A Contribution to our Knowledge of the Chemistry of Coat-Colour in Animals and of Dominant and Recessive Whiteness.

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CONTENTS.

I. Introduction.

Our knowledge of the pigments which give rise to the coat-colour of animals is very limited, and is chiefly confined to work on the melanins, an extensive bibliography of which is to be found in a monograph by Kobert* and also in a paper by von Fürth.† The dark bodies which remain as a cleavage residue after the acid hydrolysis of proteins are sometimes also called melanins, as well as the artificial products formed by the action of tyrosinase upon tyrosine.

* Kobert, 'Wiener Klinik,' vol. 27, No. 4 (1901).

† Von Fürth, 'Centralbl. für allg. Path. und pathol. Anat.,' vol. 15, p. 617 (1904).

Since the relationship of these substances to each other is still doubtful, it seems preferable to follow Gortner* in calling all the artificial black bodies humins, and to reserve the name melanin for the dark pigments occurring in the living organism.

The origin of the pigments remained in great obscurity until Landolt† suggested that melanin might be formed by a process of oxidation. But it was Bertrand's‡ discovery among plants of tyrosinase, an oxidase capable of oxidising tyrosine to a humin, that led to the present theory. Bertrand's discovery was confirmed by Biedermann§ and others who found that tyrosinase occurred among many plants and animals.

This wide distribution of tyrosinase and its obvious connection with pigment production gave rise to the current hypothesis of the mechanism of melanin formation, namely, that melanins are due to the action of an oxidase upon a colourless chromogen. This possibly takes place in two stages: (1) the formation of a cyclic compound which is split from the protein molecule by some autolytic ferment, and (2) the oxidation of the cyclic compound to a melanin by means of a specific ferment.

According to the theory of Bach and Chodat, oxidases are of a dual nature, their constituents being a peroxide (A_2O_2) and a peroxidase (P). The peroxide combines with the peroxidase to form an unstable compound (A_2PO_2). This immediately breaks down to yield

(1) An atom of "active" oxygen O.
(2) The peroxidase P.
(3) The compound A_2O.

The colourless chromogen is oxidised to a pigment by the "active" oxygen, whilst the compound A_2O is reconverted by the atmospheric oxygen to the peroxide A_2O_2, so that the cycle can be repeated indefinitely.

All attempts to ascertain the constitution of the melanin molecule have proved fruitless, mainly owing to the difficulty of isolating the pigment. The qualitative analyses do not agree in the main features and little or nothing can be deduced from the elementary analyses obtained. The mother substance of melanin has generally been supposed to be tyrosine, and Gessard‖ has shown that in one case at least tyrosine as well as tyrosinase was present in the tissues surrounding the pigment. There are, however, a number of other

* Gortner, 'Jour. Biol. Chemistry,' vol. 8, No. 4, p. 341 (1910).

† Landolt, 'Zeitschrift für Physiol. Chem.,' vol. 28, p. 192 (1899).

‡ Bertrand, 'Paris, C. R. Acad. Sci.,' vol. 122, p. 1215 (1896), and vol. 123, p. 463 (1896); 'Annales Institut Pasteur,' vol. 22, No. 5, p. 381 (1908).

§ Biedermann, 'Pflueger's Archiv für gesammte Physiol.,' vol. 72, p. 152 (1898).

‖ Gessard, 'Paris, C.R. Acad. Sci.,' vol. 136, p. 1086 (1903).

substances which might serve the purpose equally well, such as tryptophane, histidine, or the decomposition product of keratin found by Gortner,* which, though free from tyrosine, yielded Millon's reaction.

II. The Mechanism of the Action of Tyrosinase in Coloured Animals.

The work described in this paper was undertaken with a view to throwing more light upon the mechanism of the action of tyrosinase, and in it I hope to present further evidence in favour of the current hypothesis that pigmentation is the outcome of the action of an oxidase upon a colourless chromogen, and also to describe the distribution of a peroxidase in the skins of coloured rabbits. Further, I hope to show that a certain dominant white colour pattern in rabbits is due to the presence of an anti-tyrosinase or inhibitor in the skin of these animals, and that the recessive white variety is due to the lack of one or both of the oxidase and chromogen constituents of the pigment-producing system.

1. *Miss Durham's Experiments.*

At the outset of this work I was led to repeat some experiments of Miss Durham's,† carried out in 1904 for the purpose of showing the presence of a tyrosinase in the skins of young black rats, rabbits and guinea-pigs. An aqueous extract was made from the skins, and solid tyrosine as well as ferrous sulphate (as an activator) was added to the resulting reddish fluid. It was then incubated at 37° C. for some days, in the course of which dark precipitates were produced. When the skins of red guinea-pigs were used the fluid became yellow, and an orange precipitate was thrown down. In the absence of tyrosine or ferrous sulphate no coloration was produced, nor was any precipitate found. The boiled extract proved inactive.

In criticism of these experiments it seems in the first place unlikely that a black precipitate would be the first product formed. Previous investigators have found that the ferment fluid first becomes red or black on the surface where it is in contact with the air, and a precipitate only forms at the end of the reaction or on acidification. Moreover, it is not stated whether the reaction of the fluid extract was acid or alkaline. Presumably it would be acid owing to the formation of lactic acid, but it is well known that tyrosinase is most active in alkaline solutions, and that it is greatly inhibited by the presence of even a minute amount of acid. Further, it has been observed that the final colour produced by the action of tyrosinase upon tyrosine is invariably black. A reaction, therefore, which finally resulted in the pro-

* Gortner, 'Jour. Biol. Chemistry,' vol. 9, p. 355 (1911).

