$$\widetilde{S(v)} = T(v)$$

for all $v \in V_1$.

As an example of the first technique, see reference 2 where we prove that a generic n-distribution in $n + n(n-1)/2$ space is of finite type.

[1] Ehresmann, C., *Catégories topologiques et catégories différentiables*, Colloque de Géométrie différentielle globale, Bruxelles, 1959.

[2] Guillemin, V., and S. Sternberg, "Sur les systèmes de formes différentielles," *Ann. Inst. Fourier, Grenoble*, to appear.

[3] Sternberg, S., *Lectures on Differential Geometry* (New Jersey: Prentice-Hall, 1964).

DEVELOPMENTAL CHANGES AND HETEROGENEITY OF LACTIC AND MALIC DEHYDROGENASES OF HUMAN SKELETAL MUSCLES AND OTHER ORGANS

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Lactic dehydrogenase (LDH), the enzyme catalyzing the reversible reduction of pyruvate, is ubiquitous in mammalian tissues and has been shown to exist in 5 distinct molecular forms within the tissues of each mammal that has so far been studied. These isoenzymes of LDH may be separated by electrophoresis on different suitable media,[1-8] column chromatography,[9] or by coenzyme analogue specificity using nicotinamide adenine dinucleotide (NAD).[10] They differ in their catalytic and immunologic properties, amino acid compositions, and also in certain physical characteristics.[11, 12] It has been demonstrated that each LDH isoenzyme molecule is formed from four polypeptide subunits of equal size and that the subunits are divisible into two distinctive varieties by electrophoresis.[11, 12] The LDH isoenzymes are designated respectively as LDH_1 for the most rapidly migrating anodal form to LDH_5, the cathodal slowly moving variety at pH 8.6. Both LDH_1 and LDH_5 are composed of four identical subunits, each of a different type. LDH_2, LDH_3, and LDH_4 are made up of the two types of subunits in various combinations. They are thus hybrid forms.

The two pure types of LDH have also been designated as M (muscle or LDH_5) and H (heart or LDH_1) forms.[12] Recently, Kaplan and Cahn[13] reported that the composition of LDH in the various skeletal muscles of the normal chicken differs to a great extent, ranging from almost pure H type to pure M type. The red and white muscle fibers of the guinea pig, rabbit, and mouse have also been found to differ in respect to LDH isoenzyme composition.[14] In examining the isoenzyme patterns of human skeletal muscles, previous investigators[7, 15] have not taken into consideration the heterogeneous nature of this tissue. In the present paper, evidence is given for the heterogeneity of LDH and malic dehydrogenase (MDH) isoenzyme compositions of human skeletal muscles and various visceral organs.

The changes in isoenzyme pattern in these tissues during development will also be presented.

Materials and Methods.—Preparation of tissue extracts: Various human skeletal muscles and organs were obtained at autopsy within 4–10 hr after death from persons who died due to diseases other than those of neuromuscular origin. (We have evidence, derived from comparing enzyme content and isoenzyme distribution, that muscle obtained at autopsy over this interval of time does not lose much LDH enzyme when compared with freshly biopsied and processed muscle.) The tissue (100–300 mg) was homogenized with cold double-distilled water (1:4 v/v) in a small all-glass Potter-Elvehjem homogenizer for 3–5 min at 5°. Centrifugation of these homogenates was done at 0° and 2,000 *g* for 15 min and the clear supernatant fractions were used for enzyme study.

Separation of LDH and MDH isoenzymes by agar gel electrophoresis: Agar gel electrophoresis on ordinary microscope slides (25 × 75 mm) was carried out in a Spinco-Durrum type of electrophoretic cell by modification of the method of Yakulis and his associates[16] as follows: the support stand was removed from the Durrum electrophoretic cell and the cell was filled to the fluid line with barbital buffer, pH 8.6, of ionic strength 0.05. The slides containing the agar gel were placed on the two wick supports and the connection of the gel with the buffer solution was established by means of pieces of Whatman 3 mm filter paper (1″ × 1″). The agar gel was prepared by dissolving 1 gm of Ion Agar no. 2 (Consolidated Laboratories) in 100 ml of warm barbital buffer, pH 8.6, of ionic strength 0.025 and the gel thickness was maintained constant by pouring in 2

