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Conversion of cholesterol to  
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by subcellular fractions of bovine  
adrenal cortex

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Boston University

Graduate School

Dissertation

CONVERSION OF CHOLESTEROL TO ADRENOCORTICAL HORMONE  
INTERMEDIATES BY SUBCELLULAR FRACTIONS OF  
BOVINE ADRENAL CORTEX

by

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(B.Sc.Agric.; University of Reading, 1941; N.D.A. 1941)

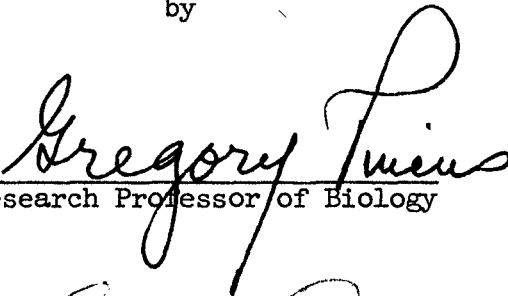
Submitted in partial fulfilment of the  
requirements for the degree of  
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**CHAPTER I**

**INTRODUCTION**

### A. A Statement of the Problem

The investigations forming the basis of this dissertation were undertaken as part of a general study on the mechanism of action of adrenocorticotrophic hormone (ACTH). While certain aspects of the conversion of cholesterol to adrenocortical steroids were of prime interest during the investigation, the experimental approach was conditioned by the desire to obtain information pertinent to the general study on ACTH action. If this has resulted in some aspects of cholesterol transformation receiving less attention than their intrinsic interest might suggest, it is because preference was given throughout the investigation to studies expressly designed to provide guidance for the general study on ACTH action.

For reasons to be examined in detail later, the role of cholesterol as a major precursor of adrenocortical steroid hormones appears to be firmly established. The biosynthetic pathways whereby cholesterol is transformed into physiologically active hormones, e.g. corticosterone, cortisol and aldosterone, involve first an oxidative scission of the cholesterol side chain with the formation of  $\Delta^5$ -pregnenolone. This transformation is enhanced by the administration of ACTH to a suitable adrenocortical preparation. From pregnenolone, progesterone is formed and then hydroxylated at specific sites on the molecule to form corticosterone, cortisol and aldosterone. In contrast to the first stage, none of these later hydroxylation reactions has been shown to be rate limiting in the absence of ACTH. However, they are known to be reactions dependent upon an adequate supply of a specific cofactor, the reduced form

of triphosphopyridine nucleotide (TPNH), and to utilize molecular oxygen as a source of the hydroxyl oxygen. In contrast very little definite information is available on the requirements for the enzymatic cleavage of the cholesterol side chain. It has been proposed that the side chain scission is preceded by hydroxylation, which raises the question whether or not it is a TPNH dependent process in common with other steroid hydroxylation systems. An understanding of the cofactor requirement for the cholesterol side chain cleavage has theoretical importance in the problem of ACTH action, partly because this step is accelerated by hormone administration and partly in view of current theories of ACTH action which invoke control of TPNH availability as an important facet of the mechanism of action of the hormone.

It seemed of prime importance to establish without any doubt whether or not TPNH was a specific cofactor in the oxidative removal of the cholesterol side chain, and the answering of this question was the first objective of the investigation.

The current theories of ACTH action referred to above place emphasis on the production of TPNH by the direct oxidative metabolism of glucose-6-phosphate. This involves dehydrogenation by a TPN specific dehydrogenase with the consequent production of TPNH. While the proposals put forward will be examined in detail in due course, for the moment it may be pointed out that these enzymes are generally regarded as being localized in the so-called soluble fraction of the cell complex. On the other hand the intracellular site of the cholesterol side chain cleavage event is not fully clarified, individual studies of the reaction having

been made on non-particulate and on mitochondrial fractions. Again it seemed important to the study on ACTH action to define more clearly the intracellular location of the early stages of cholesterol transformation and, if possible, to relate the enzymatic activity with the availability of TPNH. The investigation of these aspects of cholesterol transformation in adrenal cortex formed the second objective of the investigation.

To avoid repetition, reference to original sources have been omitted in this introduction and statement of the problem, and deferred to the following review of the pertinent literature.

B. The Status of Cholesterol as a Major Precursor  
of the Adrenocortical Steroid Hormone

The biosynthetic pathways connected with the formation of the adrenocortical secretion have been intensively investigated during the past decade and excellent reviews are available<sup>1,2,3,4</sup>. Some thirty or more steroid substances have been isolated from adrenal cortex tissue<sup>1,2</sup>, many of them probably intermediates in the biosynthesis of the adrenocortical hormones and not significant as physiological secretions of the

<sup>1</sup>Hechter, O. and Pincus, G. Genesis of the Adrenocortical Secretion. *Physiol. Revs.* 34:459, 1954.

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. Some Aspects of the Biogenesis of Adrenal Steroid Hormones. *Recent Progr. Hormone Res.* 12:79, 1956.

<sup>3</sup>Hechter, O. Conversion of Cholesterol to Steroid Hormones. in Cholesterol, ed. R. P. Cook, (New York: Academic Press, Inc., 1958), p. 309.

<sup>4</sup>Pincus, G. Recent Developments in the Study of Adrenal Cortical Steroid Biogenesis. 4th Intern. Congr. Biochem., Vienna, Austria, 1958.

endocrine tissue. The limitation of the physiologically important steroids to seven compounds may be made on the basis of a comparison of the steroid content of adrenal venous blood with that of peripheral blood<sup>4</sup>. Those steroids present in adrenal venous blood in higher concentration than in the peripheral circulation, may be regarded as belonging to the adrenocortical secretion<sup>4</sup>. Of these compounds, corticosterone and cortisol present in appreciable amounts and aldosterone, present in trace amount, appear to be the "principal" corticoids secreted by the normal animal<sup>3</sup>. The great similarity in chemical structure of these compounds, to one another and to cholesterol, present in the adrenal in high concentration, led to the early development of hypothetical pathways for their derivation from cholesterol<sup>5</sup>. In this early phase of research on corticosteroidogenesis, indirect evidence supported the hypothesis that cholesterol was a precursor of the adrenocortical steroid hormones recognized at that time. In particular the work of Long and his associates<sup>6</sup>, who showed that the cholesterol concentration in adrenocortical tissue was decreased under conditions of enhanced corticosteroid formation (ACTH administration). Direct evidence for the conversion of cholesterol to cortisol and corticosterone was first obtained in the isolated cow adrenal perfused with

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<sup>3</sup>Hechter, O. in Cholesterol, ed. R. P. Cook (New York: Academic Press, Inc., 1958), p. 309.

<sup>4</sup>Pincus, G. 4th Intern. Congr. Biochem, Vienna, Austria, 1958.

<sup>5</sup>Karrer, P. Organic Chemistry, (Elsevier, Amsterdam, 1938).

<sup>6</sup>Long, C.N.H. The Relation of Cholesterol and Ascorbic Acid to the Secretion of the Adrenal Cortex. Recent Progr. Hormone Res. 1:99, 1947.

cholesterol-4-C<sup>14</sup>(7,8). Adrenocortical tissue homogenates and subcellular fractions prepared from them were also found to convert radioactive cholesterol to radioactive cortisol and corticosterone<sup>2,9,10</sup> and from these and similar studies, reaction sequences were worked out to account for the formation of cortisol and corticosterone from cholesterol. One such scheme is presented in Fig. 1 based largely on the results of studies carried out on bovine adrenal cortex tissue<sup>3</sup>. The biosynthetic route envisaged involves a primary removal of the cholesterol side chain by cleavage between C-20 and C-22 to form  $\Delta^5$ -pregnenolone, which in turn gives rise to progesterone by transformation of the  $\Delta^5$ -3 $\beta$ -hydroxy grouping on ring A to the  $\Delta^4$ -3-ketone characteristic of the physiologically active corticosteroids. Progesterone is a key intermediary in this scheme with at least two separate synthetic pathways leading from it to the adrenocortical hormones. One pathway consists of the ordered hydroxylation of

<sup>1</sup>Hechter, O. and Pincus, G. *Physiol. Revs.* 34:459, 1954.

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. *Recent Progr. Hormone Res.* 12:79, 1956.

<sup>3</sup>Hechter, O. in Cholesterol, ed. R. P. Cook (New York:Academic Press, Inc., 1958), p. 309.

<sup>7</sup>Zaffaroni, A., Hechter, O. and Pincus, G. Adrenal Conversion of C<sup>14</sup> Labeled Cholesterol and Acetate to Adrenal Cortical Hormones. *J. Am. Chem. Soc.* 73:1390, 1951.

<sup>8</sup>Hechter, O., Solomon, M. M., Zaffaroni, A. and Pincus, G. Transformation of Cholesterol and Acetate to Adrenal Cortical Hormones. *Arch. Biochem. Biophys.* 46:201, 1953.

<sup>9</sup>Saba, N. and Hechter, O. Cholesterol-4-C<sup>14</sup> Metabolism in Adrenal Homogenates. *Federation Proc.* 14:775, 1955.

<sup>10</sup>Heard, R.D.H., Bligh, E. G., Cann, M.C., Jellinck, P.H., O'Donnell, V.J., Rao, B. G. and Webb, J. L. Biogenesis of the Sterols and Steroid Hormones. *Recent Progr. Hormone Res.* 12:45, 1956.

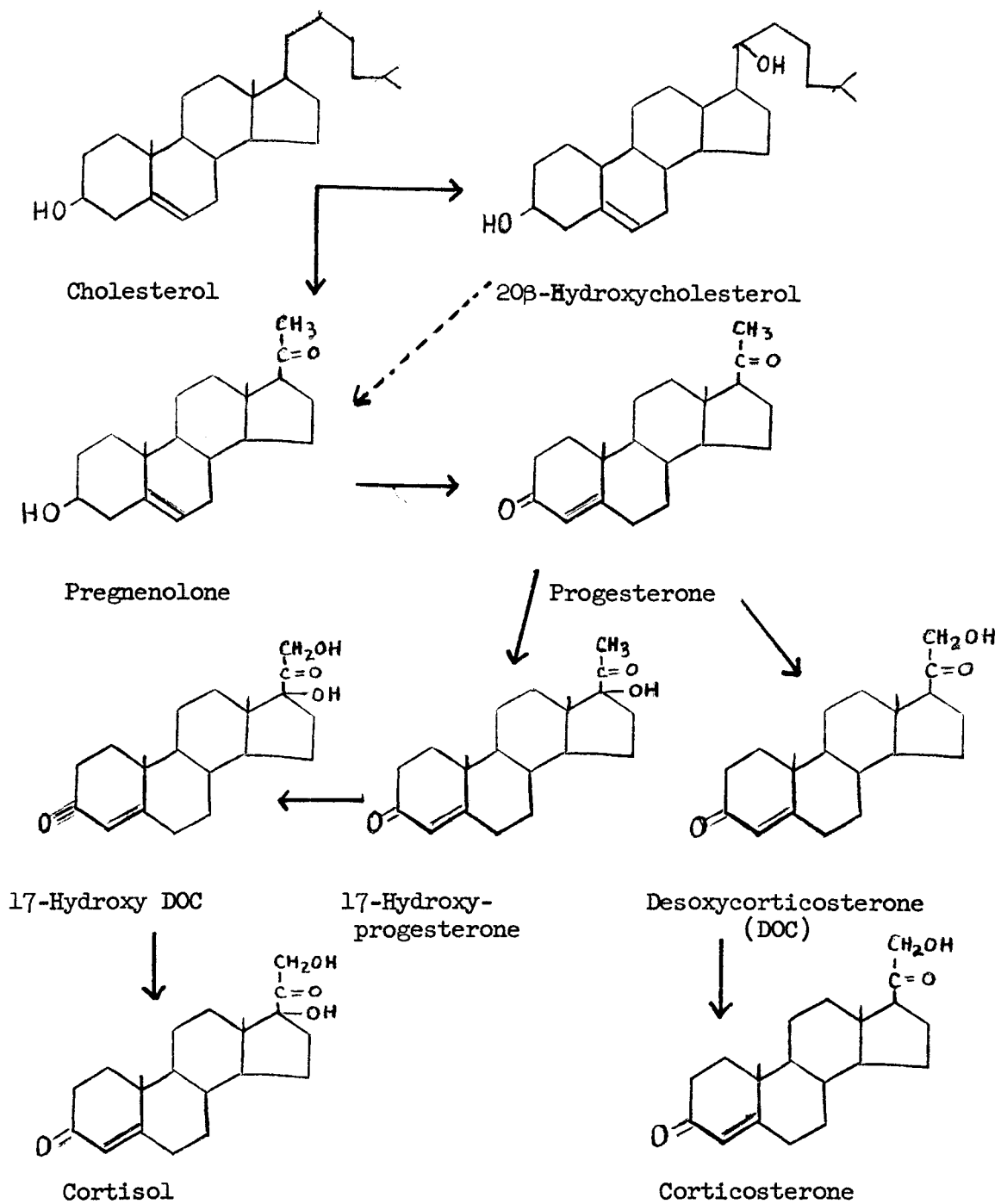


Fig. 1. The sequence of reactions in the conversion of cholesterol to adrenocortical steroid hormones. The solid lines represent established pathways.

progesterone at carbon atoms C-17, C-21, and C-11 to give rise to cortisol. The other pathway omits the hydroxylation at C-17 but includes those at C-21 and C-11<sup>1,2</sup>. The relative amounts of corticosterone and cortisol formed varies in different species; corticosterone is the main adrenal steroid found in the venous blood of the rat and rabbit, while in man, monkey, cat, and dog cortisol predominates<sup>4</sup>. The isolated bovine adrenal perfused with ACTH gives rise to approximately equal amounts of corticosterone and cortisol<sup>1</sup>.

All of the reactions involved in this picture of corticosteroid formation have been investigated using adrenal tissue preparations of differing levels of organization. Much basic information was obtained through the development of the perfused bovine adrenal technique by Hechter and his associates<sup>1,2</sup>, together with the results of studies on bovine adrenal tissue homogenates and fractions derived from them, their studies contributed largely to the overall picture presented in Fig. 1. Before discussing the detailed enzymology of the various stages in this sequence of reactions, some reference must be made to an alternative pathway of adrenocortical steroid hormone formation not necessarily involving cholesterol as a precursor.

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<sup>1</sup>Hechter, O. and Pincus, G. *Physiol. Revs.* 34:459, 1954.

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. *Recent Progr. Hormone Res.* 12:79, 1956.

<sup>4</sup>Pincus, G. 4th Intern. Congr. Biochem., Vienna, Austria, 1958.

The perfusion of  $C^{14}$ -acetate through isolated bovine adrenal glands results in the formation of  $C^{14}$ -cholesterol<sup>7,11</sup>, indicating the presence of enzymatic sequences capable of synthesizing corticosteroids from acetate via cholesterol as an intermediary. However, Heard et al.<sup>10</sup> found that a cell-free preparation of hog adrenal synthesized radioactive cortisol and corticosterone from  $C^{14}$ -acetate without the concomitant production of radioactive cholesterol. This established pathway of corticosteroid synthesis not involving cholesterol should be regarded in the light of comparative experiments carried out by Stone and Hechter<sup>12</sup>. These authors showed that the incorporation of cholesterol-4- $C^{14}$  into corticosteroids by the isolated perfused bovine adrenal is increased 1800% by ACTH administration, whereas the incorporation of  $C^{14}$ -acetate is only increased by 40%. The significance of the alternative pathway is not at the moment apparent, but it would appear very probable that cholesterol is the major adrenocortical steroid precursor.

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<sup>7</sup>Zaffaroni, A., Hechter, O. and Pincus, G. J. Am. Chem. Soc. 73: 1390, 1951.

<sup>10</sup>Heard, R.D.H., Bligh, E. G., Cann, M. C., Jellinck, P. H., O'Donnell, V. J., Rao, B. G. and Webb, J. L. Recent Progr. Hormone Res. 12:45, 1956.

<sup>11</sup>Haines, W. J. Studies on the Biosynthesis of Adrenal Cortex Hormones. Recent Progr. in Hormone Res. 7:255, 1952.

<sup>12</sup>Stone, D. and Hechter, O. Studies on ACTH Action in Perfused Bovine Adrenals: The Site of Action of ACTH in Corticosteroidogenesis. Arch. Biochem. & Biophys. 51:457, 1954.

### C. The Enzyme Systems Involved in Corticosteroidogenesis

The primary step in corticosteroid formation is represented in Fig. 1 as a scission of the cholesterol side chain between carbon atoms C-20 and C-22. This reaction has been studied in a non-particulate fraction of aged bovine adrenals incubated with cholesterol-26-C<sup>14</sup> or cholesterol-4-C<sup>14</sup>(13,14). In the former case radioactive isocaproic acid was obtained, indicating scission of the cholesterol side chain between C-20 and C-22, while  $\Delta^5$ -pregnenolone-4-C<sup>14</sup> was obtained from incubations with cholesterol-4-C<sup>14</sup>. Both DPN and ATP were required for activity to be demonstrated. Lynn et al.<sup>13</sup> suggested that 20 $\beta$ -hydroxy-cholesterol might be an intermediary in this reaction, a compound later isolated by Solomon et al.<sup>15</sup> as a C<sup>14</sup> derivative of cholesterol-4-C<sup>14</sup> after incubation of the latter compound with bovine adrenal homogenates.

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<sup>13</sup>Lynn, W. S. Jr., Staple, E. and Gurin, S. Catabolism of Cholesterol by In Vitro Systems. Federation Proc. 14:783, 1955.

<sup>14</sup>Staple, E., Lynn, W. S. Jr. and Gurin, S. An Enzymatic Cleavage of the Cholesterol Side Chain. J. Biol. Chem. 219:845, 1956.

<sup>15</sup>Solomon, S., Levitan, P., Lieberman, S. Possible Intermediates between Cholesterol and Pregnenolone in Corticosteroidogenesis. Revue Canadienne de Biologie 15:282, 1956.

Saba and Hechter<sup>9</sup> incubated the mitochondrial fraction from a 0.25M sucrose homogenate of bovine adrenocortical tissue with cholesterol-4-C<sup>14</sup> and obtained, in good yield, radioactive progesterone. The greater efficiency of the conversion in this study compared with that obtained in the non-particulate fraction suggested the presence of mitochondrial fragments in the aged preparation. Saba and Hechter<sup>9</sup> found fumarate and Mg<sup>++</sup> to be the only essential requirements of the mitochondrial preparation for conversion of cholesterol-4-C<sup>14</sup> to radioactive products.

That the stepwise degradation of the cholesterol side chain is an unlikely event in the adrenal is shown not only by the formation of isocaproic acid, but by the failure to detect 24-hydroxy (or keto)-cholesterol or 22-hydroxy (or keto)-cholesterol as intermediates in the catabolism of cholesterol.<sup>15</sup> Recently<sup>16</sup> it has been shown that a product of 22-ketocholesterol-23-C<sup>14</sup> metabolism by adrenal tissue is isovaleric acid and not isocaproic acid, indicating a preferential cleavage between C-22, 23. The failure to find 22-hydroxycholesterol as an intermediate in the cholesterol side chain scission reaction makes it difficult to maintain the most likely suggestion that hydroxylations occur at both C-20 and C-22, with subsequent splitting of the diol by a desmolase activity<sup>17</sup>.

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<sup>9</sup>Saba, N. and Hechter, O. Federation Proc. 14:775, 1955.

<sup>15</sup>Solomon, S., Levitan, P., Lieberman, S. Revue Canadienne de Biologie 15:282, 1956.

<sup>16</sup>Kautsky, G. J., Bouboulis, C. J., Becker, R. R. and King, C. G. Synthesis and Metabolism of 22-Ketocholesterol-23-C<sup>14</sup>. J. Biol. Chem. 233:1340, 1958.

<sup>17</sup>Dorfman, R. I. Comments on the Metabolism of Steroid Hormones. Cancer Research 17:535, 1956.

The second major step in the sequence outlined in Fig. 1 is the conversion of pregnenolone to progesterone. This reaction was first described as a pyridine nucleotide linked dehydrogenase by Samuels<sup>18</sup>, Byer and Samuels<sup>19</sup>, and Hayano et al.<sup>2</sup> Byer and Samuels<sup>19</sup> found the activity to be concentrated in the microsomal fraction of a 0.25M sucrose homogenate of bovine adrenal cortex tissue. The fraction used in this work included the "fluffy layer" derived from the mitochondrial pellet during the fractionation procedure. DPN was found to be an essential cofactor. The conversion of the  $\Delta^5$ - $3\beta$ -ol grouping in pregnenolone to the  $\Delta^4$ -3-ketone of progesterone is essentially an irreversible reaction, unlike most dehydrogenase activities known, as the  $\alpha$ - $\beta$  unsaturated ketones are resistant to enzymatic reduction of the ketone without prior saturation of the double bond<sup>20,21</sup>. However, the properties of the  $3\beta$ -hydroxysteroid dehydrogenase obtained as an adaptive enzyme from

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<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. Recent Progr. Hormone Res. 12:79, 1956.

<sup>18</sup>Samuels, L. T. Studies of the Enzymes Involved in the Synthesis and Degradation of the Hormone of the Adrenal Cortex. Ciba Colloquia Endocrinol. 7:176, 1953.

<sup>19</sup>Byer, K. F. and Samuels, L. T. Distribution of Steroid  $3\beta$ -ol Dehydrogenase in Cellular Structures of the Adrenal Gland. J. Biol. Chem. 219:69, 1956.

<sup>20</sup>Butenandt, A., Dannenberg, H. and Suranyi, L. A. Ber. 73:818, 1940.

<sup>21</sup>Talalay, P. and Dobson, M. M. Purification and Properties of a  $\beta$ -Hydroxysteroid Dehydrogenase. J. Biol. Chem. 205:823, 1953.

Pseudomonas testosteronii grown on a medium containing testosterone have been extensively studied by Talalay and his associates<sup>22</sup>, with the indication that the reaction proceeds in two stages: (a) the primary oxidation of the hydroxyl group by a pyridine nucleotide linked hydroxysteroid dehydrogenase, followed by (b) the enzymatic rearrangement of the double bond from  $\Delta^5$  to  $\Delta^4$ (<sup>23</sup>). The second reaction, (b), is apparently catalyzed by an enzymatic protein distinct from the dehydrogenase and designated steroid isomerase<sup>22</sup>. As isolated from Ps. testosteronii, it is one of the most active enzymes known (T.N. ca. 150,000) and has recently been obtained in crystalline form<sup>24</sup>. It appears to effect the direct transfer of hydrogen from C-4 to C-6, rather than function by way of a hydration of the  $\Delta^5$  double bond (with the formation of a 5-hydroxysteroid followed by elimination of the elements of water from positions C-4 and C-5 (cf. 25)). The isomerase reaction is essentially irreversible and thereby accounts for the non-reversibility of the conversion of the  $\Delta^5$ -3 $\beta$ -ol structure to the  $\Delta^4$ -3-ketone. The activity occurs in mammalian tissues, the richest sources in the rat being the adrenal, ovary, testis and liver<sup>22</sup>.

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<sup>22</sup>Talalay, P. Enzymatic Mechanisms in Steroid Metabolism. *Physiol. Revs.* 37:362, 1957.

<sup>23</sup>Talalay, P. and Wang, V. S. Enzymic Isomerization of  $\Delta^5$ -3-Ketosteroids. *Biochim. et biophys. acta*, 18:300, 1955.

<sup>24</sup>Kawahara, F. S. and Talalay, P. Crystalline  $\Delta^5$ -3 Ketosteroid Isomerase. *J. Biol. Chem.* 235:PC 1, 1960.

<sup>25</sup>Stern, J. and Del Campillo, A. Enzymes of Fatty Acid Metabolism. II. Properties of Crystalline Crotonase. *J. Biol. Chem.* 218:985, 1956.

Progesterone is in turn converted by hydroxylation at C-17 into 17-hydroxyprogesterone, or by hydroxylation at C-21 into 21-hydroxyprogesterone (DOC), the hydroxylation at C-21 precluding the subsequent introduction of a C-17 hydroxyl group<sup>1</sup>. The conversion of progesterone to 17-hydroxyprogesterone was first demonstrated in the adrenal by Plager and Samuels<sup>26,27</sup> using heifer glands frozen in dry ice, homogenized in Krebs' ringer medium and centrifuged at 20,000g. for 0.5 hours. The supernatant fraction was incubated with progesterone in a medium fortified by the addition of DPN, ATP and niacinamide. The C-17 and C-21 hydroxylase activities have often been found to be closely associated during fractionation<sup>2</sup>, but Ryan and Engel<sup>28,29,30</sup> obtained 21-hydroxylase activity in a particulate fraction not sedimented at 20,000g. but brought down at 105,000g. and seldom found 17-hydroxylase activity in the same

<sup>1</sup>Hechter, O. and Pincus, G. *Physiol. Revs.* 34:459, 1954.

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. *Recent Progr. Hormone Res.* 12:79, 1956.

<sup>26</sup>Plager, J. E. and Samuels, L. T. Synthesis of C<sup>14</sup>-17 hydroxy-11-desoxycorticosterone and 17-Hydroxycorticosterone by Fractionated Extracts of Adrenal Homogenates. *Arch. Biochem. & Biophys.* 42:477, 1953.

<sup>27</sup>Plager, J.E. and Samuels, L. T. The Conversion of Progesterone to 17-Hydroxy-11-desoxycorticosterone by Fractionated Beef Adrenal Homogenates. *J. Biol. Chem.* 211:21, 1954.

<sup>28</sup>Ryan, K. J. Steroid 21-Hydroxylation by Adrenal Cell Fractions. *Federation Proc.* 15:344, 1956.

