In contrast to the thoroughness with which the respiratory enzymes of such tissues as heart muscle and liver have been studied, information concerning these enzymes in the adrenal gland is scant. Such information as is available suggests that an unusual pattern for the cytochromes exists in this tissue. Cohen and Elvehjem (2) commented on the "peculiar cytochrome spectrum" of the adrenal medulla of the cow. They concluded that a very strong cytochrome c component was present, and that cytochrome a and b were absent. Huszák (3) noted that the adrenal cortex possessed a nearly normal cytochrome pattern. In the medulla he could observe no cytochrome a or c bands, but reported a strong band in the region of 559 mu which he attributed to cytochrome b. Since Huszák could demonstrate no cytochrome oxidase activity in the medulla, he felt that oxidation of substrates proceeded by way of a peroxidative mechanism. Tsou (4), on the other hand, was able to demonstrate cytochrome a and b were absent. Huszák (3) noted that the adrenal cortex possessed a nearly normal cytochrome pattern. In the medulla he could observe no cytochrome a or c bands, but reported a strong band in the region of 559 mu which he attributed to cytochrome b. Since Huszák could demonstrate no cytochrome oxidase activity in the medulla, he felt that oxidation of substrates proceeded by way of a peroxidative mechanism. Tsou (4), on the other hand, was able to demonstrate cytochrome oxidase activity in the medulla, but little if any cytochrome c. He observed the presence of a strong band at 561 mu which was referred to as cytochrome b.

In view of these divergent conclusions and in the light of newer methods and knowledge, it seemed worthwhile to reinvestigate some of the oxidative pathways in the adrenal gland. The present study deals with the adrenal medulla, and an accompanying paper deals with the cortex (5). It will be shown here that the adrenal medulla contains the regular mitochondrial cytochrome system. In addition, this tissue contains a hemochromogen which is apparently microsomal in origin and is abundantly present in the epinephrine-containing granules. The presence in the medulla of the enzymes succinate dehydrogenase, reduced di- and triphosphopyridine nucleotide-cytochrome c reductase, and transhydrogenase is also demonstrated.

EXPERIMENTAL PROCEDURE

Beef adrenal glands were obtained at a local slaughter house and brought on ice to the laboratory. The medullas were dissected from the cortical tissue and all subsequent procedures carried out with ice-cold solutions or in cold rooms at temperatures of +5 to -5°. The medullary tissue was homogenized with 0.32 M sucrose in a glass homogenizer of the Potter-Elvehjem type to yield a 20% tissue suspension. This homogenate was subjected to centrifugation to yield various fractions. First the nuclei and cellular debris were obtained by sedimentation at 800 × g for 15 minutes. The supernatant fluid so obtained was centrifuged at 15,000 × g for 20 minutes in a Servall SS-1 centrifuge to yield a precipitate and supernatant fluid B. The precipitate was washed once in cold 0.32 M sucrose and the centrifugation procedure repeated. The sediment obtained has been termed the "large granule fraction." It contains both mitochondria and epinephrine granules (6). The supernatant fluid, C, was combined with supernatant fluid B and the mixture subjected to centrifugation at 26,000 × g for 3 hours to yield a sediment which has been termed "microsomes" and a final supernatant fluid.

In some experiments the large granule fraction was fractionated further by the use of a sucrose density gradient technique in a manner similar to that employed by Blaschko, Hagen, and Hagen (6). A 15-ml Lusteroid centrifuge tube, 2 ml each of 1.5, 1.35, 1.2, 1.05, and 0.9 M sucrose were carefully layered in the order cited. A 1-ml layer of the large granule fraction suspended in 0.32 M sucrose was then placed on top of these and the tubes centrifuged at 26,000 × g for 1 hour in the Servall centrifuge. Tissue fractions formed between the 1.05 and 1.2, 1.2 and 1.35, and 1.35 and 1.5 M sucrose layers, and a pellet was obtained at the bottom of the tube. Because a clean separation of fractions was not achieved by this technique, the experiments to be reported here were performed on an upper layer and lower layer obtained by using the middle of the 1.35 M sucrose layer as the dividing point.

