ON A NEW ACID FOUND IN HUMAN URINE WHICH DARKENS WITH ALKALIES (*ALCAPTONURIA*).

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The further investigation of this subject, on which a former paper appeared in the *British Medical Journal* of November 17, 1886, has opened up a wider field of inquiry than was at first anticipated, and has led to novel and interesting results.

Among previous researches on alcaptonuria may be mentioned those of Bödeker,[1] of Ebstein and Müller,[2] and of Professor Smith[3] of Dublin. The first of these isolated a substance from the urine to which he gave the name of alcapton, and which contained nitrogen, although the presence of this element was no doubt due to impurity. Ebstein and Müller, on the other hand, found, as they supposed, pyrocatechin in the urine, and Professor Smith protocatechuic acid. In the article above referred to, I pointed out that by concentrating the urine to an eighth or a tenth, either slowly over the water-bath or by boiling, subsequently acidulating with hydrochloric acid and extracting with ether, a new body was obtained; to which I gave the name of *urrhodinic acid,* which differed widely from any of the compounds above mentioned, and the properties of which accounted for all the peculiar reactions of the urine. This method, indeed, is actually given by Méhu[4] as a means of extracting pyrocatechin from the urine, but none of those who have examined cases of alcaptonuria seem to have followed it. It may be mentioned, however, that Fleischer,[5] in his

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[1] *Annal. der Chem. und Pharm.*, Bd. cxvii. 98, 1861.

[2] Virchow's *Archiv*, Bd. lxii. S. 554, 1875.

[3] *Dublin Med. Jour.*, vol. ii. p. 465, 1882.

[4] *L'Urine Norm. et Patholog.*, p. 117. Here speaking of salicylic acid, he refers to the use of a mineral acid (sulphuric) as applicable to the extraction of oxyphenic acid (pyrocatechin) and other acids from the urine, although, in treating specially of pyrocatechin further on, he does not again mention this.

[5] *Berliner Klin. Wochenschrift.*, Sept. 1875, Nos. 39 and 40.

investigations of the urine of patients taking large doses of salicylic acid, and in which he is said to have found pyrocatechin, adopted this plan of isolating it, which he calls Bertagnini's method. But it is quite clear from his descriptions that none of the urines examined by him at all resembled that of alcaptonuria, or contained any of the remarkable substance which I have called urrhodinic acid; otherwise, he could not have failed to find it. The mass of crystals obtained by this process from the urine of alcaptonuria is, in my experience, quite unique, both in quantity and character, and, besides the urine of the horse and cow, I have examined that of patients taking large quantities of salicylic and carbolic acids. Some of the urrhodinic acid exists free in the urine, an equal volume of ether taking up about one-twentieth of the entire amount it contains. After acidulation with hydrochloric acid, however, the ether at once abstracts one-half of the peculiar substance which gives the colour with alkalies. It must be evident that we thus obtain the object of which we are in quest, whether in a pure state or not.

In further prosecuting this inquiry, I have often had, as before, the co-operation of the Rev. Mr Gibson, who has devoted much time to chemical pursuits, and who is extremely careful and accurate in his methods of investigation. Our former conclusion has been fully confirmed, but the urine has been found to yield not one, but two new acids. Some facts mentioned in my former paper, and others afterwards observed, soon made it appear that what we have called urrhodinic acid was not a true chemical compound, but a mixture of two or more substances. By keeping specimens of it exposed to heat, air, and moisture for certain periods of time, and by varying the preparation of it in various ways, it was found that its properties were not uniform. It was noticed, for instance, that some samples gave a much redder watery solution than others, and that these had less marked reducing actions and gave less colour with alkalies, besides containing sometimes a considerable quantity of a dark humus-like powder, insoluble in water. It appeared not only that there were several constituents in the mixture, but that some of these might be increased at the expense of others, thus proving a certain relationship to exist between them. We were long at a loss to understand what was taking place, and were baffled in our attempts to separate these bodies from each other. At length a more careful examination of the action of neutral lead acetate fortunately enabled us to effect the latter object. As previously stated, urrhodinic acid gives white precipitates with both neutral and basic lead acetate. It was found, however, that the neutral salt did not throw down the whole of the ingredients, but left a pale yellow filtrate which possessed the aromatic odour of the original substance, and which darkened with alkalies and exercised reducing actions as before. Further, the precipitate, if allowed to form gradually, was not white, but consisted of two distinct portions, a pale and a dark, the latter being most abundant in the redder solutions of the mixture. The dark precipitate always fell first, while the pale one formed more slowly, even in concentrated solutions; and the two differed from each other in various physical properties. It became necessary to obtain these several bodies in an isolated form, and various methods of separating them were tried. A good deal of trouble was here experienced, and many specimens were spoiled from various causes, especially during evaporation *in vacuo* over sulphuric acid, for which our apparatus, depending on a variable water pressure, was at first defective, while other samples were found at the end of the process to be too small in quantity to admit of an organic analysis and a thorough examination of the substance. We will limit the present paper chiefly to an account of the body obtained from the pale precipitate, as this has been perfectly isolated and analysed and is not only different from anything previously supposed to exist in the urine of alcaptonuria, but also, so far as known to us, new to chemistry. It assumes a definite crystalline form, and among its interesting properties must be specially mentioned its power of throwing down the black suboxide of bismuth when boiled with an alkaline solution of the nitrate, this being a new fact in alcaptonuria; while it appears to be the source whence the other bodies are derived, whether in the animal system or in the process of analysis. After various trials it was found best to prepare it as follows.