<sup>29</sup>Ryan, K. J. and Engel, L. L. Steroid 21-Hydroxylation by Adrenal Microsomes and Reduced Triphosphopyridine Nucleotide. *J. Am. Chem. Soc.* 78:2654, 1956.

<sup>30</sup>Ryan, K. J. and Engel, L. L. Hydroxylation of Steroids at Carbon 21. *J. Biol. Chem.* 225:103, 1957.

preparation. These authors used two volumes of 0.25M sucrose homogenizing media containing 0.05M Tris buffer, 0.05M KCl, 0.005M  $MgCl_2$  and 0.005M niacinamide in an early study and a 1:1 0.25M sucrose medium containing 0.1M phosphate buffer pH 6.8 and 0.04M niacinamide in later work. The total cation content of the isolation media was therefore very high and is remarked upon here because of certain observations on the use of sucrose-salt mixtures for tissue fractionation studies made during the course of the present investigation. The "microsomal" preparation, fortified with DPN and ATP was inactive in converting progesterone to 11-desoxycorticosterone or 17-hydroxyprogesterone to 11-desoxycortisol, unless supplemented with the supernatant fraction (not sedimented at 105,000g.) or a similar fraction derived from rat liver. However, the authors pointed out that the requirement for the soluble factor could be met by the addition of glucose-6-phosphate, TPN and glucose-6-phosphate dehydrogenase to the microsomal fraction, and concluded that TPNH was the specific cofactor required.

It has become clear that all the steroid hydroxylation reactions that have been extensively studied so far incorporate molecular oxygen into the steroid molecule, require metals and a specific reductant in the form of TPNH<sup>31,32</sup>. Much of the evidence leading to this conclusion was accumulated during studies of the final step in the scheme of steroidogenesis shown in Fig. 1, i.e. the introduction of the hydroxyl group

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<sup>31</sup>Dorfman, R. I. Biochemistry of the Steroid Hormones. Ann. Rev. Biochem. 26:523, 1957.

<sup>32</sup>Grant, J. K. Enzymic Hydroxylation of Steroids. Annual Repts. on Progr. Chem. (Chem.Soc. London) 52:316, 1956.

at C-11 (in the  $\beta$  orientation) into the desoxycorticosterone or 17-hydroxy-DOC molecule. The original finding that molecular oxygen was utilized for hydroxyl formation at C-11 was due to Hayano et al.<sup>33</sup> who obtained incorporation of one atom of  $O^{18}$  into the steroid molecule, when the hydroxylation was carried out in an atmosphere of radioactive oxygen. Earlier work on the  $11\beta$ -hydroxylation reaction had shown an apparent requirement for fumarate,  $Mg^{++}$ , DPN and ATP by particulate fractions of bovine adrenal cortex tissue<sup>2</sup>, though later studies on carefully prepared mitochondrial fractions showed that other citric acid cycle intermediates could replace fumarate<sup>34</sup>. Sweat and Lipscombe<sup>35</sup>, and Grant<sup>36,37</sup> both showed that the essential cofactor for this reaction was in fact the reduced form of triphosphopyridine nucleotide and that the role of fumarate was probably one of maintaining TPN in a reduced state. Most of the

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. Recent Progr. Hormone Res. 12:79, 1956.

<sup>33</sup>Hayano, M., Lindberg, M.C., Dorfman, R. I., Hancock, J.E.H. and von Doering, W. E. On the Mechanism of the C- $11\beta$ -Hydroxylation of Steroids; a Study with  $H_2O^{18}$  and  $O_2^{18}$ . Arch. Biochem. & Biophys. 59:529, 1955.

<sup>34</sup>Brownie, A. C. and Grant, J. K. The In Vitro Hydroxylation of Steroid Hormones. 1. Factors Influencing the Enzymic  $11\beta$ -Hydroxylation of 11-deoxycorticosterone. Biochem. J. 57:255, 1954.

<sup>35</sup>Sweat, M. L. and Lipscombe, M. D. A Transhydrogenase and Reduced Triphosphopyridine Nucleotide Involved in the Oxidation of Desoxycorticosterone to Corticosterone by Adrenal Tissue. J. Am. Chem. Soc. 77: 5185, 1955.

<sup>36</sup>Grant, J. K. The In Vitro Enzymic Hydroxylation of Steroids. 4. The Role of Fumarate and Triphosphopyridine Nucleotide in the Enzymic  $11\beta$ -Hydroxylation of 11-Deoxycorticosterone. Biochem. J. 64:559, 1956.

<sup>37</sup>Grant, J. K. and Brownie, A. C. The Role of Fumarate and TPN in Steroid Enzymic  $11\beta$ -Hydroxylation. Biochim. et Biophys. Acta, 18:433, 1956.

work on  $11\beta$ -hydroxylation suggests that the activity resides in the mitochondrial fraction. Tomkins<sup>38</sup> has presented evidence for the participation of at least two enzymes in the  $11\beta$ -hydroxylation system in the adrenal, one of which can be replaced by an unidentified enzyme present in liver, while further study has suggested that there may be separate enzymes for the  $11\beta$ -hydroxylation of each steroid<sup>39</sup>.

In conclusion of this review of the enzymes involved in corticosteroidogenesis, the following summary may be made. The enzymatic cleavage of the cholesterol side chain may occur in the mitochondrial fraction and the  $11\beta$ -hydroxylation activity certainly appears to be associated with particulate material having the same order of sedimentation properties as mitochondria. In contrast, the  $3\beta$ -hydroxy dehydrogenase activity appears to be a "microsomal" enzyme, though its presence in mitochondrial preparations has been implied (cf. 9), and the  $21$ , and  $17$  hydroxylase systems may be microsomal or soluble enzymes, respectively. This heterogeneous distribution of the various activities forming part of the sequence of reactions involved in corticosteroidogenesis raises the problem of how the ordered sequential reactions can be carried out in the cell. With

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<sup>9</sup>Saba, N. and Hechter, O. Federation Proc. 14:775, 1955.

<sup>38</sup>Tomkins, G. M., Curran, J. F. and Michael, P. J. Further Studies on Enzymic Adrenal  $11\beta$ -hydroxylation. Biochim. et Biophys. Acta, 28:449, 1958.

<sup>39</sup>Tomkins, G. M., Michael, P. J. and Curran, J. F. Studies on the Nature of  $11\beta$ -hydroxylation. Biochim. et Biophys. Acta, 23:655, 1957.

the initial and final reactions possibly occurring in the mitochondrial-like particulates, pregnenolone must react with the microsomal  $3\beta$ -hydroxydehydrogenase; the progesterone formed must then be transferred to soluble enzymes, or 17 and 21 hydroxylated in situ; with a final return to the mitochondrial site for  $11\beta$ -hydroxylation. This problem has been discussed by Hayano et al.<sup>2</sup> who postulated the localization of the various enzymes upon the mitochondrial surface in such a manner as to permit the ordered transformation of cholesterol to corticosterone obtaining in intact cell preparations.

#### D. Control of the Adrenocortical Secretion

The reaction of the adrenal cortex to stimulation by ACTH is manifest in growth of the tissue and a rapid increase in corticosteroidogenesis. In addition to well-established effects in vivo, ACTH has also been clearly shown to stimulate increased corticoid formation in vitro<sup>40,41,42</sup>. The mechanism whereby ACTH elicits this response has attracted considerable attention but still defies exact formulation.

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. Recent Progr. Hormone Res. 12:79, 1956.

<sup>40</sup>Hechter, O., Zaffaroni, A., Jacobsen, R. P., Levy, H., Jeanloz, R. W., Schenker, V. and Pincus, G. The Nature and the Biogenesis of the Adrenal Secretory Product. Recent Progr. Hormone Res. 6:215, 1951.

<sup>41</sup>Saffran, M., Grad, B. and Bayliss, M. J. Production of Corticoids by Rat Adrenals In Vitro. Endocrinol. 50:639, 1952.

<sup>42</sup>Haynes, R., Savard, K. and Dorfman, R. I. The Action of Adrenocorticotrophic Hormone on Beef Adrenal Slices. J. Biol. Chem. 207:925, 1954.

Stone and Hechter<sup>1,2</sup> have shown that on administration of ACTH to the isolated perfused bovine adrenal the rate of C<sup>14</sup> incorporation into corticosteroids is increased 18-fold when cholesterol-4-C<sup>14</sup> is used as the radioactive source, whereas no C<sup>14</sup> is incorporated using progesterone-4-C<sup>14</sup> as C<sup>14</sup> source. These findings have led to the suggestion that the site of action of ACTH is at the level of the transformation of cholesterol to pregnenolone, and not at any of the enzymic events concerned in progesterone hydroxylation<sup>1,12</sup>.

The in vitro action of ACTH has been demonstrated in terms of corticoid production, at the level of the perfused gland<sup>40</sup> and tissue slices<sup>41,42</sup> but not unequivocally at a level of disorganization greater than that of the tissue slice or quartered rat adrenal. This dependence on a high degree of intactness of the target tissue may be related to the rate of corticosteroid formation obtaining in the absence of ACTH<sup>2</sup>.

<sup>1</sup>Hechter, O. and Pincus, G. *Physiol. Revs.* 34:459, 1954.

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. *Recent Progr. Hormone Res.* 12:79, 1956.

<sup>12</sup>Stone, D. and Hechter, O. *Arch. Biochem. & Biophys.* 51:457, 1954.

<sup>40</sup>Hechter, O., Zaffaroni, A., Jacobsen, R. P., Levy, H., Jeanloz, R. W., Schenker, V. and Pincus, G. *Recent Progr. Hormone Res.* 6:215, 1951.

<sup>41</sup>Saffran, M., Grad, B. and Bayliss, M. J. *Endocrinol.* 50:639, 1952.

<sup>42</sup>Haynes, R., Savard, K. and Dorfman, R. I. *J. Biol. Chem.* 207:925, 1954.

The homogenized tissue is capable of high rates of steroid formation provided essential cofactors are present in adequate amount, but cannot be further stimulated by ACTH in the presence or absence of these cofactors. On the other hand, the secretion rate of a suitably perfused isolated bovine adrenal is negligible in the absence of ACTH, suggesting the necessity for maintaining a system that restrains the synthesis of corticosteroids if an action of ACTH is to be demonstrated. The manner in which ACTH brings about the increased conversion of cholesterol to pregnenolone, or removes the restraining factor, is not known, but two theories have been put forward that differ in many respects, but nevertheless contain certain elements in common.

The first theory visualizes the control of adrenocortical secretion in terms of the enzymatic regulation of TPNH production, a cofactor known to be required by some of the reactions in corticosteroid formation (cf. previous section). Haynes and Berthet<sup>43</sup> investigated the response of bovine (steer) adrenal cortex slices to ACTH and concluded that the action was a neosynthesis of corticoid rather than a release of formed hormones. Both Haynes and Berthet, and Koritz and Peron found glucose-6-phosphate (G6P) to be stimulatory to corticoid output when added to bovine adrenal slices<sup>43</sup> or quartered rat adrenals<sup>44</sup>, in the presence of added TPN. Neither TPN nor G6P were effective alone. ACTH, however, was able to further increase the corticoid output when given

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<sup>43</sup>Haynes, R. C. Jr. and Berthet, L. Studies on the Mechanism of Action of the Adrenocorticotrophic Hormone. *J. Biol. Chem.* 225:115, 1957.

<sup>44</sup>Koritz, S. B. and Peron, F. G. Studies on the Mode of Action of the Adrenocorticotrophic Hormone. *J. Biol. Chem.* 230:343, 1958.

with TPN and G6P (but cf. Eichhorn<sup>45</sup>). Freezing of the tissue abolished the response to ACTH shown by adrenal slices in vitro but did not prevent G6P plus TPN from stimulating increased formation of corticoids<sup>46</sup>. Haynes and Berthet<sup>43</sup>, finding glucose-1-phosphate (GLP) to be as effective as G6P in stimulating corticoid production in the presence of TPN, investigated the effect of ACTH on phosphorylase activity of adrenocortical slices. The phosphorylase activity of homogenates prepared from ACTH stimulated slices was found to be greater than from control tissue and an ammonium sulphate precipitate of the homogenate retained the increased activity, indicating a modification of the enzyme. No similar increase was observed in glucose-6-phosphate dehydrogenase activity. The theory was advanced that ACTH stimulated adrenal phosphorylase to provide increased GLP which in turn increased the level of G6P and hence TPNH by the direct oxidative pathway known to be highly active in the adrenal cortex<sup>47</sup>. The action of ACTH as formulated here is reminiscent of the

<sup>43</sup>Haynes, R. C. Jr. and Berthet, L. J. Biol. Chem. 225:115, 1957.

<sup>45</sup>Eichhorn, J. H. in "Aspects of Adrenal Corticosteroidogenesis, with Special Reference to Certain Intermediaries and to Factors Influencing Corticoid Formation In Vitro." Doctoral Thesis, Boston University, 1958.

<sup>46</sup>Koritz, S. B. and Peron, F. G. The Stimulation In Vitro by  $Ca^{++}$ , Freezing and Proteolysis of Corticoid Production by Rat Adrenal Tissue. J. Biol. Chem. 234:3122, 1959.

<sup>47</sup>Glock, G. E. and McLean, P. Levels of Enzymes of the Direct Oxidative Pathway of Carbohydrate Metabolism in Mammalian Tissues and Tumours. Biochem. J. 56:171, 1954.

mechanism described for the action of epinephrine and glucagon on liver cells by Sutherland and Rall<sup>48</sup>. Continuing his studies on adrenal phosphorylase, Haynes<sup>49</sup> found that adenosine-3',5'-monophosphate (3'5'-AMP) was specific stimulant to adrenal phosphorylase as it was in the case of liver<sup>48</sup>. The theory required that 3'5'-AMP would be stimulatory to corticoid formation and this indeed has been found to be the case<sup>50</sup>. Surprisingly 3'5'-AMP produced a greater response than ACTH, and further increased the output of corticoids in the presence of maximal ACTH stimulation. The observation by Noble and Papageorge<sup>51</sup> that adrenal glycogen is decreased on stimulation of the tissue by ACTH lends further support to this theory.

The second theory visualizes the regulation of corticosteroidogenesis in terms of a general theory of hormone action based on the effect of hormones to facilitate the entry of substrates into the cell, or of essential precursors or cofactors to a particular enzymatic site<sup>52</sup>. This conception of the mechanism of ACTH action has had recent support from

<sup>48</sup>Sutherland, E. W. and Rall, T. W. The Properties of an Adenine Ribonucleotide Produced with Cellular Particles, ATP, Mg<sup>++</sup> and Epinephrine or Glucagon. J. Am. Chem. Soc. 79:3608, 1957.

<sup>49</sup>Haynes, R. C. Jr. The Activation of Adrenal Phosphorylase by the Adrenocorticotrophic Hormone. J. Biol. Chem. 233:1220, 1958.

<sup>50</sup>Haynes, R. C. Jr., Koritz, S. B. and Peron, F. G. Influence of Adenosine-3',5'-monophosphate on Corticoid Production by Rat Adrenals. J. Biol. Chem. 234:1421, 1959.

<sup>51</sup>Noble, N. L. and Papageorge, F. Loss of Adrenal Glycogen in the Rat Following Stress or Treatment with Various Hormones. *Endocrinol.* 57:492, 1955.

<sup>52</sup>Hechter, O. Reflections about Hormone Action and Implications for the Cancer Problem. *Cancer Res.* 17:512, 1957.

the demonstration by Eichhorn et al.<sup>53</sup> that the entry of D-xylose into the adrenal cells of hypophysectomized rats is restricted, and that ACTH facilitates the penetration of this sugar into the cell water. If glucose were similarly affected, then the similarity in the two theories becomes apparent. In the case of facilitated glucose entry, the levels of G6P (and hence TPNH) would be increased as well as all other processes requisite for growth of the adrenocortical cell.

"Schönbaum et al.<sup>54</sup> have pointed out difficulties in connection with both theories. Specifically they point to the finding that glucose entry in vitro is not restricted under circumstances where an ACTH effect can be demonstrated in terms of increased corticoid output. The results of their experiments also show that glucose, G6P and adenosine do not stimulate corticoid output in rat adrenals in vitro but do enhance the response of the tissue to simultaneously administered ACTH. G6P and adenosine, as substances stimulatory to pentose metabolism, might be expected to increase corticoid output even in the absence of ACTH, but as Schönbaum et al. remark, it is difficult to explain the additive effects of glucose or adenosine with ACTH on the basis of activation of phosphorylase unless one postulates the formation of glycogen as an obligatory step in the metabolism of these substances.

<sup>53</sup>Eichhorn, J. H., Halkerston, I.D.K., Feinstein, M. and Hechter, O. Effect of ACTH on Permeability of Adrenal Cell to Sugar. Proc. Soc. Exper. Biol. & Med. (in press).

<sup>54</sup>"Schönbaum, E., Davidson, M., Large, R. E., Bruce, W. G. Further Studies on the Metabolism of Glucose and the Formation of Corticosteroids In Vitro. Can. J. Biochem. and Physiol. 37:1209, 1959.

## CHAPTER II

### METHODS

It is clear that the solution to the problem of the control of the adrenocortical secretion has not as yet been reached, though it seems likely that any future theory will involve the availability of TPNH to the steroid transforming systems. It is also apparent that the mechanism of ACTH action must be defined in terms of increased growth of the tissue as well as enhanced hormone output. In this respect the observed limitation to the entry of substrates into the adrenal cell in the absence of ACTH must find its explanation in the final formulation.

It is pertinent to the significance of the present investigation to point out again that in spite of the emphasis on TPNH generation in theories of ACTH action, those phases of corticosteroidogenesis responsive to ACTH are not known to be dependent upon TPNH.

The detailed description of the methodology used in the investigation is arranged in sections covering processes concerned with tissue fractionation (A), incubation of the fractions with radiocholesterol (B), separation of the products of reaction (C) and finally, details of various quantitative procedures used in the course of the investigation (D).

Subcellular fractions of bovine adrenal cortex tissue were prepared by conventional methods of tissue homogenization and differential centrifugation, the resulting fractions being "operationally" defined in terms of the centrifugal force applied and the duration of spin.

The activity of the subcellular fractions in catalyzing the conversion of cholesterol to intermediates in the biosynthetic sequence of the corticosteroids was assessed by incubating the fractions with radioactive cholesterol and estimating the degree of conversion of the tracer to radioactive products. Cholesterol labelled with carbon-14 in position 4 (ring A) was used as the tracer substance, allowing the assumption of cholesterol side chain scission on the appearance of radioactive C-21 steroids. The products of reaction were separated from the unchanged cholesterol on silicic acid columns and tentatively identified on the basis of their chromatographic behavior in paper, absorption column and partition column systems.

## A. The Preparation of Cellular Fractions of Bovine

### Adrenal Cortex

#### 1. Choice of Tissue

The large size and ready availability of bovine adrenal glands made them a convenient source of adrenal tissue particularly suited to the present study, as the cortical and medullary components are easily separable. It should be emphasized, however, that it is impossible to obtain bovine tissue in as fresh condition as that from laboratory animals, owing to the time lag between the death of the animal and removal of the tissue. In spite of this very important objection, bovine glands were preferred to those of other species largely on account of the considerable amount of information available on the formation of corticosteroids by the perfused or homogenized bovine adrenal.<sup>1,2</sup> Another factor influencing the choice of tissue, though perhaps of secondary importance, was the low cholesterol content of bovine adrenals compared with laboratory animals such as the rat. In following cholesterol changes by means of radioactive tracer techniques, it is an advantage if the degree of dilution of the tracer by endogenous substance is minimal.

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<sup>1</sup>Hechter, O. and Pincus, G. Genesis of the Adrenocortical Secretion. *Physiol. Rev.* 34:459, 1954.

<sup>2</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. Some Aspects of the Biogenesis of Adrenal Cortical Hormones. *Recent Progr. Hormone Research*, 12:79, 1956.

## 2. Collection of the Glands

Bovine adrenal glands were collected from the abattoir as soon as possible after slaughter and placed in ice-cold saline (0.9% NaCl) or 0.44M sucrose solution for transport to the laboratory. The sucrose solution was originally used when the glands were required for experiments on the effect of cations on the fractionation procedures, and was continued thereafter in the belief that the hypertonic medium might minimize the uptake of water by the chilled tissue. However, no significant differences with respect to enzymatic activity were noted in the behavior of cellular fractions prepared from glands collected in either medium.

## 3. Preparation of the Tissue

In order to separate the cortical tissue from the medulla, the glands were first slit open longitudinally and the ~~lighter~~ colored medullary tissue removed from the under-lying cortex with a scalpel. This, and subsequent operations, were carried out on a glass plate chilled with ice. The cortex tissue was then scraped off the capsule and chopped finely using razor blades held in Spencer-Wells forceps. Particular care was taken to insure that no pieces of capsule were included in the tissue mince as even small amounts of capsule made subsequent homogenization difficult.

## 4. Preparation of the Tissue Homogenate

Apart from a few preliminary experiments, hypertonic (0.44M) sucrose solutions, with or without other additions, have been used as isolation media throughout the investigation in an attempt to maintain the morphological integrity of the mitochondrial fractions as far as possible (cf. Chapter IV). In some experiments, 0.44M sucrose containing 0.01M

(ethylenedinitrilo) tetracetic acid (EDTA), M/150 phosphate buffer (K salts) ph 7.4, 0.005M potassium fumarate and 0.005M niacinamide was used as the homogenization and isolation medium. In other experiments, 0.44M sucrose was used without additions.

Aliquots (10 gms.) of the chopped tissue were transferred to an all-glass homogenizer of the Potter-Elvehjem<sup>3</sup> type (internal measurements 170mm. by 25mm.) with a loose fitting pestle (55mm. in length). Twenty-five ml. of the homogenizing medium were added and, after thorough dispersion of the suspended tissue, six passes of the motor driven pestle were made, the rate of upward and downward strokes being kept as standardized as possible. If only mitochondrial fractions were required, the homogenate was centrifuged without further preparation, the connective tissue and nuclei being spun down together, but if a full fractionation was required the homogenate was first filtered through two thicknesses of nylon cloth in order to obtain the nuclei fraction reasonably free of debris.

##### 5. Fractionation of the Tissue Homogenate

Fractionation of the homogenate was carried out by differential centrifugation in a Spinco Model L preparative centrifuge, using rotors #21, #30, and #40. The rotor chamber was maintained at 5°C. and in between centrifuge runs the samples were kept cold by standing the centrifuge tubes in ice-water.

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<sup>3</sup>Potter, V. R. and Elvehjem, C. A. J. Biol. Chem. 114:495, 1936.

The homogenate was separated into the fractions commonly accepted as being most useful for distribution studies, and the rationale for this procedure and the centrifugation conditions necessary to achieve it, were taken from deDuve and Berthet<sup>4</sup>. In general, the centrifugal conditions have been worked out during studies of rat liver tissue and the assumption that such conditions are optimal for tissues from another source cannot be made. However, in the absence of definite information on the behavior of bovine adrenal cortex tissue during centrifugation, it was decided to follow closely the scheme advocated by deDuve and Berthet<sup>4</sup>. Similar conditions were adopted by Saba and Hechter<sup>5</sup> in their study of mitochondrial fractions of bovine adrenal cortex.

The centrifugal conditions used throughout the investigation are summarized in Table 1. The fractions are defined in terms of centrifugal force and duration of spin (g-mins.), the conditions used being those advocated by deDuve and Berthet<sup>4</sup> for 0.25M sucrose homogenates with an allowance made for the greater density and viscosity of the hypertonic medium employed in these studies.\*

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<sup>4</sup>deDuve, Chr. and Berthet, J. The Use of Differential Centrifugation in the Study of Tissue Enzymes. International Review of Cytology, eds. G. H. Bourne and J. F. Danielli (New York: Academic Press, Inc., 1954), III, 225.

<sup>5</sup>Saba, N. and Hechter, O. Cholesterol- $4\text{-C}^{14}$  metabolism in Adrenal Homogenates. Federation Proc. 14:775, 1955.

\*The calculation of the values for use with 0.44M sucrose homogenates is given in the Appendix.

TABLE 1

A SUMMARY OF THE CONDITIONS USED IN THE DIFFERENTIAL CENTRIFUGATION OF  
0.44M SUCROSE HOMOGENATES OF BOVINE ADRENAL CORTEX TISSUE

Fraction g-mins.	Rotor No.	Speed r.p.m.	Centrifugal Force g.	Time mins.	Remarks
36,000	21	3,500	1,200	30	Nuclei fraction
320,000	30	13,500	16,000	20	Should contain the bulk of mito- chondria
	30	12,500	13,500	20	Used for washing the pellets
12,600,000	40	40,000	105,000	120	Microsomal frac- tion

The homogenates prepared in 0.44M sucrose containing EDTA and other additions (supplemented sucrose media) were separated into four fractions: material not sedimented at 1200g (36,000 g-mins. fraction); material not sedimented at 1200g but forming a well packed pellet at 16,000g (320,000 g-mins. fraction); material representing the remaining particulates after removal of the first two fractions and sedimented at 105,000g (12,600,000 g-mins. fraction) and finally the remaining supernatant or nonparticulate fraction.

The fractions were chosen in the hope that the bulk of each of the recognized subcellular components would appear in one fraction. Thus the 36,000 g-mins. pellet should contain the nuclei; the 320,000 g-mins. fraction, mitochondria; with the material sedimented at 105,000g probably containing microsomes. The nonparticulate fraction would then contain the soluble enzyme systems.

In the case of homogenates prepared in pure sucrose media, identical centrifugation conditions were employed to those used in the case of the supplemented sucrose media, but in the pure sucrose homogenates the 320,000 g-mins. fraction was divided into two subfractions. The full procedure for the fractionation of a pure sucrose (0.44M) homogenate is given below.