† Miss Durham, 'Proc. Roy. Soc.,' vol. 74, p. 310 (1904).

duction of an orange precipitate from tyrosine, as in the case of Miss Durham's red guinea-pigs, must have been of a totally different nature from any hitherto known. It is true that some tyrosine-oxidising ferments commence with a yellow or orange stage, but they finally turn black. Miss Durham also states that she extracted a tyrosinase of a less active nature from adult guinea-pigs, I, however, was entirely unable to obtain any ferment from four months old rabbits. Riddle* also criticises Miss Durham's work, on the grounds that her extract appeared reddish, which he claims would modify the final colour. In addition to this, an oxygen carrier like oxyhæmoglobin, which is no doubt the cause of the reddish colour, would seriously interfere with the results. Finally, Gortner† says that he has made several attempts to confirm these experiments without success. Moreover he has shown that the addition of 1 mgrm. of ferrous sulphate completely inhibits the action of tyrosinase, an observation which I have been able to confirm. It seems possible, therefore, that Miss Durham's results were due to some combination of the iron with a protein molecule, or else to some degenerative change or autolysis accompanied by pigment production, such as that called by Meirowsky‡ *post-mortem* pigment production, which he showed took place even in pieces of boiled skin if they were kept for a few days in the warmth. My own results with material obtained from the skins of young black rabbits by Miss Durham's methods are in full agreement with those of Gortner. And one must conclude that the results so obtained are not due to the action of a tyrosinase as usually understood, but to some other unexplained cause.

2. *The Peroxidase Present in the Skins of Coloured Rabbits and Mice.*

It has already been pointed out that, according to the theory of Bach and Chodat, an oxidase is of a dual nature, the two constituents being a peroxide and a peroxidase, the peroxide functioning as an activator to the peroxidase, by supplying it with oxygen from the atmosphere, which may then be transferred to the chromogen or other oxidisable body. It was therefore thought possible that not only the chromogen but also the peroxide constituent of the tyrosinase had been destroyed during the process of making the extracts from the skins. In order to test this, a very small quantity of hydrogen peroxide, which has been shown to be capable of replacing an organic peroxide of an oxidase system, was added to the extract, in addition to tyrosine, and the tube was then incubated. After 12 hours its appearance

* Riddle, 'Biol. Bulletin,' vol. 16, p. 316 (May, 1909).

† Gortner, 'Trans. Chem. Soc.,' vol. 97, p. 110 (1910).

‡ Meirowsky, 'Centralbl. für allg. Path. u. patholog. Anat.,' vol. 20, p. 301 (1909); see also Königstein, 'Wiener Klin. Wochenschrift,' No. 17, p. 616 (1910).

was totally unlike that of any tube in the former experiments. A heavy charcoal-black ring, $\frac{1}{2}$ inch wide, had formed in the upper portion of the fluid, while the lower portion remained unaltered. When the tube was shaken and allowed to incubate again, a fresh black ring formed on the surface of the fluid where it was in contact with the atmospheric oxygen. Occasionally in its earlier stages this black fluid had a reddish purple tinge, and a variable amount of black precipitate was always deposited after the lapse of some days. The results were the same if the tubes were kept at room temperature, but the darkening took place less rapidly. This seemed to be a true oxidation due to a peroxidase from the skin extracts in the presence of tyrosine and hydrogen peroxide. This reaction was accordingly always used as a method of testing for an inhibitor, in the manner afterwards described. Each experiment was repeated a number of times, and in every case with the necessary controls.

(a) *Material*—

The skins employed in these experiments were taken from a number of rabbits of the age of from two to four days, and during the course of the experiments over 200 young rabbits were employed. It was impossible to extract any ferment from older rabbits, owing probably to the increased toughness of their skins. Black rabbits were always used for the purpose of obtaining an active tyrosinase with which to test for the presence of an inhibitor. The nature of the tyrosinase in agouti, chocolate, orange, yellow, and blue rabbits, as well as in black mice, was also investigated. Since there is no true dominant white variety of rabbit, recourse was had to the type known to breeders as "English." This is a white animal with black eyes, patches of colour on the ears, face, and flanks, and a continuous line down the middle of the back. This pattern is said by Hurst* and Castle† to be dominant to self colour, a fact which was fully confirmed by breeding experiments carried out in connection with this research.

(b) *Methods*—

About eight black rabbits, preferably of the same litter, from two to four days old, were anæsthetised, killed, then carefully skinned by making an incision down the back, and the skins freed from subcutaneous tissue. The greatest care must be taken to remove completely any small blood-vessels that may be seen, otherwise the resulting fluid will be tinged with a trace of hæmoglobin which might vitiate the results. The skins were next thoroughly rinsed with water, dried with a cloth, weighed, and finely minced in a small mincing machine. The pulp was put into a mortar with about

* Hurst, 'Report Confer. on Genetics, Roy. Hort. Soc. London,' p. 114 (1906).

† Castle and Hadley, 'Amer. Nat.,' vol. 49, p. 23 (January, 1915).

half its weight of chloroform water and sufficient kieselguhr to make a good paste, and the whole mixture was thoroughly ground up for some time. The resulting black mass was finally transferred to a cheese cloth and the ferment fluid expressed by means of a hydraulic or efficient screw press. After being filtered through a soft paper, this fluid is white with a slight opalescence, provided the removal of the capillaries has been complete. The spectrum of oxyhæmoglobin cannot be detected, and the fluid does not show the presence of a trace of iron by the Prussian blue test. The extracts of the skins of all the other rabbits were prepared in exactly the same way, care being taken in the case of the English rabbits to cut away the black portions of the skin. A series of tubes was next prepared in the following manner: 2 c.c. of the ferment fluid were placed in each of a number of narrow test-tubes, together with three drops of an approximately 2-per-mille suspension of tyrosine in water, or other chromogen, and 0·1 c.c. of a 0·05-per-cent. solution of hydrogen peroxide.* This quantity of hydrogen peroxide cannot greatly be exceeded, since it has been shown by Bach† that larger amounts seriously inhibit the reaction. When an extract was to be tested for the presence of an inhibitor, it was added to the ferment fluid in the exact amount subsequently stated. Chloroform was employed as a preservative, since toluol, which was used by Miss Durham, if added in excess, rises to the surface and prevents the free access of oxygen. By means of very dilute sodium carbonate the contents of the tubes were carefully rendered faintly alkaline to litmus. The tubes were then plugged with cotton wool and incubated at a temperature of 37° C. As the reaction was found to have taken place in 12 hours the tubes were always examined after that time. An additional reason for doing this was because in some cases, when the oxidation had been slight, it was noticed that the colour had a tendency to fade after a longer period. Some idea of the amount of the pigment produced may be obtained from the following experiment:—

Ten cubic centimetres of the ferment fluid were incubated for 24 hours with an excess of tyrosine and 0·5 c.c. of a 0·05-per-cent. solution of hydrogen peroxide. This was acidified with 1 c.c. of 10-per-cent. sulphuric acid, and made up to 50 c.c. with distilled water. This solution was then titrated with potassium permanganate. It required 9·85 c.c. of a 0·2-per-cent.‡ solution to remove entirely the colour due to the melanin produced.

