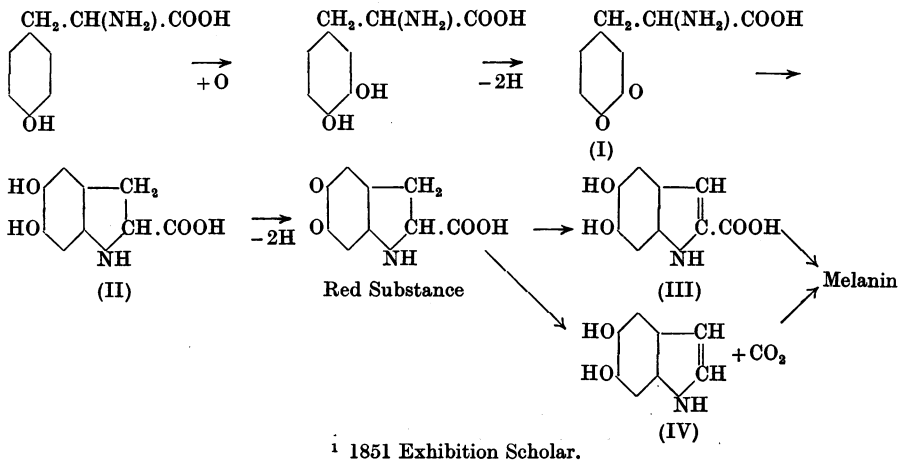
VI. A STUDY OF THE OXIDATION OF 3:4-DIHYDROXYPHENYL-*N*-METHYLALANINE WITH REFERENCE TO ITS POSSIBLE FUNCTION AS A PRECURSOR OF ADRENALINE.

By ROBERT DONALD HOSKIN HEARD[1]
and HENRY STANLEY RAPER.

From the Department of Physiology, The University, Manchester.

(*Received November 26th, 1932.*)

The close structural relationship which adrenaline bears to two amino-acids which are found in proteins suggests that either of them may logically be regarded as its parent substance. These two amino-acids, phenylalanine and tyrosine, possess almost the same carbon skeleton as adrenaline and might give rise to the latter by successive oxidation, methylation of the nitrogen atom and loss of CO_2. Investigators have from time to time unsuccessfully attempted to demonstrate a synthesis of adrenaline in this manner. Hallé [1906] exposed the adrenal glands freshly removed from the body to solutions of tyrosine and by gravimetric assay claimed to have obtained an augmentation in adrenaline content. Ewins and Laidlaw [1910] repeated and extended the experiments of Hallé, applying specific biological methods to the estimation of adrenaline. It was conclusively shown that no stage requisite to the synthesis of adrenaline from tyrosine is brought about under these conditions. The failure to obtain direct evidence of such by the action of surviving adrenal tissue does not mean that the intact organ cannot accomplish this synthesis or that the hypothesis as to the gross chemical mechanism is wrong; neither is there any evidence to suggest that all or even part of these requisite changes need take place within the adrenal gland.

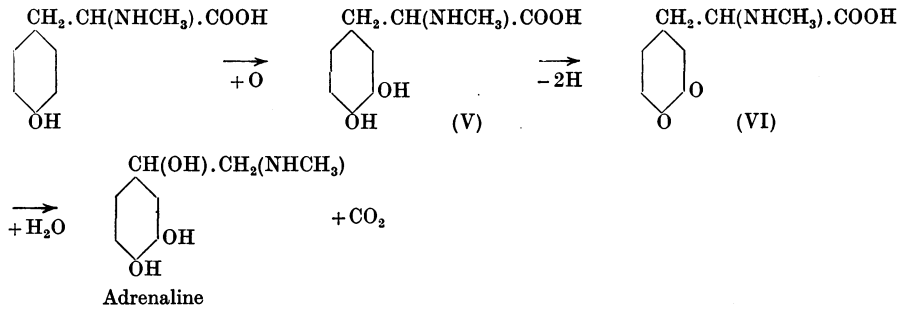


[1] 1851 Exhibition Scholar.

The isolation of *l*-3 : 4-dihydroxyphenylalanine (dopa) from the pods of *Vicia faba* by Guggenheim [1913] revealed another possible precursor of adrenaline. Renewed biological interest attached itself to this amino-acid, and the first obstacle in accepting the view that tyrosine is the parent substance of adrenaline was overcome when it was shown that dopa represents the first stage in the formation of melanin from tyrosine by the action of the enzyme tyrosinase [Raper, 1926].

The scheme of reactions given on p. 36 represents the changes which take place when tyrosine is oxidised to melanin [Raper, 1927].

The oxidation of a methylated tyrosine, which is still more closely allied to adrenaline, was found to proceed along slightly different lines from the above [Dulière and Raper, 1930]. Judging by the yields of indole derivatives corresponding to (III) and (IV) obtained from *N*-methyltyrosine on oxidation with tyrosinase, the quinone corresponding to (I) does not change immediately into the corresponding 5 : 6-dihydroxydihydroindole-2-carboxylic acid derivative (II) as with tyrosine. On allowing the red solution from *N*-methyltyrosine to decolorise *in vacuo* it was found that a small amount of pressor substance is produced. The following scheme of reactions was therefore postulated by which the quinone corresponding to (I) might conceivably give rise to adrenaline by an internal oxidation-reduction process and loss of CO_2.



The production of the 3 : 4-quinone of phenyl-*N*-methylalanine (VI) is in full accord with what is already known of the action of tyrosinase, and, with the object of investigating in more detail the changes which this quinone undergoes, the intermediate compound (V), 3 : 4-dihydroxyphenyl-*N*-methylalanine (*N*-methyldopa), has been submitted to the action of the enzyme.

The initial point of attack in the *N*-methyltyrosine molecule, when it is oxidised by tyrosinase, is the *ortho*-position to the phenolic hydroxyl group. This oxidation requires several hours. With the *ortho*-dihydric substrate oxidation takes place within a few minutes and gives rise to a greater amount of the red substance. Consequently the pressor activity of the solution from *N*-methyldopa when allowed to decolorise *in vacuo*, is considerably in excess of that obtained with *N*-methyltyrosine. Attempts to ascertain those conditions which would limit indole formation and promote greater side-chain oxidation with the production of an increased yield of pressor base met with little success. In each case it was found that the rise in blood pressure obtained on injection of the decolorised solution is not typically adrenaline-like in nature and may be imitated by administration of small doses of the ketone adrenalone. On reduction of the decolorised solution by catalytic hydrogenation a large increase in pressor activity develops. The reduced substance behaves in all respects like

adrenaline and shows the customary adrenaline reversal subsequent to administration of ergotoxine. Since no known pressor base other than a ketone of the adrenalone type exhibits this phenomenon, it is almost certain that the active substance obtained from *N*-methyldopa is adrenalone. It was found that this ketone, unlike adrenaline, is not oxidised by tyrosinase and therefore may accumulate in the reaction solution as the end-product of a secondary oxidation which occurs simultaneously with those primary oxidative changes which are concerned with the conversion of the substrate into a methylated melanin.