Absorption spectra were measured at room temperature in a Beckman model DU spectrophotometer which had been calibrated at several wave lengths by means of a mercury lamp. Cuvettes with a 1-cm light path were employed throughout. Measurements under anaerobic conditions were performed in cuvettes attached to a Thunberg-type tube in the manner previously described (7). The cuvettes were evacuated and flushed with nitrogen repeatedly to insure complete removal of oxygen. In some cases, turbid suspensions of the particles in 0.05 M phosphate buffer, pH 7.4, were employed. When this method was used, the instrument was balanced against a control cuvette containing a like amount of turbid suspension. Hence, the spectra observed with this method were "difference spectra" and represented changes in the experimental sample as compared to an untreated sample. In most cases, spectra were obtained on fractions which had been solubilized by treatment with 2% solutions of deoxycholate in 0.05 M glycylglycine buffer, pH 7.4. Centrifugation of such preparations for 1 hour at 26,000 × g yielded clear colored supernatants and a small, relatively color-
Distribution of nitrogen and catechol amines in medullary fractions

<table>
<thead>
<tr>
<th>Type</th>
<th>Nitrogen</th>
<th>Catechol amines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original homogenate</td>
<td>19.7</td>
<td>11.2</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>6.5</td>
<td>0.91</td>
</tr>
<tr>
<td>Large granules</td>
<td>4.4</td>
<td>5.28</td>
</tr>
<tr>
<td>Microsomes</td>
<td>1.0</td>
<td>0.52</td>
</tr>
<tr>
<td>Final supernatant</td>
<td>5.7</td>
<td>3.92</td>
</tr>
</tbody>
</table>

lese pellet. When this method was employed, it was found necessary to subject all particulate fractions to a preliminary wash before the deoxycholate treatment by homogenizing the fractions with either distilled water or 0.85% NaCl solution and centrifuging for 1 hour at 26,000 x g. This treatment served to remove the adrenaline from the particles without noticeably altering their cytochrome content. If adrenaline was not so removed, it was rapidly oxidized to colored products in the deoxycholate homogenates which obscured the measurement of the cytochromes.

The various tissue fractions were treated with trichloroacetic acid in an amount sufficient to yield a 7% final concentration. The suspension was centrifuged and the supernatant fluid used for the assay of the total catechol amines by the method of von Euler and Hamberg (8) at pH 6.0. The precipitate was employed for determination of total nitrogen. Digestion was performed by a micro-Kjeldahl procedure (9) and the ammonia formed was distilled and determined by nesslerization.

Transhydrogenase assays were carried out essentially by the method of Kaplan et al. (10), measuring the changes in absorption at 340 μm during the period 1 to 7 minutes after initiation of the reaction. Each cuvette contained, in a total volume of 3 ml, an aliquot of the tissue fraction, 200 μmoles of Tris buffer, pH 7.5, 10 μmoles of KCN, and 200 μmoles of nicotinamide. Also added, depending on the direction of the reaction under measurement, were either (a) 0.25 μmole of TPN, 10 μmoles of MgCl₂, 10 μmoles of glucose 6-phosphate, and enough glucose 6-phosphate dehydrogenase to reduce the TPN in 3 to 4 minutes, followed by 1.0 μmole of DPN; or (b) 0.25 μmole of DPN, 0.05 μmole of TPNH- and TPNH-cytochrome c reductase activities were assayed at 25º by measuring the change in absorbancy at 550 μm. Each cuvette contained, in a total volume of 3 ml, an aliquot of the tissue fraction, 100 μmoles of phosphate buffer, pH 7.4, 1 μmole of KCN, 0.09 μmole of cytochrome c in the oxidized form, and either 0.4 μmole of DPNH or 0.4 μmole of TPNH. A control cuvette containing all components except DPNH or TPNH was run simultaneously.

