Some speculations on the origin and evolution of NC genes have been presented elsewhere.[11]

Summary.—Evidence has been presented of recombination in *Chlamydomonas* involving two nonchromosomal genes. The two are unlinked, but each exhibits intragenic recombination between mutant alleles, giving rise to wild-type recombinants. In one of them, the acetate gene, reciprocal recombinants containing both of the parental mutant sites have also been detected. The results are interpreted as evidence of the nucleic acid composition of these nonchromosomal genes.

* Supported by grant GM-12325 of the U.S. Public Health Service.

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*TRANSCHOLESTERIN, A CHOLESTEROL-BINDING GLOBULIN: SEROLOGICAL DEMONSTRATION OF A SPECIFIC INTERACTION BETWEEN CHOLESTEROL AND SERUM GLOBULIN**

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The study to be presented was undertaken with the concept that the factors regulating the transport of cholesterol and the control of cholesterol levels in human blood are part of a homeostatic physiological system similar to haptoglobins,[1] transferrins,[2] so-called natural antibodies, and hormone carriers.[3] They constitute an immunological mechanism concerned with the preservation of the norm. Accordingly, attention turned to the use of immunological methods in an effort to determine whether specific reactions between cholesterol and serum proteins could be demonstrated. The simplicity of these methods for recognition and identification of the reactive components in serum appeared to have distinct advantages over the conventional biochemical procedures. Several immediate problems had to be met, such as a technique for putting cholesterol into a solution which is stable in aqueous medium and will diffuse in an agar or other suitable transparent gel.

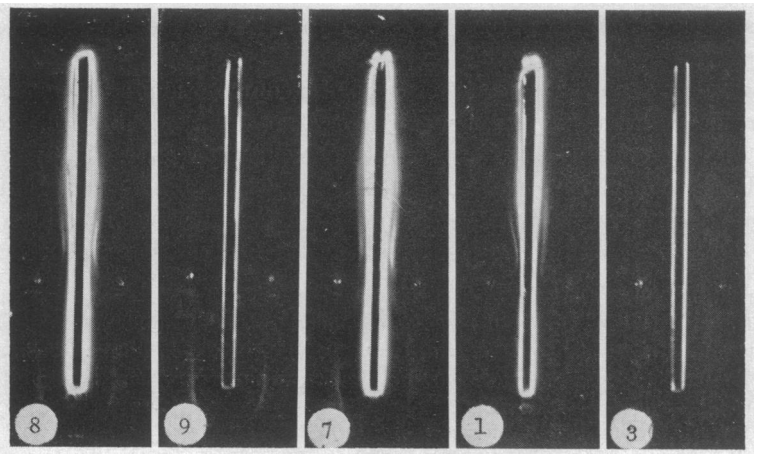

FIG. 1.—Comparison of immunoelectrophoretic patterns of *slide #1* lipoproteins with Teepol "L"-cholesterol solution in the antibody trough, *slide #3* lipoproteins with Teepol "L" in the antibody trough, *slide #7* human serum with Teepol "L"-cholesterol in the antibody trough, *slide #9* human serum with Teepol "L" in the antibody trough, and *slide #8* human lipoprotein-free serum with Teepol "L"-cholesterol in the antibody trough (dark field illumination).

The present paper describes the demonstration of a specific reaction between a serum globulin and cholesterol by means of Ouchterlony plate diffusion[4] and agar gel immunoelectrophoresis.[5] The serum component has been termed transcholesterin.

Materials And Methods.—Serum samples: Pooled and individual fresh sera were obtained from healthy blood donors.

Lipoprotein and lipoprotein-free serum preparations: Preparative ultracentrifugal flotation was carried out according to Lewis, Green, and Page[6] at solvent density of 1.21 gm per ml in a Spinco model L ultracentrifuge at 17° for 20 hr utilizing a no. 30 rotor and spinning at 30,000 rpm (79,240 × *g*). The top layer containing the serum lipoproteins was separated from the infranate. Repeated ultracentrifugation under the same conditions provided a lipoprotein preparation which was quite free from other nonlipoprotein components of the serum. The infranate, which was free of lipoproteins, was obtained in the same process. Both the lipoprotein and lipoprotein-free serum preparations were made isotonic by lyophilization and dialysis with isotonic saline. Paper electrophoresis of the isolated lipoprotein fraction and of the lipoprotein-free fraction was carried out according to Block *et al.*[7] with modifications by Lemmen and co-workers.[8] Treatment of the sample strips with Sudan Black B[9] or Oil Red O[10] resulted in staining of the lipoprotein fraction but not of the lipoprotein-free fraction. On starch gel electrophoresis also the lipoprotein-free fraction failed to stain with lipid dyes.