A concentrated solution of the mixed substances is prepared by dissolving them in a small quantity of hot, but not boiling, water, and this is filtered to remove any trace of the insoluble powder already mentioned. To the filtrate, which has a deep red colour, a saturated solution of lead acetate is now gradually added, and the dark precipitate which falls removed by repeated filtration. It is well to use several filters simultaneously for this purpose when it is desired to preserve this precipitate also, for it is so viscid that it is extremely difficult to detach it from the filter after a certain length of time. If this fractional precipitation is cautiously conducted, a specimen is at length obtained which consists of a mixture of the dark and light precipitates. The filtrate is now of a yellow colour with a tinge of brown, and any further precipitate obtainable is of a pale colour. To avoid further dilution the liquid is now transferred to a mortar, and some solid lead acetate is pounded amongst it with the pestle. In a few seconds a cream-coloured precipitate forms, consisting of the lead salt of the object of our search and some excess of lead acetate. The filtrate from this has a pale yellow colour, but all the aromatic odour of the original solution, and gives no further precipitate with neutral lead acetate, but a white precipitate with the basic lead salt.

The above precipitate is now washed on the filter with water until the washings cease to have an acid reaction. As it forms readily only in strong solutions, so it is partly soluble in water, and in our first experiments the washing was needlessly prolonged with the view of obtaining a filtrate which would give no colour with alkalies; but this was found impossible, a slight yellow colour being always produced. Neither can hot water, nor water containing glycerine be used, as these speedily dissolve the precipitate; while the use of ether is also inadmissible, owing to a reaction with the lead acetate, which involves the precipitate we wish to retain, and leads to considerable destruction and loss of it by solution in the ether. The ether, moreover, will not pass through the filter, but requires to be shaken up with the precipitate. But these procedures were found to be unnecessary, and the precipitate, simply washed as above, is next suspended in water, decomposed by H_2S, and the resulting solution either evaporated *in vacuo* over sulphuric acid, or extracted with a large quantity of ether. In driving off the latter, the last portions must be poured out in very thin layers on large watch-glasses, and the evaporation conducted at a temperature of 20° to 30° C. In either of these ways we obtain a definite compound, with a marked acid reaction, which crystallises in needles forming stellate groups of various sizes, according to the degree of rapidity with which crystallisation has taken place, and which coalesce to form a complete scale on the surface of the glass. They have a somewhat aromatic odour, and are slightly irritating to the nostrils, and before they have become quite dry are of a yellow colour, but after complete desiccation of an almost greyish tinge. If they be exposed to a temperature of 60° C. or thereby for some time, they assume a darker dull yellow colour, and they even darken somewhat at a considerably lower temperature. The substance does not appear to be essentially altered, however, although darkened, by exposure to a temperature of 40° or 45° C.; and it may easily be crystallised by slow evaporation of its watery solution in the air, but at higher temperatures it shows a tendency to lose its crystalline character, and to become pulverulent, besides changing to a darker hue. The crystals we have obtained have generally been somewhat yellow, and have furnished (at least in a concentrated form) yellow solutions; but the finest and most recent specimens procured have been of an opaque almost milk-white colour, and from this circumstance we would propose to call this body *Uroleucic Acid.*

The substance thus obtained may be further purified by recrystallisation from ether, or by reprecipitation with lead acetate, if this be thought desirable. In this way specimens which have become darker than usual may be partly recovered. The dried crystals of this purified uroleucic acid show a fixed melting point which, as the result of several trials, we have found to be about 133·3° C. They melt into a dark liquid, which boils at a temperature a few degrees higher, but no odour is given off although this be raised to 205° C.

