pounds is to interfere with normal synthesis of hydroxyproline-protein, the results presented herein further support the hypothesized general role for hydroxyproline-protein in liverwort morphogenesis. (ii) Because the action of both antagonists would logically result in the altered synthesis of hydroxyproline-protein, this experiment further supports the hypothesis that the development of three rows of leaves, rather than two, and unrestricted branching are somehow causally related to a limited formation or limited hydroxylation of hydroxyproline-protein, or both. (iii) Because changes in morphology were clearly not permanent (phenovariants reverted to their characteristic morphology) it is assumed that *Nowellia* like *Scapania* has retained, rather than obtained, the genetic capacity to form ventral leaves and to branch freely. This is considered strong support of the hypothesis that triradial symmetry with free branching is the primitive condition in the leafy liverworts and dorsiventral symmetry with restricted branching is derived (*3, 4, 7*).

Of course, even though the foregoing hypotheses are supported by work with representatives of two well-separated families of the Jungermanniales, similar evidence from experiments with representative species of several other families must be obtained before any firm conclusion concerning the role of hydroxyproline-protein in the phylogeny and morphogenesis of the leafy liverworts can be reached.

D. V. BASILE
Department of Biological Sciences,
Columbia University,
New York 10027

References and Notes

1. D. V. Basile, *Amer. J. Bot.* **54**, 977 (1967).
2. ———, *Bull. Torrey Bot. Club* **96**, 577 (1969).
3. ———, in *Current Topics in Plant Science*, J. E. Gunckel, Ed. (Academic Press, New York, 1969), p. 120.
4. R. M. Schuster, *The Hepaticae and Anthocerotae of North America* (Columbia University Press, New York, 1966), vol. 1. Phyletic schemes are on pp. 406 and 696. The relevance of branching patterns, symmetry, and leaf morphology to phylogenetic interpretations may be found in chapter 5.
5. D. V. Basile, *Bryologist* **67**, 141 (1964).
6. M. Fulford, *Phytomorphology* **6**, 199 (1956).
7. ———, *Bryologist* **68**, 1 (1965).
8. Supported by NSF grant GB-15498.

17 August 1970

Genetic Variation of Cholesterol Ester Content in Mouse Adrenals

Abstract. *Total cholesterol concentrations in muscle, liver, and plasma do not differ significantly between adult male C57BL/10J and DBA/2J mice. In the adrenal glands of these two strains, of their hybrids, and of AC mice, concentrations of free cholesterol vary by 5 percent. Adrenals from C57 mice, however, contain six times as much esterified cholesterol as adrenals from AC or DBA mice. The intermediate concentrations of cholesterol esters in F_1 hybrids suggest that the difference in this measure is inherited additively. The finding of variation in adrenal cholesterol within a species is useful for the further study of the role of cholesterol in steroidogenesis by means of genetic analysis.*

Current theories on steroid hormone production in the adrenal gland ascribe a special role to the cholesterol ester fraction. During short-term stimulation, cholesterol from the ester fraction provides substrate for the formation of the corticosteroid secreted by the gland (*1*). After prolonged stress the adrenals are depleted of the normally present lipid droplets (*2*). It was recently found that one of the early events in the mechanism of action of adrenocorticotropic hormone (ACTH) is the hydrolysis of cholesterol esters (*3*). The extent to which this pool of cholesterol contributes to the dynamics of hormone production has, however, not been studied sufficiently.

The two inbred strains of mice C57BL/10J and DBA/2J have been observed to respond differently to stress in terms of plasma corticosterone concentrations (*4*). In an attempt to find biochemical mechanisms for this apparent genetic difference in stress response, we studied the involvement of adrenal cholesterol. We report here large differences in cholesterol ester concentrations in adrenal glands of different strains of mice.

The AC mice and colonies of the pure-bred strains C57BL/10J and DBA/2J and their F_1 hybrids were maintained in our laboratory. The colonies were started from breeders purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. Litters were weaned at about 24 days after birth and housed in groups of three to eight. Breeding pans always contained two females and one male. The F_1C hybrids were derived from crossing a C57 female with a DBA male; the F_1D hybrids were derived from crossing a DBA female with a C57 male. Experiments were performed exclusively on adult male mice (10 to 20 weeks old). In experiments in which more than one type of mouse was used, the tissues were coded and analyzed without knowledge of their strain classification.

