5-16-1951

A Special Respiratory Mechanism in the Adrenal Gland of Cattle

Martin Roeder

A SPECIAL RESPIRATORY MECHANISM IN THE ADRENAL GLAND OF CATTLE, III: A MAMMALIAN TYROSINASE

A Thesis

Presented to

the Faculty of the Department of Biology

University of New Mexico

In Partial Fulfillment of

the Requirements for the Degree

Master of Science

by

Martin Roeder

June 1951

This thesis, directed and approved by the candidate's committee, has been accepted by the Graduate Committee of the University of New Mexico in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Douglas G. Humm for the patience and interest he has shown in the direction of this research and in the preparation of this thesis. I should also like to express my thanks to Dr. Richard B. Johnson and Mr. Martin W. Fleck for their able assistance in the preparation of the manuscript. I should further like to express my appreciation to the Mobley Packing Company and the Schwartzman Packing Company, both of Albuquerque, New Mexico, for supplying the adrenal glands used in this research.

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Introduction. The first definite evidence for the existence of an enzyme which catalyzes the biological oxidation of phenols was presented in 1896 by Bourquelot and Bertrand. By 1902 the latter had characterized the enzyme as acting directly on the amino acid tyrosine, and had demonstrated its action against other substances, including p-hydroxyphenylethylamine, p-cresol, and phenol. In all cases the activity of the preparation tested was dependent upon the presence of at least one phenolic hydroxyl group in the substrate molecule. The enzyme was named tyrosinase (Bertrand, 1908), and has since been described as polyphenol oxidase (Keilin and Mann, 1938; Kubowitz, 1938), and monophenol oxidase (Dalton and Nelson, 1939).

This class of enzymes is principally extracted from plant tissues, some of the most active preparations being derived from the potato Solanum tuberosum, and the mushrooms Psalliota campestris and Russula nigricans. Kubowitz (1938) using the sweet potato as a starting material, and Keilin and Mann (1938) extracting the common field mushroom, were able to obtain a highly purified polyphenol oxidase, and found the oxidase activity to be proportional to the copper content of these very pure preparations. In addition, Kubowitz (1938) found that

Introduction. The first definite evidence for the existence of an enzyme which oxidizes tyrosine was obtained by Bertrand and Bhaduri (1929) by examining the oxidation of phenols by laccase. By 1932 Bertrand had established the enzyme as acting directly on the o-dihydric tyrosine, and had demonstrated its action against other substances, including p-hydroxyphenyl ethylamine, p-cresol, and phenol. In all cases the activity of this preparation (which was dependent upon the presence of at least one phenolic hydroxyl group in the substrate molecule) was destroyed by heating. The enzyme was named tyrosinase (Bertrand, 1896), and was also described as polyphenol oxidase (Griffin and Nutt, 1923; Knowlton, 1928), and monophenol oxidase (Dalton and Nelson, 1929).

This class of enzymes is primarily extracted from plant tissues, some of the most active preparations being derived from the polypore Polyporus tuberaster, and the mushrooms Psalliota campestris and Russula nigricans. Knowlton (1928) using the sweet potato as a starting material, and Keilin and Mann (1938) extracting the common field mushroom, were able to obtain a highly purified polyphenol oxidase, and found the oxidase active by both proportional to the copper content of these very pure preparations. In addition, Knowlton (1928) found that

after dialysis a restoration of activity was achieved by the addition of copper to the solution, while several other metallic ions were ineffective. Further evidence that the oxidation of phenols by this enzyme class was dependent upon the presence of copper was furnished by Bernheim and Bernheim (1942). They observed that substances known to form complexes with copper, such as diethyldithiocarbamate and phenylthiourea, when added in concentrations comparable to the concentration of copper in the extract, would inhibit the activity of tyrosinase preparations.

The intermediate products of the reaction between tyrosinase and its substrate tyrosine have been described by several authors, and appear to be well established. (Haehn, 1921; Abderhalden and Sickel, 1923; Raper and Wormall, 1923; Mason, 1948). The scheme presented on page three represents the latest information available (Mason, 1948).

The first step in the oxidation of tyrosine by tyrosinase involves the catalytic introduction of a hydroxyl group ortho to the already existing hydroxyl group in the tyrosine molecule (Reaction one). The compound formed is dihydroxyphenylalanine (contracted as DOPA). In the second step another half mole of oxygen is consumed, and again the enzyme must be present for the

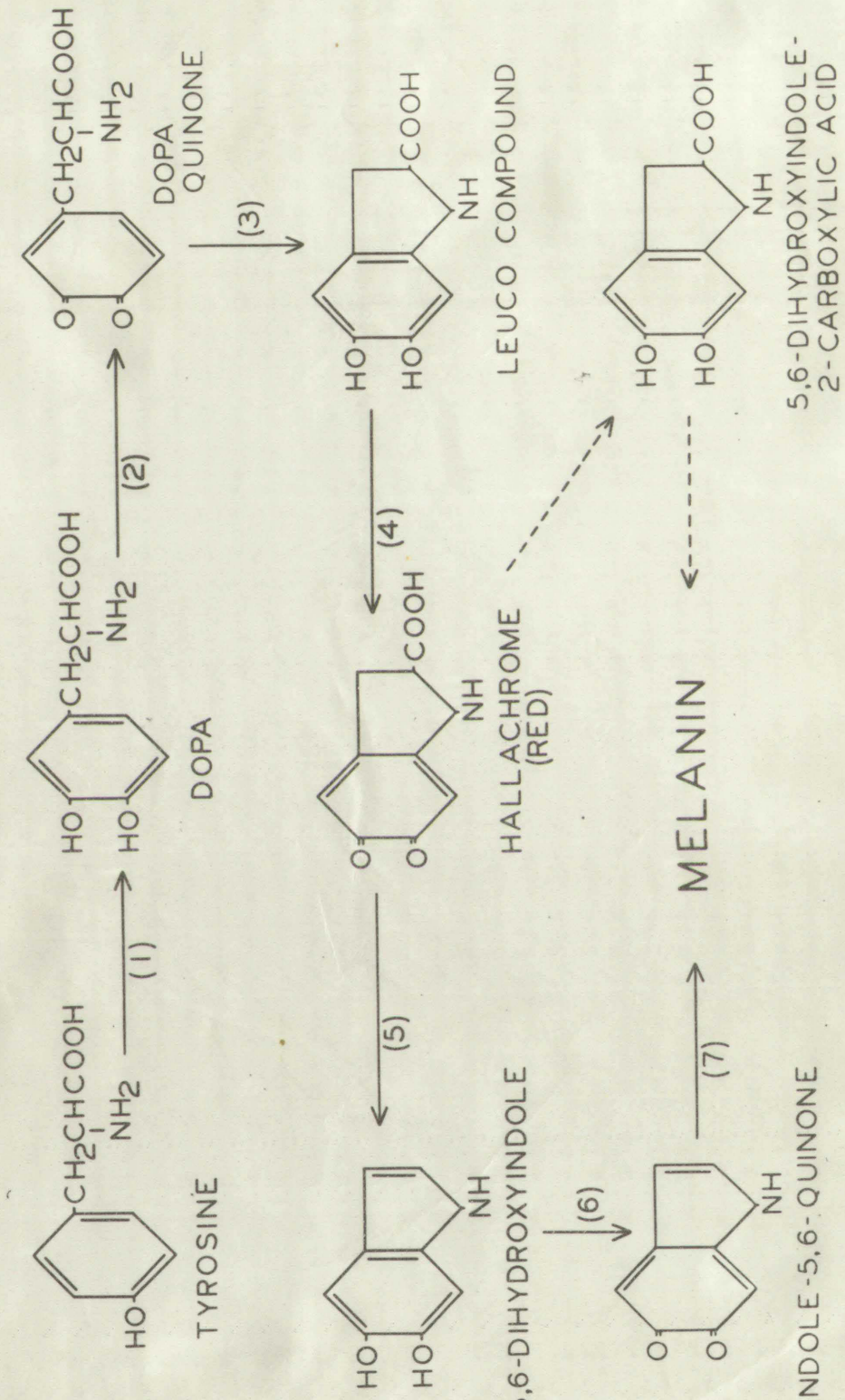

Figure I. The Conversion of Tyrosine to Melanin

conversion of the two ortho hydroxyl groups to the corresponding quinone, "DOPA quinone", (Reaction two).