We now made an ultimate organic analysis of this definite compound, which has shown it to be free from nitrogen and rich in carbon, containing, as it does, as many as nine atoms of the latter element. The average percentage of carbon, hydrogen, and oxygen obtained in the last four analyses, which agreed closely with each other, was—

C = 54·457 per cent.
H = 4·985 „
O = 40·55 „

On calculation, the lowest formula corresponding to this will be found to be $C_9H_{10}O_5$, the required percentage of the respective elements for which is—

C = 54·54
H = 5·05
O = 40·40

The error in the carbon is therefore ·088 per cent., and in the hydrogen 0·14 per cent. The amount analysed on each occasion was 10 grains, and two of the samples of the acid were prepared by evaporating a watery solution *in vacuo* over sulphuric acid, and the other two by extracting with ether. The above result was corroborated by other evidence, which appeared also to show that the above was really the rational formula of the acid, and that it was monobasic. The atomic weight of $C_9H_{10}O_5$ is 198, and when caustic soda (NaHO, atomic weight 40) was added to a solution of the acid, in the proportion of 40 parts to 198, the latter lost its acid reaction, but not before; while beyond this proportion of soda the liquid became alkaline, and darkened if exposed to the air. To avoid access of air during the combination of the acid and alkali, they were made to unite under a layer of petroleum ether, in which the former is insoluble.

It may here be mentioned that before we had isolated uroleucic acid we analysed the mixture from which it is obtained, and found its composition to correspond closely to the formula $C_8H_{10}O_6$, in which it will be observed that the hydrogen remains the same as in the above.

Uroleucic acid is very soluble in ether and alcohol, 100 parts of the former taking up 20·5, and of the latter 17·7 parts of the acid. The saturated ethereal solution in the specimen examined had a yellow colour, while the alcoholic became brownish, although it again deposited the acid unchanged. The solubility in boiling water was about 5 per cent., in cold about 4; and the acid was also freely soluble in glycerine, but insoluble in chloroform.

The following reactions and properties may be further particularised:—

A $\frac{1}{4}$ per cent. solution gives a deep reddish-brown colour with alkalies, just a little less deep than that yielded by an equal volume of the fresh urine, which must contain 1 in 500 or 600 of the acid. This colour is about five times deeper than that obtained from gallic or tannic acid, and the latter do not give the brown shade, but almost a pure red in comparison. Oxyphenic acid (pyrocatechin), on the other hand, produces three times more colour than uroleucic acid, and this is at first an intense green, and then a brown without any red.

The same solution ($\frac{1}{4}$ per cent.) gives with a 1 in 40 solution of ferric chloride a transient green colour, instantly disappearing on diffusion of the two liquids, and incapable of being rendered permanent with any proportions of the reagents. The ferric chloride, however, produces a red colour when a little is dropped on the crystals of uroleucic acid, this again giving place to the transient green when the amount of ferric chloride is increased. Lastly, when the latter is cautiously added to a strong solution of the acid, the evanescent green which first appears gives place on diffusion to a brownish or reddish colour; on further addition of the chloride, the green fades away into a pale yellow, like that of the chloride solution itself.

The same solution gives no precipitate with a saturated solution of neutral lead acetate, and even a 2 per cent. solution only a slight one. The basic acetate throws down a white precipitate, which becomes violet on exposure to the air.

The acid has an extraordinary power of reducing Fehling's solution, even when immensely diluted. It also quickly reduces solutions of mercuric nitrate, silver nitrate, and chromic acid. These are all reduced almost instantaneously by a 2 per cent. solution, and even a solution of only $\frac{1}{4}$ per cent. acts on silver in the cold in a few seconds. In the case of chromic acid there is thrown down a red precipitate, along with the green suboxide of chromium. A $\frac{1}{4}$ per cent. solution instantly decolourises a solution of potassium permanganate, without the aid of hydrochloric acid.

A most interesting fact in connection with the new acid is its undoubted power of reducing bismuth, as already stated (Löwe's bismuth test solution was used). To be able to do so, however, the solution must be of the strength of ½ per cent. or upwards; and hence the reason why this fact was not previously ascertained, the acid having never been isolated, and the urine not containing a sufficient quantity of it to manifest this reaction.

