X. THE CHROMOGEN OF MELANURIA.

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(Received November 12th, 1934.)

THE course of a melanotic sarcoma is sometimes marked by the excretion in the urine of a chromogen which readily oxidises on exposure to air to the black pigment, melanin. It has been shown by Raper [1927] that in the formation of melanin from tyrosine or 3 : 4-dihydroxyphenylalanine one intermediate product is 5 : 6-dihydroxyindole: a colourless material which rapidly oxidises to melanin in the air. It seemed possible that the chromogen might be this same indole derivative, or some closely related substance, and this view has been rendered more probable by the work of Bloch and Schaaf [1925] who showed that the production of melanin in the skin is due to the action of an enzyme, dopa-oxidase, on 3 : 4-dihydroxyphenylalanine. It has also been shown that tyrosinase is present in the skin of certain black rabbits [Onslow, 1915; Pugh, 1933] and in melanotic tumours [Gessard, 1903; De Coulon, 1920]. Clearly, if it could be demonstrated that the chromogen of melanuria is related to 5 : 6-dihydroxyindole, this would be good evidence for the view that in the higher animals melanin formation is due to the same series of changes that occur in the lower animals and plants, in which tyrosinase reacts with a suitable substrate such as tyrosine, 3 : 4-dihydroxyphenylalanine, or tyramine, 3 : 4-dihydroxyphenylethylamine.

Eppinger [1910] examined the urine from a case of melanotic sarcoma and concluded that two melanogens were present: one soluble, the other insoluble, in ether. He was able to isolate an ether-insoluble crystalline substance which appeared to be the amide of an ethereal sulphate of *N*-methylhydroxypyrrolidinecarboxylic acid. The product showed some of the properties associated with a melanogen and was considered by Eppinger to be the ether-insoluble chromogen, although in the light of recent work it seems most unlikely that a substance of the constitution suggested could be a precursor of melanin. The ether-soluble chromogenic material was further studied by Feigl and Querner [1917] who separated it into three fractions by precipitation with light petroleum and cadmium chloride. In this connection it may be noted that Thannhauser and Weiss [1922] isolated from melanotic urine an ether-soluble catechol derivative which they suggested was catecholacetic acid.

The view that tryptophan was the parent substance of the melanogen was advanced by Eppinger on his discovery that the administration of this amino-acid to the patient caused the excretion of the chromogen, as estimated by the Thormählen reaction, to rise considerably.

Our own experiments which are described below were planned to determine whether the administration to animals of the indole derivatives produced by the action of tyrosinase on tyrosine would lead to a condition of melanuria such as is encountered in melanotic sarcoma, and if this were so, whether the melanogens in the two cases had similar properties. We have also synthesised the ethereal

disulphate of 5 : 6-dihydroxyindole in order to compare its reactions with those of the melanogen, since it seemed possible that this substance might be excreted in the urine if the dihydroxyindole either arose as a metabolic product or was administered directly.

EXPERIMENTAL.

I. *The melanogen from human urine.*

Through the courtesy of Dr G. A. Harrison, of St Bartholomew's Hospital, London, who kindly furnished the melanotic urine and crude melanogen precipitate, we were able to study the chromogen excreted in human melanuria.

On exposure to air the urine slowly blackened from the surface downwards and eventually a deposit of melanin appeared. The blackening could be prevented by keeping the urine in an oxygen-free atmosphere or by the addition of a small quantity of sulphurous acid. The urine exhibited all the characteristic melanogen reactions, of which the following are the more important.

The hydrochloric acid reaction. On heating the urine with concentrated hydrochloric acid it rapidly becomes dark brown or black.

The ferric chloride reaction. The addition of a few drops of ferric chloride solution causes the urine to turn grey.

The ferricyanide reaction. Dilute solutions of ferric chloride and of potassium ferricyanide are mixed and acidified with hydrochloric acid. On the addition of a few drops of urine there is a copious formation of Prussian blue. This reaction is given to some extent by normal urine.