Immunoelectrophoresis: The procedures used in the production of purified agar, buffer solutions, preparation of agar gel-plated slides, and electrophoretic separation were described by Hirschfeld.[5] A 0.01-ml sample of serum was applied on the agar gel. Electrophoretic separation was carried out at room temperature for 2 hr employing a Shandon electrophoresis apparatus with power supply type 2523 MK II at a potential gradient of 7–8 v/cm. Under these conditions the albumin migrated about 4 cm. After the separation was completed, the slides were placed in a humid chamber; a trough was prepared in the center of the slide and was charged with 0.07 ml of either Teepol "L" (an alkyl sulfate detergent, pharmaceutical grade, obtained from Edward Gurr, Ltd., London, England) or solution of 30 mg cholesterol (Eastman Organic Chemicals, Rochester, N.Y.) per ml of Teepol "L." The charged slides were allowed to incubate at room temperature in a humid chamber for 4 hr for the precipitin line to develop before photographic records were made.

Cholesterol solution: A 30-mg sample of cholesterol was introduced with stirring into 1 ml of Teepol "L" resulting in crystal-clear solution of cholesterol for use in gel diffusion and immunoelectrophoresis.

Ouchterlony plate diffusion: The agar plate diffusion techniques of Ouchterlony[4] were used.

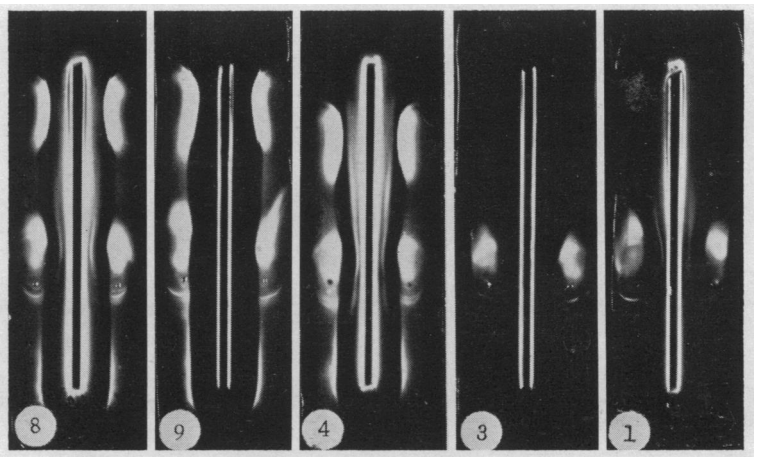

FIG. 2.—Comparison of immunoelectrophoretic patterns after acid fixation with dilute acetic acid (5%). *Slides #1, 3, 9,* and *8* are the same as those of Fig. 1, except that these are fixed with dilute acetic acid, *slide #4* human serum with Teepol "L"-cholesterol in the central trough and that slide is also fixed with dilute acetic acid (dark field illumination).

Results.—(1) *Demonstration of a reaction line between cholesterol and serum globulin:* (a) *With agar gel immunoelectrophoresis*—A precipitin-like line was demonstrated on agar gel immunoelectrophoresis of human serum, lipoproteins, and lipoprotein-free serum with cholesterol dissolved in Teepol "L." Some of the results are shown in Figure 1. The precipitin-like line produced by Teepol "L"-cholesterol and not by Teepol "L" control in the α-β globulin region of human serum, lipoprotein fraction, and lipoprotein-free serum clearly indicates a specific reaction between cholesterol and α-β globulin, also between cholesterol and the lipoprotein fraction. The line visible in the γ-globulin region is not specific because Teepol "L" control produces this line also. The interaction line observed in the presence of cholesterol is opalescent and becomes less defined and smeary when allowed to diffuse for more than 12 hr. However, after fixing the gel with dilute acetic acid, a line is visible in the albumin region in addition to the cholesterol line in the α-β globulin region. Again the Teepol "L" control line is present in the γ-globulin region. The results are given in Figure 2.