The crystals of uroleucic acid are instantly acted on and oxidised by nitric acid, with effervescence and evolution of nitrous acid fumes, and a yellow pasty substance is left. This yields to water and ether yellow solutions, from the former of which it is deposited in an apparently amorphous state, but from the latter in acicular and columnar forms. Either of these redissolved in water shows an acid reaction, and becomes slightly darker with alkalies or a drop or two of Fehling's solution; while a larger quantity of the latter turns it green, but there is no reduction of the copper on boiling. It gives a yellow precipitate with both neutral and basic lead acetates, and also with barium hydrate, besides a slight one with lime-water. Ebstein and Müller state that their pyrocatechin was oxidised to oxalic acid by nitric acid, but this does not result from the oxidation of uroleucic acid by the same reagent.

A trace of chlorine turns the crystals of uroleucic acid of a blue colour, and free exposure to the gas bleaches them completely. These blue crystals, however, do not produce a blue solution, but a yellower one than before; and the gas, when passed into a solution of the acid, also renders it more yellow. We lost several preparations of the crystals by their being thus turned blue, and spoiled, by a trace of chlorine in the atmosphere, before the cause was discovered.

A few general statements must suffice with respect to the other constituents of what we have called urrhodinic acid. The aromatic filtrate above referred to, left after removal of the pale precipitate, contains a body having also an acid reaction, and which may be obtained by precipitating with basic lead acetate; or better, (1) by extracting directly with ether, or (2) by removal of lead by H_2S, and subsequent evaporation *in vacuo* over sulphuric acid. By the last method small stellate groups of a bright yellow colour are deposited, the yellow substance, however, being apparently an amorphous deposit on a crystalline skeleton formed by a different body. Such was found to be the case by treating with ether, which dissolved the yellow ingredient readily, leaving behind a small quantity of white irregular needles, insoluble in this menstruum. The latter body, of which we obtained only $\frac{3}{4}$ of a grain, proved also very sparingly soluble in water; and we will only further remark of this solution, that it gave a bright pink colour with alkali. The ether deposits the pure yellow substance in an amorphous granular form, and the ultimate result is therefore the same as that obtained by extracting the original aromatic filtrate directly with ether, according to the other method already mentioned. This yellow body, when thoroughly dried *in vacuo*, felt hard to the touch, but on afterwards pounding it in a dry mortar it was resolved only into a pasty mass, and it could not be rendered pulverulent, however long it was kept in the vacuum. It never becomes dry in the air, as it melts and turns dark about 30° C., and below this temperature it is always viscid, so that we cannot be sure it does not retain moisture. The dark liquid boils at a low temperature, and is decomposed with the evolution of an intensely disagreeable putrid odour, below the boiling point of water. It forms a varnish-like deposit on porcelain capsules and glasses above 30° C., partly yellow but partly of a dark red colour, according to the degree of heat to which it has been exposed. In these and other respects which might be mentioned, it will be seen to correspond exactly with Bödeker's "alcapton"; the "much brown powder," (with traces of hippuric acid), which he mentions as occurring at the end of his process, and which we have also seen, being derived, as we now know, from the crystalline uroleucic acid. This amorphous body, from its yellow colour, may be distinguished meanwhile by the name of *Uroxanthic Acid.*

This amorphous substance, when rendered alkaline, gives scarcely half as much colour as the crystalline acid does, but the respective tints closely resemble each other. It may be said, generally, that it exercises the same reducing actions as the latter, with the exception that it does not reduce bismuth, while the action on the other substances is less keen and rapid. It also gives the same transient green with ferric chloride, but not the red colour when in excess. Our analysis of this substance is not yet ready for publication, but enables us to state positively that it contains considerably more oxygen than the crystalline acid.

The dark precipitate yields a body, also with an acid reaction, which by evaporation *in vacuo* over sulphuric acid, or at a low temperature in the hot-water oven, is obtained as a powder, or in the form of irregular branching crystals, but at a higher temperature in the air as a dark, oily-looking liquid. Its solution also gives a transient green colouration with ferric chloride, but not a red colour in excess like uroleucic acid. A strong solution even does not reduce bismuth, and it does not give much colour with alkalies, a solution of 2 per cent. or so only becoming brown; nevertheless, it manifests all the reducing actions already mentioned, only these are not so keen. It is very sparingly soluble in ether, which renders it tough and viscid, but takes up a small quantity, which is again deposited from it in stellate groups of partly broken, somewhat pulverulent, needles of a yellowish-brown colour, having a general resemblance in form, however, to those of the first acid. Indeed, if we decompose the dark and pale precipitates together by H_2S, and extract with ether, we get the crystalline formation of uroleucic acid, but now of a rich yellow colour.