The Ehrlich reaction. To 1 ml. urine are added 2–3 drops of a 5 % solution of *p*-dimethylaminobenzaldehyde in alcohol and 5 ml. alcohol. On the addition of 0.5 ml. concentrated hydrochloric acid a violet colour appears either in the cold or on warming. The addition of a few drops of very dilute sodium nitrite solution changes the colour to deep purple.

The Thormählen reaction. To 1 ml. urine are added a few drops of a dilute solution of sodium nitroprusside, followed by a little caustic soda. A violet-red colour is immediately produced. The addition of excess acetic acid now changes the colour to pure blue.

Both the Ehrlich and the Thormählen reactions are very delicate, although the former is considerably the more sensitive of the two. It is to be noted that both these reactions are characteristic of indole and some of its derivatives.

The urine was precipitated with lead acetate in the presence of acetic acid in order to remove phosphates and sulphates. The melanogen was then completely precipitated from the filtrate by the addition of ammoniacal lead acetate. The yield of dried melanogen precipitate was about 70 g. from 10 litres urine. It was yellowish white in colour and did not darken on keeping. 5 g. of this precipitate were ground up with 100 ml. distilled water, and a current of carbon dioxide was passed through the suspension with frequent shaking. After two hours the suspension was filtered and the precipitate washed. The filtrate was pale yellow in colour and gave intense Ehrlich and Thormählen reactions. The precipitate was again ground up with 100 ml. water and treated with carbon dioxide, the filtration and decomposition with carbon dioxide being repeated until the filtrate gave only very weak Ehrlich and Thormählen reactions.

The mixed filtrates were saturated with hydrogen sulphide to remove lead from solution, and after filtration, the liquid was concentrated to small bulk by evaporation under reduced pressure in a stream of hydrogen. The syrup so obtained was brought to dryness in a vacuum-desiccator. A brown resin remained: yield: 1.03 g. A repeat experiment gave a yield of 1.14 g.

The resin was rubbed up with methyl alcohol, in which it partially dissolved. The methyl alcoholic solution was filtered and brought to dryness in a vacuum-desiccator. This gave residue "A." The insoluble material was washed with methyl alcohol and dried in a vacuum-desiccator, giving residue "B."

The lead precipitate remaining from the carbon dioxide treatment was re-suspended in water and decomposed with hydrogen sulphide. After filtration, the liquid was concentrated to small bulk by evaporation under reduced pressure in a stream of hydrogen and finally brought to dryness in a vacuum-desiccator, giving residue "C."

Residue "A" was a brown semi-solid resin which would not crystallise; yield: 0.66 g; repeat: 0.88 g. It was further purified by redissolving in methyl alcohol, cooling the solution on ice, filtering from a small quantity of residue and again evaporating to dryness. The substance gave intense Thormählen and Ehrlich reactions and blackened when warmed with hydrochloric acid. On the addition of ether it was precipitated from methyl alcoholic solution in the form of very hygroscopic white flocks which rapidly changed to a black oil. Tollens's naphthoresorcinol test indicated the presence of a trace of glycuronate.

The material contained sulphur, nitrogen and a trace of halogen, but no phosphorus. On analysis by the method of Benedict, as modified by Denis (*i.e.* ignition with copper nitrate) the total sulphur content was found to be 2.29 %. The amount of sulphur present in the form of ethereal sulphate was found to be 1.68 %; repeat estimation: 1.60 %.

An attempt was made to effect a further purification of the melanogen by precipitating it as its mercury compound. Previous investigators have used Hopkins's reagent, 10 % mercuric sulphate in 10 % sulphuric acid, for this purpose. In view of the instability of the chromogen towards mineral acid it was thought that the sulphuric acid in Hopkins's reagent might have a deleterious effect. For this reason, mercuric acetate was used in place of mercuric sulphate.