In mildly alkaline solutions the DOPA quinone formed will undergo an internal condensation, resulting in a transfer of hydrogens from the side chain to the quinones, and linkage through the amide of the side chain to the benzene ring (Reaction three). The resulting indole is colorless, but is easily autoxidized in air at a pH over 7.0. Under these conditions the leuco compound loses two hydrogens, and is converted by reaction four into an indole quinone which is red, and which has been named hallachrome by Raper and Wormall (1923). The conversion from this product to the final product, melanin, is rapid. It involves a decarbonxylation and the consumption of one mole of oxygen per mole of tyrosine. Suggested pathways are shown in reactions five, six, and seven.

The most generally used standard for the definition of enzyme strength is that proposed by Nelson and Dawson (1944). They assumed that the enzyme has two activities, a catecholase and a cresolase one, and on this basis proposed that the activity of the enzyme must be calculated for each type of substrate. Thus one catechol unit is that amount of enzyme which will cause an oxygen uptake

of ten cubic millimeters per minute, when acting upon catechol; the same definition applies to the cresolase unit, if the substrate is changed to p-cresol.

The proposal that there is more than one activity inherent in the tyrosinase molecule has been the subject of considerable debate. Kubowitz (1938) held that the catecholase activity was proportional to the amount of copper present in the preparations. No preparation from plants has been described in which the monophenolase activity or the diphenolase activity was absent, but, depending on the method of preparation of the extract, the ratio of one to the other has varied over a wide range. Mallette (1945) has presented strong evidence that the enzyme is a single molecule in all cases; the various proportionalities and activities are ascribed to artifacts encountered in the extraction and purification of the enzyme.

A consideration of the role played by tyrosinase in the plant kingdom has led to the formulation of an interesting theory by Szent-Györgyi and Vietorisz (1931) that the enzyme acts to form a protective coat over the injured parts of plants. This coat then brings about the oxidation of phenols to quinones, which act bactericidally, and which combine with proteins to form an

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insoluble layer over the site of the injury, thus allowing the open wound to be once more shielded from the air.

While most preparations of tyrosinase in general use have been derived from plants, the presence of this enzyme has been described frequently in the animal kingdom. Gessard (1902) reported the presence of tyrosinase in the ink sac of cuttlefish, Pinhey (1930) in the blood of some crustacea, and Bhagvat and Richter (1938) have reported it as being present in the blood of some arthropods.

Metabolism of the amino acid tyrosine in various vertebrate forms has also been described. Dakin (1910) described normal tyrosine metabolism as proceeding from the amino acid to homogentisic acid. His measurements were made on urinary end products found in mammals, including man. Abderhalden and Behrens (1926) showed that neither homogentisic acid nor tyramine could be an exclusive end product of tyrosine metabolism, although Schuler, Bernhard, and Reindel (1936) have shown that an enzyme exists capable of converting tyrosine to tyramine.

Lerner (1949) showed that tyrosine metabolism in rat liver took place with a decarboxylation (presumably by tyrosine decarboxylase) and secondary amine oxidation

by a non specific amino acid oxidase, and resulted in the destruction of the benzene nucleus, with the formation of two four carbon compounds, which on analysis proved to be a ketone, and malic acid or its precursor. The colored compounds of the type described by Mason (1948) did not appear, and no melanin was formed, so presumably the system encountered here differs from the typical oxidation of tyrosine by tyrosinase, described earlier. (Figure 1).

This tyrosine decarboxylation was also observed by Clegg and Sealock (1949) in guinea pig liver and kidney slices, and appears to have been first described by Schuler et al. in 1936.

The effect of the reactions described above is to indicate that the metabolism of tyrosine can occur in the absence of a tyrosinase. Several investigations have been made, however, in which the course of study, and the results obtained, indicate a successful search for a mammalian tyrosinase.

One field in which this type of attack has been made repeatedly is that of pigment formation. Indeed, the present investigation is the outgrowth of one such attempt (Humm, communication). Onslow in 1917 reported the presence of the enzyme in the skin of colored rabbits, and described a procedure for the isolation and extraction

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of this tyrosinase, although some doubt exists as to the validity of the work (Fitzpatrick et al., 1950). It has been postulated (Arnow, 1937) that the phenomenon of sun tanning of human skin is due to the action of a tyrosinase. The presence of the enzyme in the feather germs of colored chicks has been reported by Charles and Rawles (1940). Fitzpatrick and his co-workers (1950) have described a system in human skin which seems to depend upon the presence of a tyrosinase, and which is concerned with melanin formation. According to this work the enzyme is typical in that it requires the presence of copper for its action, and is therefore inhibited by sulfhydryl reagents. The results substantiate the theory of Arnow (1937), as they show that ultraviolet radiation inhibits the action of sulfhydryl groups normally found in the skin, allowing the formation of melanin by the action of tyrosinase, according to the theory advanced. Fitzpatrick et al. have further described an extraction procedure for the preparation of tyrosinase from the Harding-Passey mouse melanoma.

From these results it seems probable that tyrosinase plays an important role in vertebrates, being necessary for several types of pigment formation. Another series of reactions have been described, however, in which the enzyme may have a different role.

These experiments rest upon the assumption that the physiological production of adrenaline must proceed from a substance having a structure closely allied to tyrosine. Schuler and Wiedemann (1935) observed that slices of the adrenal medulla of the guinea pig were able to produce adrenaline-like substances, when incubated in the presence of solutions of tyramine, while tyrosine had no such effect. In 1936 Schuler, Bernhard, and Reindel were able to show that tyrosine, if incubated with guinea pig kidney slices, could be converted to tyramine, by the action of tyrosine decarboxylase. Thus tyrosine, a normal dietary component, could serve as a precursor for the adrenaline-like substances produced by medullary slices. In 1940 Devine took issue with the claim advanced by Schuler et al. that adrenaline was produced in the kidney and adrenal, from tyrosine and tyramine. Devine objected to the color test employed by the German workers on the basis of low reliability, and in a series of determinations using a colorimetric method of greater specificity, showed that tyramine can be utilized in cattle adrenals to form adrenaline-like compounds, but that phenylethylamine is a more active substrate for these slices.

Devine succeeded in obtaining a small quantity of an adrenaline-like substance from medullary slices

when these were incubated in the presence of phenylalanine. More recently Gurin and Delluva (1947) utilized this amino acid prepared with radioactive carbon to show that the phenylalanine may be converted to adrenaline in biosynthesis.

The experiments outlined above were carried out at a pH near seven, and were not designed to show the presence or absence of any specific enzyme. Their importance to this thesis rests upon the employment by the investigators of adrenal medullary slices. This tissue has been the site of the theoretical production of adrenaline, according to a theory that has often been advanced (i.e. Baldwin, Gortner, Harrow). This theory holds that tyrosine is the normal precursor for adrenaline, and that this synthesis is dependent upon the presence of a tyrosinase located in the adrenal medulla. Thus far the theory remains unproven.