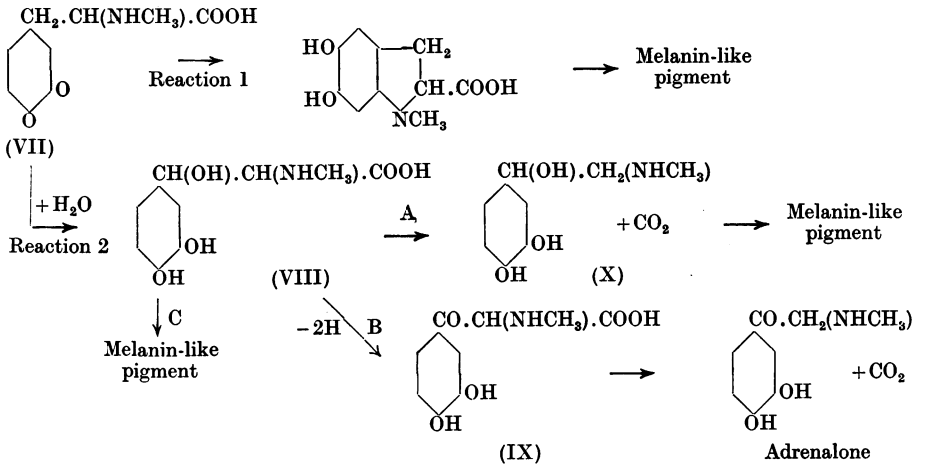
The oxidation of dopa was investigated in a similar manner, when it was found that this substance, like the methylated amino-acid, also gives rise to a pressor base which exhibits increased activity on reduction. The yield of active substance, presumably aminoacetocatechol, and the increase in activity obtained on its reduction to *nor*adrenaline are less than with *N*-methyldopa. A respirometric comparison of the rates of oxidation of these two substrates shows that both the production of the red substance and its subsequent oxidation to a melanin take place more slowly with *N*-methyldopa. Methylation of the amino-nitrogen atom diminishes the velocity of the internal oxidation-reduction process by which the 3:4-quinone of phenylalanine (I) is converted into the corresponding dihydroindole derivative (II) and thereby increases the time interval during which the quinone (I) may undergo side-chain oxidation.

It appeared conceivable that complete methylation of the amino-nitrogen atom, which prevents indole formation, would tend to promote quantitative side-chain oxidation. In the absence of a dimethylamino-acid of this type, the oxidation of hordenine with tyrosinase was investigated. This *N*-dimethyl base does not give rise to the corresponding ketone. Neither can the production of adrenalone be demonstrated on oxidation of adrenaline and 3:4-dihydroxyphenylethylmethylamine (epinine) with tyrosinase. Obviously β-oxidation in the side-chain takes place only when the terminal carboxyl group is retained; a fact which emphasises the importance of the corresponding β-hydroxy- and β-ketonic acids and may possibly explain the physiological inactivity of the precursors of adrenaline in the body.

Of the series of reactions which takes place when tyrosine is acted upon by tyrosinase, the only specific one is the introduction of a second hydroxyl group in the *ortho*-position [Dulière and Raper, 1930]. The subsequent oxidation of this product may be brought about by any enzyme or agent that gives rise to *ortho*quinones from catechol derivatives. Consequently the oxidation of dopa and its *N*-methyl derivative with silver oxide, iron and hydrogen peroxide and peroxidase and hydrogen peroxide was investigated. With the former two agents *N*-methyldopa gives rise to a solution which exhibits a typical adrenaline-like rise in blood pressure which is not increased in activity on reduction. Adrenalone, like adrenaline, is rapidly oxidised and inactivated by these agents and may not accumulate as in oxidations with tyrosinase. The pressor activity obtained from *N*-methyldopa on oxidation with silver oxide and with iron and hydrogen peroxide is almost certainly due to the presence of a small trace of adrenaline. Dopa behaves in an analogous manner, but yields a smaller amount of active substance than with tyrosinase. With peroxidase and hydrogen peroxide, which oxidise adrenalone more slowly, the evidence obtained suggests that *N*-methyldopa produces small amounts of both adrenalone and adrenaline. It was found that in this case the oxidation of dopa proceeds along different lines and gives rise to little or no pressor base.

The series of reactions which interprets these results most satisfactorily may

be represented by the following scheme which shows the changes that the 3:4-quinone of phenyl-*N*-methylalanine most likely undergoes.



The total oxygen uptake of *N*-methyldopa on oxidation with tyrosinase indicates that the greater part of the quinone (VII) is converted into a dihydroindole derivative which is subsequently oxidised to a methylated melanin (Reaction 1). A portion however gives rise to adrenalinic acid (VIII) by an internal oxidation-reduction process involving the addition of the elements of water (Reaction 2). The β-hydroxy-acid (VIII) is partially converted into a melanin-like pigment (Reaction 2 C) by a series of changes analogous to Reaction 1, whilst the remainder is oxidised to the corresponding β-ketonic acid (IX) by removal of 2 hydrogen atoms (Reaction 2 B). Adrenalonic acid (IX) gives rise to the stable ketone adrenalone on loss of CO_2. With tyrosinase the yield of adrenalone, which has been found to represent at least 3 to 7 % of the original substrate, is not necessarily a measure of the amount of the quinone (VII) which enters into Reaction 2; this may be much greater depending upon the relative velocities of Reactions 2 B and 2 C. The ketone is readily oxidised by silver oxide and by iron and hydrogen peroxide with the ultimate production of a melanin-like pigment and may not accumulate as with tyrosinase. In this case the presence of a small amount of adrenaline (X) is demonstrable (Reaction 2 A). The results suggest therefore that with tyrosinase the reactions taking place subsequent to the formation of the 3:4-quinone of phenyl-*N*-methylalanine (VII) are 1, 2 B and 2 C, the products being a melanin and adrenalone. With silver oxide or iron and hydrogen peroxide the reactions concerned are 1 and 2 A and 2 C, and the products are a melanin and adrenaline. With peroxidase and hydrogen peroxide it would appear that the reactions concerned are 2 A, 2 B, and 2 C, the products being a melanin, adrenaline and adrenalone. The active bases, adrenaline and adrenalone, are produced in small amounts because the former is oxidised by all oxidising systems employed and the latter by all except tyrosinase and atmospheric oxygen.

On perfusion of the adrenal gland with *N*-methyldopa, it has not been possible to demonstrate any production of adrenaline. The substrate is however extraordinarily stabilised on perfusion and thereafter resists oxidation by tyrosinase for a long period of time. With the unmethylated amino-acid the same phenomenon takes place. The organ has been perfused before by Schkawera

and Kusnetzow (1923), who described the presence of a remarkably stable "adrenaline-like substance" in the Ringer-Locke perfusate. They concluded that a stable precursor of the hormone was washed from the gland. In view of the resistance to oxidation of the catechol amino-acids after perfusion, this appears unlikely. Adrenaline, when added to the Ringer-Locke perfusate, was found to retain its pressor activity on remaining in the alkaline medium for many days. It is more probable therefore that the "adrenaline-like substance" of Schkawera and Kusnetzow is in reality a stabilised adrenaline. Conceivably oxidation may be delayed either by union of the catechol substrate with a suitable compound or by a continuous and complex interplay of chemical reactions constituting an oxidation-reduction system. The recent investigations of Wiltshire [1931] have shown that the presence of amino-acids delays the oxidation of adrenaline, and those of Szent-Györgyi [1928] have elucidated in part the mechanism of the oxidation-reduction system of the adrenal gland. The latter has isolated a hexuronic acid from the cortex and demonstrated the presence of a similar substance of high reduction potential in the medulla. The hexuronic acid molecule contains 2 labile hydrogen atoms which are readily released to a suitable hydrogen acceptor.