The mice were killed by decapitation. Blood was collected in small, dry, heparinized beakers. Centrifugation in a Beckman microfuge for 2 minutes yielded 0.1 to 0.3 ml of plasma. Small samples of liver and muscle (from the hind leg) were collected in one experiment. Adrenal glands were removed and freed from adhering fat. The tissues were kept ice cold and moist with isotonic NaCl. Before weighing, they were blotted briefly with filter paper.

Tracer amounts of [26-^{14}C]cholesterol and [7α-^{3}H]cholesteryl stearate were added to the tissue (100 mg of muscle or liver, 0.10 ml of plasma, or one pair of adrenals), which was then extracted twice with 3 ml of a mixture of acetone, ethanol, and ethyl ether (4:4:1, by volume) (*5*). A glass rod was used to break up the tissue in a centrifuge tube. The combined extract was evaporated with N_2. The residue was dissolved in 3 ml of a mixture of

Table 1. Weights of pairs of adrenal glands, body weights, and ratios of adrenals to body weights of pure strains of mice and hybrids (C57 mice crossed with DBA mice). For further details see text. Values shown are the means of the means obtained in n different experiments (number in parentheses) and the standard errors of the means. Because no statistically significant (at $P \leq .05$) differences were found between the two reciprocal hybrids in any of the three parameters, the values for the hybrids are pooled for the purpose of this presentation.

Strain	Adrenal glands (mg)	Body (g)	Ratio (mg/100 g)
C57BL/10J	3.0 ± 0.04 (7)	28 ± 0.6 (15)	11 ± 0.2 (4)
DBA/2J	3.1 ± 0.07 (10)	26 ± 0.3 (16)	12 ± 0.6 (5)
F_1 Hybrids	3.4 ± 0.09 (9)	29 ± 1.0 (7)	12 ± 0.3 (6)
AC	2.7 ± 0.04 (3)	21 ± 1.3 (3)	13 ± 0.9 (3)

Table 2. Cholesterol content in some tissues of two strains of mice. The numbers in parentheses are the number of separate analyses. Results are expressed as the means ± S.E.M. Experimental details are in the text and in the legend of Fig. 1.

Strain	Total cholesterol			Free cholesterol in plasma (mg/ml)	Cholesterol ester in plasma (mg/ml)
	Muscle (mg/g)	Liver (mg/g)	Plasma (mg/ml)		
C57BL/10J	0.89* (2)	2.32 ± 0.106 (8)	0.75 ± 0.040 (21)	0.25 ± 0.109 (13)	0.51 ± 0.038 (13)
DBA/2J	1.04† (2)	2.61 ± 0.164 (7)	0.75 ± 0.032 (21)	0.22 ± 0.009 (14)	0.48 ± 0.017 (14)

* Range, 0.84 to 0.94. † Range, 0.73 to 1.36.

ethanol and water (65:35) and extracted three times with 3 ml of hexane. The hexane extract was washed with water, filtered through a small column of Na_2SO_4, and reduced in volume. On thin-layer chromatography on 0.25 mm of silica gel G impregnated with Rhodamine 6G (*6*) in a mixture of toluene and ethyl acetate (9:1), free sterols were separated from sterol esters. The dye made the spots visible in daylight. The sterol and sterol ester areas were eluted three times with a mixture of benzene and methanol (19:1). The eluate was filtered through a small column of silica gel to remove eluted dye and evaporated with N_2.

The sterol ester fraction was dissolved in 2 ml of absolute ethanol. After adding 0.25 ml of saturated aqueous KOH, the mixture was kept at 72°C for 1 hour. It was then diluted with 2 ml of water and extracted three times with hexane. The hexane extract was washed with water, filtered through Na_2SO_4, and reduced in volume. Thin-layer chromatography of the saponified ester fraction and elution of sterols from silica gel was carried out as described above. As a check of the completeness of the saponification step, the sterol ester area was scraped into vials and checked for radioactivity in a scintillation counter. It always contained less than 1 percent of the original ^3H tracer dose of cholesteryl stearate.