To summarize, an enzyme exists which can convert phenols to quinones, and which has been called tyrosinase. It has been reported in both the plant and animal kingdom, and its mode of action has been clarified. In animals it may be concerned with pigment formation; a theory has been advanced that it also acts in the physiological production of adrenaline.

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It is the purpose of this paper to present indicative evidence that a tyrosinase exists in the adrenal medullas of cattle, and that this enzyme differs significantly from any previously reported tyrosinase. It is probable that the evidence will bear out the contention that tyrosine is the physiological precursor for adrenaline.

Materials and Methods. The data to be presented in this thesis were obtained by making manometric measurements, using the Warburg constant volume differential pressure manometer. The theory of an apparatus of this type is well known (i.e. Dixon, 1943) and is as follows: All quantities of gas evolved in a reaction, which is carried out in a closed system, are recorded as positive pressures, and all quantities of gas absorbed are recorded as negative pressures. Determinations are made by adjusting the volume of the reaction flask so that at the time of reading it is a constant, and a rise or fall in the open limb of the manometer is regarded as a pressure change, a rise in this limb, corresponding to an evolution of gas, is regarded as a positive reading.

In determining measurements of gaseous exchange in a reaction, gas volumes are expressed in cubic millimeters of dry gas at normal temperature and pressure, and pressure readings are made in terms of millimeters of fluid on the manometer tube.

The derivation of the equation by which the pressure reading in the manometer may be converted to gas volumes is as follows:

Let x = the amount of gas evolved in cubic millimeters at normal temperature and pressure,

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Let x = the amount of gas evolved in cubic millimeters at normal temperature and pressure,

gas absorbed will then be expressed as a negative quantity.

h= the reading value of the manometer.

Vg= the volume of the reaction chamber, including all gas space to the surface of the manometer fluid.

Vf= the volume of fluid in the reaction vessel.

T= the absolute temperature at which the reaction is carried out.

P= the initial pressure in the vessel.

Po= the normal pressure (760 millimeters of mercury in millimeters of the manometer fluid, derived thusly: if D is the density of the fluid, then Po= 760 $\frac{13.6}{D}$).

p= the vapor pressure of water at T.

α= the solubility of the gas being evolved or absorbed in the reaction (in cubic millimeters of gas at normal temperature and pressure, dissolved in one cubic milliliter of water at a pressure equal to Po).

Then the initial amount of gas contained in the reaction space is equal to $Vg \frac{273}{T} \frac{(P-p)}{Po}$, and the initial amount of gas dissolved in the reaction fluid is equal to $Vf \alpha \frac{P-p}{Po}$. The final amount of gas in the reaction space

is then equal to $Vg \frac{273}{T} \frac{(P-p \neq h)}{Po}$, and the final amount of gas dissolved is $Vf\alpha \frac{P-p \neq h}{Po}$.

The total amount of gas present can be expressed in terms of the gas initially present plus or minus the amount x, which is evolved or absorbed. Therefore:

$$(Vg \frac{273}{T} \neq Vf\alpha) \frac{(P-p \neq h)}{Po} = (Vg \frac{273}{T} \neq Vf\alpha) \frac{P-p}{Po} \neq x,$$

and one can derive the amount of gas, x, thusly:

$$x = h \left[Vg \frac{273}{T} \neq Vf\alpha \right] Po$$

The expression within the brackets is a constant for any one gas in any one vessel, provided the liquid volume and the temperature remain unchanged. This value is known as the vessel constant K, which is calculated for each vessel and manometer. When K is known, by multiplying it by the change in height h of the manometer will give the amount of gas evolved or absorbed in the reaction, over the time between measurements, expressed in cubic millimeters of dry gas at normal temperature and pressure. The expression $x = hK$ is equally valid if a second gas is present in addition to the reacting gas.

The value of K must be calculated for any gas which is to be measured. Since the two gases most usually

involved in respiratory measurement are oxygen and carbon dioxide, values for these are generally calculated as standard references.

Since one side of the manometer is exposed to the air, the manometer records changes in the barometric pressure as well as changes in pressure within the vessel. A change in the internal pressure due to variation of the temperature in the water bath surrounding the reaction vessel is also measured. A very slight change in the water temperature is sufficient to alter the reading by several millimeters, and the barometric pressure may cause a change of as much as twenty millimeters an hour, depending on the fluid used in the manometer. For this reason it is necessary to utilize a thermobarometer, which consists of a manometer fitted with a vessel containing the amount of fluid in the reaction vessels, but in which no reacting substances are placed. Temperature and pressure variations will affect all manometers alike, therefore by subtracting the change in the thermobarometer reading algebraically from the change in the reading of the other manometers, it is possible to adjust to changes not caused by the reaction being investigated. By using a mercury thermostat, sensitive to 0.05 of a degree Centigrade, it is possible to keep temperature variations to a minimum.

Such a thermostat was employed in this investigation.

Two fluids may be used in the manometer tube. The first, known as Brodie's solution is made up as follows: 23 grams of sodium chloride, five grams of sodium tauroglycholate, and a few drops of an alcoholic solution of thymol are dissolved in 500 cubic centimeters of water. The second (Krebs, 1951) employs a commercial detergent of the alkylarylpolyethoxyethanol type instead of sodium tauroglycholate to lower surface tension, and is more satisfactory. It is made up in the following manner: 44 grams of anhydrous sodium bromide, 0.3 grams of Evans blue, and one gram of the detergent (Tween 80, Triton 20, or Stergene) are diluted with water to a volume of one liter. These solutions are employed to facilitate calculation of the pressure-volume equation. Seven hundred and sixty millimeters of mercury are equal to 10,000 millimeters of these solutions.

The Warburg manometers may be read to within plus or minus one half of a millimeter, and as the average reading is about ten millimeters above or below the previous one, is accurate to within five percent.

The tissue used in these experiments were obtained from the adrenal medulla of cattle. The cattle were stunned by a blow on the head, and then sacrificed immediately according to normal abattoir procedure. The

carcasses were eviscerated, the adrenal gland secured as rapidly as possible, and the gland placed in a M/15 solution of phosphate buffer at pH 7.2 and zero degrees Centigrade. The average elapsed time between the death of the animal and the placing of the tissue in phosphate buffer was about twenty minutes. Usually the right adrenal gland was employed, one gland sufficing to furnish the tissues needed for twelve Warburg manometer vessels.

Upon arrival in the laboratory the adrenal cortex was separated from the medulla, and the latter sliced between two ground glass plates, using a modification of the free hand slicing technique of Deutsch (1936).

The sliced tissues were placed on glass, above ice, and then introduced into the Warburg vessels. The vessels contained solutions dissolved in M/15 phosphate buffer, which was adjusted to pH 9.0 by the use of small quantities of 0.1 M sodium hydroxide. The vessels were placed in the water bath, and depending on the type of experiment, were allowed to adjust to bath temperature, or were first gassed with oxygen or nitrogen, and then allowed to equilibrate.

The measurement of tyrosinase activity by the use of the Warburg manometer is an accepted method. Graubard and Nelson (1935) used this apparatus to measure the

activity of a tyrosinase preparation from plants, and manometric measurements using the Barcroft differential manometer were made by Keilin and Mann (1938) and Gregg and Nelson (1940).

All experiments to be reported were carried out with the temperature of the water bath adjusted to 37 degrees Centigrade. The vessels used had a total volume of about 20 cubic centimeters, and the amount of fluid placed in the vessels was always 3.2 cubic centimeters. The water level of the bath was kept high enough to completely cover the vessels, in order to insure against cooling while the experiment was being conducted.