Szent-Györgyi has investigated the behaviour of coupled systems and shown that adrenaline is not oxidised as long as hexuronic acid remains present in the reduced form. It is assumed that the 3:4-quinone of adrenaline functions as a suitable hydrogen acceptor and is therefore reduced to adrenaline before the intramolecular change to the indole derivative may take place. He has shown that oxidised hexuronic acid may be reduced by the action of the glutathione system of a tissue complex and therefore, in a system of normal cell constituents, this stabilisation of catechol substance may be maintained indefinitely. Under aerobic conditions hexuronic acid is irreversibly oxidised by atmospheric oxygen and consequently the process is limited. This reducing system of the adrenal gland does not bring about the reduction of adrenalone on perfusion.

The apparent absence of tyrosinase from the higher animals must not be considered an obstacle to the acceptance of the general hypothesis of the synthesis of adrenaline from tyrosine or dopa. The only specific action of tyrosinase, namely the introduction of the *ortho*-hydroxyl group, may be brought about by other agents and is known to take place in the body. Recently the synthesis of dopa from tyrosine has been accomplished by the action of iron and hydrogen peroxide [Raper, 1932], and Medes [1932] has isolated *l*-dopa from the urine of a patient suffering from faulty tyrosine metabolism (tyrosinosis). That methylation of a nitrogen atom may be brought about in the animal body has been satisfactorily proven. The occurrence in nature of *l*-*N*-methyltyrosine as the active principle (surinamine) of *Geoffroya surinaminensis* [Winterstein, 1919] is of significance; possibly *N*-methylamino-acids may be normal protein constituents. The oxidation of the *ortho*-dihydroxy-substrate is occasioned by any agent which produces *ortho*quinones from catechol derivatives. A specific dopa oxidase is known to exist in the skin [Bloch, 1916], and the occurrence of peroxidases throughout the body is widespread. There are thus reasons for believing that the agents necessary for the production of adrenaline from tyrosine by one or other of the mechanisms which are described in this paper may be encountered in the body. As to whether the complete synthesis is brought about in the adrenal gland, there is no positive evidence.

The fact that the 3:4-quinone of phenyl-*N*-methylalanine changes so readily into a dihydroindole derivative and thence to a melanin and that the reactions which lead to the production of adrenalone or adrenaline take place only to a

small extent requires further comment. The change to the indole derivative may be prevented by complete substitution of the amino-nitrogen atom as would be found in an *N*-methyl peptide. This would give more opportunity for oxidation in the side-chain. There is no reason to believe that such substitution would inhibit oxidation by tyrosinase, since Abderhalden and Schairer [1931] have shown that tyrosine-containing peptides are oxidised by tyrosinase to a melanin-like pigment. The matter will, however, be further investigated.

Experimental.

In all experiments the estimation of pressor activity was made by intravenous injection into a decerebrate cat. The animals were prepared by, and the assays carried out in conjunction with, Dr W. Schlapp, to whom we wish to acknowledge our indebtedness.

Catalytic reduction of adrenalone and N-*dimethylaminoacetocatechol.*

Adrenalone was prepared by the method of Friedmann [1906]. A small amount of the *N*-dimethyl base was synthesised in an analogous manner. In all instances the ketones were employed as the hydrochlorides which contain 1 mol. of water of crystallisation.

Hydrogenations were carried out in a Thunberg tube of 15 cc. capacity which contained finely divided palladium black (approximately 0·05 g.) suspended in an aqueous solution of the ketone (5 cc.). The tube was evacuated, filled with purified electrolytic hydrogen to a positive pressure of 3 cm. of mercury, and mechanically shaken for 10 mins., after which the absorbed gas was replaced. This operation was repeated several times until the pressure of hydrogen inside the tube remained unchanged. The suspension was finally shaken for 30 mins., the catalyst removed and the solution assayed. The increase

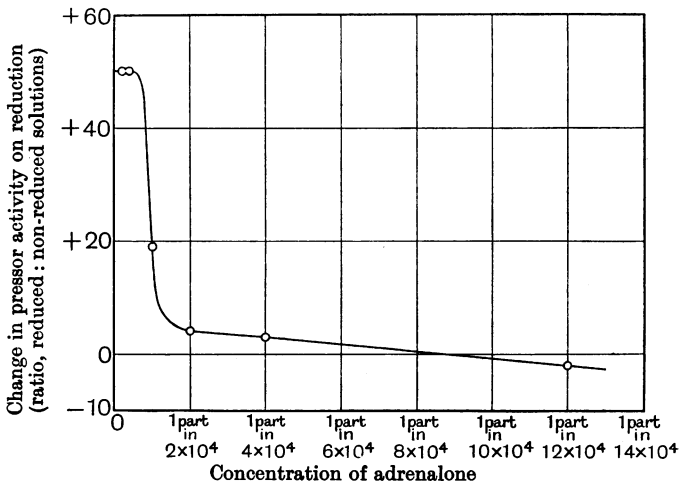


Fig. 1.

in pressor activity on reduction of adrenalone was found to be proportional to the concentration of the ketone employed. Consideration of the curve (Fig. 1) shows that in concentrations less than 1 in 4000 the amount of active substance adsorbed by the catalyst is no longer negligible and consequently the increase

in activity on hydrogenation falls off very rapidly with diminishing concentration. On shaking a solution of adrenalone (1 in 40,000) with palladium in the absence of hydrogen, it was found that the ketone was adsorbed to the extent of 50 %. The minimum concentration of adrenalone which can be detected by this method is obviously limited. The average ratio of the pressor activity of *l*-adrenaline to that of adrenalone was determined from a series of comparisons on different animals and found to be 100. Since *l*-adrenaline is approximately 1·5 times more active than the *dl*-isomeride, an increase in activity of 50 times on hydrogenation of adrenalone represents nearly quantitative reduction. The increase in activity on reduction of *N*-dimethylaminoacetocatechol (1 in 2000) was found to be 100 times. The ratio of the activity of the reduced product, *dl*-*N*-dimethyl*nor*adrenaline to that of *dl*-adrenaline is thus approximately 1:30.

Oxidations with tyrosinase.

The enzyme used in the following experiments was prepared from mealworms as decribed by Pugh [1930]; it was not dialysed before use unless so stated. Controls with the enzyme solution alone exhibited no pressor activity unless the tyrosinase had been prepared for more than a fortnight, when a histamine-like response was noted. Fresh preparations of the enzyme were therefore constantly employed and each experiment was rigidly controlled.

In most experiments the same general procedure was adopted, and unless otherwise stated the oxidation was carried out as follows. The substrate (10 mg.) was dissolved in hot water (5 cc.) and cooled, and phosphate buffer solution (4 cc.) was added. Oxidation with tyrosinase (1 cc.) was now allowed to proceed until a good red colour had developed and the enzyme had begun to precipitate. This procedure usually required from 15 to 30 mins. A few drops of 10 % acetic acid were then added to ensure complete precipitation of the enzyme, and the solution was allowed to stand for a few moments and then filtered. Decoloration of the red filtrate was carried out in one of two ways. When effected at room temperature the solution was placed in a Thunberg tube which was several times evacuated and filled with hydrogen and then set aside for 2 or 3 days. Alternatively decoloration was effected by heating in an inert atmosphere. The red solution was placed in a small flask through which hydrogen was passed until all air was expelled, when it was cautiously heated until decolorised. This phenomenon took place before the boiling-point was reached. The decolorised solution was then either tested for pressor activity immediately or divided into two equal portions and placed in Thunberg tubes. In the latter instance, one portion was catalytically hydrogenated, as described in the previous section, whilst the other was preserved under hydrogen until injections were made. When a quantity of substrate other than 10 mg. was oxidised, the amounts of water, buffer, and enzyme employed were proportional to the above values.

