine system at pH 0 and greater than that of the iodide system at pH 0 will be titrated as homogentisic acid, provided its reaction with iodine is reversible. In practice, however, this reaction is characteristic of derivatives of hydroquinone and accurate results for the total amount of *p*-quinolic substances will be obtained, unless large concentrations of catechol or catechol derivatives are also present in the solution. The method can also be applied on a micro scale and appears to be the most generally useful analytical method for homogentisic acid.

The reduction of silver ions by homogentisic acid at pH 4.4 after ether extraction is more specific than the other two methods but it is laborious and not very accurate. Its specificity is largely due to the characteristic time curve which is shown by this reaction and which can be observed colorimetrically.

It is also clear that any substance having a distribution coefficient between water and ether very different from that of homogentisic acid will be largely eliminated. The fact that even gentisic acid has a time-curve quite different from that of homogentisic acid makes it appear improbable that any other substance which might occur biologically should be mistaken for homogentisic acid, if the colour is plotted against time. The method will therefore be mainly applied, if it is desired to distinguish between homogentisic acid and some other closely related substance. The silver reduction method can also be used for the estimation of small amounts of material.

SUMMARY

1. It is shown that the colour which arises in the reaction of homogentisic acid with phosphomolybdic acid takes some time to develop to its maximum and that the rate of colour development depends on the concentration of homogentisic acid. The colour develops more slowly with *p*-hydroxyphenylpyruvic acid but it is pointed out that the difference is not sufficient for a differential estimation. It is also shown that Beer's law is only obeyed with lower concentrations of homogentisic acid. The reaction is not very specific and has to be used with caution.

2. Experimental conditions for the iodometric estimation of homogentisic acid have been defined which give a recovery of about 99 % in water and urine. Neither amino-acids such as glycine and cysteine nor keto acids like *p*-hydroxyphenylpyruvic acid interfere with the reaction. Hydroquinone and gentisic acid behave towards iodine like homogentisic acid, whilst catechol and dopa behave irregularly and might produce misleading results if present in significant amounts.

3. A new method for the estimation of homogentisic acid has been worked out. The homogentisic acid is estimated by the reduction of silver ions at pH 4.4; the silver is precipitated in the form of a sol of small and fairly uniform particle size in the presence of a gold sol and gum arabic. The time curve of the colour development is highly characteristic of the reducing compound used and permits a very specific estimation of homogentisic acid in the presence of other reducing substances.

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Studies on Alcaptonuria

2. INVESTIGATIONS ON A CASE OF HUMAN ALCAPTONURIA

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The great interest which biochemists have taken in alcaptonuria is largely due to the fact that this metabolic error may be expected to yield information on the normal metabolic pathway of aromatic amino-acids. Such an expectation is based on the assumption that the formation of homogentisic acid is a step in the normal oxidation of phenylalanine and tyrosine and that the defect of the alcaptonuric consists of an inability to break down further this intermediary metabolite. The evidence for this interpretation has been fully presented by Garrod (1923) and has been further strengthened by recent work on the experimental production of alcaptonuria in animals by dietary means. Thus in normal white rats the administration of very large doses of phenylalanine leads to excretion of small amounts of homogentisic acid (Papageorge & Lewis, 1938; Butts, Dunn & Hallman, 1938), whilst a diet deficient in methionine and cysteine (Glynn, Himsworth & Neuberger, 1945) or diets practically free of protein (Neuberger & Webster, 1947) produce a marked alcaptonuria even with a normal intake of aromatic amino-acids. It has also been shown that guinea-pigs excrete homogentisic and *p*-hydroxyphenylpyruvic acids if they are given a diet deficient in ascorbic acid but enriched by tyrosine (Sealock & Silberstein, 1940; Sealock, Perkinson & Basinski, 1941). It is reasonable to assume that these dietary deficiencies produce alcaptonuria by affecting the cellular concentration of an enzyme or other factor necessary for the normal metabolism of tyrosine and phenylalanine rather than by endowing the organism with a new type of

metabolic reaction not normally found. It may therefore be inferred that the oxidation involving homogentisic acid represents at least one pathway of the intermediate metabolism of aromatic amino-acids, probably common to all mammals.

The question as to whether all the tyrosine and phenylalanine which is catabolized passes through the homogentisic acid stage has been discussed at great length, and the earlier literature on this point is fully reviewed by Garrod (1923) and by Neubauer (1928). More recent papers indicate that at least in some cases reduction of nitrogen intake produces a disproportionate decrease in homogentisic acid output (Katsch, 1918, 1920; Braid & Hickmans, 1929; Reinwein, 1931; Sachs, 1931; Lorenz, 1937). On the other hand Lieb & Lanyar (1930) found in one case that the ratio of homogentisic acid to nitrogen in the urine remained fairly constant, although protein intake was greatly reduced. A critical examination of existing data on the output of homogentisic acid in relation to the dietary intake of precursors is rendered difficult by several factors. Most workers did not keep either the composition or the total amount of their diet constant during the experiment; they were also handicapped by the fact that the tyrosine and phenylalanine content of the proteins given was not even approximately known at the time. A further difficulty arises from the lack of statistical analysis of the basic data. The output of homogentisic acid by the alcaptonuric is subject to considerable daily variations, independently of the changes in the diet. In an attempt to obtain a greater constancy Langstein & Meyer (1903) proposed the use of the ratio of homogentisic acid to urinary nitrogen (*H*:*N*) as a measure of the rate of conversion of tyrosine and phenylalanine to homogentisic acid. The *H*:*N* ratio is certainly very useful, if the total protein intake varies from day to