For each solution placed in the vessels, a separate pipette was employed; generally the vessels were loaded with two cubic centimeters of solution in the reaction vessel proper, one cubic centimeter of solution in the side arm, and two tenths of a cubic centimeter of a five percent potassium hydroxide solution was placed in the center well, when the experiment involved the measurement of oxygen uptake. A piece of frilled filter paper was placed in this alkali solution to increase the surface area of the basic substance, and thus insure complete removal of the carbon dioxide.

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The solutions most generally employed were a 1/30

milligram percent solution of tyrosine, a M/50 solution of phenylthiourea, and a M/15 phosphate buffer at pH 9.0. Since phenylthiourea is relatively insoluble in water (41 milligrams in 100 cubic centimeters of water at 20 degrees Centigrade), it was necessary to heat the solvent slightly to obtain complete solution. Measurements of pH were made after subsequent cooling. Tyrosine is also relatively insoluble (one part per 2,491 parts of water at 20 degrees Centigrade), but it can be converted to its sodium salt, which then dissolves rapidly. This was accomplished by adding eight drops of 0.1 normal sodium hydroxide solution to every 30 milligrams of tyrosine. Solutions were made up in 25 cubic centimeter quantities, and were prepared freshly for each experiment. Only the M/15 phosphate buffer and the five percent potassium hydroxide were maintained as stock solutions.

The Beckmann pH meter was employed in all hydrogen ion measurements. All weighings were made on a Chain-o-matic analytical balance.

The solutions generally stood for about one hour before being placed in the vessels. If a longer period than an hour and a half ensued before utilization, the pH was again checked before use.

After both solutions and tissue slices were loaded

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in the vessels, these were attached to the manometers and placed on the bath. If gas was employed it was allowed to enter through the manometer stopcock and to exit through the side arm stopcock. All gases were allowed to flow through the manometers over a ten minute period, during which the manometers were agitated, to allow complete saturation of the fluid by the gas. After administration of the gas (or prior to placing the vessels in the bath if no gas were employed) the side arm stopcock was closed, and the vessels allowed to equilibrate for fifteen minutes, with the manometers open to the atmosphere. At the end of the equilibration period the manometers were set at a scale reading of 150 millimeters, and were then closed.

Readings were taken at fifteen minute intervals for a period of 45 minutes or one hour. At the end of this period the manometers were removed from the bath, and tilted so that the solution in the side arm was added to the vessel proper. The manometer was replaced in the bath as soon as possible. Readings were taken again at fifteen minute intervals for another 45 minutes to one and one half hours.

At the conclusion of the experiments the manometer stopcocks were opened, and the vessels removed from the

bath. The tissues were removed, dried of surface water by placing them on filter paper, and the wet weight was taken. Oxygen uptake (or carbon dioxide evolution) was them calculated per milligram of wet weight of the tissue slice. The graphs presented in this paper show the oxygen uptake in millimeters per milligram of tissue (wet weight) plotted against the time intervals.

Experimental Data and Discussion. The effects of the addition of solutions of phenylthiourea and tyrosine to surviving slices of the adrenal medulla of range cattle, suspended in solutions buffered at pH 9.0, are summarized in tables I-III, and graphically represented in figures 2-4. For purposes of comparison, table IV and figure five represent the endogenous respiration of similar tissue slices in buffer alone. Oxygen consumption is reported in cumulative readings taken at fifteen minute intervals, and the predump period is always forty-five minutes. In order to present the data as Q_{O2}, predump figures in the tables were multiplied by a factor of four thirds.

Figure two shows the results obtained when a 120 milligram percent solution of tyrosine is added to a slice of medullary tissue which has been incubated in a buffered solution at pH 9.0. The increase in oxygen consumption obtained after this addition is equal to 208 percent of the endogenous respiration. This figure represents the average of twelve determinations on tissues from eight animals.

Figure three shows the stimulated oxygen consumption observed when M/50 phenylthiourea solutions are substituted for the tyrosine used in calculation of figure two. These

Table I:

The effects of adding tyrosine to adrenal medullary slices, which were first incubated in buffer at pH 9.0.

No. of animals: Eight

Time of reading:	Oxygen consumption per milligram of tissue weight:	Range:	No. of Determinations:
15	0.121	0-0.57	12
30	0.406	0.127-1.21	12
45	0.832	0.171-1.7	12
60	1.69	0.55-2.61	12
75	2.19	0.88-3.95	12
90	2.56	1.27-4.65	12
105	2.75	1.3-5.2	10

Predump Q_{O2} 1.108

Postdump Q_{O2} 1.920

Time of reading	Oxygen consumption per milligram of tissue weight	Range	No. of determinations
15	0.121	0-0.37	12
30	0.405	0.127-1.21	12
45	0.743	0.121-1.7	12
60	1.49	0.37-2.61	12
75	2.19	0.38-3.38	12
90	2.96	1.37-4.69	12
105	2.75	1.3-5.2	10

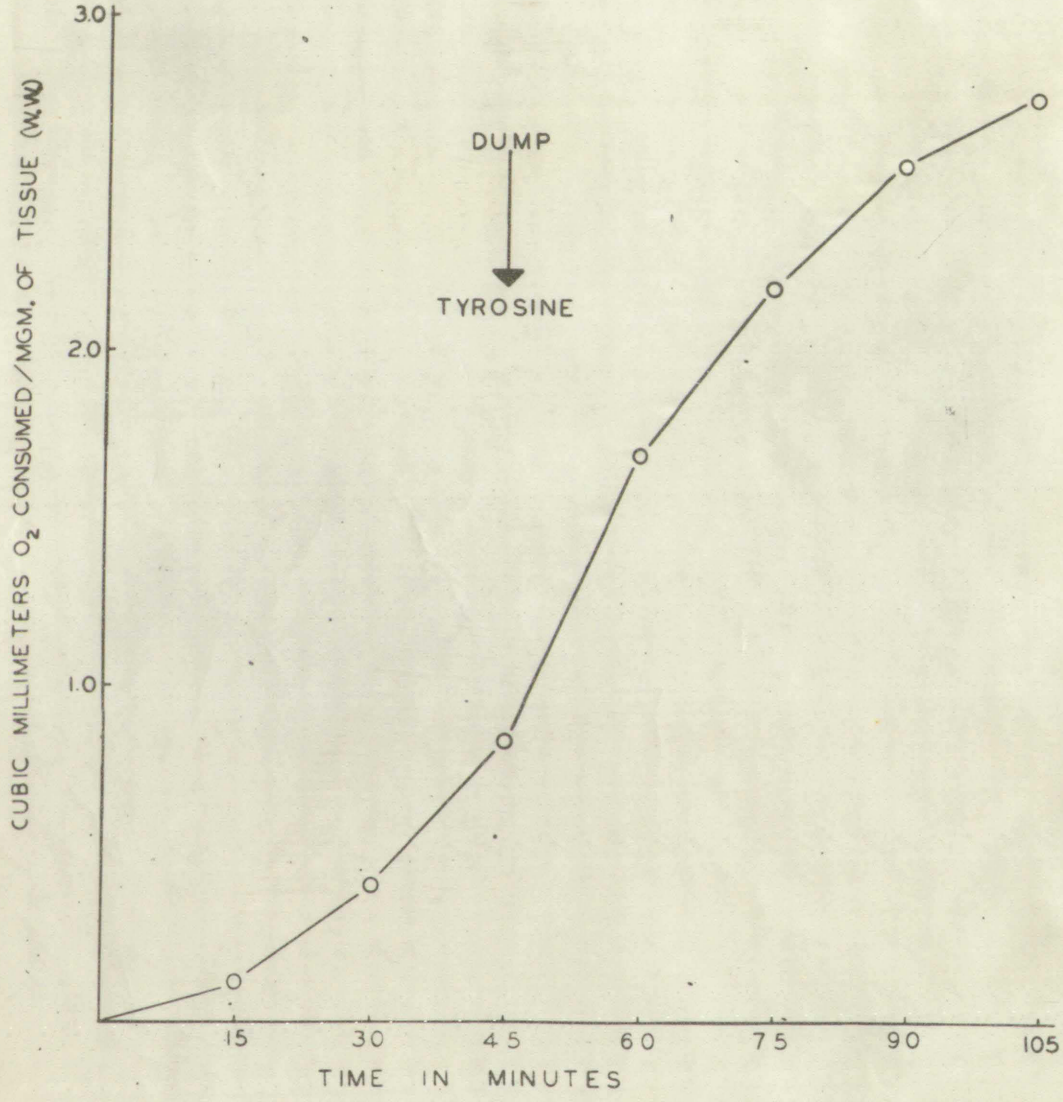

Figure 2. The effects of adding tyrosine to medullary slices; pH 9.0.