N-*Methyldopa.* *N*-Methyldopa was synthesised as already described [Heard, 1933]. Many preliminary experiments were carried out in an endeavour to ascertain those factors which influence the production of a pressor base. The reaction of the medium, which is known to have a marked influence on the velocity of the enzyme action and also on the nature of the pigments produced [Raper and Wormall, 1923], was investigated by oxidising *N*-methyldopa at p_H values 6·5, 7·0 and 7·7. The red filtrates were decolorised at room temperature for 3 days, when comparisons were made by intravenous injection. The solution oxidised at p_H 6·5, which is optimum for the production of the

red quinone, exhibited a slightly greater pressor response. The influence of reaction on the decoloration of the red substance was investigated in the following manner. *N*-Methyldopa (25 mg.) in water (22 cc.) was oxidised with tyrosinase (3 cc.), the p_H being adjusted to 6·5 by the cautious addition of a few drops of dilute ammonia (0·1 %). Aliquot samples (3 cc.) of the red filtrate were placed in six Thunberg tubes, each of which contained phosphate buffer (0·75 cc.). Decoloration was effected at room temperature over a p_H range of 4·5 to 9·5. The red substance decolorised in alkaline media overnight whereas in acid media the final disappearance of the red tint did not take place until the second day. On injection of the decolorised solutions no difference in pressor activity was noted. When decoloration was effected by heating, the yield of pressor base was increased by approximately 25 %. Consequently, in all succeeding experiments, oxidation was carried out at p_H 6·5 and the red substance was decolorised by heating in an inert atmosphere.

The above experiments were repeated several times and in each case the yield of pressor substance obtained was very small. The results were not always consistent and in a few cases injection of the decolorised solution did not cause a rise in blood pressure. Usually the pressor activity was found to be equivalent to that given by 1 in 30 millions adrenaline, but varied considerably from this value to zero. One comparison was made with the decolorised solution from *N*-methyltyrosine, which gave a pressor response equivalent to that shown by 1 in 50 millions adrenaline. On oxidation of the *ortho*-dihydric substrate a bright red solution was obtained in 20 mins. whilst with *N*-methyltyrosine only a fair red colour developed after 3 hours. In all cases the pressor responses obtained were not typically adrenaline-like in nature. The gradual rise in blood pressure, which followed a noticeable latent period, was more prolonged than that shown by adrenaline and could be imitated by administration of minute doses of the ketone adrenalone. Consequently the effect of catalytic hydrogenation on the pressor activity of the decolorised solution was investigated.

The red solution from *N*-methyldopa (15 mg.) was decolorised by heating in an atmosphere of hydrogen and divided into two equal portions. One was preserved under hydrogen until injections were made, whilst the other was reduced by catalytic hydrogenation. On removal of the catalyst previous to the injection of the reduced solution, it was noted that the latter rapidly became coloured red. Hydrogenation in this manner also effects the reduction of 5:6-dihydroxy-*N*-methylindole or its 2-carboxylic acid to the corresponding saturated dihydroindole derivative, which is rapidly oxidised by atmospheric oxygen to the red quinone. This may be adduced as confirmatory evidence supporting the structure of the indole derivative and the red substance. This experiment was repeated 8 times and in each case a large increase in pressor activity was obtained on reduction of the decolorised solution. The response became typically adrenaline-like in nature in that the rise in blood pressure was more rapid and less prolonged.

Injection of the reduced decolorised solution subsequent to the administration of ergotoxine caused a fall in blood pressure similar to that obtained with adrenaline under the same conditions. The pressor response was judged equivalent to that given by 1 in 500,000 adrenaline. Even when the original decolorised solution exhibited little or no pressor response, the presence of the ketone was strikingly demonstrated on hydrogenation when injection of the reduced solution caused a marked rise in blood pressure. Typical examples of the pressor responses obtained on injection of the decolorised solution before and after reduction are illustrated in Figs. 2 A and 2 B.

In order to measure more precisely the increase in activity on reduction, the hydrogenation of a concentrated decolorised solution was attempted. *N*-Methyldopa (150 mg.) in water (230 cc.) and phosphate buffer (52 cc.) at p_H 6·5 was oxidised with tyrosinase (18 cc.) in the usual manner. Oxygen was bubbled through the liquid which was frequently shaken until a good red solution was obtained (30 mins.). The bright red filtrate was concentrated by vacuum distillation in a stream of hydrogen until the fluid measured 15 cc.

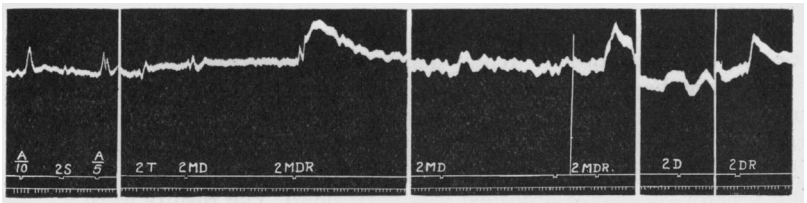


Fig. 2 A Fig. 2 B Fig. 2 C

Fig. 2. Oxidations with tyrosinase.

A/10—1 cc. of 1 in a million adrenaline.
2 MD—2 cc. of decolorised solution from *N*-methyldopa.
2 D—2 cc. of decolorised solution from dopa.
2 MDR—2 cc. of reduced decolorised solution from *N*-methyldopa.
2 DR—2 cc. of reduced decolorised solution from dopa.
2 T—Tyrosinase control.
2 S—2 cc. of physiological saline solution.

A and B illustrate the striking increase in activity developed on reduction of the decolorised solution from *N*-methyldopa. The pressor base originally present in the non-reduced solution is apparently the ketone adrenalone. C. With dopa the increase in activity on reduction is less marked. Presumably the non-reduced base is aminoacetocatechol.

approximately. The concentrate, which gave a strong indole reaction with *p*-dimethylaminobenzaldehyde, was divided into two portions. One was hydrogenated whilst the other was preserved under hydrogen until assayed. Both showed the same response on injection. It was not possible to estimate the amount of adrenalone present, as the pressor activity of the latter was masked by the presence in greater concentrations of toxic substances which caused a fall in blood pressure. The animal withstood the administration of this concentrate (1 cc.) only with difficulty. The reduced concentrate was not autoxidised with the formation of the usual red quinone, and therefore it appeared probable that hydrogenation had not taken place. A solution of adrenalone (2 cc. of 0·5 %) was added to the concentrate (2 cc.) and reduction was again attempted. The added ketone was not reduced. Obviously mealworm tyrosinase contains toxic substances which in relatively high concentrations cause a fall in blood pressure and poison the palladium catalyst. The experiment was repeated, using less enzyme (10 cc.), but the toxic substances still persisted.