The free sterol fraction and the sterol from the ester fraction were dissolved in small volumes of heptane and analyzed in a gas chromatograph. A portion of the heptane solution of each extract was assayed for radioactivity by liquid scintillation, and the recovery of ^{14}C from the free cholesterol fraction (85 to 95 percent) or ^3H from the cholesterol ester fraction (60 to 70 percent) was used to correct the mass of cholesterol determined by gas chromatography. Only a single peak with the retention time of standard cholesterol was recorded with most extracts. Occasional, additional small peaks were not further analyzed.

The free cholesterol concentration in whole adrenal glands of the different groups of mice varied only about 5 percent between the highest and lowest (Fig. 1) (*7*). In contrast, the cholesterol ester concentration in C57 was more than six times as high as that of the DBA or AC strains. The cholesterol ester values of the two reciprocal F_1 hybrids were intermediate to those of the parent strains and also differed between each other. As determined by Student's *t*-test, the differences in all groups were significant ($P < .001$).

No correlation was found between adrenal weight (Table 1) and cholesterol ester concentration. The adrenal weights of C57 and DBA mice did not differ significantly, but adrenals of the hybrids were heavier ($P < .01$). This finding rules out the possibility that differences in adrenal weight would in part compensate for differences in cholesterol ester concentration. Lastly, differences in the ratio of adrenal weight to body weight were negligible in these groups.

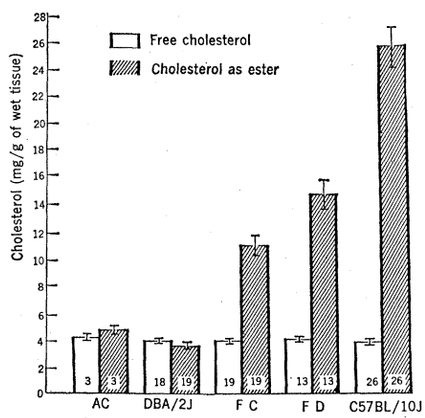

Fig. 1. Cholesterol content in whole adrenal glands of different types of adult male mice. The height of the bar indicates the mean of values of separately analyzed pairs of adrenals (the number inside the bar); the horizontal lines show the standard error of the mean. Cholesterol was analyzed by quantitative gas chromatography [hydrogen flame ionization detector, 1.8 m by 3.2 mm column of 1 percent XE-60 on Gas Chrom Q 100/120 mesh (Applied Sciences Laboratory) at 230°C and N_2 at 85 ml/min]. The retention time of cholesterol was 5 to 6 minutes, and the peak heights of standard samples (0.2 to 1.0 μg) bore a linear relation to the mass injected. Cholesterol as ester is the amount of cholesterol derived from the cholesterol ester fraction. The strains of mice and the tissue extraction procedure are described in the text.

For comparison with the adrenals, we also determined the cholesterol content of muscle, liver, and plasma in the C57 and DBA strains (Table 2). Any differences between the strains were not significant.

Large, genetically determined differences in adrenal cholesterol ester concentration (Fig. 1) have hitherto not been reported to occur within the same species. Goodman (*8*) listed one category of mammalian species, including man, rat, and others, whose adrenal glands contain 2 to 6 percent cholesterol, of which about 85 percent is esterified, and a contrasting category of species, including cattle and sheep, in which total adrenal cholesterol is less than 1 percent, with only about 50 percent esterified. The finding of the same wide variation in the single species *Mus musculus* now makes it convenient to study the pattern of inheritance of adrenal cholesterol concentrations. The results obtained with F_1 hybrids of C57BL/10J and DBA/2J are consistent with an additive mode of inheritance for adrenal cholesterol ester concentrations. Further studies in segregating (that is, F_2 and backcross) generations are needed to confirm this. An additive mode of inheritance in mice was described by Bruell (*9*) and by Yamamoto *et al.* (*10*) for serum and plasma cholesterol concentrations, respectively. The causes of the small difference between the reciprocal F_1 hybrids in cholesterol ester concentrations (Fig. 1) are not known.