results represent the average of eight experiments on tissue from five different animals, and show a stimulated oxygen uptake amounting to 189 percent of the endogenous respiration.

When tyrosine is added to slices of tissue which have been previously incubated in the presence of M/50 phenylthiourea as well as buffer at pH 9.0, the average of thirteen experiments upon tissues from eight animals shows a stimulated oxygen consumption of 400 percent. The reason for this apparently higher respiration becomes clear when one compares the endogenous respiration in the predump period, shown in figure five, to the predump respiration in these experiments, shown in figure four. In the first case the figure at forty-five minutes is 0.77 cubic millimeters of oxygen per milligram of tissue weight, while in the "tyrosine into phenylthiourea" series shown in figure four, this value is 0.46 cubic millimeters of oxygen per milligram of tissue weight.

Upon consideration of these data several different effects may be seen: first, that tyrosine alone will increase the oxygen consumption of adrenal medullary slices incubated in solutions buffered at pH 9.0; second, that phenylthiourea will also result in a stimulation under these conditions; and third, that the stimulation

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Table II:

The effects of adding phenylthiourea to medullary slices respiring in buffer at pH 9.0.

No. of animals: Five

Time of reading:	Oxygen consumed per milligram of tissue in cubic millimeters:	Range:	No. of Vessels:
15	0.03	0-0.09	8
30	0.216	0.064-0.399	8
45	0.641	0.064-1.26	8
60	1.28	0.064-2.05	8
75	1.68	0.064-2.55	8
90	1.85	0.125-2.98	8
105	1.88	0.205-3.05	6

Predump Q_{O2} .856

Postdump Q_{O2} 1.21

Table II:

The effects of adding phenylthiourea to medullary slices respiring in buffer at pH 7.0.

Time of reading	Oxygen consumed per milligram of tissue in cubic millimeters	Range	No. of Vessels
No. of animals: Five			
15	0.03	0-0.09	8
30	0.216	0.064-0.379	8
45	0.643	0.064-1.28	8
60	1.28	0.064-2.05	8
75	1.68	0.064-2.56	8
90	1.85	0.128-2.98	8
105	1.88	0.205-3.09	6

Postdump QO_2 1.21

Predump QO_2 .896

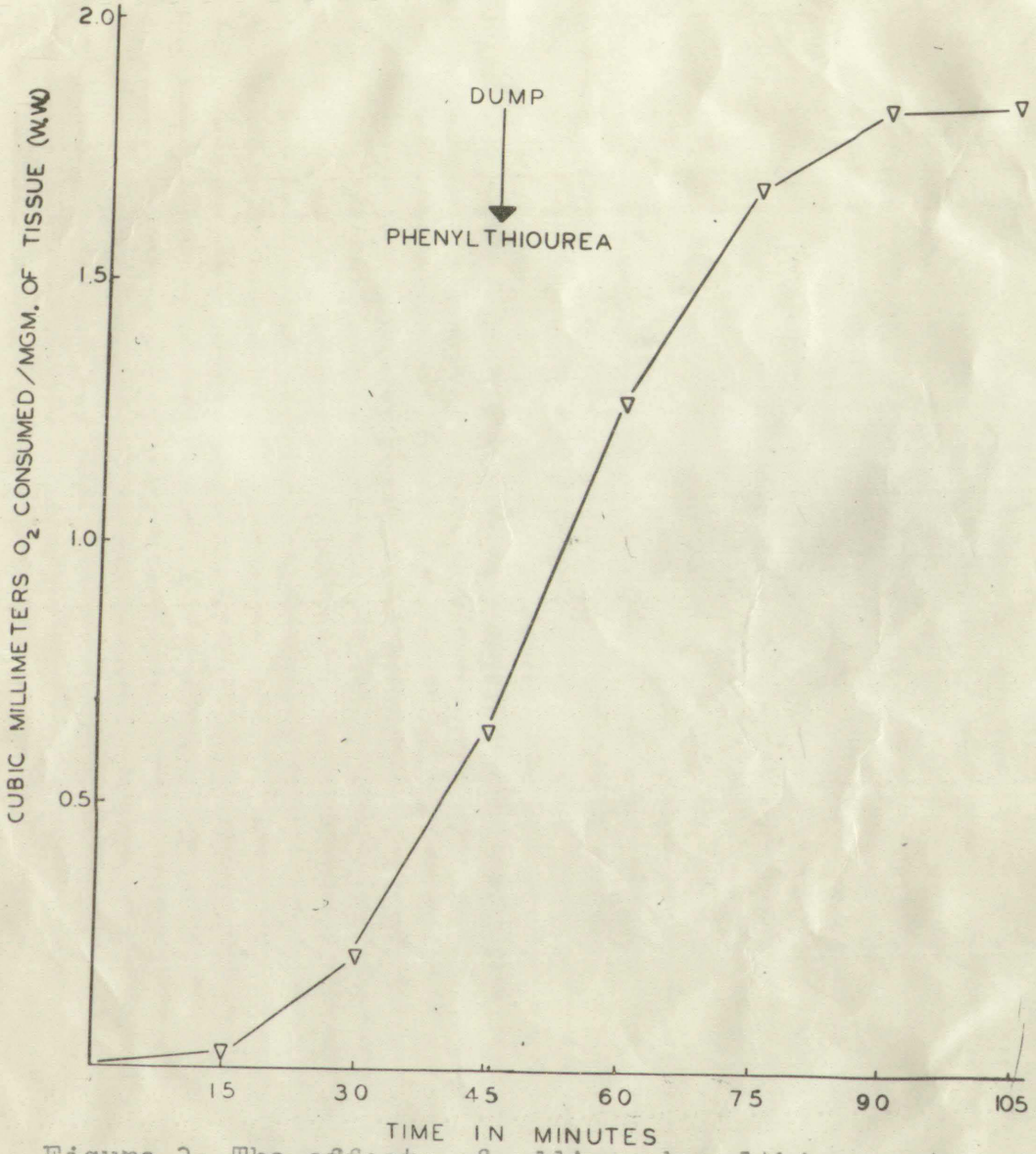

Figure 3. The effects of adding phenylthiourea to medullary slices; pH 9.0.

Table III:

The effects of adding tyrosine to medullary slices respiring in M/50 phenylthiourea and buffer at pH 9.0.