It has not been possible as yet to estimate with accuracy the amount of adrenalone produced, but a minimum figure may be deduced. The minimum dose of adrenalone to which a definite pressor response is obtained was found to vary considerably with different animals. Usually in response to an injection of 1 cc. a concentration of 1 in 30,000 was required, although in a few isolated cases 1 in 75,000 sufficed. It has been quoted above that adrenaline is 100 times more active than adrenalone; this ratio was obtained by comparison of injections which gave a rise in blood pressure of approximately 30 mm. The ketone

is not a true sympathomimetic base, and this ratio does not hold good when adrenalone is administered in amounts approaching the minimum dose. Animals often respond to the injection of 1 in 100 millions adrenaline (1 cc.), whilst we have not observed a rise in blood pressure from adrenalone in doses less than 1 in 75,000 (1 cc.). The amount of adrenalone in the decolorised solution from *N*-methyldopa (0·1 %) closely parallels the minimum quantity to which a definite pressor response is obtained. This accounts for the inconsistent results obtained on injection of the non-reduced solutions and represents a conversion of from 3 to 7 % of the substrate. On the other hand it has been shown that hydrogenation of the ketone in a concentration of 1 in 75,000 results in only a twofold increase in pressor activity, and in a concentration of 1 in 30,000 an increase of approximately 6 times. While it has not been possible to estimate with accuracy the increase in activity on reduction of the experimental solutions, it may be seen from Fig. 2 that this value is considerably in excess of the above, which suggests that as much as 15 or 20 % of the substrate may be converted into adrenalone.

Dopa. In the manner described above the oxidation of the unmethylated substrate was also investigated. It was found that dopa is more rapidly oxidised and gives rise to a less stable red substance. Injection of the decolorised solution resulted in a rise in blood pressure almost as great as that obtained with *N*-methyldopa. On hydrogenation a striking increase in activity developed (Fig. 2 C). This was however less marked than that found with the methylated substrate. The pressor activity of the reduced solution was judged equivalent to that given by 1 in a million adrenaline. The pressor bases from dopa, presumably aminoacetocatechol and *nor*adrenaline, are more active than adrenalone and adrenaline respectively. Barger and Dale [1910] record the ratio of the activity of *dl*-adrenaline to aminoacetocatechol as 23:1 and to *dl-nor*adrenaline as 1·40:1. Aminoacetocatechol is therefore three times more active than adrenalone, and consequently the yield of the ketone from dopa is approximately one-third of that obtained from its *N*-methyl derivative. Methylation of the amino-nitrogen atom diminishes the velocity of the internal oxidation-reduction process by which the 3:4-quinone of phenylalanine undergoes internal condensation in the 6-position with the production of a dihydroindole derivative, and therefore promotes oxidation in the side-chain.

Adrenalone. The hydrochloride (5 mg.) in phosphate buffer (10 cc.) at p_H 6·5 was treated with tyrosinase (1 cc.) for 30 mins., after which the enzyme was precipitated by the addition of a few drops of 10 % acetic acid and the light brown filtrate assayed. After treatment in this manner the ketone was found to have retained completely its original activity. The presence of the carbonyl group undoubtedly stabilises the hydrogen atoms of the hydroxyl groups and prevents quinone formation.

Hordenine. It appeared conceivable that a fully methylated base of this type, which is unable to undergo ring formation, might be oxidised almost quantitatively to the corresponding ketone, *N*-dimethylaminoacetocatechol. Hordenine sulphate (25 mg.) in phosphate buffer (25 cc.) at p_H 6·5 was oxidised with tyrosinase (3 cc.) for 12 hours. The solution darkened but no development of the usual red coloration took place. The enzyme was then precipitated by the addition of 10 % acetic acid and a portion of the brown filtrate, which gave an intense catechol reaction with ferric chloride, was catalytically hydrogenated. No increase in pressor activity developed on reduction. The rise in blood pressure caused by injection of the oxidised solution was approximately equivalent to that given by the original solution of hordenine sulphate.

Adrenaline. The free base (10 mg.) was dissolved in dilute hydrochloric acid (2 cc. of 1 %) and the solution neutralised to litmus by the addition of a few drops of dilute ammonia (1 %). The p_H was then adjusted to 6·5 with phosphate buffer (7 cc.), when the enzyme (1 cc.) was added. A bright red coloration developed immediately and after 30 mins. the enzyme was precipitated. The blood-red filtrate was allowed to remain under hydrogen for 3 days, when the red tint was replaced by reddish-brown and there was deposited a considerable amount of black melanin-like pigment. It was found that, regardless of extra precautions taken to remove as much oxygen as possible, this pigment formation could not be prevented. The oxidation of adrenaline does not proceed along the same lines as those of other substances already described, and it is conceivable that its 3:4-quinone is capable of bringing about anaerobic oxidation of the indole derivative to this melanin-like pigment. The solution which had stood for 3 days gave a strong indole test with *p*-dimethylaminobenzaldehyde, and on intravenous injection it was found that adrenaline when oxidised in this manner is 97 % inactivated. The residual pressor activity was not enhanced on hydrogenation of the solution, thus indicating that adrenalone was absent.

3:4-Dihydroxyphenylethylmethylamine (epinine). The hydrochloride (17 mg.) in phosphate buffer (17 cc.) at p_H 6·5 was oxidised with tyrosinase (2 cc.) for 25 mins. The red solution was decolorised at room temperature for 3 days and then assayed. Inactivation to the extent of 50 % had been effected and reduction of the decolorised solution did not increase the residual activity.

The results of these experiments with the free bases clearly indicate that β-oxidation in the side-chain occurs only when the terminal carboxyl group is present.

Oxidations with silver oxide.

It has already been established [Dulière and Raper, 1930] that dopa on oxidation with silver oxide yields the same indole derivatives as with tyrosinase. Preliminary experiments showed that adrenalone is oxidised by silver oxide and therefore it appeared of interest to ascertain whether dopa or *N*-methyldopa gives rise to a physiologically active base under these conditions.

An aqueous solution of the substrate (10 cc. of 0·1 %) was treated with freshly prepared silver oxide (approximately 0·05 g.) and vigorously shaken until a good red colour had developed. With dopa and *N*-methyldopa the solutions rapidly became bright red and then reddish-brown within 15 mins. The ketone gave rise to a more brownish solution. A drop of 10 % acetic acid and sodium sulphate solution (0·1 cc. of 10 %) were now added and the mixture was shaken to assist in coagulation of colloidal silver. After a few moments the latter was removed, after which the filtrate was heated in an atmosphere of hydrogen until the red tint was abolished. The true red solutions from dopa and its *N*-methyl derivative became colourless, whereas the more brownish solution from adrenalone only partially decolorised. On heating there was deposited more colloidal silver, which was removed previous to injection and hydrogenation. It was found that adrenalone was completely inactivated on oxidation in this manner. The decolorised solution from *N*-methyldopa produced a typical adrenaline-like rise in blood pressure equivalent to the latter in a concentration of 1 in 40 millions. Catalytic hydrogenation revealed no alteration in the nature or extent of the response. With dopa a similar rise in blood pressure of the same magnitude was obtained and again reduction of the decolorised solution did not cause any increased activity (see Fig. 3). With silver oxide the ketones are rapidly oxidised and consequently no accumulation takes place as with tyrosinase. The pressor activity is almost certainly due to the

presence in very small amounts of adrenaline and *nor*adrenaline respectively. As both these substances are rapidly oxidised by silver oxide, their existence is a transient one only.