The AC mice were included in this study because they carry the autosomal recessive adrenal lipid depletion gene (*ald*) (*11*). Adrenal lipid depletion is defined as the virtual absence of histochemical sudanophilia of adrenal slices, in contrast with the intense lipid stain of the "normal" adrenal zona fasciculata. With ACTH-stimulated adrenals of a subline of mice related to AC and carrying *ald*, Solem (*12*) noted a curtailed production in

vitro of corticosterone as compared to adrenals with the "normal" high lipid content. Since the cholesterol concentrations of DBA mice resemble those of the AC (Fig. 1), it remains to be seen whether the phenomena of low adrenal cholesterol and adrenal lipid depletion have a common genetic cause. Vicari reported (*13*) lower adrenocortical sudanophilia in young DBA mice than in young C57 mice.

The biochemical mechanism controlling ester concentration in the adrenal is still obscure. There is good evidence that blood supplies the bulk of metabolically active cholesterol and that any synthesis in the gland itself contributes relatively little (*14*). Shima and Pincus (*15*) have noted a rapid synthesis and secretion of radioactive corticosterone by the adrenals of rats infused with [7α-^{3}H]cholesterol. Nevertheless, a consideration of adrenal ester concentrations in relation to the plasma concentrations in Table 2 makes it clear that some specific sterol ester-concentrating mechanism must control the concentrations in the adrenal independently of the prevailing concentrations of cholesterol in the plasma. Dexter *et al.* (*16*) have shown that ACTH stimulates the accumulation of cholesterol by the rat adrenal when the utilization of cholesterol was blocked at the same time.

The chief interest of the findings in Fig. 1 derives from the opportunity to employ genetic analysis in the problem of correlating adrenal hormone production with cholesterol concentrations. There is a large difference between DBA/2J and C57BL/10J mice in the capacity of the liver to generate reduced nicotinamide adenine dinucleotide phosphate with implications for a number of major metabolic pathways (*17*). Bartke (*18*) has observed a much lower ratio of ester to free cholesterol in the testes of DBA than in those of C57 mice. These findings can be exploited in the study of the relation of biochemical and endocrine characters by determining their intercorrelation in segregating generations. Genetic analysis could be used as a tool for the study of the interrelationship of a number of biochemical and physiological systems which would be difficult to approach by other means.

CHARLES H. DOERING
SEYMOUR KESSLER, R. B. CLAYTON
Department of Psychiatry, Stanford University School of Medicine, Stanford, California 94305