The filtered homogenate from 20 gms. of tissue was placed in a single centrifuge tube (rotor #21) and fresh medium added to fill the tube (capacity 90 ml.). When the requisite number of tubes were filled, the head was centrifuged at 1200g for 30 mins., the time being measured from the time of attaining the required r.p.m. The supernatant was carefully decanted and the precipitate reserved for the 36,000 g-mins. fraction

(nuclei). The supernatant was then distributed between rotor #30 centrifuge tubes so that each tube contained the equivalent of 10 gms. of tissue. The volume was made up to capacity (35 ml.) with fresh medium and centrifugation carried out for 20 mins. at 16,000g, in order to sediment the 320,000 g-mins. fraction (mitochondrial). The supernatant was decanted and reserved for the isolation of later fractions. The pellet was resuspended in fresh medium with the aid of a motor driven glass-ball pestle which formed a loose fit with the walls of the centrifuge tube. The material was then sedimented at a centrifugal force 10 percent lower than that used to isolate the fraction as recommended by deDuve and Berthet<sup>4</sup> (13,500g:20 mins.). On decanting the supernatant, a part of the pellet sloughed away with it, a characteristic of the washed pellets derived from pure sucrose homogenates, but never seen in the case of supplemented sucrose homogenates. This loosely packed layer was removed as completely as possible by swirling the tubes with a small volume of fresh medium and decanting the loosened material. All the poorly packed material was collected and resedimented at 16,000g for 20 mins. to give the so-called "fluffy layer" fraction.<sup>4</sup> The firmly packed material in the 320,000 g-mins. pellet was washed once more with fresh medium, the very small amount of "fluffy" material being discarded with the supernatant. The supernatant from the original sedimentation of the 320,000 g-mins. pellets was then centrifuged in rotor #40 tubes at 105,000g for 120 mins. to sediment remaining particulate material (12,600,000 g-mins. fraction). The resulting supernatant formed the soluble fraction.

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<sup>4</sup>deDuve, Chr. and Berthet, J. International Review of Cytology, eds. G. H. Bourne and J. F. Danielli (New York: Academic Press, Inc., 1954), III, 225.

## B. Incubation of Subcellular Fractions with Cholesterol-4-C<sup>14</sup>

### 1. The Solubility of the Steroid Substrates

The incubation of aqueous tissue suspensions with lipid-soluble substrates presents a problem because of the very limited solubility of this class of substances in aqueous media. Various methods have been adopted to overcome this difficulty, such as the use of stable suspensions of the steroid in albumin<sup>6</sup>, in Tween 80 mixtures<sup>7</sup>, or the addition of the substrate in a suitable solvent which is both miscible with water and non-toxic to the tissue suspensions. Propylene glycol has been widely used for this purpose<sup>5</sup> as it is a reasonably good solvent for steroid substances and has been found to be inert under many circumstances. Some authors<sup>8</sup> recommend adding the steroid in propylene glycol solution to the tissue suspension, when the steroid precipitates out onto the surface of the particles. In the present investigation the incubation flasks were prepared before addition of the tissue suspension, so that they contained a thin film of the steroid in propylene glycol on the glass walls. This was achieved as follows:

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<sup>5</sup>Saba, N. and Hechter, O. Federation Proc. 14:775, 1955.

<sup>6</sup>Afinsen, C. B. and Horning, M. G. Enzymatic Degradation of the Cholesterol Side Chain in Cell-free Preparations. J. Am. Chem. Soc. 75: 1511, 1953.

<sup>7</sup>Whitehouse, M. W., Staple, E. and Gurin, S. Catabolism In Vitro of Cholesterol to Carbon Dioxide by Rat Liver Preparations. J. Biol. Chem. 234:276, 1959.

<sup>8</sup>Brownie, A. C. and Grant, J. K. The In Vitro Enzymatic Hydroxylation of Steroid Hormones. 1. Factors Influencing the Enzymic 11 $\beta$ -Hydroxylation of 11-deoxycorticosterone. Biochem. J. 57:255, 1954.

Propylene glycol was added to the incubation flask in sufficient quantity to give a final concentration in the medium of 0.4%. One ml. of a solution of the steroid in benzene was then added and the solvent evaporated off at 40-50°C. under a stream of nitrogen. Ten ml. of chloroform were then added to the flask and also evaporated off under nitrogen, leaving a thin film of propylene glycol steroid solution on the surface of the glass.

## 2. Incubation Media

Suspensions of the particulate fractions were prepared in buffered sucrose solutions, the tonicity of the sucrose varying with the purpose of the experiment, being either hypotonic (0.025M) or hypertonic (0.44M). The buffer used throughout was Sørensen's phosphate mixture<sup>9</sup> ( $K_2HPO_4$ - $KH_2PO_4$ ) at a final concentration of M/150 and pH 7.4. Magnesium ions were added in the form of  $MgSO_4$  and when required niacinamide was present in 0.005M concentration.

Suspensions of acetone-dried fractions were incubated in M/30 phosphate buffer, without sucrose, at a pH of 7.4 or 6.8.

Cofactors such as adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) were added to the suspension just prior to incubation in a small volume (0.5-1.0 ml.) of water. Whenever possible potassium salts were used and if necessary solutions of the cofactors were neutralized with KOH before addition to the suspension.

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<sup>9</sup>Hawk, P. B., Oser, B. L. and Summerson, W.H. Practical Physiological Chemistry, (12th ed.; Philadelphia: Blakiston Co., 1947), p23.

### 3. Incubation Technique

The particulate cellular fractions were suspended in the appropriate medium (complete except for the more labile components such as cofactors) by gentle mechanical homogenization using the glass-ball pestle employed in washing the mitochondrial pellets. The suspension was then added to the incubation flask, swirled to mix with the propylene glycol film and the cofactors added. When oxygen was used as the gas phase it was introduced by displacement with careful standardization of the rate of gas flow and the time of gassing. Incubations were carried out at 37-38°C. in a water bath equipped with a shaking device (100 oscillations per minute) and unless otherwise stated in the experimental account, the duration of incubation was 2 hours.

### C. Extraction and Purification of the Products of Reaction

#### 1. Definition of the Term "Products"

Scission of the cholesterol side chain between carbon atoms C-20 and C-22 results in C-21 steroid products which can be relatively easily separated from the unchanged cholesterol. It is convenient to make such a separation and refer to these C-21 steroids collectively as "products" when assessing the activity of a particular preparation. It must be emphasized, however, that molecular changes may, and probably do, occur within the cholesterol molecule as a result of enzymatic reactions without concomitant loss of the cholesterol side chain.<sup>10</sup> These compounds may not

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<sup>10</sup>Solomon, S. Levitan, P. and Lieberman, S. Proc. Can. Physiol. Soc. 20th Meeting, Montreal, 1956, p. 54.

be separated from the unchanged cholesterol by the same techniques used for separating cholesterol from the C-21 steroid products. With this reservation in mind, it is very useful to regard the appearance of radioactive C-21 steroids such as  $\Delta^5$ -pregnenolone and progesterone as an indication of the degree of side chain scission that has occurred, as these compounds could not have arisen without such an event having taken place.

## 2. Extraction Technique

Separation of the products of reaction and the unchanged cholesterol from the incubation medium was achieved by a single stage extraction method using a chloroform:methanol (2:1) mixture as a combined protein denaturant and lipid solvent reagent. At the end of the incubation period a volume of the extraction solvent equal to the volume of the incubation medium was added to the incubation flask and the contents well mixed. The mixture was then transferred to centrifuge tubes using a further volume of the solvent to wash out the contents of the incubation vessel. After thorough mixing of the solvent and aqueous phases in the tube with the aid of a glass rod with a flattened end, the tubes were centrifuged at 1,500 r.p.m. for 5 minutes. Two layers formed with an intermediate protein layer. The lower layer containing the extracted steroids was withdrawn by pipette and transferred to a round bottomed flask (glass joint). The extraction was repeated using one and a half volumes of the solvent and after centrifugation the lower layer was withdrawn and added to the first extract in the round-bottomed flask. The combined extracts were then taken to dryness under vacuum with a nitrogen capillary leak to avoid bumping. Provided the two layers were separated cleanly, the residue in the flask was free

from water and suitable for chromatographic analysis without further treatment. Occasionally, however, some water carried over with the solvent during the separation of the two phases, and if media containing a high concentration of sucrose were being extracted, a sticky residue of sucrose resulted after evaporation of the solvent. In these cases it was usually possible to obtain dry friable residues by the addition of a few milliliters of benzene (or toluene if much water was present) to the flask and evaporating off the water and solvent together.

### 3. Chromatographic Techniques

The separation of steroids of the polarity of progesterone (and greater) from cholesterol may be conveniently performed on columns of silicic acid. They have certain advantages over the more commonly used columns of silica gel, notably that interference and displacement effects due to phospholipids in the extracts seem to be less than in the case of silica gel columns, and furthermore their chromatographic characteristics may be varied considerably by adjustment of the water content of the silicic acid.

(a) Silicic Acid Chromatography. Silicic acid preparations usually contain a high proportion of particles of very small size, which when the material is made into columns prevent optimum rates of flow of the developing solvents. These fine particles may conveniently be removed at the same time as heavy metal contaminants by washing with strong hydrochloric acid.

Purification of silicic acid: The method adopted has been

described by Hanahan, Dittmer and Warashina.<sup>11</sup> The silicic acid (2 lbs. Mallinckrodt A.R.) was stirred into 10 liters of 10N HCl and the suspension allowed to settle overnight. The HCl was siphoned off and replaced with 10 liters of distilled water and vigorously stirred for about 5 minutes. After standing for 2-3 hours, the bulk of the silicic acid had sedimented leaving a "milky" supernatant containing the finer particles. The supernatant was siphoned off and discarded, the water wash being repeated until the wash was free from acid reaction (litmus) and the whole of the remaining material sedimented in about 30 minutes leaving a clear supernatant. To remove the last water wash, the contents of the vessel were well stirred and filtered through Whatman No. 1 paper in a 23cm. Buchner funnel. After removal of the water, air was drawn through the filter cake for approximately one hour. The filter cake of silicic acid was then transferred to a glass dish and spread in a layer not more than 1" deep and left in a drying oven at 40-50°C. overnight (ca. 16 hrs.). At this stage it was convenient to store the partly dried powder until required for a specific purpose. As the water content of the silicic acid sample markedly affects its chromatographic properties, careful standardization of the final drying process is important. For all the work in this investigation the partly dried silicic acid was placed in a glass dish (300 gms. in a 7-1/2" by 12" dish) and heated at 110°C. for 24 hours. When kept in covered containers the silicic acid retained its chromatographic properties for several months.

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<sup>11</sup>Hanahan, D. J., Dittmer, J. C. and Warashina, E. A Column Chromatographic Separation of Classes of Phospholipids. *J. Biol. Chem.* 228:685, 1957.

Preparation of the column: Three grams of the purified dried silicic acid were stirred into n-hexane to form a slurry which was poured into glass columns 0.8cm. diameter and 30cms. in length, fitted with a reservoir (100 ml.), glass wool plug and tap. The columns were then packed by means of pressure from a nitrogen cylinder.

The dried residues from the chloroform:methanol extracts were washed in the flask with 10-20 ml. n-hexane and the solvent wash removed under vacuum to remove any traces of the extraction solvents. The washed extract was then loaded onto the column using three 5 ml. portions of n-hexane, each transfer being allowed to percolate into the silicic acid before addition of the next transfer.

Development of the column: Fifty milliliters of n-hexane were then added to the reservoir and sufficient pressure from a nitrogen source applied in order to obtain a flow rate of about 5 mls. per min. The n-hexane effluent contained a considerable proportion of yellow carotenoid material and no radioactivity and was therefore discarded. The flask which contained the extract was then washed with 2-3 ml. of benzene and the benzene wash loaded onto the column. After percolation of the extract into the column, 200 ml. of benzene were added in two portions to the reservoir, each being passed through the column under pressure. The benzene effluent contained the unchanged cholesterol and products less polar than progesterone. After the last of the benzene had passed through the column, 150 ml. of ethyl acetate were added and the flow rate adjusted with the nitrogen pressure as before. The ethyl acetate extract contained all the radioactivity not accounted for in the benzene fraction and was evaporated to dryness under reduced pressure, transferred to tubes for storage,

assay of radioactivity or further purification by paper chromatography.

Measurement of radioactivity: The material eluted by ethyl acetate from the silicic acid column was dissolved in methanol and suitable aliquots placed on aluminum planchettes and the solvent removed with gentle heating. The radioactivity was measured in a windowless gas flow counter (Tracerlab, Inc., Model SC-16p). The proportion of the radioactivity added to the incubation found in the ethyl acetate fraction, expressed as a percentage, was taken as a measure of the cholesterol-4-C<sup>14</sup> transformation. Trial experiments in which 200,000 c.p.m. cholesterol-4-C<sup>14</sup> were added to similar columns showed a value of 1-3,000 c.p.m. in the ethyl acetate fraction, representing a blank value for the operation of 0.5-1.5%. A value of 1.5% was therefore subtracted routinely from all values obtained.

The extracts obtained from acetone dried fractions contained negligible amounts of endogenous cholesterol and could therefore be fractionated on smaller silicic acid columns. In these cases columns were prepared with 1 gm. of silicic acid in 0.8mm. diameter columns. Although they were prepared in n-hexane, as in the case of the larger columns, the extracts were loaded in benzene solution and the n-hexane eluant omitted. The cholesterol was eluted with 100 ml. of benzene and the products by 25 ml. ethyl acetate and 5 ml. of chloroform:methanol (2:1) collected together as one fraction.

(b) Paper Chromatography. In some instances the ethyl acetate fraction from the silicic acid columns was subjected to further separation using the paper systems described by Burton, Zaffaroni, and

Keutmann.<sup>12,13</sup>

In the experiments designed to estimate the specific activity of the radioprogestosterone formed by incubation of cholesterol-4-C<sup>14</sup> with fresh preparations of the subcellular fractions, the formamide/ligroin system was used for preliminary separation. As the radioactivity was rather low, progesterone-16-H<sup>3</sup> was added to the crude ethyl acetate extract prior to loading on paper impregnated with formamide to facilitate location of the progesterone zone. The weight of progesterone added was negligible owing to the high specific activity of the tritiated tracer. After running the papers in ligroin for 1-1/2-2 hours, they were dried in stream of warm air and the radioactive zones located using a gas flow automatic windowless paper chromatogram scanner (Atomic Instrument Co., Model RSC-5A). The zones were eluted with methanol and further purified on celite columns as described later.

The reaction products from incubations of acetone dried subcellular fractions with cholesterol-4-C<sup>14</sup> were separated by paper chromatography of the ethyl acetate eluate from silicic acid columns. Progesterone and  $\Delta^5$ -pregnenolone zones were located after application of the extracts to either formamide or propylene glycol paper and running in ligroin for 1-1/2 - 2 hours. In these separations the count was sufficiently high to dispense with the addition of tritiated tracers at this stage. After

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<sup>12</sup>Burton, R. B., Zaffaroni, A. and Keutmann, E. H. Paper Chromatography of Steroids. II Corticosteroids and Related Compounds. J. Biol. Chem. 188:763, 1951.

<sup>13</sup>Zaffaroni, A. and Burton, R. B. Identification of Corticosteroids of Beef Adrenal Extract by Paper Chromatography. J. Biol. Chem. 193:749, 1951.

location of the zones as described above, the radioactive material was eluted and further purified on celite partition columns.

The benzene eluant from the silicic acid columns was, in some experiments, chromatographed using the reversed phase paper system described by Martin<sup>14</sup>, in which Whatman No. 2 paper is impregnated with a 12% solution of odorless kerosene (B.P. 325) in skellysolve C. The extracts were dissolved in a minimal volume of ethyl acetate, applied to the paper and allowed to equilibrate in the chromatography tank, without addition of the mobile phase, for about 12 hours. Aqueous n-propanol (60%) was used as the developing solvent and the chromatogram was left to run until the solvent reached the end of the paper (ca. 12 hours). Under these conditions cholesterol showed an  $R_f$  value of 0.5,  $\Delta^4$ -cholestenone 0.3, and progesterone 0.85. The radioactive zones were located and eluted as described above.

(c) Celite Partition Column Chromatography. Further purification and characterization of the radioactive substances eluted from the paper chromatograms was achieved by the use of partition column chromatography on Celite columns impregnated with a methanol:water (5:1) stationary phase. The preparation and running of the columns was carried out essentially as described by Simpson and Tait.<sup>15</sup>

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<sup>14</sup>Martin, R. P. Reversed Phase Paper Chromatography and Detection of Steroids of the Cholesterol Class. *Biochim. Biophys. Acta*, 25:408, 1955.

<sup>15</sup>Simpson, S. A. and Tait, J. F. Recent Progress in Methods of Isolation, Chemistry and Physiology of Aldosterone. *Recent Prog. Hormone Res.* 11:183, 1955.

For the chromatography of progesterone and  $\Delta^5$ -pregnenolone zones eluted from paper chromatograms, the Celite columns were prepared as follows: 12 gms. of Celite 545 (Johns-Manville) were intimately mixed with 6 ml. of methanol:water (5:1) and then made into a slurry with skellysolve C saturated with the methanol:water mixture. The slurry was packed into 1cm. diameter columns using a perforated plate tamper to ensure uniform packing. The columns were loaded with the sample dissolved in a minimal volume of mobile phase, which consisted of skellysolve C saturated with methanol:water. Sudan 111 dye added to the sample served as a useful indicator of the position of the solvent front and all elution volumes quoted have been calculated from the appearance of the dye in the effluent. Two ml. cuts were made using a Technicon drop counter fraction collector. Progesterone was eluted by 16 ml. of mobile phase and  $\Delta^5$ -pregnenolone by 30 ml.

After the samples had been run on the celite columns they were rerun together with tritiated tracer on a second Celite column prepared as for the first columns. One ml. cuts were taken and the radioactivity in each fraction estimated in terms of count due to  $C^{14}$  (flow gas counter with micromil end window, Nuclear, Chicago, Model D-47), and count due to both  $C^{14}$  and  $H^3$  (windowless flow gas counter). Distribution diagrams of the  $C^{14}$  and  $H^3$  count were plotted to show the degree of identity of the sample with the authentic tritiated tracer substance. Further purification was achieved by rerunning the mixed sample and tritiated tracer on similar columns after acetylation. The acetylation was carried out by dissolving the mixed sample in 5 drops of pyridine and adding 3 drops of acetic anhydride. The reaction was allowed to proceed overnight at room temperature,

after which 2 ml. of methanol were added and the solvent blown off by a stream of nitrogen at 40°C. The methanol wash was repeated twice more to ensure removal of the pyridine.

The acetylated  $\Delta^5$ -pregnenolone was eluted from the Celite column with 6 ml. of mobile phase, the estimation of the relative distribution of  $C^{14}$  and  $H^3$  being carried out as described above.

#### D. Miscellaneous Methods Used in the Investigation

##### 1. The Quantitative Determination of Cholesterol.

For most of the experiments where the cholesterol content of the cellular fractions was required, a value for the total cholesterol was sufficient, i.e. free plus esterified cholesterol. For such a purpose it was convenient to dissolve the benzene fraction from the silicic acid column in anhydrous chloroform and carry out the cholesterol determination without prior precipitation with digitonin according to a modification of the method of Bloor, Pelkan and Allen.<sup>16</sup> A suitable aliquot of the chloroform solution of the sample was diluted to 5 ml. with chloroform in photometer tubes (Klett-Summerson) and 1.0 ml. acetic anhydride added. The tube was read against a blank made up of 5 ml. chloroform and 1.0 ml. of acetic anhydride in the Klett-Summerson photoelectric absorbtimeter using a violet filter #420. 0.1 ml. concentrated sulphuric acid were then added and the contents of the tube well mixed. The color was allowed to develop for 15 minutes in the dark and then read in the photometer against the

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<sup>16</sup>Bloor, W. R., Pelkan, K. F. and Allen, D. M. Determination of Fatty Acids (and Cholesterol) in Small Amounts of Blood Plasma. J. Biol. Chem. 52:191, 1922.

blank tube similarly treated. The difference between the first and second readings gave the concentration of cholesterol in the aliquot when compared with standard solutions of cholesterol treated as for the samples.

For more precise analysis of the cholesterol content, the method of Schonheimer and Sperry as modified by Sperry and Webb<sup>17</sup> was used. In this procedure the total and free cholesterol fractions were determined after digitonin precipitation. For the mitochondrial fraction, the values obtained by either method were within the same range.

## 2. The Quantitative Determination of Protein

The protein content of the fractions was determined on an aliquot of the tissue fraction suspension at the end of the incubation period. In taking the aliquot at this point rather than before incubation, two possible errors were avoided. First, the suspension at the end of incubation was in thoroughly dispersed form thus making the aliquot more representative, and secondly, it was convenient in many cases to add the whole of one mitochondrial pellet to an incubation flask containing the steroid in propylene glycol and the removal of an aliquot at this stage before thorough mixing of the suspension and steroid substrate was undesirable. Control experiments failed to show any detectable change in the protein content of the medium during incubation.

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<sup>17</sup>Sperry, W. M. and Webb, M. A. Revision of the Schonheimer-Sperry Method for Cholesterol Determination. *J. Biol. Chem.* 187:97, 1950.

The procedure adopted is due to Lowry et al.<sup>18</sup> and was carried out as follows: 1 ml. of the incubation medium was withdrawn and mixed with 1 ml. of 2N NaOH. A suitable aliquot, generally 0.2 ml. for the mitochondrial fraction, was placed in a Klett photometer tube and the volume made up to 0.5 ml. with the addition of 1N NaOH. 0.5 ml. of distilled water was then added. A standard solution of albumin (150 ugs. in 0.5 ml. water) was added to a photometer tube, followed by 0.5 ml. 1N NaOH and a blank tube containing 0.5 ml. water and 0.5 ml. 1N NaOH prepared. All the tubes received 5 ml. of a mixture of 2% sodium carbonate and a copper reagent mixed just before use in the proportion of 50 parts  $\text{Na}_2\text{CO}_3$  to 1 part of copper reagent. The copper reagent consisted of 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 1% sodium potassium tartrate. 0.5 ml. of Folin and Ciocalteu's<sup>19</sup> reagent (diluted until 1N with respect to acid) was added and the contents of the tubes mixed immediately. The colors developed were read in a Klett-Summerson photometer after 30 minutes using a red filter (#660).

### 3. The Estimation of Oxygen Uptake and Oxidative Phosphorylation

Oxygen uptake measurements were made using the conventional manometric technique of Warburg as described by Umbreit, Burris and Stauffer.<sup>20</sup> The concentration of the reactants and the conditions under which the

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<sup>18</sup>Lowry, O.H., Rosebrough, N.J., Farr, A. L. and Randall, R. J. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* 193: 265, 1951.

<sup>19</sup>Folin, O. and Ciocalteu, V. *J. Biol. Chem.* 73:627, 1927.

<sup>20</sup>Umbreit, W.W., Burris, R.H. and Stauffer, J.F. *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* (Minneapolis: Burgess Publishing Co.).

oxygen utilization was estimated are given in the procedural account of these particular experiments.

As an estimate of the capacity of the mitochondrial fractions to carry out coupled oxidative phosphorylation, the oxygen uptake and disappearance of inorganic phosphorous were measured in some experiments, the results being expressed as a ratio of atoms of phosphorous taken up to oxygen consumed. The method employed has been described by Hatefi and Lester.<sup>21</sup> The procedure consists essentially of the estimation of the inorganic phosphate remaining at the end of the incubation period during which oxygen uptake is measured manometrically, and by difference the phosphorous taken up (esterified) is calculated. A glucose-hexokinase trap is added to prevent the ATP formed from returning inorganic phosphate to the pool by the action of ATP-ase. In the presence of this "trap" the ATP forms glucose-6-PO<sub>4</sub> with the added glucose and hexokinase, and the ADP so formed is available for mitochondrial phosphorylation to ATP. The details of the procedure are included in the account of the particular experiments and the inorganic phosphate was determined by the method of Fiske and Subbarow.<sup>22</sup>

#### 4. The Estimation of Progesterone by Ultraviolet Spectrophotometry

Steroids containing a  $\Delta^4$ -3 ketone grouping show a strong absorption in the ultraviolet wave band at 240 m $\mu$ . In sufficiently purified

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<sup>21</sup>Hatefi, Y. and Lester, R. L. Studies on the Mechanism of Oxidative Phosphorylation. III Phosphorylating Particle Types from Beef Heart. *Biochim. Biophys. Acta*, 27:88, 1958.

<sup>22</sup>Fiske, C. H. and Subbarow, Y. *J. Biol. Chem.* 66:375, 1925.

samples this property allows the estimation of progesterone in concentrations as low as 5-10 ugs. per 3 ml. The extract is dissolved in purified methanol and an absorption spectrogram of the substance prepared against a methanol blank using the Beckman DK-1 recording spectrophotometer.

For the estimation of smaller amounts of progesterone advantage was taken of the intense absorption shown by the thiosemicarbazide derivative of progesterone at 299 mu. The reaction was carried out as described by Bush,<sup>23</sup> as follows:

The samples were dissolved in 0.1 ml. methanol and after the addition of 0.1 ml. of a solution of thiosemicarbazide in glacial acetic acid, the mixture was heated for 20 minutes at 95°C. After cooling to room temperature, 3 ml. of methanol were added and the absorption measured in the Beckman DK-1 spectrophotometer at 299 mu. and compared with that obtained with authentic progesterone. A high degree of purity is demanded in the reagents used. The methanol was allowed to stand over m-phenylenediamine (4 gms. per liter) for about one week, refluxed for 2 hours and then distilled through a four pear column. The thiosemicarbazide was recrystallized from water, and dried over CaCl<sub>2</sub> under vacuum at 28°C. The acetic acid was refluxed 1 hour with anhydrous chromium trioxide (3 gms. per 100 ml.) and then distilled through a fractionating column.