* For these experiments Merck's "perhydrol" was used.

† Bach, 'Ber. der Deutsch. Chem. Gesell.,' vol. 41, p. 216 (1908).

‡ According to Bach's method of estimating the amount of melanin, a 0·002-per-cent. solution of potassium permanganate was found sufficient. In the above experiment, however, this strength proved to be totally inadequate.

(c) *Properties—*

Experiment I: The Influence of Hydrogen Peroxide and of the Reaction of the Ferment Fluid.—The ferment fluid was added to each tube.

Chromogen added.	Appearance after 12 hours.		Remarks.
	Without H_2O_2.	With H_2O_2.	
None.....	—	—	Reaction faintly alkaline.
Tyrosine	—	—	Boiled.
,, ...	—	+ +	Reaction faintly alkaline.
,, ...	—	—	Reaction strongly alkaline.
,, ...	—	+	Reaction neutral.
,, ...	—	—	Reaction acid.

+ + indicates strong reaction.
+ indicates positive reaction.
— indicates no change.

From this experiment it appears that the ferment fluid lacks both a chromogen and a peroxide, for only when both these naturally occurring substances are replaced by tyrosine and hydrogen peroxide can oxidation take place; in other words, the ferment fluid contains only the peroxidase constituent of the pigment-producing mechanism. As a matter of fact, in later experiments when more practice had been obtained in extracting the ferment fluid, and when more care was taken to use the material as fresh as possible, a small amount of natural chromogen and peroxide was sometimes extracted. This was indicated by the formation of a dark grey ring in those tubes which contained either the chromogen or the peroxide, as well as in those which contained the ferment fluid alone. Tubes with a neutral reaction were only faintly grey after 12 hours, but subsequently they began to darken. Tubes with an acid reaction, however, remained permanently colourless. If alkali was present in excess, the reaction could not take place, nor did the tube containing the boiled ferment fluid show any sign of darkening. The peroxidase was found to be precipitated on saturating the fluid extract with ammonium sulphate, or on the addition of an excess of alcohol.

Experiment II: The Inhibitory Effect of Ferrous Sulphate.—The ferment fluid (prepared as previously, see pp. 40 and 41) was added to each tube, also tyrosine and hydrogen peroxide in the same quantities as in Experiment I.

Ferrous sulphate added.	Appearance after 12 hours.
None	+ +
1 mgrm.	—
0·5 „	+

+ + indicates strong reaction.
+ indicates positive reaction.
— indicates no change.

Although Miss Durham* obtained some catalytic reaction between her skin extracts and ferrous salts, yet the above experiment shows that ferrous salts inhibit the oxidation of tyrosine due to an oxidase; a result which is in agreement with Gortner's† observations upon the action of tyrosinase from meal-worms under similar conditions.

Experiment III: The Action of other Chromogens.—The ferment fluid (prepared as previously) was added to each tube.

Chromogen added.	Appearance after 12 hours.	
	Without H_2O_2.	With H_2O_2.
None	—	—
Tyrosine	—	+ +
p-cresol	+ +	+
Adrenalin*	—	+
Pyrocatechin (neutral)	+ +	+ +
Tryptophane	—	?
Pyramidone	—	—
Skatol	—	—
p-amidophenol	—	—

+ + indicates strong reaction.
+ indicates positive reaction.
— indicates no change.

* An aqueous extract of Burroughs and Wellcome's dry suprarenal glands was used.

(d) *Distribution and Nature of the Oxidase in Rabbits of Different Colours*—

An attempt was next made to discover whether the different coat-colours in rabbits were due to a corresponding difference in the oxidases which gave rise to them. For this purpose chocolate, blue and orange rabbits were experimented with. Extracts from the chocolate skins were rich in a ferment which appeared to be identical with that obtained from black rabbits. After twelve hours this ferment gave a deep black ring which gradually spread through the whole fluid, and at the end of some

* Miss Durham, 'Proc. Roy. Soc.,' vol. 74, p. 310 (1904).
† Gortner, 'Trans. Chem. Soc.,' vol. 97, p. 110 (1910).

days a black precipitate was deposited. The ferment also reacted with *p*-cresol, etc., and a dark grey colour was given when sufficient natural chromogen and peroxide had been extracted to yield the reaction without the addition of tyrosine and hydrogen peroxide. The fluid from blue rabbit skins—the dilute form of black—gave results similar in all respects, thus confirming the supposition that the pigments producing blue and black are identical. The yellow* rabbits employed carried the agouti factor, which is indicated by their white bellies. These skins yielded an extract in which no trace of coloration appeared, when either tyrosine or *p*-cresol was added as a chromogen, even after four days' incubation. Care was, of course, taken to remove the white bellies, in order to avoid the possible effects of an inhibitor similar to that occurring in the bellies of agouti rabbits (see p. 48). Many other chromogens were tried, such as tryptophane, adrenalin, pyrocatechin, pyramidone, guaiacol and other polyphenols, but no specific ferment could be detected.

Similar results were obtained when orange* rabbits were used. In these skins there is no fear of the presence of an inhibitor, as the bellies are self-coloured.