No. of animals: Nine

Time of reading:	Oxygen consumption per milligram of tissue in cubic millimeters:	Range:	No. of Vessels:
15	0.065	0-0.121	13
30	0.233	0.03-0.356	13
45	0.462	0.15-0.82	13
60	1.299	0.651-1.93	13
75	1.96	0.973-3.26	13
90	2.3	1.46-3.95	13
105	2.74	1.60-5.2	9

Predump Q_{O2} .616

Postdump Q_{O2} 2.28

Table III

The effects of adding tyrosine to medullary slices respiring in M/50 phenylthiourea and buffer at pH 9.0.

No. of animals: Nine

Time of reading:	Oxygen consumption per milligram of tissue in cubic millimeters:	Range:	No. of Vessels:
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Preburn QO_2 .616

Postburn QO_2 2.28

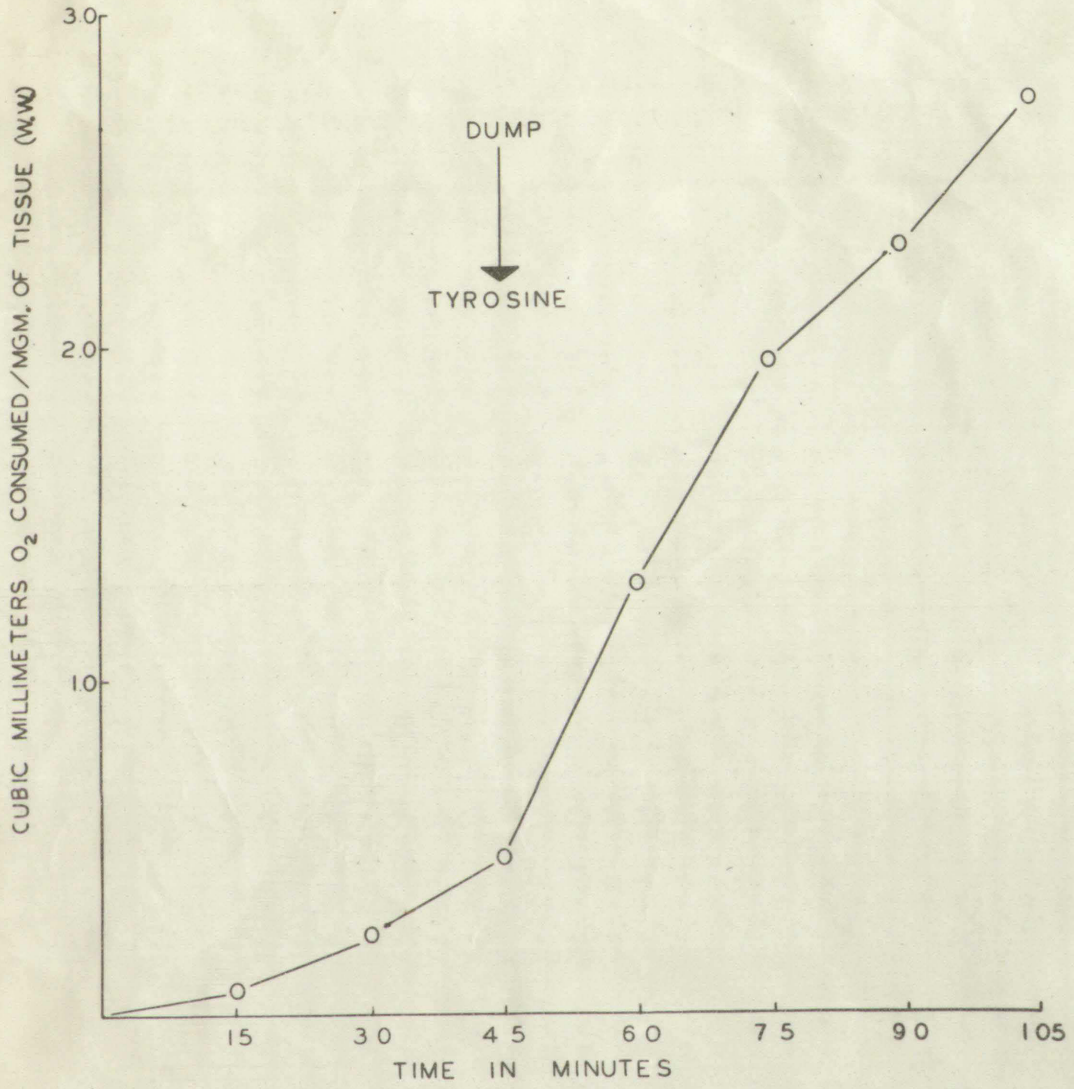

Figure 4. The effects of adding tyrosine to medullary slices in M/50 phenylthiourea; pH 9.0.

Table IV:

The respiration of medullary slices in buffer at pH 9.0.

No. of animals: Sixteen

Time of reading:	Oxygen consumption per milligram of tissue weight in cubic millimeters:	Q_{O2}	Range:	No. of Vessels:
15	0.113	0.452	0-0.57	24
30	0.37	0.74	0.064-1.21	24
45	0.77	1.04	0.064-1.7	24
60	1.21	1.21	0.69-2.49	11
75	1.08	.864	0.86-1.26	4

Table IV:

The respiration of medullary slices in buffer at pH 9.0.

No. of animals: Sixteen

Time of reading:	Oxygen consumption per milligram of tissue weight in cubic millimetres:	Q_{O_2}	Range:	No. of Vessels:
15	0.113	0.452	0-0.57	24
30	0.37	0.74	0.064- 1.21	24
45	0.77	1.04	0.064- 1.7	24
60	1.21	1.21	0.69- 2.49	11
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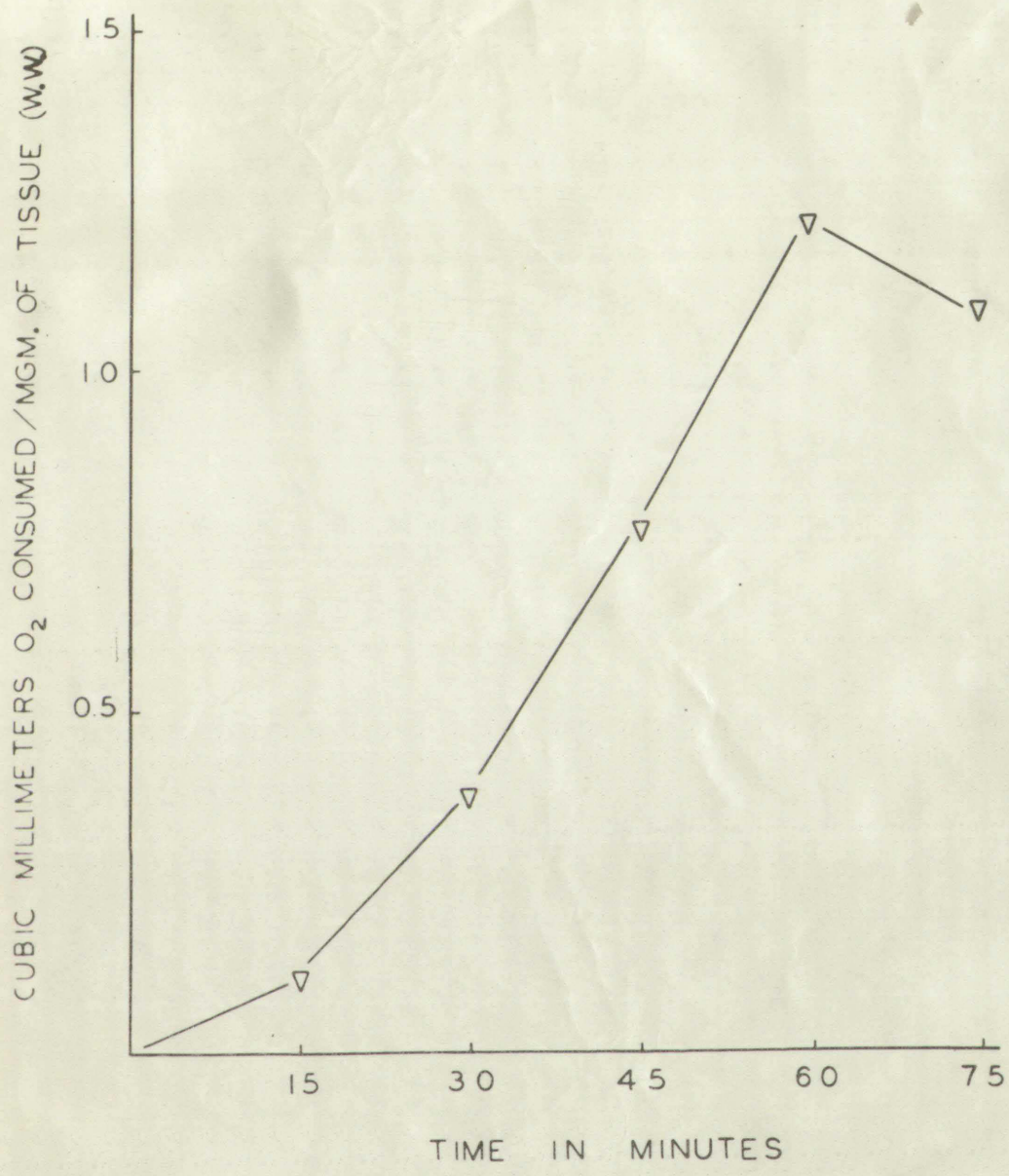