(b) *Ouchterlony plate diffusion.*—Diffusion of human serum in Ouchterlony plates was also made to determine the reality of the reaction. When the plates are fixed with dilute acetic acid, two lines are seen in the Teepol "L" control and three lines are present in the Teepol "L"-cholesterol diffusion as shown in Figure 3. The two lines in the Teepol "L" control can be compared to the albumin and γ-globulin lines represented in the immunoelectrophoretic pattern. The third line seen only in the Teepol "L"-cholesterol diffusion closest to the left central well is probably identical with that formed in the α-β globulin region of immunoelectrophoresis; the fact that the cholesterol line was closest to the central trough in immunoelectrophoresis supports this interpretation.

(2) *Staining of reaction lines:* Attempts to stain these beautiful lines with amido Schwarz 10B were unsuccessful. Oil Red O also failed to stain these lines. So far none of the commercially available lipid or protein dyes has been found to stain

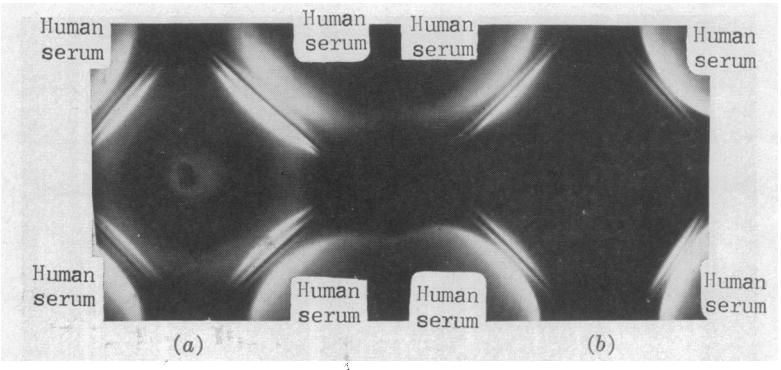

FIG. 3.—Comparisons by Ouchterlony plate diffusion of human serum in peripheral wells using (*a*) Teepol "L"-cholesterol in the left center well, and (*b*) Teepol "L" in the right center well (dark field illumination).

Teepol "L"-cholesterol lines, suggesting that cholesterol has coated the combining protein. However, the lines observed are not washed out during the staining process where the solvent for amido Schwarz 10B and the wash fluid contains 5:5:1 (v/v) of methanol:distilled water:glacial acetic acid, further supporting the conclusion that the lines observed in α-β globulin diffused with Teepol "L"-cholesterol are indeed cholesterol-globulin combinations.

(3) *Specificity of the precipitin-like reaction in the α-β globulin region:* The data in Table 1 show that of the various substances tested for the visible reaction of agar gel immunoelectrophoresis with human serum, only cholesterol dissolved in Teepol "L," dihydrocholesterol in Teepol "L" solution, and Tween 20 (1.6% solution in isotonic saline) were able to give positive reaction. Cholesterol esters with the fatty acid hooked onto the cholesterol hydroxyl group in ester linkage did not produce the precipitin-like reaction. It would seem that this reaction is specific for cholesterol molecule with its free hydroxyl as a reactive site. However, the saturation of the double bond in the cholesterol structure did not affect the capability of dihydrocholesterol to react in the α-β globulin region. Other steroid homologues such as hormones and vitamin D_3 were tested and no reaction resulted. Free fatty acids were used in order to determine if they would react with the serum globulins in this system and again negative results were obtained. No reactions were given by carbohydrate compounds. Vaccinial hemagglutinin, a product resulting from vaccinia virus infection of HeLa cells, which is a lipoprotein, did not react with either human serum α-β globulins or with Teepol "L"-cholesterol in the immunoelectrophoretic system. Negative results were obtained with cardiolipin, vitamin A, DL-α-tocopherol, folic acid, d-biotin, lecithin, lysolecithin, tripalmitin, triolein, and O-phosphoethanolamine. Detergents such as desoxycholate, triton $\times$ 100, triton $\times$ 45, pluronic L 64, sodium lauryl sulfate, glycerol monoleate, and Arlacel A were found to give no precipitin reaction.

(4) *Properties of the Tween 20 interaction line:* Tween 20 (polyoxyethylene sorbitan monolaurate) was found to yield a reaction line in the α-β globulin region. The results are presented in Figure 4. This line was stainable with Oil Red O and did not become less distinct with prolonged incubation (i.e., for time longer than 24 hr) in contrast to that of Teepol "L"-cholesterol. Since Tween 20 alone is capable

TABLE 1

SPECIFICITY OF THE PRECIPITIN REACTION

Various substances were tested for the precipitin-like line reaction on agar gel immunolectrophoresis as the reagents for human serum. All the substances were dissolved in Teepol "L" at 30 mg per ml concentration and some of the less soluble compounds were in saturated solution at less than 30 mg per ml concentration. Human sera giving good cholesterol reaction lines were used.