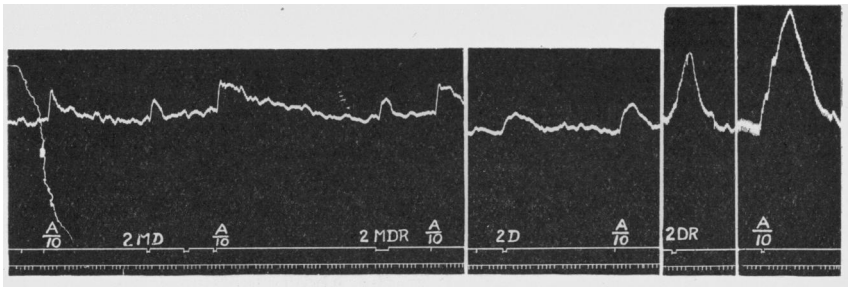


Fig. 3. Oxidations with silver oxide.

A/10—1 cc. of 1 in a million adrenaline.
2 MD—2 cc. of decolorised solution from *N*-methyldopa.
2 D—2 cc. of decolorised solution from dopa.
2 MDR—2 cc. of reduced decolorised solution from *N*-methyldopa.
2 DR—2 cc. of reduced decolorised solution from dopa.

On injection of the decolorised solutions from dopa and its *N*-methyl derivative, typical adrenaline-like pressor responses are obtained. Reduction of the solutions results in no enhanced pressor activity or alteration in the nature of the response. In the absence of the corresponding ketones, the rises in blood pressure are almost certainly due to the presence of small amounts of *nor*adrenaline and adrenaline respectively. N.B. The apparent increased activity of the decolorised dopa solution on reduction is due to increased sensitiveness of the animal as shown by the enhanced response to adrenaline given subsequently.

Oxidations with iron and hydrogen peroxide.

It is well known that hydrogen peroxide brings about oxidations of fundamental biological importance, and that ferrous salts, like peroxidases, may activate hydrogen peroxide. Dakin [1908] has shown that normal fatty acids and their phenyl derivatives may be oxidised to the corresponding β-ketonic acids by hydrogen peroxide. Similarly it appeared probable that the same agent might bring about β-oxidation of *N*-methyldopa and give rise to an active base by loss of CO_2. The behaviour of adrenalone, dopa and *N*-methyldopa was investigated.

An aqueous solution of the substrate (10 cc. of 0·1 %) was placed in a Thunberg tube and ferrous sulphate solution (0·2 cc. of 0·1 %) and the desired amount of a solution of hydrogen peroxide (1 %) were added. The amount of peroxide supplied was varied from 1 to 4 molecules per molecule of substrate. Oxidation was allowed to proceed in an inert atmosphere. Hydrogen was passed through the solution and expelled by way of the side arm of the Thunberg tube and a water-trap to the atmosphere. The solutions became deep reddish-brown within 10 mins. and on remaining under hydrogen for 24 hours the reddish tint was abolished. If oxidation is carried out in the presence of atmospheric oxygen, production of a black melanin-like pigment takes place. As with silver oxide, oxidation with hydrogen peroxide and iron inactivated adrenalone to the extent of 80 %. Injection of the solutions from dopa and *N*-methyldopa caused a typical adrenaline-like rise in blood pressure which was not altered on catalytic hydrogenation. The pressor response from *N*-methyldopa was equivalent to that given by 1 in 30 millions adrenaline and that from dopa to 1 in 40 millions adrenaline. The yield was found to remain constant regardless of the amount of hydrogen peroxide supplied during the oxidation.

Oxidations with peroxidase and hydrogen peroxide.

Peroxidase was prepared from horseradish as described by Bach and Chodat [1903]. The enzyme obtained in this manner is less active than the purified preparations recently described by Willstätter and Stoll [1918], but the former method does not involve the use of baryta, the last traces of which are difficult to remove, and consequently yields a product more suitable for injection. Catalase was obtained from beef-liver by aqueous extraction and alcohol precipitation.

The desired quantity of peroxidase was transferred to a dry Thunberg tube of 30 cc. capacity and dissolved in the recorded amount of phosphate buffer at p_H 6·5. Solutions of the substrate and hydrogen peroxide (1 %) were now added and air was immediately expelled by passing hydrogen through the apparatus in the manner described in the above section. The oxidation of adrenalone, dopa and methyldopa was investigated under the various conditions indicated in Table I. Oxidation was allowed to proceed until an optimum red coloration developed, when a trace of catalase was added to decompose any excess hydrogen peroxide. An evolution of gas on addition of catalase always occurred when more than two atomic proportions of hydrogen peroxide were employed. The Thunberg tube was then evacuated and filled with hydrogen several times. With each evacuation the solution was vigorously shaken to expel last traces of oxygen, and finally kept under hydrogen until decolorised.

Table I.

				Hydrogen peroxide			
No.	Substrate and its amount mg.	Water cc.	Buffer p_H 6·5 cc.	1 % solution cc.	Molecules per molecule of substrate	Peroxidase mg.	Time mins.
1	Dopa (5)	2·5	2·0	0·35	4·0	50	60
2	Dopa (10)	6·0	4·0	0·20	1·15	200	60
3	Dopa (5)	2·5	2·0	0·10	1·15	200	60
4	*N*-Methyldopa (10)	6·0	4·0	0·20	1·2	200	35
5	*N*-Methyldopa (10)	6·0	4·0	0·35	2·2	150	45
6	*N*-Methyldopa (5)	2·5	2·0	0·35	4·4	100	20
7	Adrenalone (2·5)	5·0	5·0	0·08	2·2	40	45

A good red colour was produced from the methylated amino-acid within a few moments and on standing under hydrogen no pigment formation took place. Using the same concentration of peroxidase it was not possible to obtain from dopa a true red colour at any stage of the oxidation (Exps. 1 and 2). After one hour the solution was dull brownish and later deposited a considerable amount of melanin. By increasing the amount of enzyme employed (Exp. 3), a more reddish solution developed, but again on standing pigment formation could not be avoided in spite of extra precautions to exclude oxygen. It is possible that in the presence of peroxidase the quinone from dopa is capable of bringing about oxidation of the indole derivative to melanin. Obviously the internal oxidation-reduction process by which the 3:4-quinone of phenylalanine is converted into the corresponding 5:6-dihydroindole derivative, and the oxidation of the latter to melanin, proceed more rapidly in the presence of peroxidase and peroxide than with tyrosinase and atmospheric oxygen or atmospheric oxygen alone. On addition of adrenalone to peroxidase, a pink coloration, which did not increase in intensity, developed immediately. No laccase was present in the peroxidase preparation as shown by the inability of the enzyme

to oxidise guaiacol in the absence of peroxide. This pink coloration is conceivably the result of a union of enzyme and substrate. *N*-Dimethylaminoacetocatechol exhibited the same phenomenon. On addition of peroxide to adrenalone and peroxidase, oxidation proceeded rapidly with the development of a dark reddish-brown solution. The reddish tint was abolished on standing under hydrogen for 24 hours. Injection of the decolorised solution showed that the ketone was inactivated to the extent of 90 %. A control solution of peroxidase and catalase alone gave no pressor response. Injection of the solutions from *N*-methyldopa caused a rise in blood pressure equivalent to that given by 1 in 2 millions adrenaline. The yield of pressor base was not affected appreciably by varying the amount of enzyme or peroxide employed for the oxidation. Reduction of the decolorised solution resulted in no enhanced pressor activity although an alteration in the nature of the response was effected (see Fig. 4).