Figure 5. Medullary slices in buffer at pH 9.0.

due to tyrosine is not inhibited by the presence of M/50 solutions of phenylthiourea.

The fact that such solutions of phenylthiourea as those employed in these experiments do not inhibit the mechanism which is responsible for the stimulated oxygen uptake due to tyrosine, is not at all in accordance to results to be expected if a classical tyrosinase were present here. A sulfhydryl reagent, such as phenylthiourea, in the concentration used, should completely inhibit the action of a copper containing enzyme, and all previously reported tyrosinases have been copper containing. If the mechanism under investigation concerns the oxidation of tyrosine by tyrosinase, this enzyme must be significantly different from any tyrosinase heretofore observed.

It is well, therefore, to consider the possibility that the oxygen uptake under observation here is not due to a tyrosinase. Another mechanism has been observed by Schuler et al. (1936) and by Clegg and Sealock (1949), which could account for the results obtained. If a tyrosinase does not exist in this gland, the same data could be the result of the action of tyrosine decarboxylase, and this possibility must be explored. If this tyrosine decarboxylase were present, the addition of tyrosine to

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medullary slices would result in a vigorous evolution of carbon dioxide, due to the decarboxylation of tyrosine by the decarboxylase. This reaction would result in the formation of tyramine, and a subsequent oxygen uptake due to the amine oxidation of tyramine by amino acid oxidase. This effect was observed by Schuler and his co-workers in guinea pig kidney slices, and by Clegg and Sealock in both guinea pig kidney and liver slices. Experimental data presented by Kline (1950) showed that at the pH used in these experiments, the kidney tissue of cattle did not show any evidence of this type of reaction. The data presented in table V and figure six show the results obtained when experiments designed to test the evolution of carbon dioxide were carried out. The addition of tyrosine to medullary slices in buffered solutions at pH 9.0, under an atmosphere of nitrogen, shows no evidence of an increase in carbon dioxide consumption, according to the data in figure six. We may thus say that at least the first step in the system under investigation does not involve the decarboxylation of tyrosine to tyramine, by the action of tyrosine decarboxylase. It may be said, therefore, that the oxygen consumption of adrenal medullary tissue slices, at pH 9.0, is capable of being stimulated by the addition of solutions of tyrosine, that this stimulation

Table V:

The effects of adding tyrosine to medullary slices incubated in buffer at pH 9.0, under an atmosphere of nitrogen:

No. of animals: Three

Time of reading:	Cubic millimeters of carbon dioxide evolved per milligram of tissue weight:	Range:	No. of Vessels:
15	0.091	0-0.18	4
30	0.106	0-0.24	4
45	0.107	0-0.25	4
60	0.118	0-0.25	4
75	0.09	0-0.25	4
90	0.03	0-0.12	4
105	0.22	0-0.62	3

Table V:

The effects of adding tyrosine to medullary slices incubated in buffer at pH 7.0, under an atmosphere of nitrogen:

No. of animals: Three

Time of reading:	Cubic millimeters of carbon dioxide evolved per milligram of tissue weight:	Range:	No. of Vessels:
15	0.091	0-0.18	4
30	0.106	0-0.24	4
45	0.107	0-0.25	4
60	0.118	0-0.25	4
75	0.09	0-0.25	4
90	0.03	0-0.12	4
105	0.22	0-0.62	3

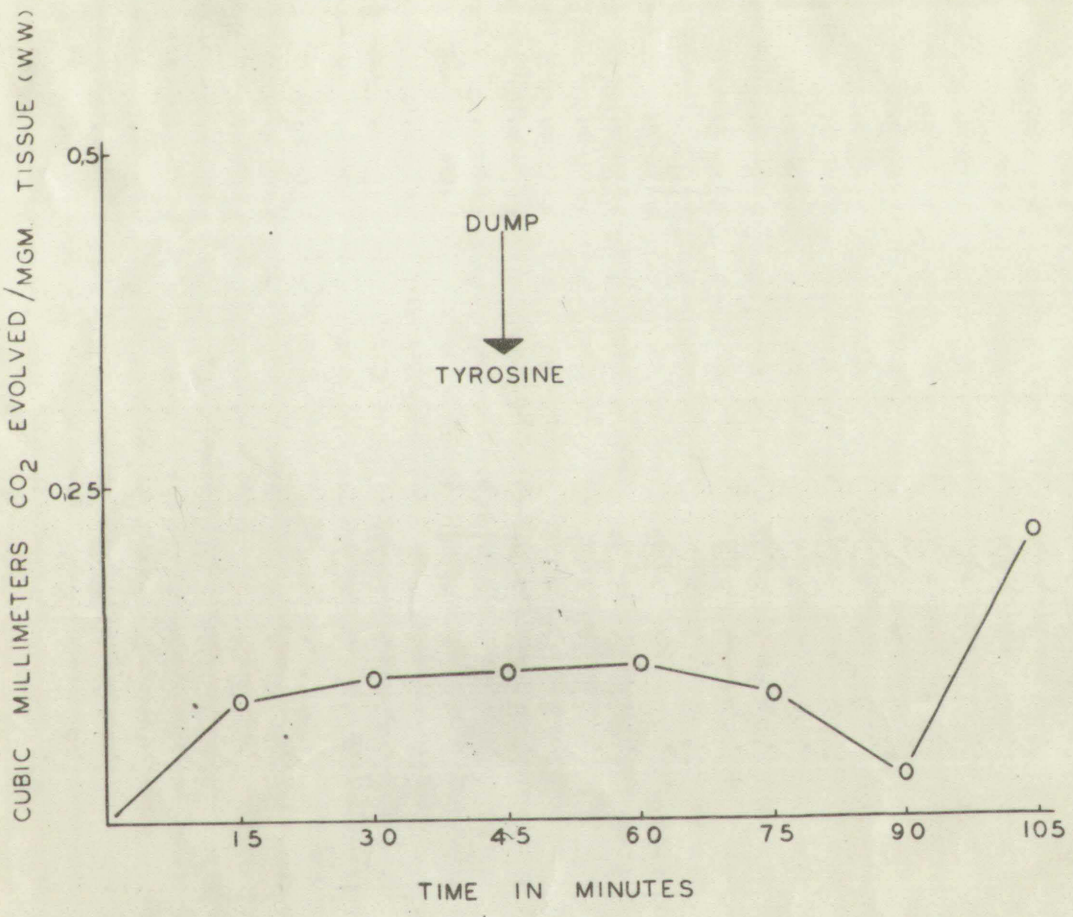

Figure 6. The effects of adding tyrosine to medullary slices in a nitrogen atmosphere; pH 9.0.

is not primarily due to the action of a decarboxylase, and that it is not inhibited by the action of phenyl-thiourea. It is possible to account for the increased consumption of oxygen by assuming the presence of a tyrosinase. If present, this enzyme must differ from any previously described tyrosinase in that it does not contain copper, as it is able to function in the presence of a sulfhydryl reagent, which has been shown to form inactive complexes with copper.