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<sup>23</sup>Bush, I. E. Use of Thiosemicarbazide in Microestimation and Recognition of Steroid Ketones. Federation Proc. 12:186, 1953.

## 5. Determination of Na<sup>+</sup> and K<sup>+</sup> by Flame Photometry

Analysis of the Na<sup>+</sup> and K<sup>+</sup> content of tissue homogenates was performed by flame photometry (Perkin Elmer: Model No. 52C) using lithium as an internal standard. Aliquots of the tissue were extracted by heating (boiling water bath) for 20 minutes and a clear extract prepared by filtration from which suitable aliquots were diluted for assay.

### E. Sources of Special Compounds Used in the Investigation

Cholesterol-4-C<sup>14</sup>: Beta Laboratories, Inc.; specific activity 4.8  $\mu\text{c./mg.}$ ; ran as a single zone on paper chromatography in propylene glycol/ligroin<sup>13</sup> and the reversed phase system of Martin<sup>14</sup>.

Progesterone-16-H<sup>3</sup>: Medical Research Council, London, England; specific activity approximately 0.3 mC/mg.

$\Delta^5$ -Pregnenolone-7-H<sup>3</sup>: generously supplied by Dr. Marcel Gut, Worcester Foundation; specific activity 20.0 mc/mg.

$\Delta^4$ -Cholestenone-4-C<sup>14</sup>: Tracerlab, Inc.; specific activity 18.4  $\mu\text{c./mg.}$ ; ran as single zone on paper chromatography<sup>14</sup>.

Diphosphopyridine Nucleotide (DPN): Nutritional Biochemicals; chromatographically pure.

<sup>13</sup>Zaffaroni, A. and Burton, R. B. J. Biol. Chem. 193:749, 1951.

<sup>14</sup>Martin, R. P. Biochim. Biophys. Acta, 25:408, 1955.

Adenosine Triphosphate (ATP): Nutritional Biochemicals; dipotassium salt  
(99% + ATP).

Triphosphopyridine Nucleotide (TPN): Sigma; by phosphorylation of yeast  
(95-100%).

Reduced Triphosphopyridine Nucleotide (TPNH): Sigma; sodium salt (80%).

Adenosine 3',5'-monophosphate: generously supplied by Dr. David Lipkin,  
Department of Chemistry, Washington University,  
St. Louis, Missouri.

Glucose-6-phosphate (G6P): Sigma; dipotassium salt (98-100%).

Glucose-6-phosphate Dehydrogenase (G6P·DH): Sigma; Type II, 800 Kornberg  
units per gram.

Hexokinase: Sigma; Type II, approximately 50,000 K.M. units per gram.

CHAPTER III

EXPERIMENTAL STUDIES

In the following account of the experimental studies the material is arranged to present, en bloc, results from a variety of experiments which provide information on the requirements of cholesterol side chain scission by one fraction of bovine adrenocortical tissue homogenates (Section A). Having established some of these requirements, the account describes experiments expressly concerned with the location of this enzymatic activity within the cell homogenate (Section B). Finally, results are presented from a number of experiments which have as a common denominator the investigation of possible relationships between cholesterol side chain cleavage activity and mitochondrial function (Section C).

A. The Requirements for Cholesterol Side Chain Cleavage  
by a Particulate Fraction Derived from Bovine  
Adrenal Cortex Tissue Homogenates

From a consideration of previous work (cf. Chapter I) it appeared likely that the mitochondrial fraction of bovine adrenocortical tissue homogenates contained the necessary enzymatic machinery for the conversion of cholesterol to early intermediates in the biosynthetic pathway of corticosteroid formation. Therefore, in the first series of experiments described in this section, the 320,000 g-min. fraction isolated from supplemented sucrose homogenates was studied from the point of view of minimal requirements for the enzymatic removal of the cholesterol side chain to form C-21 steroid products.

1. Experiments on the 320,000 g-min. Fraction  
Derived from Supplemented Sucrose Homogenates

The experiments described in this section were all carried out on fresh (intact) preparations of the 320,000 g-min. fraction in contrast to other experiments described later, where acetone dried preparations were examined for cholesterol conversion activity.

(a) Requirements for Conversion of Cholesterol-4-C<sup>14</sup> to Radioactive Products. The fraction of the cell homogenate used in these studies was isolated from a homogenate of adrenocortical tissue in 0.44M sucrose containing EDTA (0.001M); fumarate (0.005M); niacinamide (0.005M); and phosphate buffer M/150, pH 7.4. After removal of the 36,000 g-min. fraction together with cell debris, the 320,000 g-min. fraction was isolated and washed as described in Chapter II. No "fluffy layer" was obtained under these conditions. The washed pellets, each equivalent to 10 gms. of cortex tissue, were suspended in 40 ml. of 0.025M sucrose (hypotonic) containing 0.005M niacinamide and M/150 phosphate buffer pH 7.4. Other additions were made as required, Mg<sup>++</sup>, fumarate or other citric acid cycle intermediate being included in the solution used to suspend the pellet, while DPN, ATP, TPN, G6P or G6P·DH were added in a small volume of water (0.2-0.5 ml.) after addition of the suspension to incubation flasks containing cholesterol-4-C<sup>14</sup> (35 µgs., 200,000 c.p.m) in 0.15 ml. of propylene glycol. After gassing with oxygen, the suspensions were incubated with shaking at 37°C. for 2 hours. At the end of the incubation period, the unchanged cholesterol and radioactive products were extracted with chloroform:methanol (2:1) and the extracts fractionated on silicic acid columns as described in Chapter II. The radioactivity found in the ethyl

acetate fraction from the silicic acid columns was expressed as a percentage of the total count added to the incubation and a process blank value of 1.5% subtracted from each determination.

The combined results of a number of such experiments are recorded in Table 2, where the composition of the incubation medium is shown together with the percentage conversion of cholesterol-4-C<sup>14</sup> achieved. It will be seen that Mg<sup>++</sup> (0.005M) and fumarate (0.005M) together supported appreciable conversion of the radiocholesterol (9.4%) but that no conversion took place with Mg<sup>++</sup> alone. The activity found in the presence of fumarate, without added Mg<sup>++</sup> (3.2%), may have been due to the release of bound Mg<sup>++</sup> from the mitochondria in the preparation as has been shown to occur by Baltchevsky.<sup>1</sup> The addition of DPN (0.5mM) and ATP (1.0mM) to media containing fumarate and Mg<sup>++</sup> resulted in some stimulation of the cholesterol-4-C<sup>14</sup> conversion (12.4%), though TPN (0.5mM) was somewhat more effective than DPN plus ATP, supporting a conversion of 16.4%. The addition of DPN and ATP may be regarded as the equivalent of adding TPN, as in the presence of Mg<sup>++</sup> ATP can phosphorylate DPN to form TPN, provided the necessary enzyme system is present.<sup>2</sup> However, neither DPN plus ATP nor TPN are effective in supporting cholesterol-4-C<sup>14</sup> conversion in the absence of fumarate (cf. Table 2). The reduced form of triphosphopyridine nucleotide (TPNH) when added in the presence of fumarate and Mg<sup>++</sup> stimulated the reaction to much the same extent (11.6%) as DPN plus ATP or TPN,

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<sup>1</sup>Baltchevsky, H. Mitochondrial Respiratory Control and Phosphorylative Activities in a Magnesium-free Medium. *Biochim. Biophys. Acta*, 25:382, 1957.

<sup>2</sup>Kornberg, A. Enzymatic Synthesis of Triphosphopyridine Nucleotide. *J. Biol. Chem.* 182:805, 1950.

TABLE 2

THE REQUIREMENTS FOR THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup> TO RADIO-ACTIVE PRODUCTS BY THE 320,000 G-MIN. FRACTION (MITOCHONDRIAL) FROM SUPPLEMENTED SUCROSE HOMOGENATES OF BOVINE ADRENAL CORTEX TISSUE (10 GMS.)

Additions to the Basic Medium*	Cholesterol-4-C <sup>14</sup> Conversion in %
Mg <sup>++</sup> (0.005M)	0
Fumarate (0.005M)	3.2
Mg <sup>++</sup> + fumarate	9.4
Mg <sup>++</sup> + fumarate + DPN(0.5mM) + ATP(1.0mM)	12.4
Mg <sup>++</sup> + fumarate + TPN(0.5mM)	16.4
Mg <sup>++</sup> + fumarate + TPNH(0.5mM)	11.6
Mg <sup>++</sup> + DPN + ATP	1.2
Mg <sup>++</sup> + TPN	0
Mg <sup>++</sup> + TPNH	0
Mg <sup>++</sup> + TPN(0.1mM) + G <sub>6</sub> P(0.005M) + G <sub>6</sub> P·DH(1.7units)	13.1
Mg <sup>++</sup> + G <sub>6</sub> P + G <sub>6</sub> P·DH	0
Mg <sup>++</sup> + DPN + G <sub>6</sub> P + G <sub>6</sub> P·DH	0
Mg <sup>++</sup> + DPN + ATP + G <sub>6</sub> P + G <sub>6</sub> P·DH	4.3

\*Basic medium: 0.025M sucrose containing M/150 phosphate buffer pH 7.4.

but neither ATP plus DPN, nor TPN, nor TPNH were effective in supporting conversion in the absence of fumarate. Even when the TPNH was added serially throughout the incubation in an attempt to offset its rapid oxidation by mitochondrial enzymes present in the preparation, no conversion of cholesterol-4-C<sup>14</sup> was obtained.

The apparent requirement for fumarate was completely met, however, by the addition of glucose-6-phosphate (G6P) at 0.005M, its dehydrogenase (G6P·DH) 1.7 Kornberg units,<sup>2</sup> and TPN (0.1mM) in the presence of Mg<sup>++</sup>, when a conversion of 13.1% was obtained. The addition of TPN was essential for the support of cholesterol conversion by G6P and its dehydrogenase, as no conversion was obtained when TPN was omitted. The TPN could be replaced to only a limited extent (4.3%) by DPN and ATP, and not at all by DPN alone. The relative ineffectiveness of DPN and ATP in this case was probably due to the loss of ATP by the high ATP-ase activity and lack of ATP synthesis that might be expected to prevail in such a preparation incubated in the absence of an oxidizable substrate.<sup>3</sup>

It is apparent from the results of the experiments just described that the requirements for the conversion of cholesterol to products bear a close resemblance to those of the 11 $\beta$ -hydroxylase activity of mitochondrial fractions of adrenal cortex tissue discussed in Chapter I.

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<sup>2</sup>Kornberg, A. J. Biol. Chem. 182:805, 1950.

<sup>3</sup>Hunter, F.E. Jr. Oxidative Phosphorylation During Electron Transport. in Phosphorus Metabolism, eds. W. D. McElroy and B. Glass, (Baltimore: The Johns Hopkins Press, 1951), p. 297.

11 $\beta$ -Hydroxylation activity, like most of the steroid hydroxylating systems studied, has been shown to be dependent upon an adequate supply of TPNH<sup>4,5</sup> and it would appear likely that the cholesterol-4-C<sup>14</sup> conversion supported by the G6P-TPNH generating system was due to the continuous supply of this cofactor. The role of fumarate in 11 $\beta$ -hydroxylation has also been suggested to be due to its ability to generate TPNH, either via malic decarboxylase<sup>4</sup> or through the action of malic dehydrogenase and transhydrogenase.<sup>5</sup> It is probable that fumarate, or other citric acid cycle intermediates that can give rise to fumarate, may play a similar role in the cholesterol conversion system. It is clear, however, that even if both systems (i.e. fumarate and G6P) have a common mode of action as TPNH generators, they also have a fundamental difference. TPNH production by the fumarate system involves enzyme systems known to be present in mitochondria, and in this sense might be described as arising from an internal generating system. In contrast, the G6P system is certainly an external TPNH generating process. This distinction will be referred to again later in this chapter (Section C).

(b) The Replacement of Fumarate by Other Citric Acid Cycle Intermediates. The specificity of fumarate as a part of the "internal" TPNH generating system was examined by substituting other citric acid cycle intermediates for fumarate in incubations of the 320,000 g-min.

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<sup>4</sup>Grant, J. K. The In Vitro Enzymic Hydroxylation of Steroids 4. The Role of Fumarate and Triphosphopyridine Nucleotide in the Enzymic 11 $\beta$ -hydroxylation of 11-deoxycorticosterone. *Biochem. J.* 64:559, 1956.

<sup>5</sup>Sweat, M. L. and Lipscombe, M. D. A Transhydrogenase and Reduced Triphosphopyridine Dinucleotide Involved in the Oxidation of Desoxycorticosterone to Corticosterone by Adrenal Tissue. *J. Am. Chem. Soc.* 77:5185, 1955.

preparation with cholesterol-4-C<sup>14</sup> under conditions otherwise identical to those already described. The results obtained by incubating the preparation with succinate,  $\alpha$ -ketoglutarate, isocitrate or citrate in place of fumarate are shown in Table 3, where the percentage conversion of the cholesterol-4-C<sup>14</sup> is shown for each substrate tested. It can be seen that no conversion occurred in the absence of an oxidizable substrate and that both succinate (8.7%) and  $\alpha$ -ketoglutarate (8.4%) were equally as effective as fumarate (7.6%) in the absence of added cofactors. The conversion obtained with fumarate fortified with DPN and ATP (14.1%) is included for comparison and represents a greater stimulation by cofactor than that usually found, possibly reflecting a relatively lower content of endogenous cofactor in the particular preparations used in these experiments. Isocitrate supported little conversion (1.7%) unless supplemented by the addition of TPN, when the conversion (7.4%) was similar to that obtained in the presence of fumarate.

Thus it would appear that fumarate can be replaced by several other citric acid cycle intermediates and therefore is not necessarily a specific requirement of the "internal" TPNH generating system. However, it should be pointed out that both  $\alpha$ -ketoglutarate and succinate would give rise to fumarate in a preparation containing reasonably intact mitochondrial enzyme systems.

TABLE 3

THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup> TO RADIOACTIVE PRODUCTS  
 BY THE 320,000 G-MIN. FRACTION FROM SUPPLEMENTED  
 SUCROSE HOMOGENATES IN THE PRESENCE OF  
 VARIOUS CITRIC ACID CYCLE INTERMEDIATES

Additions to the Basic Medium*	Cholesterol-4-C <sup>14</sup> Conversion in %
None	0
Fumarate (0.005M)	7.6
Fumarate + DPN(0.5mM) + ATP(1.0mM)	14.1
Succinate (0.005M)	8.7
$\alpha$ -Ketoglutarate (0.005M)	8.4
Isocitrate (0.005M)	1.7
Isocitrate + TPN (0.5mM)	7.4
Citrate (0.005M)	0.9

\*Basic medium: 0.025M sucrose containing M/150 phosphate buffer pH 7.4, 0.005M niacinamide and 0.005M Mg<sup>++</sup>.

(c) Cholesterol-4-C<sup>14</sup> Conversion under Aerobic and Anaerobic

Conditions. The scission of the cholesterol side chain, being an oxidative process, it was of interest to compare the ability of the preparations to catalyze the reaction under both aerobic and anaerobic conditions.

Preparations of the 320,000 g-min. fraction were made as in the previous studies and incubated with cholesterol-4-C<sup>14</sup> in hypotonic sucrose (0.025M) containing M/150 phosphate buffer pH 7.4 0.005M Mg<sup>++</sup> and either 0.005M succinate or fumarate. One set of incubations were carried out under oxygen as in the previous experiments, another set were incubated in air and a third under nitrogen in the presence of an artificial electron acceptor<sup>6</sup>, triphenyltetrazolium chloride (TPTZ) at a concentration of 0.25%. The results obtained are shown in Table 4, where the percentage conversion of the radioactive cholesterol is given together with the substrate and gas phase used. It will be seen that in the presence of fumarate the conversion of cholesterol-4-C<sup>14</sup> in air (7.4%) was very similar to that in oxygen (8.0%) and that the values in the presence of succinate were almost identical. On the other hand, neither fumarate or succinate supported cholesterol-4-C<sup>14</sup> conversion under nitrogen and in the presence of TPTZ, despite the concurrent reduction of the tetrazolium salt to formazan as indicated by the intensity of the colored product formed.

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<sup>6</sup>Quastel, J. H. Use of Artificial Electron Acceptors in the Study of Dehydrogenases. in Methods in Enzymology, eds. S. P. Colowick and N. O. Kaplan (New York: Academic Press, Inc., 1957), IV, p. 329.

TABLE 4

THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup> TO RADIOACTIVE PRODUCTS BY  
THE 320,000 G-MIN. FRACTION FROM SUPPLEMENTED SUCROSE  
HOMOGENATES UNDER AEROBIC AND ANAEROBIC CONDITIONS

Additions to the Basic Medium*	Gas Phase	Cholesterol-4-C <sup>14</sup> Conversion in %
Fumarate (0.005M)	Oxygen	8.0
Succinate (0.005M)	"	8.7
Fumarate	Air	7.4
Succinate	"	7.4
Fumarate + TPTZ(0.25%)	Nitrogen	0
Succinate + TPTZ	"	0

\*Basic medium: 0.025M sucrose containing M/150 phosphate buffer  
pH 7.4 and 0.005M Mg<sup>++</sup>

Thus it would appear that aerobic conditions are required for the conversion of cholesterol-4-C<sup>14</sup> to radioactive products by the 320,000 g-min. fraction.

The results so far obtained indicated that the conversion of cholesterol-4-C<sup>14</sup> to radioactive products by the 320,000 g-min. fraction from supplemented sucrose homogenates, was stimulated by the presence of an active TPNH generating system, which could be either (1) external, such as the glucose-6-phosphate system, or (2) internal, as in the fumarate supported reaction. In the case of the fumarate supported reaction, the pyridine nucleotide cofactors did not have to be added for conversion of radiocholesterol to occur, and if they were involved in cholesterol side chain scission, must have been contained in the preparation. The possibility existed that the reaction cholesterol to products might be stimulated by a TPNH generating system, solely by virtue of increased hydroxylation of C-21 steroids formed from the cholesterol-4-C<sup>14</sup> by processes not dependent upon TPNH. Therefore it was necessary to obtain preparations of the 320,000 g-min. fraction depleted of their cofactor content in order to be able to demonstrate a dependence on added pyridine nucleotide and its maintenance in the reduced form for the conversion of cholesterol to products. Attempts to prepare such a fraction are described in the following section.

2. Attempts to Deplete the 320,000 g-min.  
Fraction of Endogenous Cofactor

Three different approaches to the problem of removing the endogenous cofactors from the 320,000 g-min. fraction derived from supplemented sucrose homogenates, are described in the present section. Not all were successful, but each yielded some information on the nature of the enzyme complex concerned with cholesterol conversion.

On incubation in the absence of an oxidizable substrate, mitochondrial preparations have been shown to loose pyridine nucleotide cofactors to the incubation medium<sup>7</sup>, and the first attempts to reduce the cofactor content of the 320,000 g-min. fraction were made along these lines.

A 320,000 g-min. fraction from 40 gms. of adrenal cortex tissue was incubated in 80 ml. of 0.025M sucrose containing M/150 phosphate buffer pH 7.4 (no  $Mg^{++}$ ), for 40 minutes at 37°C. under oxygen. After this "ageing" period the suspension was distributed between four rotor #30 tubes and the volume made up to capacity (35 ml.) with fresh medium. After centrifuging at 35,000g for 30 minutes, the pellets from each of the four tubes were suspended separately in 40 ml. of 0.025M sucrose containing M/150 phosphate buffer pH 7.4, 0.005M fumarate and 0.005M  $Mg^{++}$  and incubated with cholesterol-4-C<sup>14</sup> (35  $\mu$ gs., 200,000 c.p.m.) at 37°C. for 2 hours. DPN, ATP, or TPN were added as required just before incubation.

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<sup>7</sup>Hunter, F. E. Jr. and Ford, L. Inactivation of Oxidative and Phosphorylative Systems in Mitochondria by Preincubation with Phosphate and Other Ions. J. Biol. Chem. 216:357, 1955.

The results obtained are shown in Table 5 where the percentage cholesterol- $4\text{-C}^{14}$  conversion is given together with the composition of the incubation medium. It will be seen that appreciable conversion (9.6%) occurs in the presence of fumarate without added cofactor and while some stimulation occurs on adding DPN (11.8%), or especially TPN (15.0%), it is apparent that the "ageing" procedure has not depleted the preparation of its endogenous cofactor content sufficiently to prevent cholesterol- $4\text{-C}^{14}$  conversion.

In another attempt, the 320,000 g-min. fraction was extracted with 50% aqueous glycerol, a medium which has been used to extract ATP from muscle preparations without damage to the protein moiety as judged by the ability of ATP added to the extracted muscle to restore contractility.<sup>8</sup> The 320,000 g-min. fraction from 40 gms. of adrenal cortex tissue was suspended in 28 ml. of 60% aqueous glycerol and left at  $-10^{\circ}\text{C}$ . for 5 days. After thawing, the suspension was centrifuged at 35,000g for 2 hours. The pellet was very ill defined and some of the material was discarded with the supernatant. The material remaining was then suspended in 35 ml. of 0.44M sucrose containing 0.005M fumarate, 0.005M niacinamide and M/150 phosphate buffer pH 7.4, and centrifuged again at 8,000g for 40 minutes to remove glycerol contained in the sediment. The resulting well-packed pellet was then resuspended in 0.025M sucrose containing 0.005M fumarate, 0.005M niacinamide and M/150 phosphate buffer pH 7.4. Aliquots (40 ml.)

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<sup>8</sup>Szent-Györgyi, A. Chemistry of Muscular Contraction, (New York: Academic Press, 1953).

TABLE 5

THE EFFECT OF "AGEING" ON THE COFACTOR REQUIREMENT OF THE  
 320,000 G-MIN. FRACTION FROM SUPPLEMENTED SUCROSE HO-  
 MOGENATES FOR CONVERSION OF CHOLESTEROL-4-C<sup>14</sup>  
 TO RADIOACTIVE PRODUCTS

Additions to Basic Medium*	Cholesterol-4-C <sup>14</sup> Conversion in %
None	9.6
DPN (0.5mM)	11.8
DPN + ATP (1.0mM)	9.7
TPN (0.5mM)	15.0

\*Basic medium: 0.025M sucrose containing M/150 phosphate  
 buffer pH 7.4, 0.005M Mg<sup>++</sup> and 0.005M fumarate.

were added to incubation flasks containing cholesterol-4-C<sup>14</sup> (35 ugs., 200,000 c.p.m.) and DPN, ATP, TPN, or TPNH were added just before incubation of the suspension for 2 hours at 37°C. under oxygen. The results from this experiment are shown in Table 6, where the percentage conversion of the cholesterol-4-C<sup>14</sup> is given together with the composition of the incubation medium and the endogenous cholesterol content of the suspension at the end of the incubation period. It is apparent that the glycerol extraction reduced the conversion of cholesterol-4-C<sup>14</sup> in the presence of fumarate without added cofactor (1.0%), and to some extent the ability of DPN plus ATP to stimulate the fumarate supported conversion has been reduced (2.1%). TPN and TPNH both stimulated the cholesterol-4-C<sup>14</sup> conversion (5.3 and 4.1%, respectively). However, the values for the endogenous cholesterol content of the preparation ranged from 0.36-0.41 mgs. per pellet derived from 10 gms. of adrenal cortex tissue, showing that there had been a considerable loss on extraction with glycerol, as values for intact preparations normally fell within the range 1.4-1.6 mgs. per 10 gms. tissue equivalent. This finding will be further discussed after the presentation of the next series of attempts to deplete the preparations of their endogenous cofactor.

In the third series of experiments the 320,000 g-min. fractions were prepared from supplemented sucrose homogenates as before and then acetone dried. In order to obtain acetone powders of the pellets, they were suspended in 0.15M KCl (5 ml. per pellet from 10 gms. of tissue) and the suspension poured into 10 volumes of cold acetone (-10°C.). The resulting precipitate was collected on filter paper in a chilled buchner, washed twice with cold acetone and dried in vacuo over calcium chloride.

TABLE 6

THE EFFECT OF 50% AQUEOUS GLYCEROL EXTRACTION ON THE COFACTOR REQUIREMENT OF THE 320,000 G-MIN. FRACTION FROM SUPPLEMENTED SUCROSE HOMOGENATES FOR THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup> TO RADIOACTIVE PRODUCTS

Additions to the Basic Medium*	Cholesterol (Endogenous) mgs.	Cholesterol-4-C <sup>14</sup> Conversion in %
None	0.36	1.0
DPN (0.5mM) + ATP (1.0mM)	0.40	2.1
TPN (0.5mM)	0.41	5.3
TPNH (0.5mM)	0.41	4.1

\*Basic medium: 0.025M sucrose containing 0.005M Mg<sup>++</sup>, 0.005M niacinamide and M/150 phosphate buffer pH 7.4.