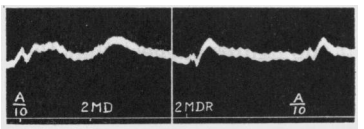


Fig. 4. Oxidations with peroxidase and hydrogen peroxide.

A/10—1 cc. of 1 in a million adrenaline.
2 MD—2 cc. of decolorised solution from *N*-methyldopa.
2 MDR—2 cc. of reduced decolorised solution from *N*-methyldopa.

Reduction of the decolorised solution from *N*-methyldopa results in no enhanced pressor activity. The response becomes more typically adrenaline-like in nature; this indicates the presence of both adrenalone and adrenaline in the original non-reduced solution.

The rise in blood pressure became more rapid and less prolonged, closely approximating to that caused by adrenaline. Since adrenalone is oxidised by peroxidase and hydrogen peroxide, it is unlikely that an accumulation of any appreciable amount of the ketone would occur, but this change in the nature of the pressor response after reduction certainly suggests that a small amount is present. On reduction of adrenalone in a concentration of approximately 1 in 90,000, a similar alteration of the pressor response without any additional rise in blood pressure takes place. With dopa (Exp. 3) the decolorised solution exhibited only a very slight, if any, pressor activity. The amount of red substance present at any given stage in the oxidation of dopa is small and consequently the formation of a pressor base, which presumably occurs simultaneously with the intramolecular change which the quinone undergoes during decoloration, is limited.

Respirometer experiments.

These were carried out in a modified form of Haldane's blood-gas apparatus in the manner previously described [Dulière and Raper, 1930]. In order to eliminate errors due to inequality in temperature, the blood-gas apparatus as well as the conical flasks and connecting tubings were immersed in a water-bath designed for this purpose. The bath was electrically heated and maintained at a temperature of 20·5°, ± 0·2°.

Solutions used. All substrates (5 mg.) except adrenaline were dissolved in water (5 cc.) and phosphate buffer (5 cc.) at p_H 6·5. The reaction flask contained 4 cc. of this solution and a further 2 cc. of buffer. With adrenaline the free base (5 mg.) was dissolved in dilute hydrochloric acid (5 cc. of 0·05 %) and 2 cc. of this solution were added to 4 cc. of buffer in the reaction vessel. In each instance

tyrosinase (1·4 cc.), which had previously been dialysed for 2 days against phosphate buffer (p_H 8), was employed. Phenylurethane was added as an antiseptic. Readings were taken periodically until oxygen uptake finally ceased and were corrected for barometric pressure and temperature. They are expressed in terms of the number of atoms of oxygen absorbed per molecule of substrate (Table II).

Table II.

Substrate (2 mg.)	No. of exps.	O_2 absorbed at N.T.P. cc.	O_2 uptake. Atoms per molecule of substrate (average)
Dopa	2	0·426–0·430	3·87
N-Methyldopa	2	0·428–0·430	4·04
Adrenaline	1	0·607	4·95
Adrenalone	1	0	0

The methylated substrate is seen to utilise more oxygen than dopa but as this difference is not great the figures suggest that the main reaction proceeding is the same as that with tyrosine. One experiment with *N*-methyldopa was carried out in the presence of glycine (0·03 %). Wiltshire [1931] has shown that amino-acids delay the oxidation of adrenaline in very dilute solution, but the addition of glycine did not affect the total oxygen uptake or rate of oxidation in our experiments. Adrenaline utilised 5 atoms of oxygen, which is in agreement with the figure of 5·3 previously recorded using undialysed tyrosinase [Dulière and Raper, 1930]. It is known that certain *ortho*-quinones are capable of bringing about oxidative deamination of amino-acids [Happold and Raper, 1925] and recently Kisch [1931] has shown that the quinone of adrenaline is particularly active in this respect. It was thought that the high oxygen uptake of adrenaline observed by Dulière and Raper might have been due to the oxidation of extraneous amino-acids in the enzyme preparation and consequently their experiment was repeated using dialysed tyrosinase.

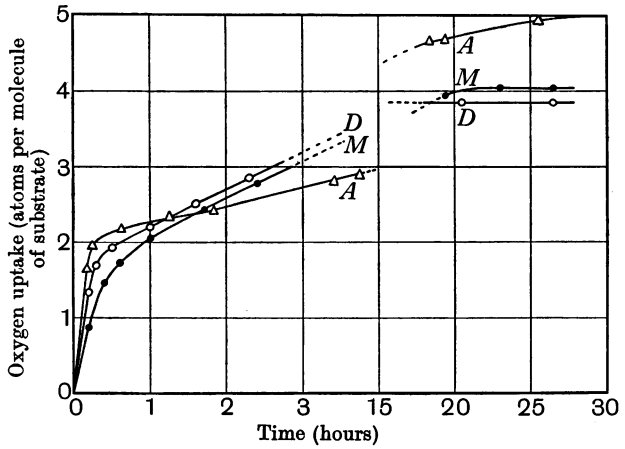


Fig. 5. A = Adrenaline; M = *N*-methyldopa; D = dopa.

Consideration of the rate of oxidation in these experiments (Fig. 5) shows that 2 atoms of oxygen per molecule of substrate are rapidly taken up within the first hour after which oxidation proceeds much more slowly. This is in

accordance with the view that dopa requires 2 atoms to convert it into 5:6-dihydroxyindole and a further 2 atoms to convert this into melanin. Methylation of the N atom diminishes the velocity of both stages of the oxidation; *i.e.* the speed of the two internal oxidation-reduction processes, which both the 3:4-quinone of phenylalanine and the 5:6-quinone of the dihydroindole derivative undergo, is diminished. With adrenaline the initial stage of the oxidation takes place much more rapidly than with dopa, whereas the reaction velocity of the second stage is considerably less. In the absence of the carboxyl group it is known that the speed of the 2nd intramolecular change is greatly diminished [Dulière and Raper, 1930].

Perfusion of the adrenal gland.

The technique adopted in the perfusion of this organ was essentially that described by Schkawera and Kusnetzow [1923]. The glands of a cow, together with a large piece of surrounding fatty tissue which encased 6 or 8 inches of both the aorta and vena cava, were obtained from the abattoir directly after the slaughtering of the animal and conveyed to the laboratory in an ice-bath. The blood vessels were then carefully dissected from the surrounding tissue and cannulae inserted in the adrenal arteries. In some cases it was possible to locate a main vessel leading directly from the aorta to the gland, whilst in others it was necessary to trace smaller branches back from the organ until these united and formed a vessel large enough to accommodate the cannula. The vena cava was opened, exposing the adrenal vein or veins, and the preparation set up in the usual liver perfusion apparatus. The opened flap of the vena cava was placed over the drain tube of a shallow bakelite basin and the whole preparation was contained in a metal box, the temperature of which was maintained at 37°. The perfusing fluid, warmed to 37°, was fed by gravity through the organ at a pressure of 1 metre. The perfusion was in operation within 3 to 4 hours after the death of the animal. On completion of each experiment, methylene blue was injected to determine the extent of perfusion. In most instances this represented about 10 to 20 % of total gland tissue. It is possible that only branches of the two main adrenal arteries described by Schkawera and Kusnetzow [1923] were perfused, although occasionally there appeared to be more than two independent arterial systems irrigating the gland. Methylene blue does not stain the tissues immediately; a freshly exposed surface remains pale for some minutes, after which time the medulla becomes blue, followed after a longer interval by the cortex. Conceivably the dye is reduced to the leuco-form by the reducing systems of the gland.