Additional evidence may be adduced to support the possibility that a tyrosinase is present in the adrenal medullary tissue of range cattle. Raper and Wormall (1925) describe the reaction between tyrosinase and tyrosine as giving rise to color in a definite manner. At pH eight the reaction produces a red color, and this color slowly changes to black. A color change of this type has been reported in all cases of tyrosinase action upon tyrosine. This color production is typical of the tyrosine-melanin conversion reported earlier (figure one). The product which produces the red color is called hallachrome, melanin is responsible for the black. In the production of adrenaline-like substances, products have been isolated which closely resemble hallachrome in their chemical structure; these are called adrenachromes.

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They have a pink-red color, and resemble hallachrome so closely that they have been postulated in the conversion of tyrosine to adrenaline.

In all cases, in the experiments reported herein, when tyrosine was added to medullary slices under aerobic conditions, a color change from colorless to pink, and then to a brown black was observed. This color change may have been due to the autoxidation of DOPA, but the production of DOPA depends upon the oxidation of tyrosine. Respiration in buffer at the same pH, without added substrate, was never observed to yield this color, and respiration under anaerobic conditions did not allow color development.

This evidence is no more than suggestive, yet when it is added to the observations of oxygen consumption, strongly suggests the possibility that a tyrosinase is concerned with this reaction. It may be, if this is the case, that the enzyme could be concerned with the production of adrenaline, from tyrosine. If the enzyme tyrosinase is present, it is the first reported tyrosinase capable of functioning in the presence of phenylthiourea, and accordingly does not require the presence of copper, as the reagent is known to form inactive complexes with copper. Further, this tyrosinase is unusual in that it

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The above data indicate that the phenomena reported by Kline (1950) and Watland (1950) are concerned with the presence of the tyrosinase being reported here. Indeed, all data for pH optima were taken from the paper by Watland (1950). Several conjectures may be made concerning a tentative description of a system in which this tyrosinase could be employed in the production of a physiologically active hormone, adrenaline.

According to all reports, the production of this hormone is limited to times of emergency, and is not produced in an organism not confronted with a situation which places a stress upon it. Some mechanism must then be present which, first, prevents the formation of all but extremely small amounts of adrenaline under normal circumstances, and second, acts as a trigger to allow the production of the hormone in times of stress. The following explanation may be the proper one, in any case, it agrees with all observed data.

Normally in the adrenal cortex, a large amount of ascorbic acid is present. In a fright reaction this vitamin immediately disappears from the gland, and is not again found here until the period of stress is past

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(Bacon, communication). It is well known that this acid can act as a hydrogen donor or acceptor, and it could quite conceivably act in the adrenal medulla to reduce any DOPA quinone formed from the oxidation of tyrosine by tyrosinase. In this case the equilibrium would be shifted away from the production of DOPA, and no reaction products past the quinone could be observed. In fright, however, the acid disappears, and the reaction would be free to proceed. The disappearance of the acid from the cortex may be due to the possibility that the oxidized form, dehydro ascorbic acid, is acting in the medulla as an emergency hydrogen acceptor, allowing that tissue to contract an oxygen debt in times of stress. It is possible that this explanation serves to clarify the effect of phenylthiourea in the experiments described herein. Phenylthiourea could act to tie up the ascorbic acid as an ascorbigen, and when this occurred a reaction between the residual tyrosine in the gland, and tyrosinase could proceed, and thus result in the stimulated oxygen consumption observed upon the addition of phenylthiourea. It is known that ascorbic acid appears naturally as an ascorbigen, in combination with the compound glutathione. This latter substance derives its activity from the presence of sulfhydryl groups within its molecule; it seems logical to assume that phenylthiourea, a sulfhydryl reagent could take its place.

Normally, however, one does not think of a physiological system that takes place within the cell, as varying greatly from pH 7.2-7.4. The fact that tyrosinase, as described above, would not function measurably below pH eight would have to be accounted for. I am indebted to Mr. Dean C. Watland for the following explanation, which serves to explain the pH optima observed. If the adrenal medulla is treated as a postganglionic fibre of the sympathetic nervous system, it would be cholinergic, that is, it would produce acetyl choline, upon stimulation. This compound would be destroyed by the action of the enzyme cholinesterase, and would be broken into acetic acid and choline. The former could be metabolized through the so-called seven carbon cycle of Krebs, and the choline would remain temporarily in the gland. Since choline is a quartenary ammonium base, this would serve to raise the pH locally.

The explanation may be extended further. One of the steps in the production of adrenaline appears to be a methylation. Choline does serve as a fine methylating agent. Then upon the production of adrenaline, choline would disappear, and the pH would drop, forcing the mechanism into quiescence, unless the stimulus were to be repeated, in which case more adrenaline could be produced.

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Summary and Conclusions. The effect of adding tyrosine solutions to slices of adrenal medullary tissue incubated in solutions buffered at pH 9.0 was investigated. The tyrosine addition was made in the presence and in the absence of phenylthiourea solutions, phenylthiourea being employed as an inhibitor of copper containing enzymes. Both tyrosine and phenylthiourea were found to cause a stimulation on the oxygen uptake of medullary tissue, under the conditions employed. No evolution of carbon dioxide due to the addition of tyrosine was observed, in tissues incubated under an atmosphere of nitrogen. A color production in cases of tyrosine stimulation was observed, this phenomenon did not occur in normal respiration. The data are discussed in the light of previous experiments, with the view toward explaining them in terms of a mammalian tyrosinase concerned with the production of adrenaline.

On the basis of the data it is concluded:

1. That the stimulated oxygen consumption observed is not due to the presence of a decarboxylase in medullary slices.
2. That a tyrosinase is definitely indicated as being present in the adrenal medulla of range cattle.
3. That this tyrosinase differs from any previously described, in that sulfhydryl

Summary and Conclusions. The effect of adding tyrosine solutions to slices of adrenal medullary tissue incubated in solutions buffered at pH 7.0 was investigated. The tyrosine addition was made in the presence and in the absence of phenylthiourea solutions, phenylthiourea being employed as an inhibitor of copper containing enzymes. Both tyrosine and phenylthiourea were found to cause a stimulation on the oxygen uptake of medullary tissue, under the conditions employed. No evolution of carbon dioxide due to the addition of tyrosine was observed, in tissues incubated under an atmosphere of nitrogen. A color production in cases of tyrosine stimulation was observed, this phenomenon did not occur in normal respiration. The data are discussed in the light of previous experiments, with the view toward explaining them in terms of a mammalian tyrosinase concerned with the production of adrenaline.

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3. That this tyrosinase differs from any previously described, in that sulfhydryl

reagents do not inhibit its action.

4. That the system discussed is probably concerned with the physiological production of adrenaline.

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