Sucinate oxidase activity was determined by measuring manometrically the oxygen consumed by the various fractions in a total volume of 3 ml, containing 100 μmoles of phosphate buffer, pH 7.4, 0.03 μmole of cytochrome c, and 100 μmoles of succinate. Because of the large adrenaline content of the medullary fractions, the oxygen consumed by the control flasks containing no succinate was quite large. Since the oxidation of epinephrine was minimal in the absence of cytochrome c, it was felt desirable to use a method not requiring cytochrome c. For this reason, the succinic dehydrogenase activity of the fractions was determined in some of the later experiments. This was measured as the amount of oxygen consumed in the presence of 100 μmoles of phosphate buffer, pH 7.4, 100 μmoles of succinate, 3.2 mg of brilliant cresyl blue, and 3 μmoles of KCN in a total volume of 3 ml (11). The oxygen consumption of control flasks containing no substrate was negligible with this assay. Both succinic oxidase and succinic dehydrogenase assays were run at 37º.

When attempts were made to measure cytochrome oxidase activity, by the manometric procedure with p-phenylenediamine as substrate, a large oxygen uptake was obtained. This activity was not diminished by boiling the tissue fraction, and was only slightly inhibited by cyanide. Since it seemed likely that the large epinephrine content of the medullary fractions might in part be responsible for this oxygen uptake, epinephrine and p-phenylenediamine were incubated singly and together in the absence of tissue. Each substrate alone consumed a small amount of oxygen, but when both were present together, a large uptake occurred. Cytochrome oxidase activity in the medulla was therefore investigated by measuring the decrease in absorption of reduced cytochrome c at 550 μm in the presence of an aliquot of the tissue in 3 ml containing 100 μmoles of phosphate buffer, pH 7.4, and 0.06 μmole of reduced cytochrome c.

The amount of cytochrome c present in the medulla was determined on the whole tissue by the method of Rosenthal and Drabkin (12). Recovery assays were run by adding known amounts of cytochrome c to samples of the medulla. At the same time, beef heart muscle was analyzed for its content of cytochrome c, and recovery studies were made on this tissue as well.

The pyridine nucleotides employed were the purest preparations obtainable from Sigma Chemical Company or Pabst Laboratories. Cytochrome c was a sample kindly supplied by Eli Lilly and Company. The deoxycholate was purified as described previously (7).

RESULTS

The distribution of protein nitrogen and catechol amines in the various fractions obtained by differential centrifugation is shown in Table 1.

Absorption Spectra of Large Granule Fraction—The absorption spectra of a 2% deoxycholate extract of the large granules showed an absorption maximum at 550 μm and a Soret band at 422 μm (Fig. 1). The 559-μm band was not diminished on shaking in air, but disappeared on addition of ferricyanide. When cytochrome was added to this preparation under anaerobic conditions, the absorption at 559 μm was greatly intensified, and in addition, there was an indication of a small peak at 605 μm. A secondary band was visible at 527 μm, and the Soret band shifted to 427 μm. The addition of dithionite to this preparation further intensified the absorption and shifted the peaks to 560, 530, and 428 μm. When a spectrum was plotted for the difference in absorption obtained with dithionite and cytochrome, maxima at 430, 532, and 563 μm were found. These results suggest the presence in the large granule fraction of cytochromes b and a + a₁ as well as a large amount of a cytochrome absorbing at 559
Ascorbate and epinephrine produced the same picture as did cysteine, reducing the 559 μm cytochrome and cytochromes a + a₃, but not cytochrome b. DPNH and TPNH readily reduced all of the cytochromes to approximately the same extent as dithionite, but succinate was ineffective as a reducing agent in the presence of deoxycholate.

When turbid suspensions of the large granule fraction were investigated in the Beckman instead of deoxycholate-clarified preparations, all of the cytochrome components appeared to be reduced in 15 minutes by DPNH, TPNH, glutamate, malate, and succinate (Fig. 2). No reduction occurred in the absence of substrate. It should be pointed out that the 559 rmp peak seen in untreated deoxycholate-solubilized preparations would presumably be present in both the control and experimental cuvettes when turbid preparations were studied and would therefore not be detected in the difference spectra being discussed here. When both succinate and antimycin A were added to these turbid preparations, under aerobic conditions, only cytochrome b was reduced (Fig. 2), and this proved a useful tool for studying the presence of cytochrome b in the various medullary fractions. On the other hand, DPNH in the presence of antimycin A reduced not only cytochrome b, but also the 559 μm cytochrome suggesting the existence of a pathway from DPNH to the 559 μm cytochrome which is not sensitive to antimycin A.