0.52 g. material "A" was dissolved in 20 ml. distilled water and completely precipitated by the addition of a 2 % solution of mercuric acetate in 2½ % acetic acid (20 ml. of reagent required). The yellowish white precipitate was filtered off on paper pulp, washed, suspended in water and decomposed with hydrogen sulphide. After filtration, the liquid was concentrated to small bulk by distillation under reduced pressure in a stream of hydrogen and finally taken to dryness in a vacuum-desiccator. This gave residue "D." The filtrate remaining after the removal of the precipitate was saturated with hydrogen sulphide and, after filtration from mercuric sulphide, brought to dryness as before, giving residue "E."

The substance "D" was a dark brown solid which easily powdered on being rubbed; yield: 0.18 g. The powdered material showed no crystalline form: it was very hygroscopic and readily soluble in water, giving a brown solution which gave a fair Ehrlich reaction, good ferric chloride and hydrochloric acid reactions, but no Thormählen reaction. The solution was very acid to litmus. The solid was also soluble in methyl and ethyl alcohols, but not in ether, benzene, light petroleum, acetone or chloroform. The amount of sulphur present in the form of ethereal sulphate was found to be 2.86 %. The dark colour of the product, the weakness of the Ehrlich and absence of the Thormählen reactions suggested that a considerable decomposition of the melanogen occurred in the mercuric acetate precipitation process, and for this the acidity of the solution was, perhaps, responsible.

The residue "E" was a pale yellow viscid syrup which could not be crystallised; yield: 0.24 g. Amount of sulphur present as ethereal sulphate: 0.99 %.

Since the Ehrlich and Thormählen reactions were absent and the ferric chloride reaction weak, it was concluded that the substance was not a melanogen.

The material "B" was a pale pink powdery solid which was not hygroscopic; yield: 0.37 g.; repeat: 0.26 g. It was readily soluble in water and in dilute acids and alkalis, insoluble in methyl and ethyl alcohols, benzene, light petroleum and acetone. The substance was purified by dissolving in hot dilute ethyl alcohol, filtering rapidly and cooling. An emulsion formed which eventually solidified into flocks. The product was almost white in colour, entirely amorphous in appearance, gave a weak Ehrlich, but no Thormählen reaction, and no coloration with neutral or acidified ferric chloride. The pale pink coloration of the solid was probably due to a trace of melanogen and this would account for the positive Ehrlich reaction (which is more sensitive than the other melanogen tests). In aqueous solution it was acid to litmus but not to Congo red. On heating, it charred with a peculiar odour, melted and finally burnt completely away. It contained sulphur and nitrogen but no phosphorus. From the ready solubility in water it followed that the substance could not be cystine: whilst the absence of a reaction with sodium nitroprusside after boiling with caustic soda proved that it was no derivative of cysteine. Since it gave no coloration with ferric chloride it could not be a melanogenic substance. The presence of a sulphate group, ethereal or otherwise, could not be detected, so it was concluded that the sulphur existed as so-called "neutral sulphur." The only compounds containing neutral sulphur which have hitherto been found in urine and which resemble the substance "B" are antoxyproteic, oxyproteic, alloxypoteic and uroferric acids. Their principal reactions have been described by Bondzyński and Panek [1902] and by Bondzyński *et al.* [1905]. On applying these reactions to "B," it was clear that it was not a proteic acid. Its further examination was deferred.

The substance "C" was a yellowish white amorphous material; yield: 0.07 g. It was almost insoluble in water, but dissolved in dilute caustic soda, from which it was precipitated by sulphuric acid. Its suspension in water was faintly acid to litmus and did not decolorise bromine water. No nitrogen was detected, but traces of sulphur and halogen were present. The presence of a trace of melanogen probably accounted for the appearance of weak Thormählen, Ehrlich and ferric chloride reactions. As the material was evidently not a chromogen it was not further investigated.