Aliquots of the acetone dried preparation, equivalent to 10 gms. of adrenal cortex tissue were homogenized in 40 ml. of 0.025M sucrose containing 0.005M  $Mg^{++}$  and M/150 phosphate buffer pH 7.4, with 0.005M fumarate added in certain cases. The suspensions were then incubated with cholesterol-4- $C^{14}$  (35  $\mu$ gs, 200,000 c.p.m), DPN, ATP, TPN, G6P, and G6P dehydrogenase being added where required. Incubation was carried out at 37°C for 2 hours under oxygen. The conversion of cholesterol-4- $C^{14}$  obtained with this preparation is shown in Table 7 together with the endogenous cholesterol values obtained at the end of the incubation period. As in the case of the glycerol extracted preparations, the activity with fumarate without added cofactor (0.6%) has been greatly reduced. DPN plus ATP stimulated the conversion to some extent (4.3%) and TPN (7.8%) or the G6P "external" TPNH generating system (6.6%) showed greater stimulation. In the acetone dried preparations the loss of endogenous cholesterol (0.16 to 0.24 mgs. per 10 gm. equivalent) is even more marked than in the case of the glycerol extracted preparations.

In spite of a considerable reduction of the endogenous cholesterol content of the preparations by glycerol or acetone treatment, the values obtained for the conversion of added cholesterol-4- $C^{14}$  in the presence of a TPNH generating system are of the same order as those found for the intact preparations. This was unexpected, as a reduction in dilution of the added radiotracer should be reflected in a correspondingly greater conversion of the cholesterol-4- $C^{14}$ . That the expected enhancement of the conversion of the radiotracer did not occur, might have been due to several factors, but of these, two may be considered here. If, in the intact preparations, the added cholesterol-4- $C^{14}$  did not mix with the endogenous

TABLE 7

THE COFACTOR REQUIREMENTS OF THE 320,000 G-MIN. FRACTION AFTER  
 ACETONE DRYING FOR THE CONVERSION OF CHOLESTEROL-  
 $4\text{-C}^{14}$  TO RADIOACTIVE PRODUCTS

Additions to the Basic Medium*	Cholesterol (Endogenous) mgs.	Cholesterol- $4\text{-C}^{14}$ Conversion in %
None	0.24	0
Fumarate (0.005M)	0.21	0.6
Fumarate + DPN(0.5mM) + ATP(1.0mM)	0.18	4.3
Fumarate + TPN (0.5mM)	0.16	7.8
G6P(0.005M) + G6P·DH(1.7 units) + TPN(0.1mM)	0.21	6.6

\*Basic medium: 0.025M sucrose containing M/150 phosphate buffer,  
 0.005M  $\text{Mg}^{++}$  and 0.005M niacinamide.

pool of cholesterol, then alterations in endogenous cholesterol levels need not necessarily affect the tracer conversion. Another explanation is that the enzymatic activity concerned with certain stages of the conversion is damaged by the process used to remove the endogenous cofactor.

At this stage of the investigation it became apparent from parallel studies that the ionic content of the supplemented sucrose medium used to isolate the 320,000 g-min. fraction affected the distribution pattern during differential centrifugation. Thus the studies on the requirements for cholesterol conversion were continued using an acetone dried 320,000 g-min. fraction derived from pure sucrose homogenates.

### 3. Acetone Dried Preparations of the 320,000 g-min. Fraction Derived from Pure Sucrose (0.44M) Homogenates

The fraction used in these experiments was obtained from homogenates of adrenal cortex tissue in 0.44M sucrose, without any addition. Apart from the use of a salt-free isolation medium, the 320,000 g-min. fraction and the acetone powders derived from it were prepared as described in previous sections.

A weight of powder equivalent to 5 gms. of adrenal cortex tissue was used for each incubation rather than the 10 gm. equivalent pellets used in the study of the intact preparations. The powder was homogenized in M/15 phosphate buffer pH 7.4 (5 ml. per 5 gm. equivalent) and added to incubation flasks containing cholesterol-4-C<sup>14</sup> (17.5 ugs., 100,000 c.p.m.) in 0.07 ml. propylene glycol. Mg<sup>++</sup>, fumarate, or glucose-6-phosphate were added as ml. of 0.05M solution and TPN, or G6P dehydrogenase in 0.2 ml. of water. The final volume was made up to 10 ml. with water and incubation

carried out in air at 37°C. for 2 hours. Extraction of the unchanged cholesterol and radioactive products was performed as previously described except that proportionally less solvent was used in extracting the lower incubation volume. Silicic acid chromatography of these extracts is described in Chapter II.

The combined results from a number of experiments carried out to determine the cholesterol conversion by the acetone dried preparation are shown in Table 8. It will be seen that fumarate in the presence of  $Mg^{++}$  supported a conversion of 37.0% of the added cholesterol-4- $C^{14}$ , but that  $Mg^{++}$  alone (1.3%) or fumarate without  $Mg^{++}$  (0.5%) were quite ineffective. Neither TPN (29.6%) nor the external G6P system (30.1%) showed any stimulation over the fumarate supported conversion. It is apparent from the results of these experiments that if pyridine nucleotides were concerned in the fumarate supported conversion of cholesterol, they were present in adequate amount in the acetone dried 320,000 g-min. fraction prepared from pure sucrose homogenates. The expected enhancement of the conversion of the radiotracer on reduction of the endogenous cholesterol content of the preparations did occur under these conditions, as evidenced by the finding of a conversion value about six times that obtained from preparations derived from supplemented sucrose homogenates. Evidence to be presented in a later section suggested that the low conversion values obtained with the fraction from supplemented homogenates might have been due to a low enzyme content of the preparations.

TABLE 8

THE REQUIREMENTS OF ACETONE DRIED 320,000 G-MIN. FRACTIONS FROM  
0.44M SUCROSE HOMOGENATES FOR CHOLESTEROL-4-C<sup>14</sup> CONVERSION  
TO RADIOACTIVE PRODUCTS

Additions to the Basic Medium*	Cholesterol-4-C <sup>14</sup> Conversion in %
Fumarate (0.005M)	0.5
Mg <sup>++</sup> (0.005M)	1.3
Mg <sup>++</sup> + fumarate	37.0
Mg <sup>++</sup> + fumarate + TPN (0.5mM)	29.6
Mg <sup>++</sup> + G6P(0.005M) + G6P·DH(1.7 units) + TPN(0.1mM)	30.1
Mg <sup>++</sup> + TPN (0.5mM)	0

\*Basic medium: M/30 phosphate buffer pH 7.4 containing  
0.005M Mg<sup>++</sup>.

The acetone dried 320,000 g-min. fraction prepared from pure sucrose homogenates proved a very useful preparation for further study of the cofactor requirements, and were found to retain their activity for several weeks if stored at  $-10^{\circ}\text{C}$  in a desiccator.

4. Dialysis of Acetone Dried 320,000 g-min.  
Fractions from 0.44M Sucrose Homogenates

In an attempt to reduce the endogenous cofactor content of the acetone dried 320,000 g-min. fraction from pure sucrose homogenates, aliquots equivalent to 15 gms. of tissue were suspended in 10 ml. phosphate buffer M/15, pH 7.4, and dialyzed against 250 ml. of similar buffer for 2 hours in the cold room and then transferred to 4 liters of distilled water overnight. The dialyzed suspensions were well mixed and aliquots of the suspension equivalent to 5 gms. of tissue added to incubation flasks containing cholesterol-4- $\text{C}^{14}$  (17.5 ugs., 100,000 c.p.m.).  $\text{Mg}^{++}$ , fumarate, or G6P were added as 1 ml. of 0.05M solution when required, and TPN, ATP, or adenosine-3',5'-monophosphate added in 0.2 ml. of water before making the volume up to 10 ml. with distilled water. Incubation and extraction procedures were carried out as described in the previous section. The results obtained with this dialyzed preparation are shown in Table 9 (a), where the percentage conversion of cholesterol-4- $\text{C}^{14}$  is given together with the additions to the phosphate- $\text{Mg}^{++}$  medium. It can be seen that the preparation was inactive with fumarate and that no stimulation occurred upon adding TPN together with fumarate, or in the presence of the external TPNH generating system (G6P).

TABLE 9

THE EFFECT OF DIALYSIS ON THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup> TO  
 RADIOACTIVE PRODUCTS BY ACETONE DRIED 320,000 G-MIN.  
 FRACTIONS FROM 0.44M SUCROSE HOMOGENATES

Additions to the Basic Medium*	Cholesterol-4-C <sup>14</sup> Conversion in %
(a) Fumarate (0.005M)	0.6
Fumarate + TPN (0.5mM)	0
Fumarate + adenosine-3',5'-monophosphate	2.6
G6P(0.005M) + G6P·DH(1.7 units) + TPN(0.1mM)	0.5
G6P + G6P·DH + TPN + ATP(1.0mM)	0.2
G6P + G6P·DH + TPN + adenosine-3',5'-monophosphate	0.2
(b) Fumarate (0.005M)	0.3
Fumarate + TPN (0.5mM)	22.6
Fumarate + DPN (0.5mM)	5.4
Fumarate + DPN + TPN	28.3
G6P(0.005M) + G6P·DH(1.7 units) + TPN(0.1mM)	35.3
G6P + G6P·DH + TPN + DPN(0.5mM)	26.0
G6P + G6P·DH	9.4

\*Basic medium: M/30 phosphate buffer pH 7.4 containing 0.005M Mg<sup>++</sup>.

However, when attempting to modify the dialysis conditions in order to be able to add to the dialyzed preparation an aliquot of the dialysate equivalent to the amount of dialyzed suspension used in the incubation, it was observed that under these conditions of restricted dialysis, the preparation was no longer fully inactivated. The acetone dried preparation equivalent to 35 gms. of tissue, was suspended in 3 ml. of phosphate buffer M/15, pH 7.4, and dialyzed against 30 ml. of the same buffer for 8 hours followed by dialysis against distilled water (4 l.) for a further 12 hours. The dialyzed suspension was made up to 35 ml. with M/15 phosphate buffer pH 7.4 and 5 ml. aliquots incubated with cholesterol-4-C<sup>14</sup> as in the case of the extensively dialyzed preparations. The degree of cholesterol conversion by this partially dialyzed preparation is shown in part (b) of Table 9, together with the composition of the medium. It will be seen that the preparation did not catalyze cholesterol-4-C<sup>14</sup> conversion in the presence of fumarate alone, but considerable activity (22.6%) was obtained on the addition of TPN to the fumarate fortified medium. DPN was relatively ineffective in replacing TPN (6.9%) and only slightly augmented the stimulation by TPN (28.3%). The activity supported by the glucose-6-phosphate system (35.3%) was somewhat reduced by the addition of DPN (26.0%). The G6P and its dehydrogenase were able to react with endogenous TPN to a certain extent as evidenced by a conversion of 9.4% in the absence of added TPN (cf. intact preparations). In no case was it possible to restore the activity of the partially or extensively dialyzed preparations by the addition of the dialysate.

Considerable variation in behavior of individual acetone dried preparations with respect to their inactivation on dialysis was observed with many variables affecting the activity. The concentration of powder inside the dialysis sack in terms of total phosphate volume inside and outside the sack, the duration of dialysis in phosphate (but not apparently in water), and whether or not phosphate dialysis was followed by dialysis against water, were all factors influencing the activity of the dialyzed preparations. It is clear, however, from the results of incubations of the partially dialyzed preparations, that the cholesterol conversion system requires TPN and a system capable of maintaining this cofactor in the reduced form.

5. The Solubilization of the Cholesterol Conversion Activity in Acetone Dried Preparations of the 320,000 g-min. Fraction

While preparing suspensions of the acetone powders derived from the 320,000 g-min. fraction in phosphate buffer M/15 pH 6.8, it was observed that the cholesterol conversion activity could be solubilized quite easily. The use of buffer at the lower pH was initiated in order to better preserve pyridine nucleotides contained in the preparation as they are known to be more stable at acid pH values.<sup>9</sup>

The acetone dried 320,000 g-min. fraction equivalent to 20 gms. of tissue was homogenized in 24 ml. M/15 phosphate buffer pH 6.8 and centrifuged at 105,000g. for 60 minutes in the Spinco L centrifuge. The

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<sup>9</sup>Horecker, B. L. and Smyrniotis, P.Z. Glucose-6-phosphate Dehydrogenase. in Methods in Enzymology I, eds. S. P. Colowick and N. O. Kaplan, (New York: Academic Press, Inc., 1955), p. 323.

sedimented material (insoluble fraction) was then resuspended in 24 ml. of the buffer containing  $Mg^{++}$  (0.005M). Aliquots (6 ml.) of the suspension were incubated with cholesterol-4- $C^{14}$  (17.5 ugs., 100,000 c.p.m.) in the presence of either the G6P external TPNH generating system, with and without DPN, or with fumarate alone, or with TPN, or TPN plus DPN. The clear supernatant (soluble fraction) was treated similarly except that  $Mg^{++}$  was added together with the other constituents. In all cases the final incubation volume was made up to 10 ml. with distilled water. Incubation and estimation of the degree of cholesterol-4- $C^{14}$  conversion was carried out as in previous experiments. The results are shown in Table 10 where it will be seen that the soluble fraction catalyzed the conversion of cholesterol-4- $C^{14}$  to radioactive products in the presence of fumarate alone (22.8%) and showed no requirement for added cofactor as judged by the lack of stimulation on addition of TPN (22.4%) or TPN plus DPN (23.5%). The conversion supported by the G6P-TPNH generating system was somewhat lower (17.9%), which might have been a reflection of the pH 6.8 buffer used, as the G6P dehydrogenase had a pH optimum at about 7.4<sup>9</sup>, or the inhibitory action of phosphate.<sup>9</sup> In contrast to the activity of the soluble fraction, the insoluble material was inactive under all conditions tested. Another similar experiment carried out under similar conditions indicated that about 70% of the activity of the original acetone powder was recovered in the soluble fraction.

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<sup>9</sup>Horecker, B. L. and Smyrniotis, P. Z. in Methods in Enzymology I, eds. S. P. Colowick and N. O. Kaplan, (New York: Academic Press, Inc., 1955) p. 323.

TABLE 10  
 THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup> TO RADIOACTIVE PRODUCTS BY A  
 SOLUBLE EXTRACT OF ACETONE DRIED 320,000 G-MIN. FRACTION

Additions to the Basic Medium*	Cholesterol-4-C <sup>14</sup> Conversion (%)	
	Soluble Fraction	Insoluble Residue
Fumarate (0.005M)	22.8	0
Fumarate + TPN (0.2mM)	22.4	0
Fumarate + TPN + DPN (0.5mM)	23.4	0
G6P(0.005M) + TPN(0.1mM) + G6P·DH(1.7 units)	17.9	0

\*Basic medium: M/15 phosphate buffer pH 6.8 containing 0.005M Mg<sup>++</sup>.

Results obtained on the fresh 320,000 g-min. fraction showed that the external TPNH generating system (G6P) required the addition of exogenous TPN for cholesterol conversion activity, as apparently structural barriers restricted the reaction of G6P with the endogenous cofactor (cf. Table 2). The efficiency of the external system without added TPN was therefore tested using the solubilized activity from acetone dried 320,000 g-min. fractions under similar experimental conditions to those previously described for this fraction. The results obtained are shown in Table 10A where it can be seen that the external system was still dependent upon added TPN to a considerable extent, though a significant activity in the absence of TPN was obtained (6.8%).

TABLE 10A

CHOLESTEROL CONVERSION BY THE NON-PARTICULATE FRACTION OF ACETONE  
DRIED 320,000 G-MIN. FRACTIONS IN THE ABSENCE OF ADDED TPN

Additions to Basic Medium*	Cholesterol-4-C <sup>14</sup> Conversion (%)
Fumarate (0.005M)	23.6
G6P (0.005M) + G6P·DH (1.7 units) + TPN (0.1mM)	21.9
G6P + G6P·DH	6.8

\*Basic medium: M/15 phosphate buffer pH 6.8 containing 0.005M Mg.

6. The Nature of the Radioactive Products Formed from Cholesterol-4-C<sup>14</sup> by Acetone Dried Preparations of the 320,000 g-min. Fraction

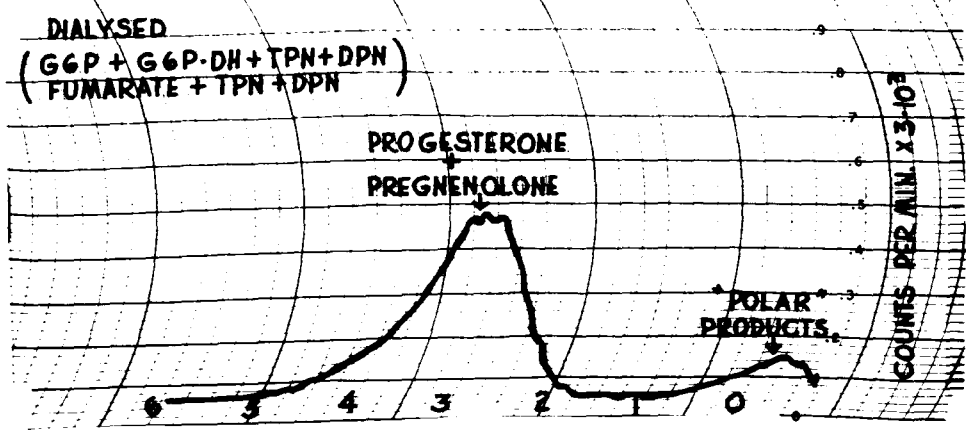
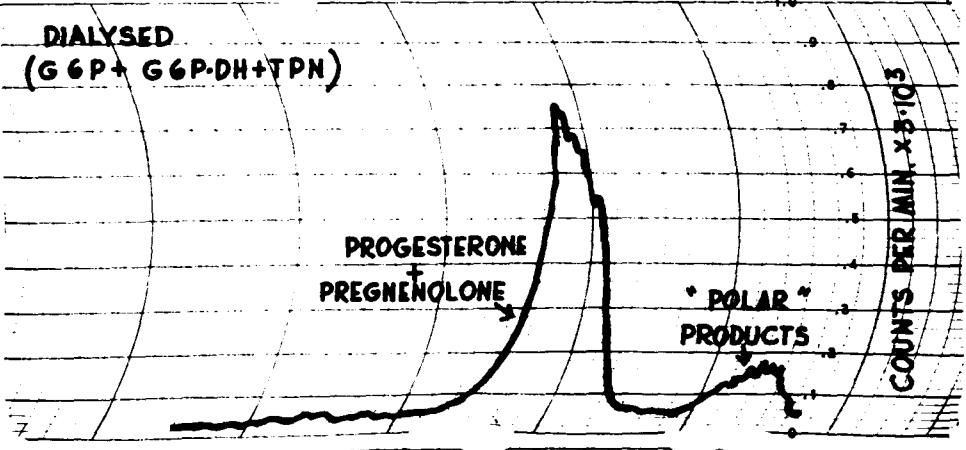
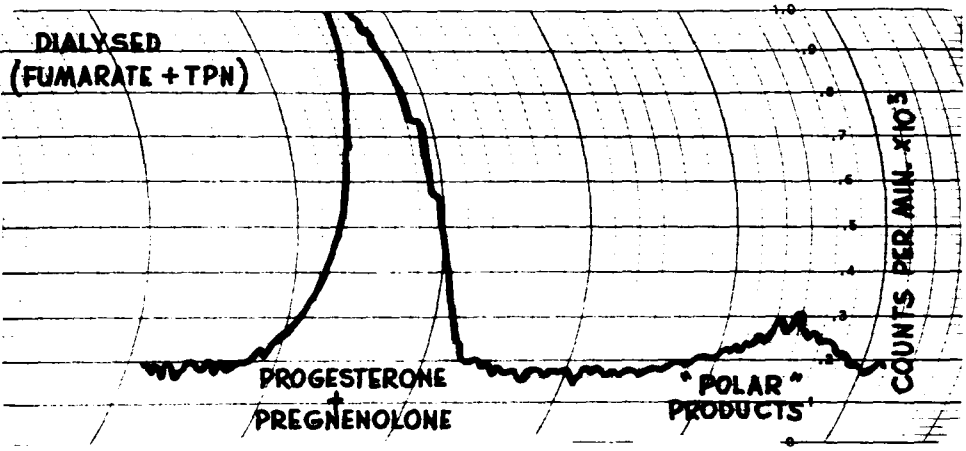
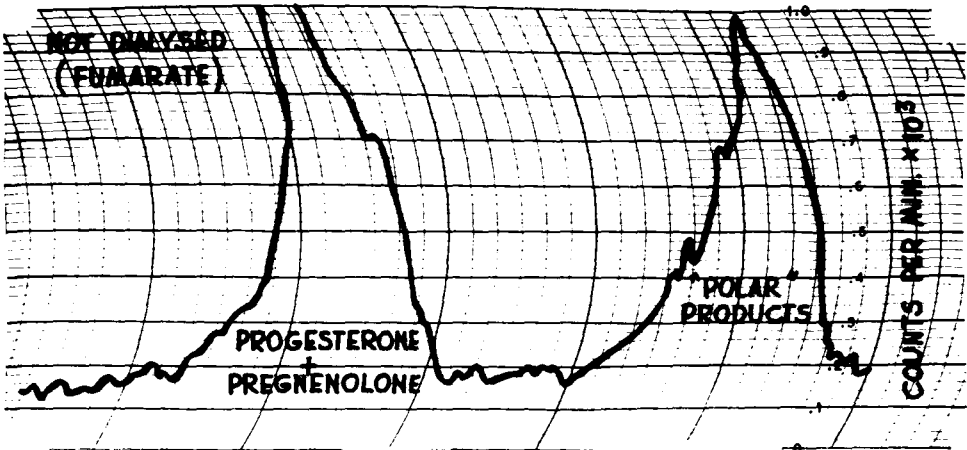
It has been demonstrated that in order to obtain radioactive products from cholesterol-4-C<sup>14</sup> the 320,000 g-min. fraction requires the presence of adequate amounts of TPNH. It has not as yet been demonstrated that these products are indeed C-21 steroids or to what extent, if any, the steroid ring has been hydroxylated. In order to relate the TPNH requirement to the enzymatic cleavage of the cholesterol side chain, the presence of C-21 steroids such as progesterone, or preferably  $\Delta^5$ -pregnenolone in the products fraction had to be confirmed.

The products of reaction (ethyl acetate fraction) obtained from cholesterol-4-C<sup>14</sup> on incubation with acetone dried preparations of the 320,000 g-min. fraction derived from pure sucrose homogenates were fractionated using the ligroin-formamide paper system of Zaffaroni and Burton.<sup>10</sup> The ethyl acetate fractions, pooled from a number of experiments were loaded on paper impregnated with formamide:methanol (3:1) and run in ligroin for 6 hours at 70°F. On scanning the papers, two radioactive zones were located, equal in intensity, one moving in an equivalent manner to authentic  $\Delta^5$ -pregnenolone or progesterone, and the other remaining on or near the starting line (Fig. 2). This slow moving zone represented material considerably more polar than progesterone and was therefore discarded. The fast running zone was eluted with methanol and loaded on

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<sup>10</sup>Zaffaroni, A. and Burton, R. B. Identification of Corticosteroids of Beef Adrenal Extract by Paper Chromatography. J. Biol. Chem. 193:749, 1951.