References and Notes

1. J. K. Grant, K. Griffiths, M. Lowe, *Mem. Soc. Endocrinol.* **17**, 113 (1967); L. D. Garren, *Vitamins Hormones* **26**, 119 (1968); S. I. Sulimovici and G. S. Boyd, *ibid.* **27**, 199 (1969); H. L. Moses, W. W. Davis, A. S. Rosenthal, L. D. Garren, *Science* **163**, 1203 (1969); L. D. Garren, W. W. Davis, G. N. Gill, H. L. Moses, R. L. Ney, R. M. Crocco, *Excerpta Med. Int. Congr. Ser. No. 184* (1969), pp. 102–114.
2. R. A. Knouff, J. B. Brown, B. M. Schneider, *Anat. Rec.* **79**, 17 (1941); G. Popják, *J. Pathol. Bacteriol.* **56**, 485 (1944); G. Sayers, M. A. Sayers, E. G. Fry, A. White, C. N. H. Long, *Yale J. Biol. Med.* **16**, 361 (1944); G. E. Bergner and H. W. Deane, *Endocrinology* **43**, 240 (1948); D. Glick and M. J. Ochs, *ibid.* **56**, 285 (1955); C. T. Ashworth, G. J. Race, H. H. Mollenhauer, *Amer. J. Pathol.* **35**, 425 (1959); C. Riley, *Biochem. J.* **87**, 500 (1963).
3. W. W. Davis and L. D. Garren, *Biochem. Biophys. Res. Commun.* **24**, 805 (1966); W. W. Davis, *Fed. Proc.* **28**, 701 (1969); S. Ichii, N. Okada, A. Ikeda, *Endocrinol. Jap.* **17**, 83 (1970).
4. S. Levine and D. M. Treiman, *Endocrinology* **75**, 142 (1964).
5. J. J. Kabara, *Meth. Biochem. Anal.* **10**, 263 (1962).
6. J. Avigan, D. S. Goodman, D. Steinberg, *J. Lipid Res.* **4**, 100 (1963).
7. The application of Student's *t*-test to free cholesterol concentrations revealed that the C57 versus DBA values ($P < .05$) and C57 versus pooled hybrid values ($P < .01$) differed significantly.
8. D. S. Goodman, *Physiol. Rev.* **45**, 747 (1965).
9. J. H. Bruell, *Science* **142**, 1664 (1963).
10. R. S. Yamamoto, L. B. Crittenden, L. Sokoloff, G. E. Jay, Jr., *J. Lipid Res.* **4**, 413 (1963).
11. K. Arnesen, *Acta Pathol. Microbiol. Scand., Suppl.* **109**, 1 (1956); *Proc. XIV Scand. Congr. Pathol. Microbiol.* (Universitetsforlaget, Oslo, 1964), pp. 15–16; K. Molne, *Acta Pathol. Microbiol. Scand.* **75**, 37 (1969); the adrenal lipid depletion gene is described in the introduction of the latter paper.
12. J. H. Solem, *Acta Pathol. Microbiol. Scand.* **70**, 25 (1967).
13. E. M. Vicari, *Anat. Rec.* **86**, 523 (1943).
14. H. Werbin and I. L. Chaikoff, *Arch. Biochem. Biophys.* **93**, 476 (1961); A. A. Krum, M. D. Morris, L. L. Bennett, *Endocrinology* **74**, 543 (1964); A. J. Borkowski, S. Levin, C. Delcroix, A. Mahler, V. Verhas, *J. Clin. Invest.* **46**, 797 (1967).
15. S. Shima and G. Pincus, *Endocrinology* **84**, 1048 (1969).
16. R. N. Dexter, L. M. Fishman, R. L. Ney, G. W. Liddle, *ibid.* **81**, 1185 (1967); R. N. Dexter, L. M. Fishman, R. L. Ney, *ibid.* **87**, 836 (1970).
17. M. Lindberg, J. G. M. Shire, S. Kessler, R. B. Clayton, *Fed. Proc.* **29**, 900 (1970).
18. A. Bartke, personal communication.
19. We thank Dr. J. G. M. Shire for helpful suggestions and discussions, Mrs. J. Richards for technical assistance, and Prof. K. Arnesen for arranging the shipment of the AC mice from his colony. Supported by PHS grants MH 10976 and HD 00801 and a grant-in-aid from the American Heart Association.

24 August 1970

Similarity and Limited Multiplicity of Membrane Proteins from Rough and Smooth Endoplasmic Reticulum

Abstract. *During electrophoresis on acrylamide gel 30 to 45 percent of the protein of hepatic microsomal membranes migrates as a single band corresponding to a molecular weight of 52,000. Rough and smooth microsomal membranes exhibit essentially identical electrophoretic patterns. Different findings by previous workers may be the result of contamination of the membranes by adsorbed and entrapped nonmembrane protein.*

Recent studies (*1, 2*) suggest that most animal cell membranes contain many different proteins instead of a single predominant protein, as had been proposed earlier (*3*). Some of these studies also indicate that there is considerable variation in the protein composition of different membranes, even when closely related membranes from the same tissue are compared. However, an alternative possibility that these findings represent contamination of the membranes by extraneous proteins has not been excluded.

Indeed, evidence from our laboratory and others (*4, 5*) suggests that hepatic microsomal membranes, as normally isolated, contain relatively large quantities of protein that do not appear to be required for their structural or functional integrity. A procedure for selectively removing these proteins with salt and detergent has been briefly described (*4*). We have examined the protein composition of membranes both before and after removal of these proteins.

Rough and smooth microsomes, isolated from rat liver by the method of Dallner *et al.* (*6*), were washed sequentially with 0.14*M* sodium chloride, 1.0*M* sodium chloride, and 0.1*M* sodium carbonate with 0.1*M* sodium bicarbonate (8 ml of each solution per fraction from 1 g of liver). This procedure removes membrane-attached ribosomes and large amounts of adsorbed protein without extracting significant amounts of phospholipid or several microsomal enzymes that have been examined (*4*). Finally, in order to remove protein that appears to be trapped within the closed vesicles, we washed the membranes once with half the above volume of 0.075 percent sodium deoxycholate, *p*H 7.6.

Unwashed and washed microsomal fractions were analyzed by electrophoresis on polyacrylamide gel in a sodium dodecyl sulfate (SDS) system by means of a modification of a meth-