II. *The production of melanuria by the administration of 5 : 6-dihydroxyindole.*

The method of Raper [1927] was used for the preparation of a solution of 5 : 6-dihydroxyindole from tyrosine by the action of tyrosinase. The solution obtained on the oxidation of 2 g. tyrosine was concentrated to about 10 ml. by evaporation under reduced pressure in a current of hydrogen, filtered from unchanged tyrosine, and its neutrality ensured towards Congo red. The liquid was then immediately injected subcutaneously into a rabbit. The urine excreted thereafter for about 30 hours had the typical properties of a melanotic urine: after the lapse of this period the excretion of a normal urine was resumed.

The colour of the "melanotic" rabbit urine was normal when fresh, but on standing, the urine blackened from the surface downwards. It gave a good hydrochloric acid reaction and very strong ferric chloride, Thormählen and Ehrlich reactions. On the other hand, there was no alteration in appearance on the addition of caustic soda, even on boiling. This immediately proved that no 5 : 6-dihydroxyindole was excreted unchanged, for this substance is very sensitive to alkali and, even very dilute solutions of it blacken when caustic soda is

added. The urine also gave a good positive reaction on applying Tollens's test for glycuronates; normal rabbit urine gave this reaction but not so well.

The separation process previously used with the human melanotic urine was applied, with a small modification, to the rabbit urine. To the fresh urine was added sufficient dilute acetic acid to give an acid reaction to litmus, followed by an excess of 20 % lead acetate. The acid lead precipitate was filtered off and washed, and the filtrate and washings were mixed and rendered alkaline by the addition of dilute ammonia, whereupon a yellow precipitate was thrown down. This was filtered off and washed. A sample of each precipitate was suspended in water, decomposed with hydrogen sulphide, the solution filtered and a current of air passed to remove hydrogen sulphide.

The solution from the basic lead precipitate gave strong Thormählen, Ehrlich and ferric chloride reactions, whilst neither the solution from the acid lead precipitate nor the filtrate remaining from the two precipitations gave these reactions. Evidently the basic lead precipitate contained the whole of the chromogen. It was dried in a vacuum-desiccator to obtain it in a stable condition. By the injection into rabbits of the 5 : 6-dihydroxyindole prepared by the action of tyrosinase on 12 g. tyrosine, a total yield of 19.33 g. dry melanogen precipitate was secured from a bulk of 1188 ml. urine.

5 g. dried precipitate were ground up with 150 ml. distilled water, and the suspension was saturated with carbon dioxide and mechanically shaken for 4 hours. The suspension was then filtered, the precipitate resuspended in water and the saturation with carbon dioxide repeated until the filtrates ceased to give the melanogen reactions. Lead was removed from the mixed filtrates by the passage of hydrogen sulphide in the presence of ammonia. After filtration, the solution was evaporated to small bulk under reduced pressure in a stream of hydrogen and finally brought to dryness in a vacuum-desiccator. The product obtained was a brown resin which was completely soluble in absolute methyl alcohol and gave all the melanogen reactions; yield: 0.91 g. It thus corresponded to the substance "A" from human melanotic urine. No substance equivalent to "B" was found.

The material was redissolved in 20 ml. distilled water, and 30 ml. mercuric acetate reagent were added. The precipitate was filtered off, washed, suspended in water, mixed with a little baryta paste and saturated with hydrogen sulphide. When absorption of the gas ceased, the liquid was filtered from mercuric sulphide. It was found that the presence of barium in the solution complicated the further treatment of the product, so ammonium carbonate was added to precipitate the barium and the liquid refiltered. The filtrate was evaporated to dryness in the usual way. The residue (corresponding to "D" from human melanotic urine) smelt strongly of ammonium acetate. This was driven off by re-dissolving in 250 ml. water and repeating the evaporation to dryness. The product was a hard, pale brown, amorphous solid; yield: 0.19 g. Its solution in methyl alcohol deposited fern-shaped crystals of ammonium chloride. To remove this impurity, 0.11 g. of the material was dissolved in water with an equal weight of potassium carbonate and the solution allowed to evaporate in a vacuum-desiccator. The dry residue was extracted with absolute methyl alcohol (in which potassium carbonate and chloride are insoluble) and the solution brought to dryness in a desiccator. The resulting potassium salts formed a pale brown, very hygroscopic, amorphous solid, soluble in absolute methyl alcohol, but not in ether, light petroleum or benzene; yield: 0.11 g. It gave intense Ehrlich, Thormählen, and ferric chloride reactions and also Ehrlich's diazo-reaction. The material was analysed quantitatively for sulphur. (Found: total sulphur,

2.48 %; sulphur in form of ethereal sulphate, 1.96 %.) It was apparently stable towards alkalis, but on warming with hydrochloric acid it blackened with formation of melanin.