Substances	Reaction line at α-β globulin region	Substances	Reaction line at α-β globulin region
Dihydrocholesterol	+	Tween 20*	+
Cholesteryl oleate	−	Oleic acid	−
Cholesteryl stearate	−	Stearic acid	−
Cholesteryl laurate	−	Palmitic acid	−
Cholesterol	+	Glycerol	−
		Triolein	−
		Tripalmitin	
Androsterone	−	Inositol	−
Vitamin D_3	−	Cardiolipin	−
Aldosterone	−	Vitamin A	−
Epiandrosterone	−	DL-α-Tocopherol	−
Progesterone	−	Folic acid	−
Cortisone	−	d-Biotin	−
Pregnanediol diacetate	−	Lecithin (purified)	−
		Lysolecithin	−
		O-Phosphoethanolamine	−
		2-Phosphoglyceric acid	−
Vaccinial hemagglutinin	−	Ribose-5-phosphate	−
		Ribose	−
D-Glucosamine	−	Mannose	−
N-Acetyl Glucosamine	−	Fructose	−
		Mannitol	−
Glucuronic acid	−	Hexose di-phosphate	−
Teepol "L"	−		

* 1.6% solution in isotonic saline.

of being stained by most of the well-established lipid and protein stains, the complex formed with serum globulin remains stainable. The staining capacity of this complex for Oil Red O would suggest that the interaction is due to the enveloping effect of Tween 20 on serum globulin. In other words, the globulin molecule remains inside a shell made up of Tween 20 molecules. Conversely, the nonstaining cholesterol-protein complex would represent a complex with the protein in the middle and cholesterol covering the protein, thus explaining why ordinarily stainable protein, after complexing with cholesterol, is no longer reached by protein stains.

(5) *Isolation and characterization of the cholesterol-binding protein:* Preliminary isolation of the cholesterol-binding protein has been carried out by zone electro-

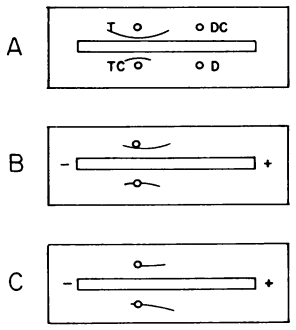

FIG. 4.—Comparisons by agar gel plate diffusion and by immunoelectrophoresis of human serum. *Slide A:* agar gel diffusion with human serum in the central trough and various substances in the adjacent wells are *T*, Tween 20 (1.6% in isotonic saline); *TC*, cholesterol dispersion in Tween 20; *D*, desoxycholate (1.6% in isotonic saline); and *DC*, cholesterol dispersion in desoxycholate. *Slide B:* immunophoresis of human serum with cholesterol dispersion in Tween 20 in the central trough. *Slide C:* immunophoresis of human serum with Tween 20. All the agar gel slides are stained with Oil Red O.