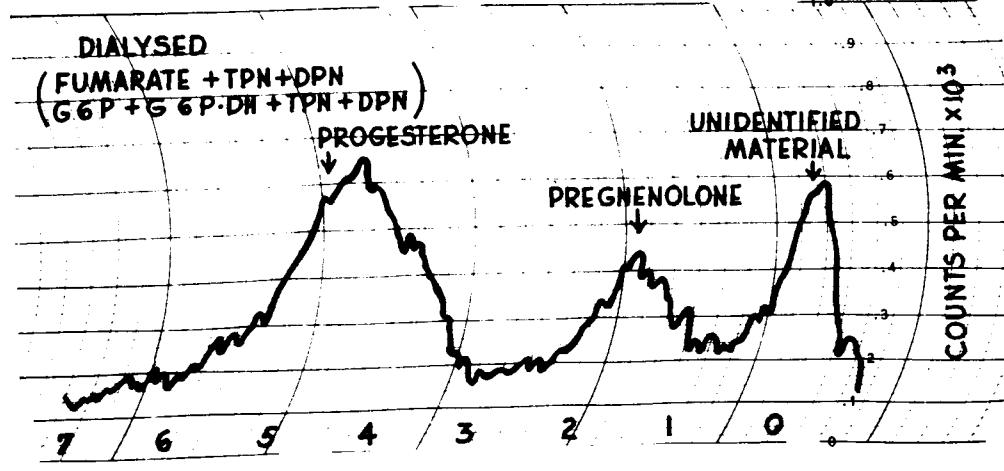
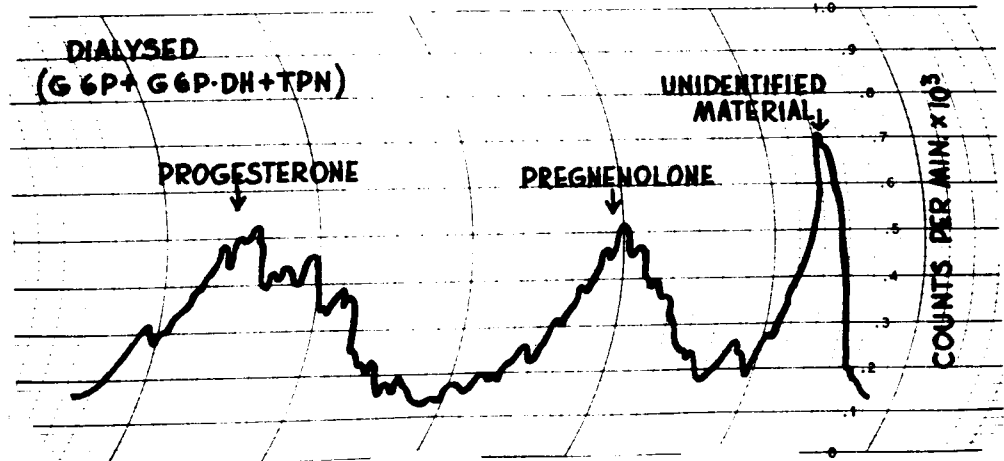
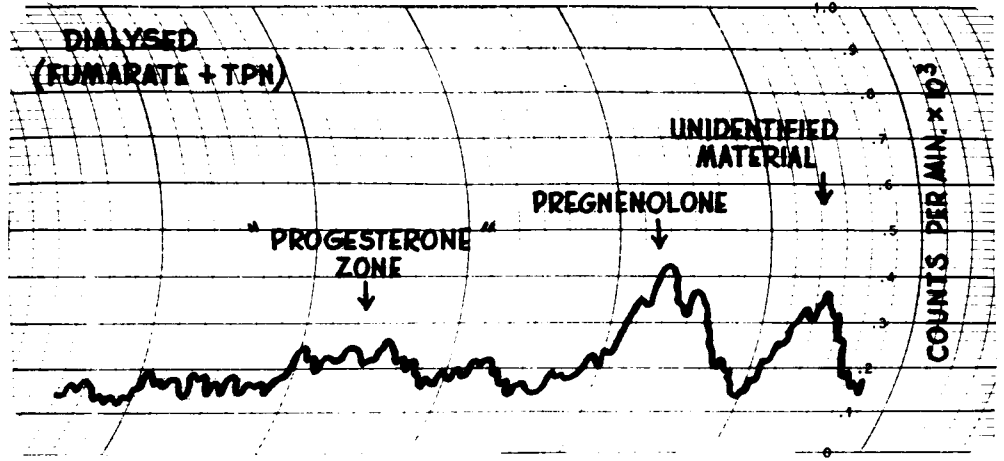
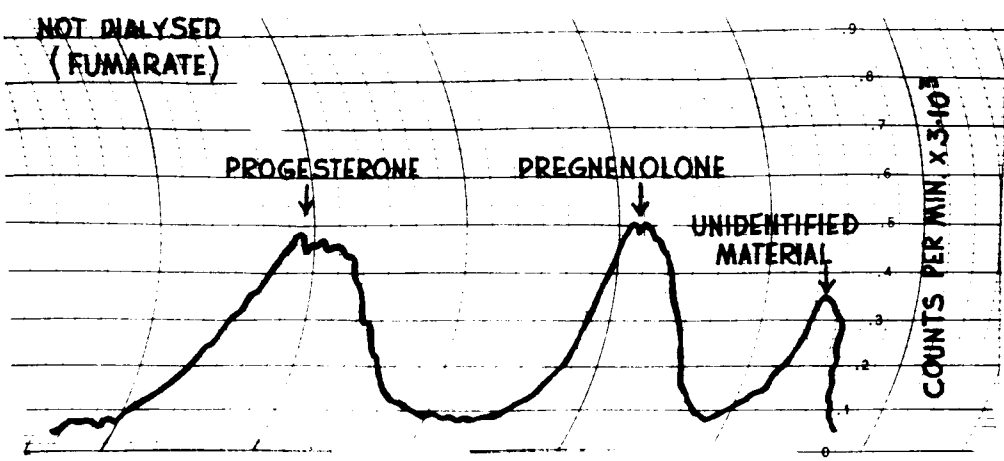
**Fig. 2. Radiochromatograms of the products of cholesterol- $4\text{-C}^{14}$  conversion by acetone dried 320,000 g-min. fractions run in ligroin/formamide systems.**



paper impregnated with propylene glycol:methanol (1:1) and run in ligroin for 1-1/2 - 2 hours, or until the solvent front had just reached the end of the paper. On scanning the paper two radioactive zones were located, one showing similar running properties to  $\Delta^5$ -pregnenolone ( $R_f$  0.15) and the other faster zone behaving as authentic progesterone ( $R_f$  0.40) (Fig.3). The two zones were eluted separately with methanol and reserved for further characterization on Celite partition columns.

When the same procedure was carried out on ethyl acetate fractions derived from incubations of partially dialyzed acetone powders of the 320,000 g-min. fraction, in the presence of fumarate plus TPN, or G6P, TPN and G6P dehydrogenase with and without DPN, several differences were noted. On chromatography in the ligroin-formamide system, the radioactivity in the more polar (slower) zone was, at the most, only 20% of the fast zone (Fig. 2). When the fast zone was eluted and run in the ligroin-propylene glycol system, the distribution of radioactivity varied with the nature of the TPNH generating system and the presence or absence of DPN. From incubations of the partially dialyzed preparations with fumarate plus TPN, the extracts showed considerably more radioactivity in the slow zone ( $\Delta^5$ -pregnenolone) than in the fast zone (progesterone). The two zones were approximately equal in radioactive content when the G6P-TPNH generating system was employed, while the presence of DPN enhanced the radioactivity found in the fast zone compared to that in the slow zone (Fig. 3). The radioactive zones from the ligroin-propylene glycol papers were eluted and further characterized on Celite columns.

Fig. 3. Radiochromatograms of the combined progesterone and pregnenolone zones from ligroin/formamide chromatograms (Fig. 2) run in ligroin/propylene glycol.



The fast zones, suspected to be progesterone, were run on the Celite columns as described in Chapter II, the radioactivity being eluted in a single zone with 16 ml. of mobile phase. The radioactivity eluted was then acetylated and rerun on a similar column when the material again appeared in a single peak eluted by 16 ml. of mobile phase.

Similarly the slow zones suspected to contain  $\Delta^5$ -pregnenolone were eluted in a single peak from the Celite columns by 30 ml. of mobile phase. The eluted material was then mixed with authentic  $\Delta^5$ -pregnenolone-7- $H^3$  and rerun on a Celite column, the radioactivity due to  $C^{14}$  +  $H^3$  being assayed in each fraction using a gas flow counter and that due to  $C^{14}$  alone on a micromil end window counter. The distribution of radioactivity due to  $C^{14}$  +  $H^3$  and  $C^{14}$  alone is shown in Table 11, where it will be seen that there is a very similar distribution of both the  $C^{14}$  (unknown sample) and the  $H^3$  radioactivity (authentic  $\Delta^5$ -pregnenolone-7- $H^3$ ). The individual fractions from the column were pooled, acetylated and rerun on a Celite column using the same solvent system. In this case the radioactivity ran with the solvent front and was completely eluted with 6 ml. of mobile phase. The radioactivity due to each tracer was determined as before and the distribution is also shown in Table 11. Again there was no evidence of separation of the unknown sample and authentic compound. While the procedures described do not permit positive identification of the products derived from cholesterol-4- $C^{14}$ , their chromatographic behavior strongly indicates the presence of  $\Delta^5$ -pregnenolone and progesterone and the presence of such compounds is consistent with previous findings on the nature of the products of cholesterol-4- $C^{14}$  conversion by mitochondrial

TABLE 11

DISTRIBUTION OF A MIXED SAMPLE OF  $\Delta^5$ -PREGNENOLONE-7- $H^3$  (AUTHENTIC)  
AND  $\Delta^5$ -PREGNENOLONE-4- $C^{14}$  (UNKNOWN) IN EFFLUENTS FROM CELITE  
PARTITION COLUMNS BEFORE AND AFTER ACETYLATION OF SAMPLE

	Volume of Effluent ml.	% Distribution in Effluent	
		$C^{14} + H^3$	$C^{14}$
(A) Before Acetylation	22	3	6
	23	13	13
	24	19	21
	25	23	20
	26	21	19
	27	12	11
	28	5	7
	29	4	3
	(B) After Acetylation	1	16
2		42	42
3		22	21
4		13	12
5		5	6
6		2	1

preparations<sup>11</sup>.

The preceding account of the radioactive products obtained from cholesterol-4-C<sup>14</sup> refers to those compounds separated from the radioactive cholesterol on silicic acid chromatography. It is conceivable that enzymatic modification of the cholesterol molecule could give rise to substances intermediate in the cholesterol to  $\Delta^5$ -pregnenolone transformation. Such compounds might possibly be eluted from the silicic acid columns by benzene, and thus would not occur in the "product" fraction (ethyl acetate). Only a limited amount of investigation was carried out on the nature of the benzene eluate, but it is certain that in some cases the radioactivity was not homogenous. When the benzene eluate from silicic acid columns was applied to kerosene paper and run in 60% n-propanol as described by Martin<sup>12</sup> (cf. Chapter II), on some occasions two radioactive zones were located. A slower moving zone ( $R_f$  0.5) was observed in all cases and probably corresponded to unchanged cholesterol. In benzene fractions derived from incubations of intact 320,000 g-min. fractions or whole acetone powders derived from them, a second faster moving zone ( $R_f$  0.85) was seen. So far, this second zone, as yet unidentified, has not been observed in extracts derived from incubations of cholesterol-4-C<sup>14</sup> with the soluble fraction of acetone powders of the 320,000 g-min. fraction. It was present in extracts from incubations of

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<sup>11</sup>Saba, N. and Hechter, O. Cholesterol-4-C<sup>14</sup> Metabolism in Adrenal Homogenates. Federation Proc. 14:775, 1955.

<sup>12</sup>Martin, R. P. Reversed Phase Paper Chromatography and Detection of Steroids of the Cholesterol Class. Biochim. Biophys. Acta, 25: 408, 1955.

cholesterol-4-C<sup>14</sup> with partially dialyzed acetone powders in the presence of TPN, but it cannot be said that extensively dialyzed acetone powders (which did not give rise to products in the ethyl fraction) did not produce a second zone. The significance of these findings must await further experimentation, but it may be pointed out that 20 $\beta$ -hydroxy-cholesterol might be expected to behave in a similar manner on chromatography and it has been shown to arise from cholesterol-4-C<sup>14</sup> incubated with adrenal preparations<sup>13</sup>.

7. Cholestenone-4-C<sup>14</sup> as a Substrate for the Non-particulate Fraction of Acetone Dried 320,000 g-min. Fractions

Evidence has been presented in the introductory review that cholesterol conversion to progesterone proceeds by way of  $\Delta^5$ -pregnenolone. The finding that the products of cholesterol-4-C<sup>14</sup> conversion included  $\Delta^5$ -pregnenolone was therefore to be expected. However, it was of interest to see if an alternative pathway through  $\Delta^4$ -cholestenone might not occur. In this case, the enzymes capable of converting  $\Delta^4$ -cholestenone to progesterone should be present. In order to test this possibility,  $\Delta^4$ -cholestenone-4-C<sup>14</sup> was incubated with the soluble extract of acetone dried 320,000 g-min. fractions and the products of conversion (ethylacetate eluate) examined for progesterone. At the same time an estimate of the effect of  $\Delta^4$ -cholestenone on the conversion of cholesterol-4-C<sup>14</sup> was investigated. The results of two such experiments are given in Table 12 ,

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<sup>13</sup>Solomon, S., Levitan, P. and Lieberman, S. Possible Intermediates between Cholesterol and Pregnenolone in Corticosteroidogenesis. *Revue Canad. de Biol.* 15:282, 1956.

TABLE 12

THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup> IN THE PRESENCE OF  $\Delta^4$ -  
 CHOLESTENONE AND THE CONVERSION OF  $\Delta^4$ -CHOLESTENONE-4-C<sup>14</sup>  
 TO RADIOACTIVE PRODUCTS BY A SOLUBLE EXTRACT OF  
 ACETONE DRIED 320,000 G-MIN. FRACTION

Substrate	Conversion to "Products" (%)
Cholesterol-4-C <sup>14</sup> (17.5 $\mu$ gs)	34.2; 24.3
$\Delta^4$ -Cholestenone-4-C <sup>14</sup> (15 $\mu$ gs)	13.8; 7.8
Cholesterol-4-C <sup>14</sup> + $\Delta^4$ - cholestenone (15 $\mu$ gs)	18.2; 15.1
Cholesterol-4-C <sup>14</sup> + $\Delta^4$ - cholestenone (150 $\mu$ gs)	11.7; 12.4

where it will be seen that  $\Delta^4$ -cholestenone-4-C<sup>14</sup> was converted to a limited extent into radioactive substances eluted from silicic acid by ethyl acetate. However, on chromatography in the formamide/ligroin system, the products were clearly not progesterone, but represented far more polar material. In the presence of an approximately equal concentration of  $\Delta^4$ -cholestenone (15 ugs.), cholesterol-4-C<sup>14</sup> conversion was inhibited. In one experiment the conversion was decreased from 34.2% to 18.2% and in the other from 24.3% to 15.1%, but on increasing the level of  $\Delta^4$ -cholestenone tenfold (150 ugs.), little further inhibition occurred (11.7 to 12.4%).

From these findings it seems unlikely that  $\Delta^4$ -cholestenone is an alternative intermediary in the conversion of cholesterol to progesterone, and furthermore, the affinity of the enzyme complex for cholesterol would appear to be greater than that for cholestenone.

B. The Intracellular Distribution of the Cholesterol  
Conversion Activity

The results so far described were obtained using one particular fraction of the adrenocortical tissue homogenate, chosen because of its known ability to convert cholesterol-4-C<sup>14</sup> to radioactive C-21 steroids.<sup>11</sup> Having established that the enzymatic scission of the cholesterol side chain required TPNH as a specific cofactor, it was then possible to investigate other fractions of the tissue homogenate for activity in the presence of the external TPNH generating system (G6P). The external system was used rather than the internal (fumarate) because it could not be assumed that all the fractions contained the enzymes required for TPNH generation by citric acid cycle intermediates.

From the behavior of the so-called "fluffy layer" it appeared that the distribution of subcellular fractions might be different in pure sucrose isolation media from those obtained from tissue homogenized in supplemented sucrose solutions. As a preliminary to full distribution studies, it was necessary to investigate the influence of supplementation of the sucrose isolation media on the behavior of cell fractions.

1. The Effect of Cations on the Appearance  
of the "Fluffy Layer" Fraction

The fluffy layer fraction forms on washing the 320,000 g-min. pellet derived from pure sucrose homogenates, but does not arise when the sucrose homogenate is supplemented by EDTA, fumarate, niacinamide and

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<sup>11</sup> Saba, N. and Hechter, O. Cholesterol-4-C<sup>14</sup> Metabolism in Adrenal Homogenates. Federation Proc. 14:775, 1955.

phosphate buffer. As similar findings had been made in preliminary experiments using supplemented 0.25M sucrose in which the EDTA was absent and  $Mg^{++}$  present in the supplements, it seemed unlikely that the addition of EDTA was responsible for the observed differences between the supplemented and unsupplemented sucrose media. A consideration of the other supplementary additions revealed that the total cation ( $K^+$  plus  $Na^+$ ) content of the original suspension of the tissue (20 gms. per 90 ml. of medium) was increased by an amount of cation equal to 26.7mM when supplemented sucrose was employed. Furthermore, an analysis by flame photometry of the  $K^+$  and  $Na^+$  content of the chopped tissue showed that the total concentration of these two cations in the tissue suspension was on the average 28mM. Thus in the case of pure sucrose homogenates the pellet was isolated from a suspension 28mM with respect to cation, and washed with pure sucrose. On the other hand in the supplemented sucrose homogenates the pellet was isolated from double the cation concentration and washed with sucrose containing 26.7mM cation. As the fluffy layer only appeared on washing the pellets, it seemed possible that its appearance was related to the decreased cation content of the suspending medium. If this were true, then one might expect the fluffy layer to appear during the initial sedimentation of the 320,000 g-min. fraction if the cation content of the tissue were reduced prior to homogenization. The following experiment was therefore carried out to determine the effect of washing the adrenal cortex tissue in hypertonic or isotonic media prior to homogenization, on the cation content of the tissue, the behavior of the fluffy layer and the activity of the 320,000 g-min. fraction on incubation with cholesterol- $4-C^{14}$ .

The tissue mince was prepared from glands collected in ice-cold 0.44M sucrose rather than 0.9% NaCl, in order to avoid adding cations to the tissue. Aliquots (10 gms.) of the chopped tissue were suspended in 250 ml. ice-cold 0.44M or 0.25M sucrose or glucose, and allowed to stand in the cold with occasional stirring for 5 minutes. The tissue was then collected on a nylon cloth filter and resuspended in 250 ml. of fresh medium for a further 5-minute period. Finally the tissue was allowed to remain in a third wash medium for 1 hour in the cold and recovered on the nylon filter as before. After homogenization of the washed tissue in 0.44M sucrose, aliquots (1 ml.) were taken for estimation of  $K^+$  and  $Na^+$  content and the 320,000 g-min. fraction prepared as previously described. A heavy fluffy layer fraction sloughed off from the pellet on decanting the supernatant after the initial sedimentation, but little further loose material appeared during the washing process. An aliquot of the same tissue mince was used to prepare the 320,000 g-min. fraction after homogenizing in 0.44M sucrose without a pre-washing treatment. The treated and control pellets were suspended in 0.025M sucrose containing 0.005M  $Mg^{++}$ , 0.005M niacinamide and M/150 phosphate buffer pH 7.4 and incubated with cholesterol-4- $C^{14}$  (35  $\mu$ gs., 200,000 c.p.m.) in the presence of G6P, G6P dehydrogenase, and TPN as previously described. The results of this experiment are shown in Table 13, where the nature of the tissue wash medium is recorded with the  $K^+$  concentration (mM) of the tissue homogenate, the protein and cholesterol content (in mgs. per 10 gms. of tissue) of the 320,000 g-min. fraction and the percentage conversion of the cholesterol-4- $C^{14}$ . It will be seen that the level of  $K^+$  in the washed tissue homogenate was reduced to 10% of the control level, each wash medium being

TABLE 13

THE EFFECT OF PREWASHING ADRENOCORTICAL TISSUE ON THE POTASSIUM  
 CONTENT OF THE HOMOGENATE AND THE CHOLESTEROL CONVERSION  
 ACTIVITY OF THE 320,000 G-MIN. FRACTION

Wash Medium	K <sup>+</sup> mM	Cholesterol mgs.	Protein mgs.	Cholesterol-4-C <sup>14</sup> Conversion (in %)
0.44M Sucrose	2.8	0.81	68.8	14.3
0.25M "	3.3	0.87	74.5	16.2
0.44M Glucose	3.1	0.50	51.6	22.4
0.25M "	2.6	0.65	58.4	23.6
Unwashed Tissue	26.0	0.75	61.5	13.6

equally effective in removing the cation. The 0.44M or 0.25M sucrose wash media had little effect on the protein and cholesterol content of the 320,000 g-min. fraction or on its ability to convert cholesterol-4-C<sup>14</sup> to radioactive products (14.3; 16.2%). Some loss of protein and cholesterol occurred on washing with the glucose media and the cholesterol-4-C<sup>14</sup> conversion was correspondingly increased (22.4; 23.6%).

From these results it appeared that the cation content of the tissue homogenate considerably affected the behavior of at least one fraction of the homogenate, and further experiments were carried out to examine the effect of K<sup>+</sup> on the distribution of the cholesterol conversion activity in homogenates prepared from washed tissue. By the use of the washed tissue it was expected that any differences between the absence and presence of the cation would be accentuated.

## 2. The Effect of KCl on the Distribution of Protein, Cholesterol and the Cholesterol-4-C<sup>14</sup> Conversion Activity

The effect of K<sup>+</sup> on the fractionation procedure was examined by comparing fractions obtained from a homogenate prepared in sucrose containing KCl (50mM) with those from a pure sucrose homogenate. In each case the tissue was prewashed with 0.44M sucrose as described in the previous experiment. Two 10 gm. aliquots of the washed tissue were homogenized individually in either 0.44M sucrose or 0.44M sucrose containing 50mM KCl. After filtering the homogenates through nylon cloth, the various fractions were isolated as described in Chapter II. A heavy fluffy layer appeared on sedimenting the 320,000 g-min. fraction from the pure sucrose homogenate but no loosely packed material was obtained from

the sucrose-KCl homogenate, even during the washing of the 320,000 g-min. pellet. The particulate fractions were suspended in 40 ml. of 0.025M sucrose containing 0.005M  $Mg^{++}$ , 0.005M niacinamide and M/150 phosphate buffer pH 7.4 and added to flasks containing cholesterol-4- $C^{14}$  (35  $\mu$ gs., 200,000 c.p.m.) in 0.15 ml. propylene glycol. G6P (100  $\mu$ moles), TPN (20  $\mu$ moles) and G6P dehydrogenase (1 unit) were added just before incubation at 37°C for 2 hours. The soluble fractions received 5 ml. each of 0.05M  $Mg^{++}$ , 0.05M niacinamide and M/15 phosphate buffer pH 7.4, and were incubated in the presence of G6P, TPN and G6P dehydrogenase as in the case of the particulate fractions. Aliquots (1 ml.) were taken for protein estimation at the end of the incubation period and the endogenous cholesterol content was determined on the benzene eluate from the silicic acid columns. Cholesterol-4- $C^{14}$  conversion was estimated by the standard procedure.

The results of this experiment are shown in Table 14, where the protein and cholesterol content of each fraction is given together with the percentage cholesterol-4- $C^{14}$  conversion. It will be seen that the addition of KCl to the sucrose homogenizing medium not only suppresses the appearance of the fluffy layer, but also increases the amount of protein sedimented in the 36,000 g-min. fraction at the expense of the other fractions. The effect of the KCl addition is even more marked in the case of cholesterol where there was a shift of this substance to the heavier particulate fractions, with the 36,000 g-min. fraction from the KCl-sucrose homogenate showing a value of 10.1 mgs. per 10 gms. tissue equivalent against a value of 2.5 mgs. in the same fraction prepared from the pure sucrose homogenate. The highest cholesterol-4- $C^{14}$  conversion was obtained

TABLE 14

THE DISTRIBUTION OF THE CHOLESTEROL-4-C<sup>14</sup> CONVERSION ACTIVITY IN SUBCELLULAR FRACTIONS OF PURE SUCROSE AND SUCROSE-KCL HOMOGENATES

Fraction	Homogenizing Medium					
	0.44M Sucrose			0.44M Sucrose + 50mM KCl		
	Protein mgs.	Cholesterol mgs.	Cholesterol-4-C <sup>14</sup> Conversion %	Protein mgs.	Cholesterol mgs.	Cholesterol-4-C <sup>14</sup> Conversion %
36,000 g-min.	152.0	2.5	3.7	265.0	10.1	1.7
320,000 g-min.	56.0	0.7	16.9	30.2	1.5	14.0
Fluffy layer	72.0	2.1	9.7	--	--	--
12,600,000 g-min.	43.4	4.3	0	18.0	1.7	0
Soluble	74.3	1.7	6.2	76.2	1.5	1.6

in the 320,000 g-min. fraction from both homogenates with the fluffy layer showing appreciable activity (9.7%). The 12,000,000 g-min. fraction was inactive in either case but some activity was obtained with the soluble and 36,000 g-min. fractions derived from the pure sucrose homogenate.

The altered sedimentation properties of the cholesterol, observed on addition of KCl to the sucrose isolation medium, emphasized a basic problem in the estimation of cholesterol conversion activity in different fractions by means of radiotracer techniques. In the experiment just described, the 36,000 g-min. fraction from the pure sucrose homogenate contained 2.7 mgs. of endogenous cholesterol and converted 3.7% of the added cholesterol-4-C<sup>14</sup> while the same fraction from the KCl-sucrose homogenate contained 10.1 mgs. of cholesterol and converted only 1.7% of the added cholesterol-4-C<sup>14</sup>. The lower conversion in the presence of greater amounts of cholesterol might be expected on the basis of tracer dilution, but this relationship between radiocholesterol conversion and cholesterol content did not hold over all the fractions and could not be expected to if the fractions contained variable amounts of enzymatic activity. Clearly, it was not possible to use the cholesterol-4-C<sup>14</sup> conversion value as a measure of the relative activity of different fractions unless the degree of mixing was known in each case, when the total cholesterol converted could be calculated. In the next section an experiment will be described, which to a certain extent indicated the degree of mixing of the radiotracer with the endogenous pool.

3. The Specific Activity of Radioprogesterone Isolated from Incubations of Various Sub-cellular Fractions with Cholesterol-4-C<sup>14</sup>

The specific activity of a radioactive C-21 steroid product derived from radiocholesterol incubated with a tissue fraction is related to the endogenous cholesterol content, the degree of mixing of tracer with the endogenous pool, and on the extent to which the product arises from alternative precursors. If one assumes complete mixing of tracer and endogenous material and postulates a single precursor for the radioactive product, then the specific activity of the product may be calculated from the cholesterol content and the amount of radioactivity added. Conversely, if one knows the specific activity of the product, some idea of the validity of the two assumptions may be formed. It was convenient to isolate the radioprogesterone formed from cholesterol-4-C<sup>14</sup> during incubation with various fractions prepared from the pure sucrose homogenate used in the previous experiment, and to estimate the weight and radioactivity of this product of cholesterol conversion.