Substances corresponding to the products "C" and "E" from human melanotic urine were also obtained; yields: 0.03 g. and 0.72 g. respectively. As these substances gave the melanogen reactions very weakly or not at all they were not examined any further.

III. *The administration of 5 : 6-dihydroxyindole-2-carboxylic acid.*

Having produced a typical melanotic urine by the injection of 5 : 6-dihydroxyindole into rabbits, it was of interest to see if the injection of a closely related compound, 5 : 6-dihydroxyindole-2-carboxylic acid, would lead to a similar result. This acid is a colourless material which readily oxidises to melanin on exposure to air, and Raper [1927] has shown that it arises in the tyrosinase-tyrosine reaction under certain conditions. A solution of 5 : 6-dihydroxyindole-2-carboxylic acid was prepared according to the method described by Raper and concentrated to about 10 ml. by evaporation under reduced pressure in a stream of carbon dioxide. After filtration from unchanged tyrosine, the liquid was injected subcutaneously into a rabbit. It was noted that the solution showed some marked differences in its behaviour from a solution of 5 : 6-dihydroxyindole. While the Ehrlich, ferric chloride, and hydrochloric acid reactions were given in the usual way, the Thormählen reaction was negative and on the addition of alkali the colour of the solution changed to brown but did not become black. The substance was precipitated by basic lead acetate and by Hopkins's reagent. Following the administration of the carboxylic acid the rabbit excreted a urine which was not a typical melanotic urine, though it resembled one in certain respects. The urine darkened on exposure to air and gave the Ehrlich, ferric chloride and hydrochloric acid reactions. On warming with caustic soda, however, the colour changed to brown—melanotic urine is stable to alkali—whilst the Thormählen reaction was negative. The chromogenic substance could be completely precipitated from the urine by basic lead acetate and by Hopkins's reagent. On decomposing the lead or mercury precipitate with hydrogen sulphide, the solution obtained gave the same reactions as the original urine. These properties strongly suggest that the 5 : 6-dihydroxyindole-2-carboxylic acid was excreted unchanged. In any case, it is obvious that the substance is not produced in human melanuria and is unlikely to be the precursor of the melanogen.

IV. *The separation process applied to normal rabbit urine.*

In order that an indication might be gained of the extent to which the chromogenic substances were separated from other constituents of urine by the precipitation process used in the foregoing experiments, normal rabbit urine was worked up by the same method. 8.29 g. basic lead acetate precipitate were prepared from 400 ml. urine and treated with carbon dioxide as previously described. Corresponding to the substance "A" a very hygroscopic pale brown amorphous solid was obtained, containing sulphur and nitrogen; yield: 1.53 g. The Thormählen, Ehrlich and ferric chloride reactions were negative, but the ferricyanide reaction was strongly positive. A solution of 1.38 g. of this material on precipitation with the mercuric acetate reagent gave products corresponding to "D" and "E"; yields: 0.10 g., 1.25 g., respectively. The former substance was a brown gummy residue completely soluble in water and absolute methyl alcohol, insoluble in ether and benzene, and containing nitrogen and sulphur.

The Ehrlich, Thormählen, ferric chloride and biuret reactions and tests for the presence of tryptophan, indoxyl, allantoin, and ethereal sulphates were all negative. Ehrlich's diazo-reaction succeeded, whilst on applying Tollens's test for glycuronate the substance gave a positive, though weak, reaction.