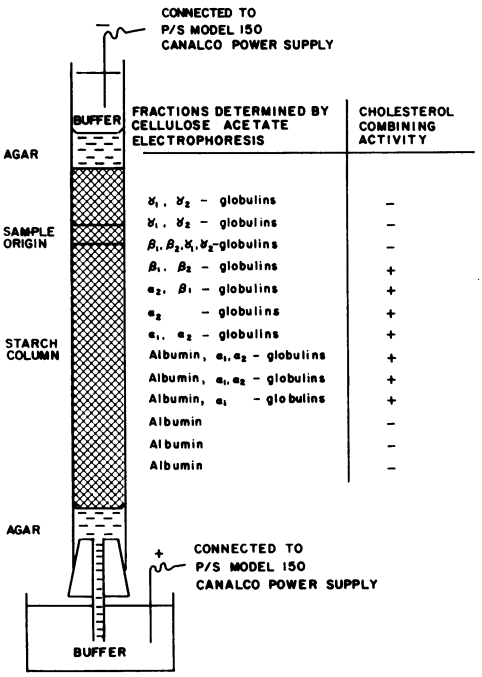

FIG. 5.—Separation of lipoprotein-free serum proteins on vertical zone electrophoresis packed with potato starch powder (Baker analyzed reagent) saturated with buffer as medium. Buffer 5 consisted of: 8.845 gm diethylbarbituric acid; 6.533 gm sodium acetate; and 5.815 gm Tris (hydroxymethyl) aminomethane dissolved in distilled water, adjusted to pH 8.10, and finally made up to 1 liter with distilled water. A 300-mg sample of lipoprotein-free serum dissolved in buffer was applied onto the starch column (2 × 28 cm). Length of time taken for electrophoresis was 28 hr with two intermittent changes of fresh buffer. Agar gel made with the buffer was used to compartmentalize the starch medium and also to serve as salt bridge. Temperature, 22–25°C. Upon completion of electrophoresis, the starch column was extruded, cut into 1-cm fractions, eluted, reduced to small volume by lyophilization, dialyzed against 0.154 *M* sodium chloride, identified by cellulose acetate electrophoresis, and finally tested for cholesterol-combining activity.

phoresis on starch column as shown in Figure 5. After the lipoprotein-free serum was subjected to electrophoresis for 28 hr, the column of starch was pushed out the tube with a rubber plunger and 1-cm fractions collected, eluted with isotonic saline, lyophilized, and dialyzed against isotonic saline. The fractions were identified by means of cellulose acetate electrophoresis.[11] Later, these fractions were tested for cholesterol-combining activity. All the fractions containing α-globulins gave positive results with cholesterol solution and one fraction of β-globulin close to α_2-globulin was also positive. All manipulations were performed at room temperature where the α-β-globulins appear to be stable. Aging the sera at 4°C for up to 2 months did not affect the precipitin reaction.

Discussion.—In 1935 Brunelli[12] carried out experiments to demonstrate the binding of steroid hormones to serum proteins and protein fractions *in vitro*. He was able to show that estrogen added to serum did not dialyze through a collodion membrane. This he thought was due to binding of estrogen to serum proteins. Later, Szego and Roberts[13] confirmed this finding with ultrafiltration studies and by precipitation with organic solvents of a protein-estrogen combination from the serum of women. Various steroid substances were found by Westphal[14] to interact with serum albumin and the binding affinity was increased with the introduction of alkyl group into the steroid and decreased by electron-attracting groups (−OH; = O; and halogen). Early indications for participation of serum globulins in steroid interaction have been obtained for corticosteroid,[15] estrogens,[16] and 17-ketosteroids.[17]

Hageman and Gould[18] in 1951 showed that the cholesterol alcohol component of the red cell was in rapid equilibrium and exchange with the cholesterol alcohol component of plasma. The cholesterol of the blood is carried as part of a lipoprotein complex and approximately two thirds of the serum cholesterol is contained

in the density 1.019–1.063 fraction of serum lipoproteins.[19] Recently, Roheim and co-workers[20] have presented results showing complete equilibration of free cholesterol with serum lipoproteins but none for esterified cholesterol *in vitro*.

In vitro studies involving binding of cholesterol onto serum lipoproteins have been conducted by Avigan[21] who, by an ingenious method of coating celite particles with cholesterol-C^{14} and then by passing serum through this cholesterol-coated celite column, was able to show the binding of cholesterol onto serum lipoproteins. Whereat and Staple[22] found that cholesterol-C^{14} in a Tween 20 dispersion readily exchanged with the cholesterol of plasma lipoproteins. Cholesterol has very limited solubility in any aqueous medium and, in order to be soluble in serum, a transport system is required.

Two types of transport system for cholesterol in the serum are considered. One involves the serum lipoproteins. The lipoproteins have been implicated in the solubilization of added cholesterol[21] in serum. However, the lack of specificity with respect to the kinds of lipid bound to lipoproteins would suggest that the phenomenon is due to physical solution of the lipids by the lipid phase of the lipoproteins rather than by an interaction with specific sites on the protein moiety of the molecule. Recent studies by Scanu and Hughes[23] have demonstrated that the protein of α_1-lipoproteins delipidated by an alcohol-ether extraction procedure has great avidity under both *in vitro* and *in vivo* conditions for lipids similar to those extracted. Oncley[24] has considered the protein moiety of a lipoprotein to be an apolipoprotein with much more highly developed specificity for lipid binding and he has thought of the fully conjugated lipoprotein as consisting of an apolipoprotein monolayer around a lipid core. It is quite possible that some of the cholesterol is transported as apolipoprotein-lipid-cholesterol complexes.