The ethyl acetate eluates from the silicic acid columns were applied to ligroin-formamide papers, run in ligroin for 24 hours, and the overflow of the mobile phase from the ends of the papers collected. These overflows contained the progesterone relatively free from more polar products, and were then mixed with authentic progesterone-16-H<sup>3</sup> (0.04 μgs., 24,000 c.p.m) and again applied to formamide papers. This time the mobile phase was allowed to run to the end of the paper (4 hours) when the papers were removed and dried in a current of warm air. The radioactive zone was located by a gas flow chromatogram scanner (cf. Chapter II), and eluted with methanol. After removal of the solvent, the dried residue

was dissolved in pyridine and acetylated with acetic anhydride overnight (cf. Chapter II). After removal of excess reagent, the sample was again chromatographed on the ligroin-formamide system. The radioactive zone from this last chromatogram was eluted and counted for radioactivity due to  $C^{14}$  in an end window counter which did not record the activity due to the added tritiated tracer. The progesterone content of these samples was estimated by the thiosemicarbazide reaction (cf. Chapter II), the unknown samples being read against blanks prepared from eluates of chromatography paper taken through the same procedure (including the acetylation stage) and compared with a sample of authentic progesterone. The specific activity of the samples was expressed in terms of counts per minute (c.p.m.) per micromole ( $\mu$ mole) of progesterone. The values found for the specific activity of the progesterone isolated from incubations of the subcellular fractions derived from the pure sucrose homogenate used in the experiment described in section (2) are shown in Table 15. The cholesterol content and its calculated specific activity, assuming complete mixing for each fraction is given together with the weight ( $\mu$ gs.), radioactivity (c.p.m.) and specific activity (c.p.m./ $\mu$ mole) of the progesterone found. It will be seen that only in one fraction (fluffy layer) was the specific activity of the progesterone (325 c.p.m./ $\mu$ mole) in the same range as that calculated for the cholesterol (42.0 c.p.m./ $\mu$ mole). The value for the 36,000 g-min. fraction is of doubtful significance in view of the very low count of radioactivity in the sample and in the 12,000,000 g-min. fraction no count due to  $C^{14}$  was recorded. The discrepancy between the calculated cholesterol specific activity (128.5 c.p.m./ $\mu$ mole) and that found for progesterone (8.8 c.p.m./ $\mu$ mole)

TABLE 15

THE SPECIFIC ACTIVITY OF PROGESTERONE-4-C<sup>14</sup> DERIVED FROM INCUBATIONS  
OF VARIOUS SUBCELLULAR FRACTIONS WITH CHOLESTEROL-4-C<sup>14</sup>

Fraction	Cholesterol mgs.	Calculated Specific Activity of Cholesterol-4-C <sup>14</sup> cpm x 10 <sup>3</sup> /umole	Progesterone-4-C <sup>14</sup>		
			ugs.	cpm.	cpm x 10 <sup>3</sup> / umole
36,000 g-min.	3.52	34.5	17.8	12	0.2*
320,000 g-min.	0.65	128.5	6.5	180	8.8
Fluffy layer	2.06	42.0	18.0	1848	32.5
12,600,000 g-min.	4.28	20.4	9.4	-	-
Soluble	1.72	50.3	6.9	168	7.7

\* Not significant due to low radioactivity level.

in the 320,000 g-min. fraction was considerable, indicating either incomplete mixing or alternative synthetic pathways or both. For the present purpose it is not important which of these factors ~~was~~ responsible for the discrepancy, but the finding that the relationship ~~was~~ a variable from fraction to fraction clearly showed the non-validity of assessing distribution of cholesterol conversion activity on the basis of radiotracer conversion.

#### 4. The Distribution of Cholesterol-4-C<sup>14</sup> Conversion Activity in Acetone Dried Fractions from 0.44M Sucrose Homogenates

By taking advantage of the fact that acetone drying of the tissue homogenate fractions removes the endogenous cholesterol it was possible to continue the use of radiotracer techniques in determining the distribution of the cholesterol conversion activity. The usual fractions were prepared from 0.44M sucrose homogenates by methods already described except that after sedimentation the 36,000 g-min. fraction was resuspended in 0.44M sucrose and centrifuged at 1200g for 15 minutes. A heavy "fluffy layer" was obtained and treated as a separate fraction. The fractions were then suspended in 0.15M KCl, precipitated with cold acetone and subsequently washed and dried. The activity of these acetone powders was assessed by incubating amounts of the preparations equivalent to 5 gms. of adrenal cortex tissue with cholesterol-4-C<sup>14</sup> in a manner similar to that described for the incubation of acetone dried 320,000 g-min. fractions in section A of this chapter. The external TPNH generating system was used (G6P, TPN and G6P dehydrogenase). The conversion obtained for each fraction is shown in Table 16 together with the protein equivalent of the

TABLE 16

THE DISTRIBUTION OF CHOLESTEROL-4-C<sup>14</sup> CONVERSION ACTIVITY IN ACETONE  
DRIED SUBCELLULAR FRACTIONS OF A PURE SUCROSE HOMOGENATE

Fraction	Protein mgs.	Cholesterol-4-C <sup>14</sup> Conversion (%)	Potency cpm/mg pro- tein x 10 <sup>3</sup>	% of Total Activity
36,000 g-min.	28.2	42.0	1.49	22.9
Fluffy layer derived from 36,000 g-min.	25.1	50.4	2.01	27.4
320,000 g-min.	5.9	35.7	6.05	19.5
Fluffy layer derived from 320,000 g-min.	4.2	51.5	12.55	28.0
12,600,000 g-min.	6.4	2.4	0.38	1.3
Soluble	3.3	1.3	0.39	0.7

powder used in the incubation, from which the potency in terms of c.p.m. per mg. protein was calculated. Finally the distribution of activity in terms of the percentage of the total activity found is tabulated in the last column. It will be seen that the 12,000,000 g-min. fraction and the soluble fraction showed negligible activity. The cholesterol conversion activity appeared to be distributed between the 36,000 and 320,000 g-min. fractions and the fluffy layers derived from them, with the fluffy layer of the 320,000 g-min. fraction showing the highest potency.

The four active fractions were then extracted with M/15 phosphate buffer pH 6.8 and centrifuged at 105,000g. for 60 minutes to remove particulate matter. The soluble extracts were then incubated with cholesterol-4-C<sup>14</sup> in the presence of (a) fumarate, (b) fumarate + TPN, (c) succinate + TPN, (d) G6P + G6P·DH + TPN. The degree of conversion obtained from each fraction with the various TPNH generating systems is shown in Table 17. The conversion in the presence of the external TPNH generating system (G6P) was similar in all four fractions except that the value for the 320,000 g-min. fraction was abnormally low. Similarly, the conversion with fumarate and TPN suggested a uniform distribution, the activity in each case being derived from the equivalent of 5 gms. of tissue. However, only in the case of the 320,000 g-min. fraction was the activity independent of added TPN. Succinate + TPN did not support activity indicating a loss of some enzymatic step in the metabolism of this citric acid cycle intermediate. Similar loss of function was observed in other experiments where  $\alpha$ -ketoglutarate was used in place of fumarate.

TABLE 17  
 CHOLESTEROL-4-C<sup>14</sup> CONVERSION BY SOLUBLE EXTRACTS OF ACETONE DRIED  
 SUBCELLULAR FRACTIONS

Fraction	Soluble Protein (mgs)	% Cholesterol-4-C <sup>14</sup> Conversion in Presence of			
		Fumarate	Fumarate + TPN	Succinate + TPN	G6P + G6PDH + TPN
36,000 g-min.	48.3	0	17.9	1.0	20.3
Fluffy layer derived from 36,000 g-min.	41.5	0	20.0	0	24.7
320,000 g-min.	9.2	13.5	22.9	0	14.5
Fluffy layer derived from 320,000 g-min.	6.2	0.5	23.7	0.7	27.6

C. The Inhibitory Effect of Hypertonic Sucrose Incubation Media on Various Activities of Subcellular Fractions of Bovine Adrenal Cortex

Early in the investigation it was observed that the conversion of cholesterol-4-C<sup>14</sup> by 320,000 g-min. fractions was lower in hypertonic incubation medium (0.44M sucrose) compared to that in the usual hypotonic (0.025M) sucrose medium. This observation was of interest because of the known preservative effect of hypertonic sucrose on the morphological integrity of mitochondria<sup>12,13</sup>. If the maintenance of mitochondrial structure was responsible for the reduced cholesterol conversion in hypertonic medium, it was important from the point of view of answering the question whether or not the cholesterol conversion enzyme complex was truly part of the mitochondrial structure. Therefore, in the following series of experiments, various parameters of mitochondrial activity have been examined in relation to cholesterol conversion and the tonicity of the sucrose incubation medium.

1. The Inhibitory Action of Hypertonic Sucrose on Cholesterol-4-C<sup>14</sup> Conversion

Following the observation that cholesterol conversion was reduced in hypertonic incubation media, many of the experiments described in previous sections were conducted in two parts. One set of incubations was carried out in 0.025M sucrose medium and another set from the same batch of tissue incubated in 0.44M sucrose medium. In this way comparable

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<sup>12</sup>Lehninger, A. L. Physiology of Mitochondria. in Enzymes: Units of Biological Structure and Function, ed. O. H. Gaebler (New York: Academic Press, 1956) p 217.

<sup>13</sup>Witter, R. F. and Cottone, M. A. A Study of Some Factors Involved in the Swelling of Isolated Mitochondria. Biochim. Biophys. Acta, 22:364, 1956.

values were obtained for the conversion of cholesterol-4-C<sup>14</sup> by the 320,000 g-min. and other fractions, in hypotonic and hypertonic media. The results from these experiments are shown collectively in Table 18, where the values for conversion of cholesterol-4-C<sup>14</sup> by the 320,000 g-min. fraction from supplemented or pure sucrose homogenates, the fluffy layer fraction and also acetone dried fractions, are given for incubations carried out in 0.025M and 0.44M sucrose media, using either the internal or external TPNH generating system. As a measure of the degree of inhibition by the hypertonic medium the ratio (percent conversion by hypertonic:percent conversion in hypotonic) times 100 was calculated and is also shown in Table 18. It will be evident that in all the intact tissue fractions the conversion is lower in the 0.44M sucrose medium and that the inhibition is independent of the nature of the TPNH generating system used. When acetone powders of the 320,000 g-min. fraction, or the non-particulate fraction derived from them, were incubated in 0.44M sucrose containing M/15 phosphate in addition, only a slight inhibition was observed in some cases.

The failure of hypertonic sucrose incubation media to inhibit cholesterol conversion by acetone dried preparations, suggested that the phenomena was not due to a non-specific inhibition of the enzymes involved in cholesterol conversion due to the increased molar concentration. On the assumption that the high sucrose content of the medium was maintaining some form of structural integrity related to the cholesterol conversion enzyme complex, some aspects of mitochondrial function were examined to see if the high sucrose media had an effect parallel to that on cholesterol conversion activity.

TABLE 18

THE INHIBITORY EFFECT OF HYPERTONIC SUCROSE INCUBATION MEDIA ON  
 CHOLESTEROL-4-C<sup>14</sup> CONVERSION BY VARIOUS CELL FRACTIONS

Fraction	TPNH Generating System Used	Cholesterol-4-C <sup>14</sup> Conversion (%)		
		0.025M Sucrose	0.44M Sucrose	$\frac{0.44M}{0.025M} \times 100$
320,000 g-min. (Supplemented sucrose)	Internal	11.6	4.1	35.4
	External	12.4	3.9	31.4
320,000 g-min. (Pure sucrose)	Internal	22.9	9.0	39.3
	External	30.4	11.4	26.6
Fluffy layer (from 320,000 g-min. fraction)	Internal	20.5	4.4	21.5
	External	18.3	4.5	24.6
Whole acetone dried 320,000 g-min. fraction	Internal	53.0	42.3	79.8
	"	23.6	20.9	88.5
Soluble extract of acetone dried 320,000 g-min. fraction	Internal	23.6	22.5	95.4
		35.1	40.5	115.2

2. The Effect of Hypertonic Sucrose on the Oxygen Uptake and Oxidative Phosphorylation of the 320,000 g-min. Fraction

Preparations of the 320,000 g-min. fraction were isolated from supplemented sucrose media as previously described and the pellets suspended in either 0.025M or 0.44M sucrose at a concentration of the equivalent of 60 gms. of tissue in 10 ml. of sucrose solution. Warburg flasks, kept at 0°C. by placing on a chilled glass plate, were prepared as follows: each flask received 1.0 ml. of 0.88M or 0.05M sucrose; 0.2 ml. 0.25M fumarate or 0.1 ml. 0.2M pyruvate plus 0.1 ml. 0.2M malate; 0.8 ml. of a "trap" solution made up by mixing immediately before use, 4 ml. 0.2M phosphate buffer pH 7.4, 2 ml. 0.05M ATP, 2 ml. 0.05M MgSO<sub>4</sub>, 4 ml. 9% glucose, 4 ml. distilled water and 4.3 mgs. hexokinase (Sigma Type V). After addition of 1.0 ml. of either the hypotonic or hypertonic suspension, the flasks were allowed to equilibrate at 30°C. for exactly 8 minutes before oxygen uptake measurements were made every six minutes. After 30 minutes the flasks were removed from the water bath and 2 ml. aliquots of the incubate rapidly removed and added to 8 ml. of 10% trichloroacetic acid. The precipitated proteins were removed by centrifuging and aliquots of the supernatant (1 ml.) taken for assay of inorganic phosphate by the method of Fiske and Subbarow<sup>14</sup>. The respiration rate in the presence of fumarate, or pyruvate plus malate, expressed in  $\mu$ -atoms per hour per mg. of tissue fraction protein are recorded in Table 19. The amount of

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<sup>14</sup>Fiske, C. H. and Subbarow, Y. J. Biol. Chem. 66:375, 1925.

TABLE 19

THE EFFECT OF HYPERTONIC SUCROSE ON THE OXYGEN UPTAKE AND P/O RATIO OF THE 320,000 G-MIN. FRACTION

	0.025M Sucrose		0.44M Sucrose	
	Fumarate	Malate+Pyruvate	Fumarate	Malate+Pyruvate
Oxygen uptake uatoms/hr./mg.	2.82	2.76	1.74	1.74
Phosphate uptake uatoms/hr./mg.	1.38	2.58	1.44	1.38
P/O ratio	0.49	0.94	0.82	0.81

phosphate taken up (in  $\mu$ -atoms/hr./mg.) is also shown in Table 19, the values representing the difference in inorganic phosphate levels before and after incubation. The initial level was determined by precipitating aliquots of media with trichloroacetic acid immediately after addition of the tissue suspension. It will be seen that the oxygen uptake in the presence of both substrates is lower in the hypertonic than in the hypotonic medium, but the degree of phosphate "esterification" as indicated by disappearance of inorganic phosphate, is not affected in the fumarate oxidation and apparently reduced in the case of pyruvate plus malate oxidation. The ratio  $\mu$ -atoms oxygen consumed: $\mu$ -atoms phosphate taken up (P/O ratio) also shown in Table 19, reveals the most pertinent finding of the experiment, which is the very low level of oxidative phosphorylation shown by these preparations in both media. Similar measurements of the P/O ratio of another "slaughter house" tissue (bovine heart muscle) made by Hatefi and Lester<sup>15</sup> gave values of 2.0 or greater for pyruvate plus malate oxidation. In view of the very low level of oxidative phosphorylation in the adrenal preparations, even in hypertonic medium, it seemed unlikely that the inhibitory action of hypertonic sucrose on cholesterol conversion could be ascribed to a maintenance of coupled oxidative phosphorylation by the mitochondria contained in the preparation.

The extent to which oxidative phosphorylation was concerned in the cholesterol conversion may be assessed by examining results from

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<sup>15</sup>Hatefi, Y. and Lester, R. L. Studies on the Mechanism of Oxidative Phosphorylation III. Phosphorylating Particle Types from Beef Heart. *Biochim. Biophys. Acta*, 27:88, 1958.

experiments where the 320,000 g-min. fraction was incubated in the presence of  $\alpha$ -dinitrophenol (DNP), a potent inhibitor of oxidative phosphorylation<sup>16</sup>, or azide, an inhibitor of terminal oxidation enzymes<sup>17</sup>.

The results from two experiments in which the 320,000 g-min. fraction derived from supplemented sucrose homogenates was incubated with cholesterol-4-C<sup>14</sup> under standard conditions, except that in some cases DNP or azide were present in the incubation medium, are shown in Table 20. In Experiment A, the effect of DNP ( $10^{-4}$ M) on cholesterol-4-C<sup>14</sup> conversion was investigated in the presence of the internal (fumarate) TPNH generating system in both hypotonic and hypertonic incubation media. It will be seen that DNP had no inhibitory action on cholesterol-4-C<sup>14</sup> conversion in either medium. In Experiment B, the effect of DNP ( $2 \times 10^{-4}$ M) and azide ( $10^{-3}$ M) on cholesterol-4-C<sup>14</sup> conversion was examined using the external (G6P) TPNH generating system in hypotonic medium. Again there was no evidence of inhibition by either DNP or azide; in fact the former substance appeared stimulatory under these conditions.

The results from these experiments taken together with the results from the examination of respiratory and phosphorylative activities of the tissue preparation, indicated that the inhibitory action of hypertonic sucrose on cholesterol conversion could not have been entirely due to inhibition of respiration or oxidative phosphorylation as neither activity was essential for cholesterol conversion to take place.

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<sup>16</sup>Loomis, W. F. and Lipman, F. J. Biol. Chem. 173:807, 1948.

<sup>17</sup>Chance, B. Techniques for the Assay of the Respiratory Enzymes. in Methods in Enzymology, eds. S. P. Colowick and N. O. Kaplan (Vol. IV, New York: Academic Press, Inc., 1957) p. 273.

TABLE 20

THE EFFECT OF DNP AND AZIDE ON THE CONVERSION OF CHOLESTEROL-4-C<sup>14</sup>  
BY THE 320,000 G-MIN. FRACTION

Expt.	Inhibitor	TPNH Generating System Used	Cholesterol-4-C <sup>14</sup> Conversion (%)	
			0.025M Sucrose	0.44M Sucrose
A	None	Internal	14.4	4.7
	DNP ( $10^{-4}$ M)	"	14.0	6.0
B	None	External	8.7	---
	DNP( $2 \times 10^{-4}$ M)	"	15.7	---
	Azide( $10^{-3}$ M)	"	6.4	---

3. The Influence of Sucrose Tonicity on the Specific Activity of the Radioactive Products of Cholesterol-4-C<sup>14</sup> Conversion

As typical activities of mitochondria, i.e. oxidative phosphorylation and respiration, did not seem to be involved in the sucrose inhibition of cholesterol conversion, another facet of the system was examined. On a previous occasion (Section A), mention has been made of the problems arising from the use of a radiotracer to estimate metabolic activities in the presence of appreciable amounts of endogenous compound. In the event of incomplete mixing of the cholesterol-4-C<sup>14</sup> with the endogenous cholesterol pool in hypertonic medium, an apparent reduction in the conversion of cholesterol might be observed if the endogenous cholesterol was metabolized preferentially. To test this possibility, the specific activities of certain radioactive products of cholesterol-4-C<sup>14</sup> conversion by a 320,000 g-min. fraction of supplemented sucrose homogenate were determined. The values for progesterone were obtained by methods already described and those for 11 $\beta$ -hydroxyprogesterone, corticosterone and cortisol were found using similar methods, but with the paper chromatography systems appropriate to the compound in question. Thus the 11 $\beta$ -hydroxyprogesterone was isolated on propylene glycol/ligroin:toluene (1:1), corticosterone on propylene glycol/toluene, and cortisol on formamide/chloroform systems as described by Zaffaroni and Burton<sup>10</sup>. The values obtained are recorded in Table 21, where it will be seen that the specific

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<sup>10</sup>Zaffaroni, A. and Burton, R. B. Identification of Corticosteroids of Beef Adrenal Extract by Paper Chromatography. J. Biol. Chem. 193:749, 1951.

TABLE 21

THE EFFECT OF THE SUCROSE TONICITY OF THE INCUBATION MEDIUM ON THE SPECIFIC ACTIVITIES OF PRODUCTS OF CHOLESTEROL-4-C<sup>14</sup> CONVERSION

Product*	Specific Activity (cpm $\times 10^3$ / $\mu$ mole)			x 100
	Hypotonic (0.025M Sucrose)	Hypertonic (0.44M Sucrose)	$\frac{\text{Hypertonic}}{\text{Hypotonic}}$	
Progesterone	30.9	17.7	57.4	
11 $\beta$ -Hydroxyprogesterone	28.9	17.6	61.0	
Corticosterone	36.8	25.4	69.0	
Cortisol	41.9	29.1	69.5	

\* Determined by U.V. absorption at 240 m $\mu$ .

activities of the products isolated from incubations in hypotonic medium are close to that calculated for the total cholesterol of the incubated sample ( $47.0 \times 10^3$  c.p.m./ $\mu$ mole), indicating a high degree of mixing of radiotracer and endogenous cholesterol. However, the specific activities of the products isolated from incubations in hypertonic medium are only about 40-50% of the theoretical value for complete mixing. This reduced level of radioactivity in the products could be due to their production from a precursor other than cholesterol, or to a failure of the added cholesterol- $4\text{-C}^{14}$  to enter the metabolic pool to the same extent as in hypotonic incubation media. As there is no valid reason to assume an alternative pathway of product synthesis in the hypertonic medium to that obtaining in the hypotonic medium, the second conclusion is to be preferred. In this case, the reduction of the cholesterol- $4\text{-C}^{14}$  conversion in hypertonic media could well be due, at least in part, to the failure of the added cholesterol- $4\text{-C}^{14}$  to enter the sites of enzymatic reaction. The mechanism of such an effect of hypertonic sucrose cannot be determined from the data available, but by analogy with the effect of hypertonic media on mitochondrial integrity already mentioned, an action of the sucrose in maintaining some state of structural order in an enzyme complex could be postulated.

The nature of the products of cholesterol conversion obtained from incubations of the 320,000 g-min. fraction of supplemented sucrose homogenates, deserves some comment. The finding that cortisol was one of the products implies the presence of 17 and 21-hydroxylating systems in this preparation in addition to the enzymes responsible for progesterone production. The significance of this finding will be discussed in due course.

CHAPTER IV

DISCUSSION AND SUMMARY

A statement of the problem has been made in the introductory chapter of the dissertation but for the purpose of discussing the results of the experimental studies the aims of the investigation may be recapitulated in the form of three questions:

- (a) Is TPNH an essential cofactor in the enzymatic cleavage of the cholesterol side chain by adrenocortical preparations?
- (b) What is the intracellular location of the enzyme complex?
- (c) What information, if any, can be derived from the study with respect to control mechanisms of the adrenocortical secretion?

The following discussion attempts to answer these questions within the framework of the experimental results presented in Chapter III.

#### A. The Cofactor Requirement for Cholesterol Side Chain

##### Cleavage

Certain subcellular fractions of bovine adrenal cortex tissue have been found to catalyze the conversion of cholesterol-4-C<sup>14</sup> to radioactive products when incubated with the radiotracer in buffered sucrose media containing Mg<sup>++</sup> and fumarate. A cleavage of the cholesterol side chain between carbon atoms C-20 and C-22 results in the formation of a C-21 steroid. As  $\Delta^5$ -pregnenolone and progesterone have been identified, on the basis of their chromatographic behavior, as products of cholesterol conversion by these adrenal cortex preparations, it may be assumed that side chain scission did occur.

The 320,000 g-min. fraction, whether derived from supplemented or pure sucrose homogenates, required only  $Mg^{++}$ , fumarate and aerobic conditions in order to cleave the cholesterol side chain. The preparations could utilize succinate or  $\alpha$ -ketoglutarate as efficiently as fumarate in supporting cholesterol conversion, indicating the presence of mitochondrial activity. Further evidence that the preparation contained mitochondria was afforded by their ability to support respiration in the presence of pyruvate and malate and other citric acid cycle intermediates. However, inhibitors of mitochondrial oxidative, phosphorylative activities such as dinitrophenol and azide, did not inhibit the cholesterol conversion activity.

The demonstration that the citric acid cycle intermediates could be replaced by a TPNH generating system in the form of G6P, G6P·DH and TPN, strongly suggested that the role of fumarate was also that of a TPNH generating system. Similar observations have been made for the fumarate supported  $11\beta$ -hydroxylase activity of adrenocortical mitochondrial fractions by Sweat and Lipscomb<sup>1</sup> and by Grant<sup>2</sup>. The first authors presented evidence for the participation of a transhydrogenase activity such as that described by San Pietro, Colowick and Kaplan<sup>3</sup>, in the fumarate

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<sup>1</sup>Sweat, M. L. and Lipscombe, M. D. A. Transhydrogenase and Reduced Triphosphopyridine Nucleotide Involved in the Oxidation of Deoxycorticosterone to Corticosterone by Adrenal Tissue. *J. Am. Chem. Soc.* 77:5185, 1955.

<sup>2</sup>Grant, J. K. The In Vitro Enzymic Hydroxylation of Steroids. 4. The Role of Fumarate and Triphosphopyridine Nucleotide in the Enzymic  $11\beta$ -Hydroxylation of  $11$ -Deoxycorticosterone. *Biochem. J.* 64:559, 1956.

<sup>3</sup>San Pietro, A., Kaplan, N. O. and Colowick, S.P. Pyridine Nucleotide Transhydrogenase VI. Mechanism and Sterospecificity of the Reaction in *Pseudomonas Fluorescens*. *J. Biol. Chem.* 212:941, 1955.

supported reaction, and suggested that fumarate gave rise to malate and that the DPNH formed by malic dehydrogenase activity reacted with TPN and the transhydrogenase to generate TPNH. Grant and Mongolkul<sup>4</sup>, however, found the transhydrogenase activity of bovine adrenal cortex to be very low and favored Grant's<sup>2</sup> suggestion that fumarate functioned by providing malate for the TPN-linked malic decarboxylase, with consequent TPNH generation. Little in the way of evidence has been obtained in this investigation to help distinguish between these two possibilities, beyond the observation that DPN showed only slight indications of the inhibitory action on TPNH function that might be expected in the presence of an active transhydrogenase. However, rat adrenal cortex tissue appears to show high transhydrogenase activity<sup>5</sup> and the question of which reaction predominates may be answerable in terms of species difference or the relative stability of the two systems to post mortem changes.