III. The Cause of Dominant Whiteness.

It is well known that white animals and flowers may be divided into two distinct classes—albinos or recessive whites and dominant whites. Dominant whites when crossed with coloured varieties invariably throw white offspring in the first generation, that is to say, they behave as a dominant to coloured varieties. Such dominant whites exist among various domestic animals, as for instance the White Leghorn fowl. The other type, albino, behaves as a simple recessive when crossed with coloured varieties, giving nothing but coloured offspring in the first generation. These albinos occur among most kinds of domestic animals, and anomalously among wild species. A further difference between the two forms lies in the fact that the eyes of dominant whites are generally more or less heavily pigmented, whereas the eyes of albinos are pink and almost entirely devoid of pigment. For the sake of clearness, albinos will be called "recessive whites" throughout the rest of this paper. Animals that are partially white or piebald may also be placed under one or other of these categories. In these animals the pattern, or, speaking more correctly, the white portions of it, are usually recessive to colour, but in certain cases, such as the "English"

* For the yellow rabbits I was indebted to Mr. J. Hammond, and for the orange rabbits, which correspond in the chocolate series to yellow rabbits in the black series, to Prof. R. C. Punnett, whose paper in the 'Journal of Genetics,' vol. 2, No. 3, p. 235 (November, 1912), gives a full account of this relationship.

rabbit, they are dominant. In man,* white and spotted negroes have for a long time been well known, but their genetic behaviour is far more complicated than that of most animals.† A simple and most interesting case, however, has lately been described, of a family of spotted negroes,‡ possessing a white skin pattern which apparently behaves as a dominant to the normal black type. It would be of the greatest interest to know whether the white skin of these individuals contains a pigment-inhibiting substance such as the one about to be described.

These two forms of whiteness and partial whiteness are visibly, and have hitherto been chemically, indistinguishable, being capable of differentiation by breeding experiments only. The chief problem therefore which presented itself was to discover whether any chemical basis underlay this difference. I propose to present evidence of the fact that dominant whiteness may be caused by the presence of an inhibitor of the pigment-producing oxidase, that recessive whiteness may be caused by the absence of either or both of the chromogen and oxidase constituents of the pigment-producing system, and finally, to make some suggestions as to the probable cause of variation in coat-colour.

1. *The "White Melanin" Theory.*

Two distinct theories have been advanced to account for dominant whiteness. The announcement by Spiegler§ that he had isolated a greyish substance from white horse-hair, which he called "white melanin," seemed to offer one possible explanation, namely, that a specific white pigment body, behaving as a dominant to the pigment of other colours, was present in the hair, so that the two forms of whiteness were due to the presence or absence of a "white melanin." This hypothesis has been criticised by Gortner.‖ He attempted to isolate this pigment by hydrolysing various keratin structures with 10-per-cent. sodium hydroxide and subsequently precipitating with hydrochloric acid. From black wool he obtained 2·45 per cent. of melanin, but from the feathers of dominant and recessive white fowls he obtained respectively 0·195 and 0·155 per cent. of a greyish brown substance. Since the yield of the grey substance from the white animals was so much smaller than the melanin from the black wool, and since in this and in other

* Pearson, Nettleship, and Usher, 'Drapers' Company Research Memoirs,' Biom. Ser. 8, 1911.

† Davenport, Publication by the Carnegie Instit., Washington, U.S.A. (1913).

‡ Simpson and Castle, 'Amer. Nat.,' vol. 47, p. 50 (January, 1913).

§ Spiegler, 'Hofmeister's Beiträge zur chem. Physiol. u. Pathol.,' vol. 4, p. 40 (1904).

‖ Gortner, 'Amer. Nat.,' vol. 44, p. 497 (August, 1910).

experiments the percentage yield from the dominant and the recessive whites was so similar, Gortner concluded that dominant whites do not possess a "white melanin" which is lacking in recessive whites; and, further, that the greyish brown substance derived from the feathers was not a pigment body at all, but merely a decomposition product of the keratin. The mere fact that the percentage of the grey substance was no larger in the dominant than in the recessive white feathers does not seem in itself to be a very strong argument in favour of the absence of the white pigment body. If this existed it might easily have been hydrolysed by the strong alkali used (10 per cent.).

But Gortner is undoubtedly correct in denying the pigment nature of the greyish brown substance. I have obtained it by Spiegler's methods from the hair of albino rabbits and white sheep, and find it to be an admixture of cholesterine and cholesterine esters from the surface of the hair and a substance resembling meta-protein derived from the hydrolysis of the keratin. The properties of this substance will be dealt with in a separate communication.

2. *The Inhibitor Theory.*

Having discarded the theory of a "white melanin," Gortner* suggested that an inhibitor might be present. He assumed that normal pigment formation is due to the oxidation of a chromogen (tyrosine) induced by the action of an enzyme (tyrosinase), and suggested that the tyrosine gives rise to (or is replaced by) a closely related substance which acts as an inhibitor or anti-tyrosinase. This substance is deposited in the epithelial cells of the skin, and the potentiality to reproduce it is transmitted to all the offspring. In support of this theory he was able to show that certain dihydroxyphenols which carry the hydroxyl groups in the meta-position to each other, such as orcin and phloroglucin, inhibit the action of certain tyrosinases upon tyrosine and other compounds. Later, Keeble and Armstrong† were of the opinion that the dominant white variety of *P. sinensis* contains an inhibitor, after the destruction of which by hydrogen cyanide the petals give strong oxidase reactions with suitable reagents. Miss Wheldale‡ also regards the pale shades of certain flowers which are dominant over the deeper shades as due to the presence in the petals of deoxidising substances such as tannin or sugar, and Atkins§ has observed and precipitated anti-oxidases in numerous

* Gortner, 'Jour. Biol. Chemistry,' vol. 10, No. 2, p. 113 (1911).
† Keeble and Armstrong, 'Journal of Genetics,' vol. 2, p. 277, No. 3 (November, 1912).
‡ Wheldale, 'Progressus Rei Botanicæ,' vol. 3, p. 457 (1910).
§ Atkins, 'Sci. Proc. Roy. Dublin Soc.,' vol. 14, Nos. 7 and 8 (1913).

plant juices. Lastly, Gortner* claims to have modified the pigmentation of the larvæ of *Spelerpes bilineatus*, by subjecting the eggs to a dilute solution of orcin, and to have increased the pigmentation by subjecting them to a dilute solution of tyrosine. Moreover, the Mendelian explanation of dominant whiteness, involving the existence of a factor which inhibits pigment-formation, even in the presence of the full pigment-producing mechanism, is sufficiently convincing to give great support to the chemical evidence available.