Sucrose Density Gradient Fractionation of Large Granules—
When the large granule fraction was subdivided by sucrose density gradient centrifugation, the catechol amines were concentrated in the lower layer (Table II). The absorption spectra of the two layers as measured in buffered 2% deoxycholate extracts are shown in Fig. 3. In order to obtain an approximation of the content of the various cytochrome components in these two layers, as well as in the original large granule fraction, base lines were drawn to correct for the nonspecific absorption of the preparations, as shown in Fig. 3. Both upper and lower layers showed some absorption at 559 rmp on reduction with cysteine. However, when the density above the base line at 559 μm was expressed per milligram of nitrogen in the fraction (Table II), it was found that the layer richest in catechol amines contained a larger concentration of the 559 μm cytochrome than did the upper layer. When the amount of cytochrome b present in each fraction was estimated from the difference in reduction at 563 μm obtained with dithionite and cysteine, expressed per milligram of nitrogen in the fraction, the upper layer was found to have the larger concentration of cytochrome b (Table II). Similarly, this layer contained larger amounts of cytochromes a plus a₃ (Fig. 3). The greater abundance of cytochrome a + a₃ and b in the upper layer suggest that mitochondria are concentrated in this layer. Thus, the large granule fraction is apparently composed of two types of particles, granules containing catechol amines and those containing mitochondria. Although a variety of differential centrifugation techniques were tried, none of those used was capable of achieving a complete separation of the two types of particles.

### Table II

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Experiment No.</th>
<th>Large granules</th>
<th>Upper layer</th>
<th>Lower layer</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol amines (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>0.39</td>
<td>1.98</td>
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<td>0.89</td>
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<td>0.31</td>
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<td>0.51</td>
<td>1.94</td>
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<tr>
<td>Absorbancy at 559 μm (cysteine)</td>
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<td></td>
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<tr>
<td>3</td>
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<tr>
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<td>0.034</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome b (absorbancy at 563 μm in difference spectrum)</td>
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<td>9†</td>
<td>0.032</td>
<td>0.010</td>
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</table>

* Turbid suspension reduced with cysteine.
† Turbid suspension reduced with succinate in presence of antimycin A (aerobic).
Cytochrome c Determination—Because of the large absorption given by the 559 μm cytochrome on reduction with cysteine, it was difficult to determine whether cytochrome c was present in the medullary particles. In the direct extraction procedure for cytochrome c on whole medullary tissue, it was possible to demonstrate the presence of an absorption spectrum typical of cytochrome c with maxima at 520 and 550 μm on reduction with dithionite. With a micromolar extinction coefficient of 28.1 for the reduced form at 550 μm, the following values were obtained on three separate determinations: 10 × 10⁻⁴, 17 × 10⁻⁴, and 5.6 × 10⁻⁴ μmoles of cytochrome c per g of wet tissue. These values may be compared with concentrations of 161 × 10⁻⁴ and 200 × 10⁻⁴ μmoles per g of beef heart determined at the same time. The average recovery of added cytochrome c (see "Experimental Procedure") was 80% for medullary tissue, and 95% for heart muscle, and the values cited above were corrected for these recoveries.

Absorption Spectra of Microsomes—The absorption spectra of deoxycholate-solubilized microsomes are shown in Fig. 4. The untreated preparation again had some absorption at 559 μm, 527 μm and 420 μm. Cysteine and dithionite reduction intensified the absorption at 559 μm and 527 μm and shifted the Soret-band to 427 μm. There was little evidence for the presence of cytochrome b as determined by the difference between the amount of reduction obtained with cysteine and dithionite. There is a suggestion of the presence of a trace of cytochrome a + a₁ as judged by the absorption at 605 μm. The main cytochrome component of the microsomal fraction appears to be the 559 μm cytochrome.

Relative Concentrations of Cytochromes—In order to obtain an approximation of the relative amounts of each of the cytochromes present in the large granule fraction, the absorbancy above the base line was measured at 559 μm on reduction with cysteine, at 563 μm in the dithionite less cysteine difference spectrum, and at 605 μm on reduction with cysteine. The averages of these absorbancies were compared with the absorbancy of cytochrome c at 550 μm in the whole tissue extract. The ratios of the densities were cytochrome c to cytochromes a + a₁ to cytochrome b to cytochrome at 559 μm, 1:1.4:5.5:9.7.