A second transport mechanism is visualized as the cholesterol-combining globulin demonstrated in the present studies. The data presented in this report clearly support the concept of a specific type of binding of lipoprotein-free serum globulin for cholesterol. The isolation of a cortisol-binding α_1-glycoprotein from plasma of diethylstilbesterol-treated men[25] further demonstrates the importance of a trace protein for the specific interaction with one class of steroid hormones. Analogous to transferrin, the cortisol-binding protein has been designated "Transcortin" by Slaunwhite and Sandberg.[26] The existence of this protein, transcortin, in human serum with a great avidity for cortisol *in vitro* during equilibrium dialysis has provided the fact that this specific protein may be involved in the physiological control and transport of this hormone. Similarly, the capacity of α-β globulin to bind cholesterol specifically may be the primary means by which cholesterol is being transported to the various tissues and organs where it is being transformed into biologically active steroid hormones[27] and also metabolized[38] into conjugated products to be excreted. On that basis, the name of "transcholesterin" is proposed for this component. There is a possibility that transcholesterin may actually constitute a system of several proteins rather than a single one. This is suggested by the broad cholesterol interaction line which is visible in the α-β globulin region of the serum immunoelectrophoresis. Furthermore, the spreading of the transcholesterin activity in the whole spectrum of serum α-β globulins when partially separated by zone electrophoresis on starch also points to that possibility. The electrophoretic mobility, nondializability through visking casing, and specificity of

interaction, all in all, indicate that the transcholesterin is macromolecular in nature with certain definite tertiary configurational binding sites expected of a protein.

The mechanism of *in vitro* interaction of cholesterol with transcholesterin is not fully established. However, that the structure of the cholesterol-transcholesterin complexes could be similar to the one proposed by Folch-Pi[29] for proteolipids consisting of a protein core covered by a lipid shell may be supported by the failure of the complex to be stained by protein stains. The specificity of the immunological type of reaction between cholesterol-transcholesterin would clearly require the stereochemical configuration of cholesterol and the specific steric structure of the individual protein, providing available reactive groups for binding of the cholesterol. Furthermore, Van der Waals forces, hydrogen bonding, and various forms of hydrophobic bonding may likely occur between hydrocarbon chains of cholesterol and aliphatic branched chains of a number of amino acids present in the transcholesterin. Thus, the direct binding of cholesterol to specific sites of the protein molecule appears responsible for the combination of cholesterol with transcholesterin.

In atherosclerosis, where there is a tendency to deposit cholesterol in plaques in vessel walls, the capacity of the transcholesterin to carry smaller or larger amounts of cholesterol may actually be the primary determining factor in the homeostatic control of serum cholesterol level.

Summary.—With an immunoelectrophoretic technique in agar gel involving the use of human serum, a broad reaction line was observed in the α-β globulin region after diffusion with cholesterol dissolved in Teepol "L." Human lipoprotein-free serum, obtained by ultracentrifugation in a salt solution of density 1.21, when subjected to immunoelectrophoresis produced the same result. Hence, this system which may constitute a class of proteins is designated as transcholesterin. Lipoproteins, likewise, produced a cholesterol reaction line under the same conditions. These lines were not stained with either protein or lipid stains, whereas the line formed with Tween 20 was stainable. The proposed structure of the cholesterol-transcholesterin complex consisting of a protein core covered by a surface layer of cholesterol would be consistent with the negative staining property of this complex. The findings provide evidence that in human sera there is a specific cholesterol-binding globulin which may serve as a specific transport system for cholesterol.

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*A MECHANISM FOR THE REACTIONS OF CALCIUM WITH MITOCHONDRIA**

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The uptake of either calcium or manganese by isolated mitochondria is now recognized to consist of a burst of respiration, a cycle of oxidation-reduction of cytochrome *b* and the pyridine nucleotides, and the production of hydrogen ions.[1-5] Cation accumulation is an energy-dependent reaction supported either by substrate oxidation or ATP hydrolysis[1] leading to the conclusion that a high-energy intermediate of oxidative phosphorylation is involved. The nature of this involvement is not clear. Both Saris[5] and Chappell[2, 6] have emphasized the importance of hydrogen ion ejection as an early response to cation addition. These observations have led to the proposal that the primary basis for cation transport is an outwardly directed H^+ pump,[6, 7] the hydrogen ions being generated during the flow of reducing equivalents through the respiratory chain with the added assumption of a membrane impermeable to H^+. This proposal is an extension of that initially advanced by Davies and Krebs,[8] by Mitchell,[9] and by Robertson.[10]

If an H^+ pump does exist, one of its measurable properties should be a stoichiometry between H^+ production and electron transport, i.e., oxygen consumption. One of the difficulties in obtaining a clear insight into this relationship has been the