IV. The Tyrosinase-Inhibitor.

1. *The Presence of the Inhibitor in the Skins of English Rabbits.*

Experiment IV: The Effect of the Inhibitor in English Rabbit Skins.—2 c.c. of the ferment fluid (prepared as previously, see pp. 40 and 41), tyrosine, and hydrogen peroxide were added to each tube.

No.	Substance added.	Appearance after 12 hours.
1	None	+
2	0·5 c.c. English extract	—
3	1 c.c. English extract (boiled)	+
4	Precipitate obtained from half saturation of 1 c.c. English extract with ammonium sulphate	+
5	Filtrate from precipitate in No. 4	—
6	Precipitate obtained from full saturation of 1 c.c. English extract with ammonium sulphate	—
7	Filtrate from precipitate in No. 6	+

\+ indicates positive reaction.
— indicates no change.

The English extract alone had no action on tyrosine, either before or after the addition of hydrogen peroxide.

As little as 20 per cent. of the English extract was found to be sufficient completely to inhibit the oxidation due to the tyrosinase.

It is seen that the inhibitor cannot be precipitated by half saturation with ammonium sulphate, but that it can be precipitated by full saturation.

In Nos. 4 and 6 the precipitate was dissolved in the same volume of water as that of the extract taken.

The fact that ammonium sulphate alone is not the cause of the inhibition is shown by the result of No. 7.

Attempts further to purify the inhibitor obtained from the ammonium sulphate precipitate have so far failed. The chief cause of this is the inherent

* Gortner, 'Ohio Nat.,' vol. 13, No. 3, p. 49 (1913).

instability of the inhibitor, which spontaneously decomposes in 48 hours at room temperature.

2. *Distribution of the Inhibitor in the Skins of Agouti and Yellow Rabbits.*

An attempt was made to extract a ferment fluid from agouti rabbits. On two occasions a large number of tubes were prepared from these rabbits, but in no single case was there any sign of oxidation. The most probable explanation of this seemed to be that the white belly of the wild rabbit which is well known to be dominant over self-colour, contained sufficient inhibitor to prevent oxidation. The skin of the bellies of some more agouti rabbits was therefore separated from the skin of the backs, and an extract prepared from each portion. It was now found that the skin of the backs contained an active tyrosinase apparently similar in all respects to that of black rabbits, whereas the skin from the bellies contained so much inhibitor that 20 per cent. of the extract was sufficient to prevent oxidation taking place in the ferment fluid extracted from the backs. The white bellies of yellow rabbits were found to contain a similar inhibitor.

It seems indubitable, therefore, that whenever rabbits, and probably also other animals, have dominant white coats, or coats which have a white pattern that behaves as a dominant to self-colour, the dominance is produced by the presence of an inhibitor or anti-tyrosinase in the skin of the animals in question which can prevent any colour being produced by the existing chromogenic system.

V. The Cause of Recessive Whiteness.

1. *The Distribution of Enzyme and Chromogen.*

Having shown that dominant whiteness is caused by the presence of a chemical inhibitor or anti-tyrosinase, it remained to indicate the cause of recessive whiteness. It is clear that recessive whiteness cannot be due to the same factor as dominant whiteness, for it is logically impossible that a form of whiteness caused by an inhibitor can be recessive to colour, since it is not conceivable that the union of two germ-cells, one carrying potentially a chemical inhibitor and the other the full mechanism of pigment-production, could result in a pigmented animal; unless, indeed, the potentially pigmented germ-cell also contained a factor which neutralised the effect of the inhibitor. Such a supposition is hardly an economy of hypotheses.

If, then, recessive whiteness is not caused by an inhibitor, it must be due to the lack of one or both of the factors necessary for pigment-production,

namely, the enzyme and the chromogen. Gortner* has shown that in the case of the Colorado Potato-beetle, the colour pattern of the elytron is due to a restriction of the chromogen to the coloured areas, and that the enzyme is secreted over the entire surface. This distribution of chromogen was made evident by the fact that the pigmentation did not become general when an unpigmented elytron was placed in a solution of tyrosinase, whereas a solution of tyrosine caused the elytron to become pigmented over its entire surface. But in the case of rabbits the distribution is, as will be shown, different, the tyrosinase being restricted to the pigmented areas, and entirely absent from the white areas. It was easy to demonstrate the absence of a tyrosinase, but it was not so simple to do this in the case of a chromogen, owing to the difficulty attending its extraction. An attempt was therefore made, by means of a microscopical examination of a number of white hairs, to discover whether an unoxidised chromogen was present in any of them.

2. *On the Presence of Granules in Certain White Hairs, and the Possibility of their Chromogenic Nature.*

It was observed that the medullary cells of some white hairs† contained groups of small granular bodies, which may be the same as the small, conspicuously stained bodies in colourless hair, described by Nathusius,‡ and believed by him to be structurally related to pigment granules. They could be stained with hot aqueous solutions of methyl green or methyl violet, but these stains were not permanent. Much better results were obtained by using Nissl's methylene blue diluted with four volumes of water. The hairs were placed in this solution and heated in a water-bath for 30–60 minutes, according to the coarseness of the hairs, after which they were allowed to remain in xylol for about an hour, in order to expel the air from the vacuoles and to remove the excess of stain. With fine hairs, such as mouse hairs, it was found necessary to dilute the stain twenty times to prevent the medullary cells from becoming stained too deeply. Treated in this manner the granular bodies appeared exactly like groups of bright blue pigment granules. They were about the same size ($1{\cdot}5\mu$) as normal granules, and were situated in groups of the same appearance and in the same position within the medullary cells. In the case of white hairs from the belly of wild rabbits, in which a little pigment is present, normal black pigment granules were found interspersed here and

* Gortner, 'Amer. Nat.,' vol. 45, p. 743 (December, 1911).