Hemochromogen Formation—In the course of reducing a deoxycholate preparation of medullary particles with a reaction mixture containing, among other things, 0.07 m nicotinamide, a strong intensification of the absorption and a shift of the absorption maximum to 557 μm was noted. Since the customary procedures for forming the pyridine hemochromogen use a strongly alkaline solution, the possibility of forming such hemochromogens under the milder conditions of a pH 7.4 deoxycholate-glycylglycine solution was investigated. It was found that under these conditions, nicotinamide readily yielded a hemochromogen with the medullary cytochromes absorbing maximally at 557 μm, whereas pyridine, at a concentration of 0.07 m also, yielded its characteristic hemochromogen with an absorption maximum at 558 μm. Similar results were obtained with deoxycholate extracts of liver microsomes.

Effect of Carbon Monoxide on Absorption Spectra—In order to investigate the possibility that the medullary spectra might in part be caused by hemochromogens derived from hemoglobin degradation, deoxycholate-solubilized preparations reduced with dithionite were equilibrated with carbon monoxide and their visible spectra observed. No alteration was seen in the absorption spectra. Although a change in the band at 605 μm with carbon monoxide might be expected, none was observed. Such a change would, however, be very difficult to detect because of the small amount of cytochrome oxidase present.

Medullary Enzymatic Activities—In Table III, the activities of...
the medullary enzymes measured are presented for the large granules, for the upper and lower layers from a sucrose density gradient fractionation of the large granules, and the microsomes. In order to make the activities presented in Table III more directly comparable, they are expressed as microequivalents of the substrate measured per hour; that is, micromoles of cytochrome c, micromoles of DPNH or TPNH × 2, and micromoles of oxygen × 4. It may be seen that succinate oxidase and dehydrogenase activity is highest in the upper layer of the large granules. This localization is thus the same as that found for DPNH at 340 mp, 6.22

The activity of the former system is, however, some 100 times greater than the latter. The catechol amine-containing granules would appear to be low in DPNH-cytochrome c reductase activity. Transhydrogenase activity is greatest when measured in the direction TPNH + DPN → DPNH + TPN. The activity of this enzyme would also seem to be highest in the fraction in which the mitochondria appear to be concentrated.

Effect of Inhibitors—Succinic oxidase activity of the medullary large granule fraction was completely inhibited by antimycin A at a concentration of 2 μg per ml, by 5 μg per ml of SN 5949, and 10⁻³ m KCN. Similarly, the cytochrome c oxidase activity of these granules, assayed spectrophotometrically, was completely inhibited by 10⁻³ m KCN. However, neither the DPNH-cytochrome c reductase nor the TPNH-cytochrome c reductase activity of the medulla was inhibited by 2 μg per ml of antimycin A, or by 20 μg per ml of SN 5949.

### DISCUSSION

The most striking aspect of the cytochrome pattern of the adrenal medulla is the presence of a relatively large amount of a compound which shows in the reduced state an absorption band centered at 559 mp. The exact localization of this component in the organelles of the cells is difficult to determine. It is definitely present in the microsome fraction in which the other cytochrome components are absent or scant. It is also present in abundance in a large granule fraction. This fraction may be subdivided in two. One of these, which is very rich in catechol amines, also contains more of the 559 mp compound, but is low in content of the cytochromes usually associated with the mitochondria. The other subfraction contains the bulk of the mitochondrial cytochromes and is richest in succinate oxidase and dehydrogenase activity. It is, however, not free of the component absorbing at 559 mp. It should be noted, however, that this subfraction contains an appreciable amount of catechol amines and is thus undoubtedly not free of the granules carrying these materials. These findings suggest that this cytochrome component is localized chiefly in the microsomes or rather in the endoplasmic reticulum from which the microsomes are derived. The data obtained do not permit one to state that it is absent from the mitochondria. The fact that the large granules which are rich in catechol amines also contain this microsomal cytochrome makes it tempting to suggest that these granules are derived from the endoplasmic reticulum. They would thus bear the same relation to the endoplasmic reticulum of the adrenal medulla as the zymogen granules of the pancreas bear to the endoplasmic reticulum of this gland (13).