Oxygenated Ringer-Locke solution was perfused for 4 hours and the pressor activity of each quarter-hour fraction estimated. All fractions gave the same response (1 in 200,000 adrenaline). On re-perfusion, the activity summed proportionally and the rate of perfusion decreased. The latter phenomenon may be ascribed to constriction of blood vessels by the active substance of the perfusate. Thus, Ringer-Locke solution (210 cc.) required 17 mins. to pass through the gland in the first instance (conc. = 1 in 200,000 adrenaline), 21 mins. in the second instance (1 in 100,000) and, when perfused the third time, required 35 mins. On attempting to obtain a still more potent perfusate, the vessels were constricted so much as to render maintenance of temperature of the entering fluid almost impossible. When assayed colorimetrically by the method of Folin, Cannon and Denis [1913] the perfusate showed a greater concentration of adrenaline than that found by injection. This indicates the presence of catechol substance which is either physiologically inactive or less active than

adrenaline. The remarkable stability of the active substance of the perfusate, as described by Schkawera and Kusnetzow [1923], was confirmed. The alkaline perfusate retained its pressor activity when kept in an ice-chest for many days and was not inactivated on boiling (10 mins.). Since no known sympathomimetic base other than a ketone of the adrenalone type resists oxidation to an extent even approaching this, the perfusate (10 cc.) was reduced by catalytic hydrogenation, but no increase in pressor activity developed. The "adrenaline-like" substance of the perfusate was slowly oxidised by tyrosinase and by hydrogen peroxide when activated with either peroxidase or a trace of ferrous salt. It gave rise to a red solution with no subsequent deposition of black pigment. With silver oxide, a deep yellow solution was obtained. It was found that the perfusate was capable of delaying the oxidation of adrenaline for a long period of time. Adrenaline hydrochloride (1·2 mg.) was added to the perfusate (60 cc.), which was then incubated at 37° for 4 hours. The decrease in activity of this solution was very slight whereas a control containing adrenaline in Ringer-Locke solution was completely inactivated. On standing for 5 days at room temperature the activity of the former fell to a value equal to that of the perfusate alone which itself had decreased in activity by 50 %. Thus, added adrenaline is remarkably stabilised although it is more readily destroyed than the "adrenaline-like" substance originally present in the perfusate. In view of these facts it is more probable that this "adrenaline-like" substance is in reality stabilised adrenaline and not a stable precursor of the hormone as concluded by Schkawera and Kusnetzow [1923].

N-Methyldopa (100 mg.) in Ringer-Locke solution (100 cc.) was twice perfused through the gland, but no increase in pressor activity above that shown by a control perfusate could be demonstrated. The substrate was however remarkably stabilised on perfusion. In alkaline Ringer-Locke solution *N*-methyldopa rapidly undergoes autoxidation and the solution becomes deep brownish-black in 30 mins. The perfusate only slightly darkened overnight and did not become black until 3 days had elapsed. In the presence of tyrosinase no formation of the usual red quinone took place and only a trace of black pigment was deposited overnight. With dopa a similar stabilisation took place on perfusion. In the introduction to this communication, the manner in which this stabilisation may be effected has been discussed. Adrenalone in Ringer-Locke solution (150 cc. of 0·1 %) was perfused, but no evidence indicating reduction of the ketone by the reducing systems of the gland was obtained.

Summary.

1. As a result of the action of tyrosinase, 3:4-dihydroxyphenyl-*N*-methylalanine (*N*-methyldopa) yields a small amount of pressor base, the activity of which increases very greatly on reduction. It has been concluded that this pressor base is the ketone adrenalone which is not oxidised by the enzyme. Similarly 3:4-dihydroxyphenylalanine (dopa) yields a smaller amount of pressor substance, presumably aminoacetocatechol.

2. Production of a pressor base takes place as the result of a secondary oxidation of the β-carbon atom in the side-chain, which occurs simultaneously with the main series of reactions concerned with the formation of indole derivatives and subsequently of melanin from tyrosine. Methylation of the nitrogen atom diminishes the reaction velocity of the intramolecular change by which the indole derivative is produced and promotes β-oxidation in the side-chain.

3. Side-chain oxidation occurs only when the terminal carboxyl group is retained.

4. The above substrates, on oxidation with silver oxide and with iron and hydrogen peroxide, yield pressor bases which are not increased in activity on reduction. These are most likely adrenaline and *nor*adrenaline respectively. The corresponding ketones are also oxidised by these agents. On oxidation of *N*-methyldopa with peroxidase and hydrogen peroxide, the evidence suggests that both adrenaline and adrenalone are produced.

5. *N*-Methyldopa does not give rise to adrenaline on perfusion through the surviving adrenal gland but on the contrary is stabilised against subsequent oxidation. In the same manner, the oxidation of dopa and of adrenaline is delayed. The possible significance of this stabilisation and the synthesis of adrenaline *in vivo* are discussed.

We wish to express our thanks to the Government Grant Committee of the Royal Society for a grant in aid of this work.

REFERENCES.

Abderhalden and Schairer (1931). *Fermentforsch.* **12**, 329.
Bach and Chodat (1903). *Ber. deutsch. chem. Ges.* **36**, 600.
Barger and Dale (1910). *J. Physiol.* **41**, 19.
Bloch (1916). *Z. physiol. Chem.* **98**, 226.
Dakin (1908). *J. Biol. Chem.* **4**, 419.
Dulière and Raper (1930). *Biochem. J.* **24**, 239.
Ewins and Laidlaw (1910). *J. Physiol.* **40**, 275.
Folin, Cannon and Denis (1913). *J. Biol. Chem.* **13**, 477.
Friedmann (1906). *Hofmeister's Beitr.* **8**, 113.
Guggenheim (1913). *Z. physiol. Chem.* **88**, 266.
Hallé (1906). *Hofmeister's Beitr.* **8**, 276.
Happold and Raper (1925). *Biochem. J.* **19**, 92.
Heard (1933). *Biochem. J.* **27**, 54.
Kisch (1931). *Biochem. Z.* **242**, 1.
Medes (1932). *Biochem. J.* **26**, 917.
Pugh (1930). *Biochem. J.* **24**, 1442.
Raper (1926). *Biochem. J.* **20**, 735.
—— (1927). *Biochem. J.* **21**, 89.
—— (1932). *Biochem. J.* **26**, 2000.
—— and Wormall (1923). *Biochem. J.* **17**, 1454.
Schkawera and Kusnetzow (1923). *Z. ges. exp. Med.* **38**, 37.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
Willstätter and Stoll (1918). *Liebig's Ann.* **416**, 21.
Wiltshire (1931). *J. Physiol.* **72**, 88.
Winterstein (1919). *Z. physiol. Chem.* **105**, 20.