The product corresponding to "E" was a hygroscopic syrup containing nitrogen and sulphur and giving a weak ferric chloride reaction, Ehrlich's diazo-reaction and Tollens's test for glycuronates. It was not an ethereal sulphate. Only a minute amount of material corresponding to the substance "B" from human melanotic urine was isolated, and although 0.09 g. of a substance corresponding to "C" was obtained, it was a yellow resin which did not resemble in any way the product in the human case.

These experiments show that a substance is excreted in normal rabbit urine which cannot be separated from the melanogen by any of the processes applied by Eppinger and later workers, for in its solubility and precipitation reactions it behaves in the same way as the chromogenic material. Whether this substance is excreted in human urine has not yet been tested, but the possibility must be borne in mind.

V. Preparation of the ethereal sulphate of 2 : 6-dihydroxyindole.

This was carried out by a method similar to that used by Jolles and Schwenk [1915] for making indoxyl-potassium sulphate. 2 g. tyrosine in 4 l. water were oxidised by means of tyrosinase at p_H 6.5 as described previously [Raper, 1927]. When the maximum red colour had developed, 1 ml. of 10 % acetic acid was added and the solution filtered. The filtrate was evacuated in large filter flasks and well shaken to remove dissolved gases. It was then kept under hydrogen until the red colour had disappeared, this indicating the formation of 5 : 6-dihydroxyindole. After adding a few ml. of sulphurous acid and a little capryl alcohol dissolved in ethyl alcohol the solution was concentrated by distillation under reduced pressure in an atmosphere of hydrogen. During this concentration a second similar batch of material was prepared and this was evaporated in the same flask as the first. When the liquid had been reduced in volume to about 300 ml., unchanged tyrosine (1.37 g.) which had separated out was filtered off. The filtrate was concentrated further to about 20 ml. and again filtered. The filtrate was taken to dryness and the residue extracted with 30 ml. of warm pyridine. Only a small portion dissolved. This pyridine solution containing 5 : 6-dihydroxyindole was added slowly to a mixture of 30 ml. pyridine and 4 ml. chlorosulphonic acid containing 20 ml. of chloroform. It was allowed to stand at room temperature for 2 days. An equal volume of water was now added and the liquid distilled under reduced pressure to remove chloroform and pyridine. 11.5 g. caustic potash dissolved in a minimum volume of water were added and the potassium sulphate which separated was filtered off. The filtrate was at once saturated with carbon dioxide and the residual pyridine removed by distillation *in vacuo*. To the pigmented solution, which now measured 35 ml., 40 ml. absolute alcohol were added. This precipitated some potassium sulphate and carbonate as well as pigment, which were filtered off and washed with 50 % alcohol. To the filtrate an equal volume of alcohol was added which removed principally potassium sulphate. The precipitate was extracted with boiling 95 % alcohol and the extract added to the filtrate. The two were now concentrated by distillation *in vacuo* and finally in a vacuum-desiccator. A little KCl separated and was filtered off. The filtrate now measured 10 ml. It gave all the melanogen reactions of melanotic urine except that with $FeCl_3$ in the cold. It did, however, darken on heating with $FeCl_3$ and HCl. The filtrate was now precipitated with 100 ml.