† Onslow, 'Knowledge,' New Series, vol. 11, Part V, p. 161 (May, 1914).

‡ Nathusius, 'Archiv für mikroskop. Anat.,' vol. 43, pp. 152, 153 (1894).

there among the blue granules, as if now and then one of them had become oxidised. In other hairs, such as those of white mice, the medullary cells appeared bright blue and entirely devoid of granules. Whether or not these bodies represent an unoxidised chromogen there is not sufficient evidence to say. Their presence or absence was not correlated either to dominant or recessive whiteness, but their occurrence seemed rather to be specific in nature, as may be seen from the following Table:—

White hairs containing granular bodies.	White hairs lacking granular bodies.
Albino rabbit.	Albino mouse.
Dutch rabbit.	Piebald mouse.
Angora rabbit.	*Mus sylvaticus* (belly).
English rabbit.	Piebald rat.
Himalayan rabbit.	Agouti rat (belly).
Agouti rabbit (belly).	Red squirrel (belly).
Mountain hare (winter coat).	Ermine (winter coat).
White cat.	Albino guinea-pig.
Border terrier	Piebald guinea-pig.
White foxhound.	White sheep.
Tricolor foxhound.	
Arctic fox (winter coat).	

White hairs from a white horse, a white goat, a white Pomeranian, and an albino wolf were also examined. It was very difficult to determine whether they contained any granules, for although a number of particles were plainly visible within the medulla, they did not group themselves in the form of pigment granules, and their shape and distribution were irregular.

VI. The Absence of Tyrosinase-Inhibitor and of Tyrosinase in the Skins of Recessive White Rabbits and Mice.

1. *Materials and Methods.*

Owing to some difficulty in obtaining albino and black rabbits simultaneously, preliminary experiments were made upon a variety of rabbits known as Black Dutch. These rabbits have the hindquarters pigmented and the forequarters white, patches of pigment on the head and ears, and pigmented eyes. The special convenience of such half-black, half-white animals was that the black portions of the skin, when removed from the white, could be used to procure an active tyrosinase with which to test for the presence of an inhibitor in the white portions. Recessive white rabbits with pink eyes were also tested on several occasions to make sure that absence of colour was caused, in their case as well as in that of the piebalds, by the lack of tyrosinase. Further, experiments were made with extracts

from recessive white mice, which were tested with an active tyrosinase procured from black mice of the same age, as well as with that from black rabbits. These extracts behaved in every way like those from the recessive white rabbits. The method of preparing the extracts was similar to that already described, except that the mice, on account of their relative immaturity at birth, were kept till they were more than a week old, and their skins were chopped with a knife instead of being passed through a machine.

Before ascertaining the absence of tyrosinase in recessive whites, it was necessary first to show experimentally what has already been concluded logically, namely, that no tyrosinase inhibitor can be present in recessive whites. This was clearly shown to be the case by the following experiment.

2. *Distribution of Tyrosinase.*

Experiment V: The Absence of a Tyrosinase-Inhibitor in Recessive White Rabbits.—2 c.c. of the ferment fluid (prepared as previously, see pp. 40 and 41) from black rabbits was added to each tube.

No.	Substances added.	Appearance after 12 hours.	
		Without H_2O_2.	With H_2O_2.
1	Tyrosine	—	+ +
2	Tyrosine + 2 c.c. of the extract from recessive white rabbits	—	+
3	Tyrosine + 2 c.c. normal saline solution	—	+
4	*p*-cresol + 4 c.c. extract from recessive white rabbits	+	+

+ + indicates strong reaction.
+ indicates positive reaction.
— indicates no change.

As much as 50 per cent. of the extract from recessive whites was powerless to prevent darkening, though as little as 20 per cent. of the extract from English rabbits was sufficient to inhibit the reaction completely.

No. 3 was prepared as a control by substituting normal saline for the recessive white extract, in order to indicate the effect of dilution upon the ferment fluid.

The absence of an inhibitor was confirmed by using the more delicate reagent *p*-cresol in a solution of ferment diluted with two volumes of recessive white extract, and also by testing the precipitates formed by saturation with ammonium sulphate and by the addition of an excess of

alcohol. The aqueous solutions of these precipitates were powerless to inhibit an active tyrosinase solution.

Experiment VI: The Absence of Tyrosinase in Recessive White Rabbits.—The extract from recessive white rabbits (prepared as previously, see above) was added to each tube.

No.	Substance added.	Appearance after 12 hours.	
		Without H_2O_2.	With H_2O_2.
1	None	–	–
2	Tyrosine	–	–
3	*p*-cresol	–	–
4	Adrenalin	–	–

\+ indicates positive reaction.
– indicates no change.

This experiment clearly shows that the extract from recessive white rabbits not only lacks a tyrosinase but also an enzyme capable of oxidising *p*-cresol or adrenalin, although Meirowsky* reports the presence of an adrenalin-oxidising enzyme in extracts from the normal human skin. The results obtained with white mice and the white areas of the Dutch-rabbits were similar in all respects to those given by the recessive white rabbits in Experiments V and VI, and clearly show that recessive whiteness is due to the absence of at least tyrosinase, and possibly of chromogen as well.

3. *The Effect upon the Skin Extracts of Dihydroxyphenols.*

The presence of what might possibly have been a second specific enzyme, with properties differing from those of tyrosinase, was observed very early in the course of the experiments, but to avoid confusion the account of it has been reserved until now. An experiment was performed to see whether phloroglucin and similar dihydroxyphenols inhibited the reaction of tyrosinase in the manner described by Gortner.†

Experiment VII: The Effect of the Extract from Black Rabbits upon Dihydroxyphenols.—Ferment fluid (prepared as previously) and tyrosine were added to each tube.

* Meirowsky, 'Centralbl. für allg. Path. u. pathol. Anat.,' vol. 20, p. 301 (1909); and Münchener Med. Wochenschrift,' vol. 58, No. 19, p. 1005 (1911).

† Gortner, 'Jour. Biol. Chemistry,' vol. 10, No. 2, p. 113 (1911).