Whatever the mechanism of the TPNH generation by mitochondrial enzymes, one can clearly differentiate such internal generation from external TPNH generation by G6P, G6P·DH and TPN. In order that fumarate may function as an internal TPNH generating system, the preparation must contain either TPN or at least a system capable of forming TPN. In the latter case the TPN forming reaction might be dependent upon fumarate

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<sup>2</sup>Grant, J. K. Biochem. J. 64:559, 1956.

<sup>4</sup>Grant, J. K. and Mongolkul, K. Hydrogen Transport in Ox-adrenocortical Mitochondria in Relation to Steroid Hydroxylation. Biochem. J. 69:36P, 1958.

<sup>5</sup>Koritz, S. B. and Peron, F. G. 1st International Congress of Endocrinology, Copenhagen, 1960. Personal communication of accepted abstract.

metabolism, but for reasons to be considered later this is an unlikely situation. In the event that TPN is present in the preparation, some form of 'binding' of the cofactor must be assumed to account for its stability. This conclusion may be reached after considering the findings that "aged", acetone dried or solubilized preparations still do not require exogenous TPN in addition to fumarate to effect cholesterol conversion. After dialysis in phosphate buffer, however, the acetone dried preparations can no longer support cholesterol conversion without addition of both TPN and fumarate, affording clear evidence of the participation of the cofactor in the cholesterol conversion reaction.

The external generation of TPNH by addition of G6P and G6P·DH is, in contrast to the fumarate system, dependent upon externally supplied TPN. Intact preparations of the 320,000 g-min. fraction from supplemented or pure sucrose media do not catalyze the conversion of cholesterol in the presence of the G6P system minus TPN. Even in acetone dried preparations the conversion of cholesterol under these conditions is considerably reduced. Either one must assume some form of structural barrier to the reaction of G6P and its dehydrogenase with the internal TPN, or one must consider that TPN, as such, does not occur in the preparation, but is generated by fumarate metabolism. The small but distinct activity shown by acetone powders in the presence of the external system minus TPN does suggest that TPN is contained in the preparation, becoming available to a certain extent following damage to the enzyme-cofactor complex by the drying process.

The conception of a cofactor-enzyme complex associated with both cholesterol conversion and mitochondrial activity is of considerable interest in the question of the intracellular location of the cholesterol conversion activity, which will now be discussed, and of possible significance in terms of control mechanisms.

#### B. On the Intracellular Location of the Cholesterol

##### Side Chain Cleavage Enzymes

The discussion of the TPNH requirement for cholesterol side chain cleavage just concluded was based on evidence obtained from studies of the 320,000 g-min. fraction. In certain cases the fraction was isolated from homogenates prepared in supplemented sucrose while in others, pure sucrose media were used. Although the preparations from either type of homogenate both required TPNH for cholesterol conversion, they differed in several other respects. The activity in terms of radioactive cholesterol conversion was greater in the pure sucrose preparation and apparently not entirely due to the much lower cholesterol content compared to the supplemented sucrose preparation. Acetone dried pure sucrose preparations were also more active than those from supplemented sucrose homogenates and furthermore contained a higher proportion of "bound" cofactor, as they were independent of added TPN for fumarate supported cholesterol conversion. There were also differences in the nature of the radioactive products of cholesterol conversion formed by these two preparations. Both were able to convert cholesterol to progesterone and  $11\beta$ -hydroxyprogesterone but the supplemented sucrose preparation contained enzymes capable of

21- and 17-hydroxylation as substances chromatographically similar to corticosterone and cortisol were obtained. As well as these differences in cholesterol conversion by the 320,000 g-min. fractions, the supplementation of the sucrose homogenizing medium by fumarate, phosphate, EDTA and nicotinamide, brought about considerable changes in the distribution of protein, and particularly cholesterol, between the various other fractions. In large measure the effects of the supplements could be duplicated by the addition of cations ( $K^+$  or  $Na^+$ ) to the sucrose.

One effect of the addition of 50mM cation to the medium was to increase the sedimentation of protein and particularly cholesterol into the low speed, or nuclei, fraction. When comparative experiments were carried out to determine the distribution of cholesterol conversion activity in fractions prepared from pure or salt-sucrose homogenates, evaluation of the results was complicated by this shift of cholesterol to the lower sedimenting fractions. Under conditions where variable mixing of tracer cholesterol with the endogenous pool occurred, the cholesterol-4- $C^{14}$  conversion values were not an accurate guide of activity. Continuation of the distribution studies using acetone dried fractions derived from pure sucrose media seemed to be the simplest solution of the problem. However, on examination of such fractions for cholesterol conversion activity, it was found to be distributed over several fractions ranging from the low speed nuclei fraction to the fluffy layer of the mitochondrial pellet. An approximately equal proportion of the total activity was found in each active fraction, but in terms of activity per mg. protein, the mitochondrial, and even more so, the fluffy layer derived from it showed the highest potency. In no case could

appreciable activity be demonstrated in the microsomal (12,600,000 g-min.) or soluble fractions.

Another effect of the addition of cations to the sucrose medium was the suppression of the appearance of the "fluffy layer". The significance of the "fluffy layer" may be related to the work of Green and his group<sup>6</sup> on bovine heart sarcosomes, where "light" and "heavy" mitochondrial fractions are distinguished by techniques similar to those used to separate the "fluffy layer" from the mitochondrial pellet in the present investigation. In the studies on the heart sarcosomes it was shown that the "light" mitochondrial layer contained partially disorganized particles which, although capable of supporting high rates of succinate oxidation, did not carry out oxidative phosphorylation to the same extent as the "heavy" fraction. It is pertinent to consider whether the presence of cations during homogenization and isolation prevented the appearance of the "fluffy layer" by protection of particulate structures, or by agglutination of small particles into larger forms that sedimented more easily. Such "clumping" effects of saline media have long been recognized<sup>7</sup>. If the cation effects on the behavior of the "fluffy layer" were due to salt-induced agglutination of the damaged particles, then there may be sufficient cation normally present in the tissue to bring about similar effects, as reducing the tissue cation content by washing prior to homogenization, allowed the appearance of the "fluffy layer"

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<sup>6</sup>Ziegler, D., Lester, R. and Green, D. E. Oxidative Phosphorylation by an Electron Transport Particle from Beef Heart. *Biochim. et biophys. Acta*, 21:80, 1956.

<sup>7</sup>Hogeboom, G. H., Scheider, W. C. and Palade, G. E. Cytochemical Studies of Mammalian Tissues. 1. Isolation of Intact Mitochondria from Rat Liver; Some Biochemical Properties of Mitochondria and Submicroscopic Particulate Material. *J. Biol. Chem.* 172:619, 1948.

during the initial sedimentation rather than on washing the pellet with pure sucrose.

During the process of homogenizing tissues, a variety of conditions can contribute to the artificial distribution of enzymes<sup>8</sup>. In addition to the agglutination of particulates by salt already mentioned, fragmentation of structures may occur with secondary absorption on other particles with varying sedimentation properties. Such redistribution of activity is exemplified by Beinert's<sup>9</sup> studies on cytochrome c liberated by fragmentation and secondarily absorbed on other sites. This type of artificial redistribution of enzymatic activity has been found to occur most easily in pure sucrose media and least of all in saline homogenates<sup>9,10</sup> Cunningham et al.<sup>11</sup>, attempting to find a compromise between agglutination (salt) and absorption (sucrose) proposed the use of salt-sucrose media very similar to those employed in the supplemented sucrose solutions in

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<sup>8</sup>de Duve, Chr. and Berthet, J. The Use of Differential Centrifugation in the Study of Tissue Enzymes. *International Review of Cytology*, eds. G. H. Bourne and J. F. Danielli (New York: Academic Press, Inc. 1954), III, 225.

<sup>9</sup>Beinert, H. The Extent of Artificial Redistribution of Cytochrome c in Rat Liver Homogenates. *J. Biol. Chem.* 190:287, 1951.

<sup>10</sup>Berthet, J., Berthet, L., Applemans, F. and de Duve, C. Tissue Fractionation Studies. 2. The Nature of the Linkage Between Acid Phosphatase and Mitochondria in Rat Liver Tissue. *Biochem. J.* 50:182, 1951.

<sup>11</sup>Cunningham, L, Griffin, A. C. and Luck, J. M. Effect of a Carcinogenic Azo Dye on Liver Cell Structure. Isolation of Nuclei and Cytoplasmic Granules. *Cancer Res.* 10:194, 1950.

the present study. With the understanding that the homogenate, whether prepared in pure sucrose or salt-sucrose, probably contained an artificial distribution of activity, one may consider the criteria generally used for deciding upon the intracellular site of an enzyme activity.

In some instances the activity may be so firmly associated with structural elements that its solubilization is a matter of great difficulty. This is true, for example, of the succinoxidase of mitochondria, or the aryl sulphatase of the microsomes<sup>8</sup>. While the microsomal 21-hydroxylase of Ryan and Engel<sup>12</sup> behaves in such a manner, this cannot be said of the cholesterol side chain cleavage activity, or for that matter, of the 11 $\beta$ -hydroxylase. Both these activities may be extracted from acetone powders by buffer or salt solutions.

Another approach may be made through the association of the enzyme activity with systems of known intracellular location. In the present instance it may be profitable to consider the relationship of the cholesterol side chain cleavage enzymes to the internal TPNH generating system, as this is clearly a mitochondrial function. The two most potent acetone dried fractions, in terms of cholesterol conversion, were the mitochondrial and "fluffy layer" fractions from pure sucrose homogenates. These fractions showed several differences in behavior. Fresh preparations of both fractions could generate TPNH from succinate, indicating mitochondrial activity, and both showed a restricted availability of TPN for

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<sup>8</sup>de Duve, Chr. and Berthet, J. International Review of Cytology, eds. G. H. Bourne and J. F. Canielli (New York: Academic Press, Inc., 1954), III, 225.

<sup>12</sup>Ryan, K. J. and Engel, L. L. Hydroxylation of Steroid at Carbon 21. J. Biol. Chem. 225:103, 1957.

the G6P external TPNH generating system. The cholesterol conversion in both preparations was also similarly inhibited by high sucrose concentrations, but in the case of the "fluffy layer", the entry of cholesterol-4-C<sup>14</sup> was apparently achieved more easily than in the mitochondrial fraction as judged by the specific activity of the radioactive progesterone formed. Acetone dried mitochondrial and "fluffy layer" fractions showed a difference in the level of "bound" TPN as the solubilized form of the "fluffy layer" fraction was dependent upon externally added cofactor for cholesterol conversion activity, whereas the mitochondrial fraction was not. It is possible that the higher potency of the "fluffy layer" fraction was related to the greater degree of mitochondrial disorganization which might be expected in this fraction. A disruption of the mitochondrial complex might well release enzyme activity which was then secondarily absorbed on the nuclei fraction elements and further dispersed on washing this fraction with pure sucrose. It is interesting to note that the mitochondrial fraction from supplemented sucrose homogenates allows almost complete mixing of the added radioactive tracer with the endogenous cholesterol and also yields acetone powders requiring added TPN for cholesterol conversion activity. In other words, the mitochondrial fraction prepared in the presence of cations resembles the "fluffy layer" fraction in most of its properties other than its sedimentation behavior.

On the basis of the considerations just discussed, it would seem to be reasonable to conclude that the cholesterol side chain cleavage activity was associated closely with the mitochondrial fraction. However, this conclusion should be considered in connection with the other enzymatic activities concerned in cholesterol conversion to adrenocortical steroid hormones.

The identification of the cholesterol side chain cleavage reaction with the mitochondrial fraction would mean that both the initial and final (11 $\beta$ -hydroxylation) steps in the corticosteroidogenetic sequence have the same intracellular location. It was pointed out in the introductory chapter that the other enzymes of the sequence have been assigned to either the soluble or microsomal fractions. It is clear that a 3 $\beta$ -hydroxydehydrogenase activity is firmly associated with the cholesterol side chain cleavage activity, as progesterone is found in all cases of active conversion by intact preparations. The solubilized activity from acetone dried mitochondrial preparations did not always give rise to progesterone, but in this case the limiting factor appeared to be DPN and not the dehydrogenase itself. It is interesting to note that Byer and Samuels<sup>13</sup> included the "fluffy layer" from the mitochondrial pellet into their microsomal fraction which they showed to contain the dehydrogenase activity. The microsomal nature of the 21-hydroxylase activity described by Ryan and Engel<sup>12</sup> is more evident as they obtained a definite separation of 21 and 11-hydroxylase activities and furthermore used highly salted sucrose media which would prevent the formation of a "fluffy layer". These authors also found the 21-hydroxylase activity to be insoluble and inseparable from the microsomal matrix in an active form. If the 17-hydroxylase can be regarded as a microsomal enzyme, then the activities concerned in the conversion of cholesterol to cortisol occupy two loci in the cell structure; mitochondria and microsomes. It is not difficult to visualize an association of these

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<sup>12</sup>Ryan, K. J. and Engel, L. L. J. Biol. Chem. 225:103, 1957.

<sup>13</sup>Byer, K. F. and Samuels, L. T. Distribution of Steroids 3 $\beta$ -ol Dehydrogenase in Cellular Structures of the Adrenal Gland. J. Biol. Chem. 219:69, 1956.

components in situ, with linkages too frail to withstand the processes of cell rupture, but close enough to permit the ordered transformation of cholesterol to cortisol. Such an association of enzymes with the mitochondria has been postulated by Hechter and his associates<sup>14</sup> to account for the observed specific orderliness of the steroid transformation reactions.

### C. On the Action of ACTH and Cholesterol Side Chain Cleavage

From the point of view of the general study on ACTH action referred to in the introductory chapter, the most interesting finding of the investigation was the demonstration that cholesterol side chain cleavage did indeed require TPNH as an essential cofactor. Apart from the fact that this supported the view that hydroxylation might precede side chain scission in the conversion of cholesterol to pregnenolone, it meant that the only step in the synthesis of corticoids from cholesterol shown to be enhanced by ACTH, was also TPNH dependent. The question that arises is, of course, the origin of the TPNH in the cell. In vitro it can arise, as we have seen, from either an external system (G6P) or from mitochondrial activity (internal system). A concept of ACTH action involving the increase of G6P levels, whether by activation of phosphorylase or increased substrate entry, does not necessarily imply TPNH production by direct

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<sup>14</sup>Hayano, M., Saba, N., Dorfman, R. I. and Hechter, O. Some Aspects of the Biogenesis of Adrenal Steroid Hormones. Recent Progr. Hormone Res. 12:79, 1956.

oxidation of G6P (G6P dehydrogenase) in preference to glycolytic metabolic pathways leading to increased mitochondrial TPNH production or for that matter, the reverse. The complexity of the intracellular distribution of the enzyme systems involved in corticosteroidogenesis is such that one could envisage either pathway of TPNH production as participating in the reaction. It is clear, however, that an increase in G6P levels is not adequate to explain many of the findings in relation to ACTH action. It does not explain the additive action of ACTH on tissue already stimulated by addition of G6P and TPN<sup>15</sup> or why ACTH does not enhance the hydroxylation of progesterone<sup>16</sup>. The present work does not provide evidence to determine the site of TPNH generation for steroid hydroxylation reactions in vivo but it does underline another aspect of the problem of control of the corticoid synthesis and that is the availability of cholesterol for the side chain scission event. The studies have shown that cholesterol is not always free to enter into the site of side chain cleavage and it is not unreasonable to postulate a mechanism of ACTH action involving increased entry of cholesterol into the reaction sequence. It is clear, however, that most of the evidence on ACTH action requires an increase in

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<sup>15</sup>Koritz, S.B. and Peron, F.G. Studies on the Mode of Action of the Adrenocorticotrophic Hormone. J. Biol. Chem. 230:343, 1958.

<sup>16</sup>Stone, D. and Hechter, O. Studies on ACTH Action in Perfused Bovine Adrenals: The Site of Action of ACTH in Corticosteroidogenesis. Arch. Biochem. & Biophys. 51:457, 1954.

TPNH as part of the mechanism, whether due to increased phosphorylase activity<sup>17</sup> or substrate entry into the cell<sup>18</sup>. A mechanism involving increased availability of TPNH and a "permissive" action on cholesterol entry into the site of action, bears some relationship to recent ideas on the action of insulin on muscle where glucose entry into the cell is facilitated by the hormone and in addition a "directive" effect on glycogen synthesis is evident<sup>19,20</sup>.

A preliminary account of the experiments demonstrating the TPNH requirement of the cholesterol side chain cleavage reaction has been published<sup>21</sup> and further comments on the reaction have been accepted in abstract form<sup>22</sup>.

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<sup>17</sup>Haynes, R. C. Jr. and Berthet, L. Studies on the Mechanism of Action of the Adrenocorticotrophic Hormone. *J. Biol. Chem.* 225:115, 1957.

<sup>18</sup>Hechter, O. Reflections about Hormone Action and Implications for the Cancer Problem. *Cancer Res.* 17:512, 1957.

<sup>19</sup>Chain, E. B., Beloff-Chain, A. and Pocchiani, F. Selected Scientific Papers. *Instituto superiore di Sanita* 1:393, 1956.

<sup>20</sup>Norman, D., Menozzi, P., Reid, D., Lester, G. and Hechter, O. Action of Insulin on Sugar Permeability in Rat Diaphragm Muscle. *J. Gen. Physiol.* 42:1277, 1959.

<sup>21</sup>Halkerston, I.D.K., Eichhorn, J. and Hechter, O. TPNH Requirement for Cholesterol Side Chain Cleavage in Adrenal Cortex. *Arch. Biochem. & Biophys.* 85:287, 1959.

<sup>22</sup>Halkerston, I.D.K., Eichhorn, J. and Hechter, O. Products of Cholesterol Metabolism in Soluble Extracts of Acetone-dried Bovine Adrenocortical Mitochondria. Abstracts 44th Federation Meeting, 1960.

## APPENDIX

The derivation of a factor relating the centrifugal forces required to sediment subcellular fractions in 0.25M sucrose, with those required for similar particles in 0.44M sucrose.

From de Duve and Berthet<sup>1</sup>:

$$R_{av} \cdot \log_{10} \frac{R_{max}}{R_{min}} = \frac{5686.96 r^2 (d_p - d_m)}{v_m} \int_0^T g_{av} \cdot dT \dots \dots (i)$$

where  $R_{max}$  and  $R_{min}$  are the distances from the bottom and top of the centrifuge tube to the center of rotation and  $R_{av}$  is the mean of these values.

For a particle of radius  $r$ ; density  $d_p$ , in a medium of density  $d_m$  and viscosity  $v_m$ ; for  $T$  seconds.  $g_{av}$  is the average centrifugal field.

For a 0.25M sucrose homogenate ( $g_{av}^{25}$ ):

substituting in (i)  $d_m = 1.034$ ;  $v_m = 1.97$  (Anderson<sup>2</sup>).

$$R_{av} \cdot \log_{10} \frac{R_{max}}{R_{min}} = \frac{5686.96 r (d_p - 1.034)}{1.97} \int_0^T g_{av}^{25} \dots \dots (ii)$$

For a 0.44M sucrose homogenate ( $g_{av}^{44}$ ):

substituting in (i)  $d_m = 1.080$ ;  $v_m = 2.50$  (Anderson<sup>2</sup>).

$$R_{av} \cdot \log_{10} \frac{R_{max}}{R_{min}} = \frac{5686.96 r^2 (d_p - 1.080)}{2.50} \int_0^T g_{av}^{44} \dots \dots (iii)$$

For a given particle the ratio of the centrifugal forces required for equivalent sedimentation in 0.25M and 0.44M sucrose is given by:

$$\text{from (ii) and (iii)} \quad \frac{d_p - 1.034}{1.97} \int_0^T g_{av}^{25} = \frac{d_p - 1.080}{2.50} \int_0^T g_{av}^{44} \dots \dots (iv)$$

$$\text{from (iv)} \quad \frac{g_{av}^{44}}{g_{av}^{25}} = \frac{(d_p - 1.034) 2.5}{(d_p - 1.080) 1.97}$$

$$\text{assuming a particle density of } 1.15^1 \quad g_{av}^{44}/g_{av}^{25} = 2.03.$$

<sup>1</sup>de Duve, Chr. and Berthet, J. The Use of Differential Centrifugation in the Study of Tissue Enzymes. *International Review of Cytology*, eds. G. H. Bourne and J. F. Danielli (New York: Academic Press, 1954) III, 225.

<sup>2</sup>Anderson, N. G. Techniques for the Mass Isolation of Cellular Components. in *Physical Techniques in Biological Research*, ed. G. Oster (New York: Academic Press, 1956) III, 300.

## AN ABSTRACT OF THE DISSERTATION

### Conversion of Cholesterol to Adrenocortical Hormone Intermediates by Subcellular Fractions of Bovine Adrenal Cortex

The sequence of reactions concerned in the formation of adrenocortical steroids from cholesterol includes the cleavage of the cholesterol side chain between C-22, 23 to form  $\Delta^5$ -pregnenolone. This step is accelerated by adrenocorticotrophic hormone (ACTH) administration, whereas the later hydroxylations of progesterone to form corticosterone and cortisol are not. These hydroxylations are known to be dependent upon triphosphopyridine nucleotide (TPNH), but the cofactor requirement for cholesterol side chain cleavage has not been defined. In view of the dominant role proposed for TPNH by currently held theories of the mechanism of action of ACTH, it is important to know if the only ACTH responsive step in the corticosteroidogenic sequence requires TPNH as an essential cofactor.

The conversion of cholesterol to C-21 steroids was investigated in subcellular fractions of bovine adrenal cortex tissue incubated with cholesterol-4-C<sup>14</sup> and the extent of conversion estimated by separating the radioactive products from unchanged cholesterol-4-C<sup>14</sup> on silicic acid columns. The radioactive products were tentatively identified on the basis of their chromatographic behavior in paper and partition column systems. The subcellular fractions were prepared by conventional differential centrifugation techniques with 0.44M sucrose, with and without ionic additions, for homogenization and isolation media. Nuclei, mitochondrial, microsomal and soluble fractions were prepared from the ionic-sucrose media and in the case of pure sucrose homogenates the "fluffy layer".

of the mitochondrial pellet was treated as an additional fraction.

The mitochondrial fraction from either homogenate required only  $Mg^{++}$  and fumarate for cholesterol conversion activity. Succinate or  $\alpha$ -ketoglutarate could replace fumarate and aerobic conditions were necessary. The requirement for citric acid cycle intermediates could be met by the "external" TPNH generating system, glucose-6-phosphate (G6P), its dehydrogenase (G6P·DH) and TPN. Acetone dried mitochondrial fractions from pure sucrose homogenates were fully active with fumarate and  $Mg^{++}$ , but after restricted dialysis against phosphate buffer, TPN was required in addition to fumarate. The activity could be obtained in a soluble, non-particulate form by extraction of the acetone dried mitochondrial fraction with M/15 phosphate buffer pH 6.8, followed by centrifugation at 105,000g. The products of cholesterol-4- $C^{14}$  conversion by the dialyzed or soluble extract of acetone dried mitochondrial fractions were largely accounted for by  $\Delta^5$ -pregnenolone and progesterone, with the relative amounts of the two intermediates depending upon the endogenous level of DPN in the preparation or the presence of this cofactor in the medium.

Distribution studies based on the activity of acetone dried fractions were run in order to avoid problems connected with unequal mixing of tracer and endogenous cholesterol. The results indicated that approximately equal amounts of the total activity were present in the nuclei, mitochondrial and "fluffy layer" fractions. The microsomal and soluble fractions were inactive. The highest activity per mg. protein was found in the "fluffy layer" with the mitochondrial fraction next, a result possibly related to the greater ease of cholesterol-4- $C^{14}$  entry into the disorganized structure of the "fluffy layer".

Soluble extracts from the acetone dried fractions were active on addition of  $Mg^{++}$ , fumarate and TPN, but only the mitochondrial fraction gave acetone powders active in the absence of TPN. The "bound" cofactor in fresh mitochondrial or "fluffy layer" fractions was not available for TPNH generation by G6P and its dehydrogenase and only reacted to a limited extent in acetone dried preparations.

High concentrations of sucrose inhibited cholesterol conversion by particulate preparations, but not the solubilized activity from acetone powders. The effect was possibly due to a reduced entry of cholesterol-4- $C^{14}$ , as the specific activities of the products of conversion were reduced, while TPNH generation by either the "internal" or "external" systems was inhibited to an equal extent.

The possible relationships of the findings to ACTH action are discussed.



#### AUTOBIOGRAPHY

The writer, Ian Donald Kermode Halkerston, was born August 8, 1921, in Uganda Protectorate, of an Australian mother and Scottish father, Dorothy M. and Donald M. Halkerston. At the age of 7 months, his parents returned to England where he was educated at Price's School, Faneham, Hants; Truro School, Cornwall; and the University of Reading, Berks. In 1941 he graduated in Agricultural Science (Reading) and passed the Royal Society of Agriculture's National Diploma examinations (N.D.A.). The next six years were occupied in service with the War Agricultural Executive Committees as an advisory and technical officer. Early in 1947

an opportunity to carry out biochemical research came with an appointment to the Endocrinology Research Department of Bristol Mental Hospitals, under the direction of Dr. Max Reiss. There followed a period of research in various field of Endocrinology, including preparative and assay work with pituitary hormones, the analysis of urinary steroids in connection with the diagnosis and treatment of mental patients, and sundry biochemical studies related to the clinical program of the department. In 1956, accompanied by his wife and two daughters, the writer came to the Worcester Foundation for Experimental Biology to work with Dr. Oscar Hechter on problems connected with the action of hormones, and during this period enrolled as a doctoral candidate with the Department of Biology of Boston University.