alcohol and the white crystalline precipitate dried *in vacuo*. It contained the substance giving the melanogen reactions as well as potassium chloride and sulphate. After several trials it was found that the inorganic potassium salts could be separated by converting them all into the sulphate and precipitating this with 70 % alcohol in which the 5 : 6-dihydroxyindole potassium sulphate was moderately soluble. The precipitate containing the melanogen was dissolved in water and a slight excess of silver sulphate added. The solution was left overnight and the silver chloride filtered off. The filtrate was made faintly alkaline with potassium carbonate to remove the excess of silver, filtered through pulp and concentrated to 60 ml. by distillation *in vacuo*. Absolute alcohol was added to make the concentration 70 % and the solution left for some hours to allow K_2SO_4 to separate. This was filtered off and the filtrate concentrated by distillation *in vacuo* and then in a vacuum-desiccator. A small amount of amorphous material was filtered off and the solution was then returned to the desiccator and taken to dryness. The residue was dissolved in 2 ml. water and allowed to evaporate slowly in a desiccator over sulphuric acid at the ordinary pressure. A white crystalline substance separated which gave the Ehrlich and Thormählen reactions but still contained a little K_2CO_3 . This was removed by redissolving in water, adding the mother-liquor and diluting to 20 ml. The solution was boiled and 5 % sulphuric acid added until it was exactly neutral to litmus. After cooling, 3 vols. of alcohol were added and after standing the K_2SO_4 was filtered off. The filtrate was taken to dryness *in vacuo*. The residue was dissolved in 1 ml. hot water, and 5 ml. hot alcohol were added. On cooling rosettes of needles separated out which were dried *in vacuo*. They weighed 63 mg. Analysis showed that they were the expected dipotassium sulphate of 5 : 6-dihydroxyindole. (Found by micro-analysis, N, 4.15; S, 16.00 %. $C_8H_5N(SO_4K)_2$ requires N, 3.6; S, 16.6 %.) A trace of the substance dissolved in a few ml. water gave strong Ehrlich and Thormählen reactions, but it did not darken with ferric chloride except when heated in strongly acid solution. The latter behaviour is unlike that of the melanogen of melanotic urine which rapidly darkens in the cold when ferric chloride alone is added.

An attempt was made to prepare the ethereal sulphate from the products of the enzyme action which had not dissolved in warm pyridine as described above. The residue insoluble in pyridine was heated in a water-bath under a reflux condenser with a mixture of 15 ml. pyridine, 75 ml. chloroform and 4 ml. chlorosulphonic acid for 10 hours. On working up the product in the same way as described for the pyridine-soluble material practically no substance giving the Ehrlich and Thormählen reactions was obtained.

DISCUSSION.

It is very probable that the melanogen produced by the administration of 5 : 6-dihydroxyindole to rabbits is identical with the chromogen of human melanuria. The artificially produced melanotic urine possessed all the characteristic properties of human melanotic urine, whilst the chromogenic material could be separated from both the human and rabbit urines by the same method and showed the same colour reactions, precipitation reactions and solubility in organic solvents in each case. Unfortunately, no conclusions could be drawn from an analysis of the products as they were certainly not pure. It has been demonstrated that a non-chromogenic material, exactly similar to the melanogen in its solubility in organic solvents and precipitation with salts of lead and mercury, occurs in normal rabbit urine and must therefore have been admixed

with the rabbit melanogen; it may also have been present along with the human melanogen.

In their general properties the human and rabbit melanogens we have studied closely resemble the ether-insoluble chromogens described by Eppinger [1910], Feigl and Querner [1917], and Fürth and Kaunitz [1932]. The fact that some preparations of the chromogen obtained by Fürth and Kaunitz failed to give the Thormählen reaction was possibly due to a partial decomposition of the melanogen; in the present investigation preparations have been made which decomposed so far as to lose the Thormählen reaction without loss of the others. On the other hand, Eppinger's crystalline product failed to show the Ehrlich reaction while it still gave the Thormählen reaction. As we have already noted, however, there is some reason for doubting whether this substance was actually a melanogen. The existence of ether-soluble chromogens may also be questioned. Previous investigators have inferred their presence from the fact that methyl alcoholic solutions, from which the melanogen has been precipitated by ether, may retain the chromogen reactions. The melanogen is very soluble in methyl alcohol and it is reasonable to suppose that the addition of ether will not remove it completely from solution.