None of the three compounds above noticed have been found to have any action on polarised light, but we may repeat our observations on this point with the more perfectly isolated bodies recently obtained.

The fourth constituent of our original mixture, the insoluble powder, exists in very small quantity in freshly-prepared samples, and its formation appears to depend on exposure to air and moisture. It dissolves in ether with a red, and in alkalies with a brown colour, and the latter solution does not reduce Fehling's copper test if the powder has been thoroughly separated from the other ingredients.

Our latest observations appear to show that both the insoluble powder and the acid which forms the dark precipitate do not exist in the urine as such, but are produced in the course of the analysis or after exposure to the air. Quite recently we found that by acidulating the urine with hydrochloric acid beforehand none of the red body was obtained, and very little insoluble powder, which, moreover, had only a yellow colour, and dissolved easily in boiling water. At the same time the yield of uroleucic acid was increased, and as much as 20 grains was obtained from 33 ounces of urine. This may seem surprising after the statement in my previous paper that urrhodinic acid decomposes during the evaporation of its watery solution, and indeed it was a surprise to ourselves. But that error arose chiefly from the result of Bödeker's process, the final evaporation in which yielded little but powder and the amorphous "alcapton." Further, we have now evaporated down solutions of the free acid, both slowly over the water-bath and by boiling, and also solutions of the neutral combination of the acid with sodium, and have found that the body yielding the dark precipitate may be obtained by the latter of these two procedures, although it has not in all cases been identical in shade with that obtained from the urine. Even as derived from the latter, moreover, it has also varied somewhat in depth of colour in specimens prepared at different times. Neither of these two procedures, however, has yielded any of the amorphous aromatic substance, which we therefore conclude must occur as such in the fresh urine, although it probably owes its origin in the animal system to the same source.

It will be allowed that the foregoing account quite agrees with and explains the chief results of Bödeker, whose alcapton was at all events one of the bodies actually existing in the urine, although he did not isolate it in a pure state. It is remarkable that the urine should contain two substances differing so much from each other in certain respects, and yet with such a family likeness in their reactions; and it is clear how this circumstance, coupled with the transformations which one of them may undergo, has increased the difficulty of isolating and identifying them. It has generally been believed by authorities that the urine in Ebstein and Müller's case must have been of the same character as in that of Bödeker, although these investigators lay stress on two points as proving a difference, viz., that their pyrocatechin did not reduce silver nitrate in the cold without the aid of alkali, and had no reducing power on chromic acid at all. But if we evaporate down the fresh urine without acidulation and extract with ether, we can get precisely the same result. If we shake up the unconcentrated fresh urine with ether we get a small quantity of the crystalline and amorphous acids which crystallises readily in stellate groups, as if one substance only. But after evaporation the result is quite different, and varies according to the temperature to which the liquid has been exposed, and the extent to which the concentration is carried. We have repeatedly evaporated down 40 ounces of urine to determine this point. When this has been reduced to about an eighth the other deposits the two bodies on a watch-glass generally in four concentric zones, the central one consisting of a darkish powder, which has scarcely any reducing power; the second and fourth formed mainly by granular masses of the amorphous substance; and the third zone (between the two latter) containing a few brown circular crystals representing the crystalline acid. When the concentration is carried on till the urine is reduced to a syrup the ether may deposit nothing but an oily liquid in the centre of the glass, round which a few very fine stellate groups may form slowly in the course of a day or two. This deposit has often an alliaceous odour, and may have so far lost its characteristic properties that it cannot reduce nitrate of silver, and chromic acid, if at all, only after prolonged boiling. Further, this deposit gives a pronounced green with alkalies before becoming reddish-brown, and Ebstein and Müller expressly mention this fact, although the urine in their case, as in other cases of alcaptonuria, did not do so. It may be finally noticed that the transient green colour observed by Professor Hartley in Professor Smith's case, with the crystals isolated by him, when treated with ferric chloride, was the characteristic reaction of uroleucic acid; whereas protocatechuic acid would have produced an intense and permanent green. The urine in alcaptonuria does not contain a trace of pyrocatechin or protocatechuic acid.