Phenol added.	Appearance after 12 hours.	
	Without H_2O_2.	With H_2O_2.
None	—	+ +
Phloroglucin	+ +	+
Orcin	+	+
Resorcin	+	+
Phenol	—	—

+ + indicates strong reaction.
+ indicates positive reaction.
— indicates no change.

In every case in which a di-phenol was added, the ring that appeared after 12 hours was coloured more or less deeply yellow. This result suggested either that tyrosinase oxidises these di-phenols yellow, or that the di-phenols inhibit tyrosinase, and are themseves acted upon by a specific ferment or some other agent present in the solution. To prove that the oxidation was not due to tyrosinase, the skin extracts from both dominant and recessive white rabbits were tested with phloroglucin, etc., and in both cases the results were similar to those given by the coloured skins, as is shown by the following experiment.

Experiment VIII: The Effect of the Extracts from Dominant and Recessive White Rabbits upon Dihydroxyphenols.—The extracts were prepared as before, and to each tube was added the phenol to be tested.

Extract.	Phenol added.	Appearance after 12 hours.
Black rabbits	Phloroglucin	+
Recessive white rabbits	,,	+
English rabbits	,,	+
,, ,,	Orcin	+
,, ,,	Resorcin	+
Recessive white rabbits	Hydroquinone (reaction neutral)	+ +
,, ,, ,,	Pyrogallol (reaction neutral)	+ +
,, ,, ,,	Phenol	—

+ indicates a yellow ring.
+ + indicates a brown ring.
— indicates no change.

The fact that the recessive white extract, containing no tyrosinase, behaves in a way similar to that from black rabbits, proves that the effect on phloroglucin cannot be attributed to a tyrosinase. The reaction must be due, therefore, either to an enzyme which is specific for poly-phenols, but which has no action on tyrosine, or else to the presence of organic colloidal material

in the fluid extract, which has no effect upon mono-phenols, but which readily oxidises the more complex phenols.

Now the extract was observed to be very thermostable, and this made it doubtful whether a true enzyme were present. Bertrand* has, however, described a *laccase* capable of oxidising phenols, but with no action on tyrosine, which could be subjected to a temperature of 70° C. for 15 minutes without losing its activity. Moreover, Gortner† has described a similar enzyme which he separated from tyrosinase by bringing the mixture to boiling point, at which temperature the tyrosinase is destroyed and an active enzyme remains which oxidises quinol. "This oxidase," he adds, "is much more resistant to heat than tyrosinase, and may be heated at 100° C. for some minutes without losing much of its activity. Prolonged heating, however, gradually causes it to lose its oxidising power." This enzyme was obtained from meal-worms, but a similar one was also extracted from the tissues of various vertebrates, and notably from the skin of albino rats.

In order to test the effect of temperature on my skin extracts the following experiment was made with recessive white rabbits.

Experiment IX: The Effect of Temperature on the Oxidation of Phloroglucin by the Recessive White Extract.—The recessive white extract (prepared as previously) was added to each tube, as well as phloroglucin.

In each case the extract was heated for a period of 10 minutes at the temperature stated. Before heating, the fluid was as usual made faintly alkaline to litmus, so that little or no coagulation took place.

No.	Temperature.	Appearance after 12 hours.
1	37° C.	+
2	78	+ +
3	82	+ +
4	90	+

+ + indicates strong reaction.
+ indicates positive reaction.
— indicates no change.

The increase in the oxidation of Nos. 2 and 3 after a slight heating agrees with Gortner's statement that "long-continued boiling causes a gradual loss in the oxidising power, although a very short heating seems to increase the activity." In view of this great resistance to heat it seems extremely doubtful whether an enzyme, as generally understood, can be responsible for

* Bertrand, 'Paris, C.R. Acad. Sci.,' vol. 123, p. 463 (1896).
† Gortner, 'Trans. Chem. Soc.,' vol. 97, p. 110 (1910).

this oxidation. More probably, both in this case and in that described by Gortner, the poly-phenols are catalysed by some organic colloidal material in the fluid extract and not by a specific enzyme at all.

VII. A Possible Cause of Colour Variation.

The foregoing experiments call attention to one or two points which are worthy of further consideration, since they throw some light on the nature of the cause determining the difference between the various coat-colours of rabbits.* Chodat has shown how the colour due to the action of tyrosinase upon tyrosine and certain phenols is modified by the presence of amino-acids or polypeptides. He suggests, therefore, that the colour of a given pigment depends on the action of a particular oxidase upon different combinations of a phenol group, and an amino or colour-modifying group. Before accepting such a theory it must be clearly shown that such variations in colour are really due to a difference of the quality and not of the quantity of the pigment present. The colours concerned in the case of rabbits and mice are generally divided into black, chocolate and yellow, many of the other varieties, such as agouti and blue, being caused by different combinations and concentrations of these three colours. The so-called black pigment, however, appears chocolate by strong transmitted light, and bright yellow in very dilute solutions. Moreover, chocolate pigment appears black in concentrated solutions and yellow in dilute solutions. It is possible, therefore, that these differences of colour may be due to the concentration of the pigment in the granules and to the manner of their distribution rather than to any real qualitative difference in the pigment. Now the properties of the tyrosinase extracted from chocolate rabbits appeared identical with those of the tyrosinase from black rabbits, which seems to support this view. On the other hand, no tyrosinase could be extracted from yellow and orange rabbits, which suggests that an oxidase of some other nature is present. This failure to find tyrosinase might equally well be explained on the supposition that a very small amount of tyrosinase is necessary to produce a yellow colour in the hair, and that this was lost to such an extent during extraction that the remainder could not give a yellow colour *in vitro*. The observation of Miss Durham's† that the pigment of black, chocolate, and yellow hairs showed a marked difference in solubility when the entire hair was treated with alkali, appears at first

* Chodat, 'Archives des Sciences physiques et naturelles,' 4th period, vol. 33, p. 70 (1912).

† See paper by Bateson, 'Proc. Zool. Soc.,' vol. 2, p. 71 (1903).

sight to be opposed to this view. When, however, purified samples of the pigment were used, I was quite unable to detect any such difference.