The foregoing experiments strongly suggest that the chromogen is a simple derivative of 5 : 6-dihydroxyindole. The latter substance shows many of the properties associated with the melanogen, including the ready oxidisability to melanin and the positive Ehrlich and Thormählen reactions—the latter reaction is not given by such closely related compounds as the 2-carboxylic acid and the *N*-methyl derivative. In the body it is converted into the chromogen; that this is not a general property of 5 : 6-dihydroxyindole derivatives is shown by the fact that the 2-carboxylic acid fails to produce a melanogen under such conditions. The stability of the chromogen towards alkali proves that it cannot be 5 : 6-dihydroxyindole itself. Now phenolic and similar hydroxylic compounds are excreted in the urine chiefly in combination with sulphuric or glycuronic acid. 2-Hydroxyindole, for example, is excreted as indican. It is therefore probable that if 5 : 6-dihydroxyindole were to arise in the body it would be excreted in the form of an ethereal sulphate or glycuronate. Such derivatives would be stable towards alkalis and readily hydrolysed by acids to 5 : 6-dihydroxyindole which would then oxidise to melanin. The behaviour of the melanogen would be explained by this hypothesis and there is a certain amount of evidence that it represents the actual state of affairs. In the present investigation a small percentage of ethereal sulphate was always associated with the chromogen and accounted for about 80 % of the total sulphur content. The dipotassium disulphate of 5 : 6-dihydroxyindole is, however, more stable than the chromogen of melanuria; it does not darken with ferric chloride unless heated in strongly acid solution. It is, of course, quite possible that the monosulphate is a more unstable compound. Glycuronates were always found with the melanogen, though it must be remembered that, in the experiments with rabbits at least, the melanogen could not be separated from a glycuronate occurring in normal urine.

The close relationship between the chromogen of melanuria and 5 : 6-dihydroxyindole is in agreement with the view that melanin is formed in the higher animals by the same series of changes that take place when tyrosine is oxidised by tyrosinase *in vitro*. Fürth and Kaunitz [1932] have pointed out that on this basis it is easy to account for the appearance of a catechol derivative in some melanotic urines by supposing that the decomposition of the 3 : 4-dihydroxyphenylalanine which is the first oxidation product of tyrosine can take place

along two paths: either (i) there is oxidation to the quinone, followed by ring closure and the formation of 5 : 6-dihydroxyindole as previously described by Raper [1927], or else (ii) there is oxidation of the side chain in the usual way, leading to the formation of catecholacetic acid.

SUMMARY.

1. The chromogen of human melanuria has been studied and its separation from other constituents of urine by precipitation with lead acetate and mercuric acetate is described.

2. The administration of 5 : 6-dihydroxyindole to rabbits causes the excretion of a melanogen in the urine. The properties of this melanogen suggests its identity with the human melanogen.

3. 5 : 6-Dihydroxyindole-2-carboxylic acid does not produce a condition resembling human melanuria on administration to rabbits and is unlikely to be a precursor of the melanogen.

4. There is evidence that the chromogen is a simple derivative of 5 : 6-dihydroxyindole, possibly an ethereal sulphate or glycuronate.

5. The synthesis of 5 : 6-dihydroxyindole dipotassium disulphate is described.

A part of the expense of this investigation has been defrayed by a grant from the Government Grant Committee of the Royal Society for which the authors express their thanks.

REFERENCES.

- Bloch and Schaaf (1925). *Biochem. Z.* **162**, 181.
Bondzyński, Dombrowski and Panek (1905). *Z. physiol. chem.* **46**, 83.
— and Panek (1902). *Ber. deutsch. chem. Ges.* **35**, 2959.
De Coulon (1920). *Compt. Rend. Soc. Biol.* **83**, 1451.
Eppinger (1910). *Biochem. Z.* **28**, 181.
Feigl and Querner (1917). *Deutsch. Arch. klin. Med.* **123**, 107.
Fürth and Kaunitz (1932). *Biochem. Z.* **253**, 231.
Gessard (1903). *Compt. Rend. Acad. Sci.* **136**, 1086.
Jolles and Schwenk (1915). *Biochem. Z.* **69**, 467.
Onslow (1915). *Proc. Roy. Soc. Lond. B*, **89**, 36.
Pugh (1933). *Biochem. J.* **27**, 475.
Raper (1927). *Biochem. J.* **21**, 89.
Thannhauser and Weiss (1922). *Kongr. inn. Med.* **34**, 156.