The presence of a microsomal cytochrome has been reported...
amine was found to reduce the 550 mμ cytochrome in turbid suspensions would seem to differentiate it from the liver microsomal cytochrome. However, in experiments which we have conducted with turbid suspensions of liver microsomes, it has been found that though succinate does not reduce the cytochrome of carefully purified microsomes, it will do so if a small amount of liver mitochondria are added. It thus seems likely that during succinate oxidation in the mitochondria, either a metabolite is produced which diffuses out to reduce the microsomal hemochromogen or reduction occurs by direct contact of the microsomal and mitochondrial particles.

Previous investigators (6) have noted the existence of two types of particles in the medullary large granule fraction, a denser, more rapidly sedimenting granule containing the bulk of the tissue epinephrine and a second particle in which the succinate oxidase of the tissue is localized. The present work has confirmed the presence of these two particles and has demonstrated that in addition to succinate oxidase, a full complement of the usual mitochondrial cytochromes is present in the lighter particles. The total content of mitochondrial cytochromes in the adrenal medulla is, however, not large. For example, the cytochrome c content of beef adrenal medulla as determined by direct analysis is only about 8% of that found in beef heart. In addition, succinate dehydrogenase and DPNH-cytochrome c reductase activities are low as compared with rat liver (14). Thus the enzymes which are involved in the transport of electrons to oxygen are not abundant in the adrenal medulla.

On the other hand, it is of interest to note that the content of the TPNH-generating enzymes, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, has been reported by Glock and McLean (17) and Kelly et al. (18) to be higher in the adrenal medulla. As shown in this study, the TPNH-cytochrome c reductase activity of this tissue is very low. Indeed, it is less than 1% of the activity of the DPNH-cytochrome c reductase activity of either the large granule or microsomal fraction. The transhydrogenase activity of the tissue is, however, higher than any reported by Kaplan et al. (10) for a variety of tissues.

The total capabilities of the tissue for oxidation of TPNH by way of the transhydrogenase reaction are about 40 μeq (electrons) per g of tissue per hour as measured at 37°. The amount oxidized by way of the cytochrome c reductase pathway is negligible by comparison. The data of Kelly et al. (18) indicate that as measured at 25°, beef adrenal medulla is capable of producing 400 μeq per hour per g by the glucose 6-phosphate dehydrogenase reaction alone. Corrected to 37°, this may be assumed to approximate 800 μeq per hour per g. The medulla thus has far less capacity to funnel electrons from TPNH to oxygen than it has for the production of TPNH. This tissue is thus well adapted to conserve TPNH for use in reductive synthetic processes, such as perhaps the formation of the catechol amines.

**Summary**

The predominant cytochrome of the adrenal medulla is a compound showing an absorption band at 550 mμ upon reduction with cysteine, adrenaline, ascorbate, and reduced diphenpho- and reduced triphosphopyridine nucleotide. Upon differential centrifugation of homogenates of the tissue, this hemochromogen is found in a microsomal fraction and in the granules rich in catechol amines. A clean separation of mitochondria and the granules containing catechol amines could not be achieved. A fraction was obtained however, in which an enrichment of cytochrome b + cytochromes a + a3 could be demonstrated spectroscopically. Cytochrome c in the whole medulla was extracted and determined quantitatively. Its content is only 8% of that found in beef heart. The quantitative distribution of the enzymes succinate dehydrogenase, reduced diphenpho- and reduced triphosphopyridine nucleotide cytochrome c reductase, and transhydrogenase in the various fractions of the medulla has been measured. It was found that the content of enzymes in the medulla capable of carrying electron transport from reduced triphosphopyridine nucleotide to oxygen was greatly exceeded by the values reported in the literature for its content of enzymes capable of producing reduced triphosphopyridine nucleotide. This relationship is discussed in regard to the use of reduced triphosphopyridine nucleotide by the gland for reductive synthetic purposes.

**REFERENCES**