In order to put this hypothesis to a further test, a rough experiment was devised as follows:—A few decigrams of pigment were prepared from black, chocolate, and yellow rabbits, by treating the hair according to Gortner's* method of extracting melanin, with a 0·2-per-cent. sodium hydroxide solution. The three amorphous black preparations resulting appeared identical, and their solubilities were found to be similar, that is to say, they were easily soluble in alkalies, slightly less soluble in dilute acids (N/20 HCl) and insoluble in strong acids and saturated ammonium sulphate solution; 10 mgrm. of each of the purified preparations were then dissolved in 10 c.c. of 5-per-cent. sodium hydroxide, and the solutions compared in a Dubosq colorimeter. To the eye these solutions appeared a fairly uniform yellow colour, and colorimetrically the average difference between yellow and chocolate and between chocolate and black was not more than 18 per cent. This difference can be partly accounted for by the slight variation in the colour of the solutions and the consequent difficulty of comparison, and partly by the admittedly unsatisfactory method of extracting the pigment. The similarity in the colour of the solutions is the more striking in view of the contrast in the appearance of bright yellow and of black rabbits, a difference which cannot be less than several hundred per cent. On the other hand, it is conceivable that during extraction the yellow and chocolate pigments may have been decomposed by the dilute alkali and converted into black pigment.

There is one other class of colours among rabbits which deserves attention, namely, the so-called dilute colours, such as blue, which is the dilute form of black. The pigment which gives rise to this colour is identical in appearance with that of the black rabbit, both microscopically and in solution. The tyrosinase which it is possible to extract also behaves like that of the black rabbits. To account for this difference in colour it has been suggested that the pigment granules are deposited far less abundantly in the blue hair than in the black.† A number of careful microscopical examinations have, however, convinced me that the amount of pigment deposited in the distal portion of the hairs is not appreciably less in blue and other dilute animals than it is in black.‡ The basal portions, on the other hand, contain very few granules, but the colour of an animal is given by the distal portion of the hair alone, since this is the only part of the

* Gortner, 'Jour. Biol. Chemistry,' vol. 8, No. 4, p. 342 (1910).

† Bateson, 'Mendel's Principles of Heredity,' p. 83.

‡ Onslow, 'Knowledge,' New Series, vol. 11, Part V, p. 161 (May, 1914).

hair to be seen when the coat is lying flat. The blue appearance must therefore be due to some other cause, and I believe the fact to be that in blue hairs the pigment is entirely confined to the medulla, whereas in black and other intense colours there are many hairs in which granules are distributed throughout the fibrils of the cells of the cortex. These granules within the cortex absorb the light which in dilute hairs passes through and is reflected by the air-cells or vacuoles which occupy the medulla, thus causing an increase in the white light and a consequent dilution of the colour observed. Dilute colours therefore, and perhaps other colours also, depend rather upon the distribution and intensity of the pigment than upon its chemical composition.

VIII. Summary and Conclusions.

1. Miss Durham's evidence for the existence of a tyrosinase in the skins of vertebrates is inconclusive.

2. A peroxidase can, however, be extracted from the skins of certain coloured rabbits and mice, which behaves like a tyrosinase towards tyrosine in the presence of hydrogen peroxide. It can be precipitated from solution by saturation with ammonium sulphate or by an excess of alcohol.

3. The peroxidase present in agouti, chocolate, and blue rabbits is indistinguishable in its reactions from that present in black rabbits; but no peroxidase could be extracted from yellow and orange rabbits.

4. Spiegler's "white melanin" is not a pigment substance; nor is it the cause of dominant whiteness, which is due, as has been suggested by Gortner, to the presence of an inhibitor or anti-tyrosinase in the skin.

5. *Dominant whiteness* in the English rabbit is due to the presence of a *tyrosinase-inhibitor* in the skin, which destroys the activity of tyrosinase; and the dominant white bellies of yellow and agouti rabbits are due to the same cause. The inhibitor can be precipitated by saturation with ammonium sulphate, and is destroyed by boiling or by standing for 48 hours.

6. *Recessive whiteness* in rabbits and mice is due to the *lack of the enzyme unit* of the pigment-producing system, for no tyrosinase or anti-tyrosinase could be extracted from their skins. There is not sufficient evidence to decide whether a chromogen is present or not.

7. The presence of an unoxidised chromogen might, however, serve to explain the occurrence of certain colourless granular particles which are found in the medullary cells of the hairs of some white animals. These particles are microscopically visible when stained, and in appearance very closely resemble coloured pigment granules.

8. The capacity of both white and coloured skin extracts to oxidise

dihydroxyphenols, but not mono-phenols, is more probably due to the catalysing effect of organic colloidal material than to a true enzyme as stated by Gortner. The extreme resistance to high temperatures shown by these extracts excludes the presence of an enzyme as generally understood.

9. Variations in coat-colour are due probably to a quantitative rather than to a qualitative difference in the pigment present, for the pigments isolated from black, chocolate, and yellow rabbits show very little difference either in the depth of their colour or in their chemical behaviour.

10. Blue and the other dilute coat-colours are not caused by a lack of pigment in the medulla, but by the absence of granules in the cortex, which, being present in the intense colours, absorb the light which in the dilute colours is reflected from the vacuoles.

In conclusion, the writer of this paper wishes to acknowledge his indebtedness to Mr. S. W. Cole for his invaluable suggestions and help throughout the course of the experiments, and to Prof. F. G. Hopkins for his kindness in revising the paper.

The Transmission of Infra-red Rays by the Media of the Eye and the Transmission of Radiant Energy by Crookes and other Glasses.

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(Report of Experiments carried out for the Glassworkers' Cataract Committee of the Royal Society.)

(From the Physiological Laboratory, Cambridge.)

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Our experiments were designed to obtain evidence on the following points:—

(1) In what amount do the infra-red radiations of different wave-length gain access to the deeper structures of the eye, the lens being particularly considered?

(2) What percentage of these radiations is absorbed in transmission through the lens?

The apparatus is shown in fig. 1; it consisted of a standard constant deviation Hilger spectrometer, which was modified in the following manner.