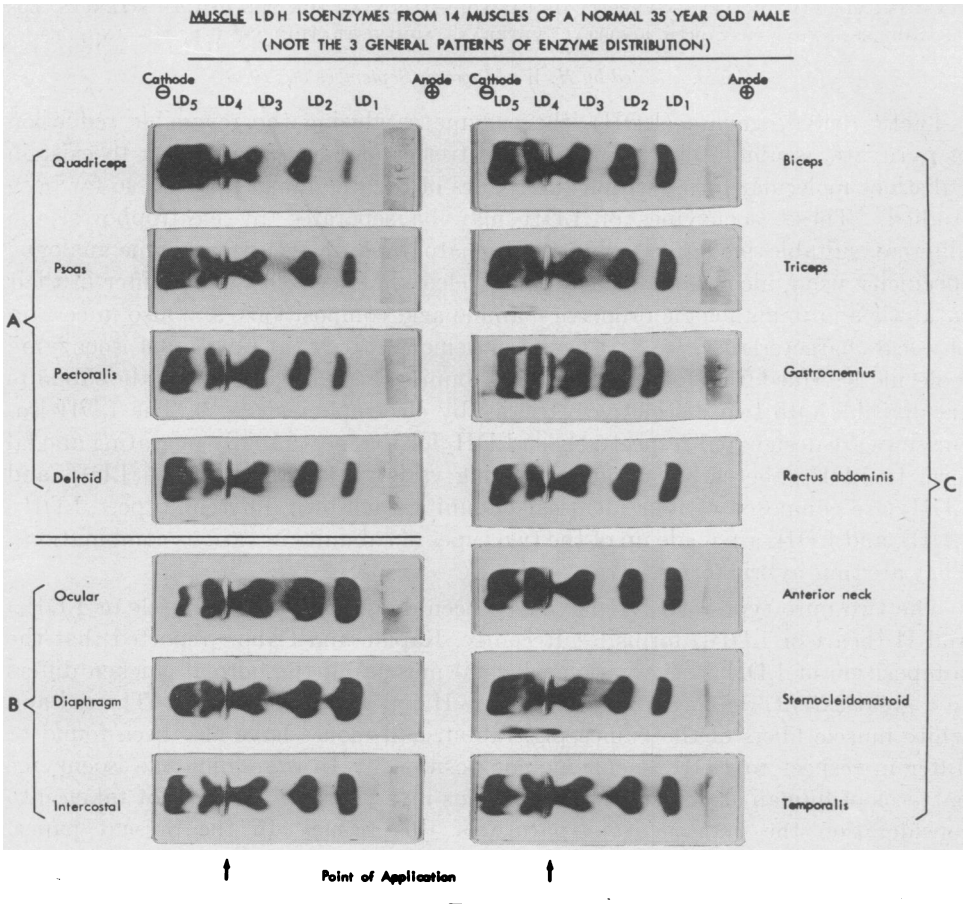

FIG. 1.

ml of hot agar solution to each slide. The agar gel was allowed to set for 5 min and a small groove (1 × 10 mm) was made with a cutter which was fashioned from 2 single-edged razor blades. Ten μl of the tissue extract can be placed in this groove. Six slides could be easily subjected to electrophoresis simultaneously with good separation of the isoenzymes. A potential gradient of 6.5 volts/cm was maintained for 90 min at room temperature.

Localization of LDH and MDH isoenzymes: After electrophoresis, the LDH isoenzymes were localized on the gel by incubating the slides with a substrate medium as described by Yakulis and his associates[16] and modified in the following manner: the slides were placed in a petri dish containing 10 ml of substrate medium which consisted of: phosphate buffer, pH 7.4, 0.1 *M*, 5 ml; sodium *DL*-lactate, 1 *M*, 1 ml; NAD, 10 mg/ml, 0.50 ml; phenazine methosulfate, 1 mg/ml, 0.50 ml; nitro-blue tetrazolium (Nutritional Biochemicals), 1 mg/ml, 2.5 ml; double-distilled water, 0.50. These were then incubated for 90 min at 37° in the dark. The distribution of MDH isoenzymes was determined in a similar manner, except that sodium *DL* malate was used in the substrate medium instead of sodium lactate. After the incubation was over, the slides were washed repeatedly with ethanol:acetic acid:water (5:1:5 v/v), until the background of the gel was clear. These were then covered with filter paper and dried at 37°. After drying, the slides could be stored indefinitely in the dark. They were then photographed and a semiquantitative idea about the proportional distribution of each LDH isoenzyme could be obtained by visual inspection of the stained zones.

Assay of LDH: Total LDH activity of the muscle extracts were determined by the method of Cabaud and Wroblewski.[17]

Results.—LDH isoenzyme patterns of adult human muscle: In Figure 1 are compared the LDH isoenzyme patterns of 14 muscles of a normal adult human male. It can be seen that every muscle examined contains all five LDH isoenzymes but each in somewhat different proportions. A similar set of patterns was also found in another normal adult. In general, these patterns could be grouped into three distinct classes as follows: (*a*) predominant M type (i.e., LDH_5) with a steady decline of activity in the other isoenzymes toward the anodal pole; (*b*) predominant H type (i.e., LDH_1) with a gradual diminution of activity toward the cathodal end of the spectrum; and (*c*) the mixed type of LDH, found in the majority of muscles examined, where nearly equal amounts of enzyme activity are present in all fractions of the isoenzyme spectrum.

From Table 1, it is apparent that the total LDH activity per gm wet weight also varies considerably from one muscle to another.

LDH isoenzyme patterns of muscles obtained from a newborn male child: In Figure 2 are shown the LDH isoenzyme patterns from various muscles of a 2-day old male child. It is evident that in all the muscles examined most of the enzyme activity is of the H type. One can observe the differences that exist between the LDH compositions of muscle from this newborn and from adult muscle by comparing specific muscles in Figures 1 and 2 or in the lower part of Figure 4.

TABLE 1

TOTAL LDH ACTIVITY OF 14 MUSCLES OF A NORMAL HUMAN ADULT

Muscle	Units/gm of wet muscle*	Muscle	Units/gm of wet muscle*
Quadriceps	112,500	Biceps	80,500
Psoas	117,000	Triceps	57,500
Deltoid	64,500	Gastrocnemius	67,000
Pectoralis	98,000	Rectus abdominis	144,000
Ocular	102,000	Anterior neck	58,000
Diaphragm	130,000	Sternocleidomastoid	130,000
Intercostal	40,500	Temporalis	89,500

* Each unit is equivalent to that amount of LDH that would cause a decrease in $O.D._{340}$ of 0.001/min in a reaction mixture of 3 ml volume as described by Wroblewski and La Due.[22]

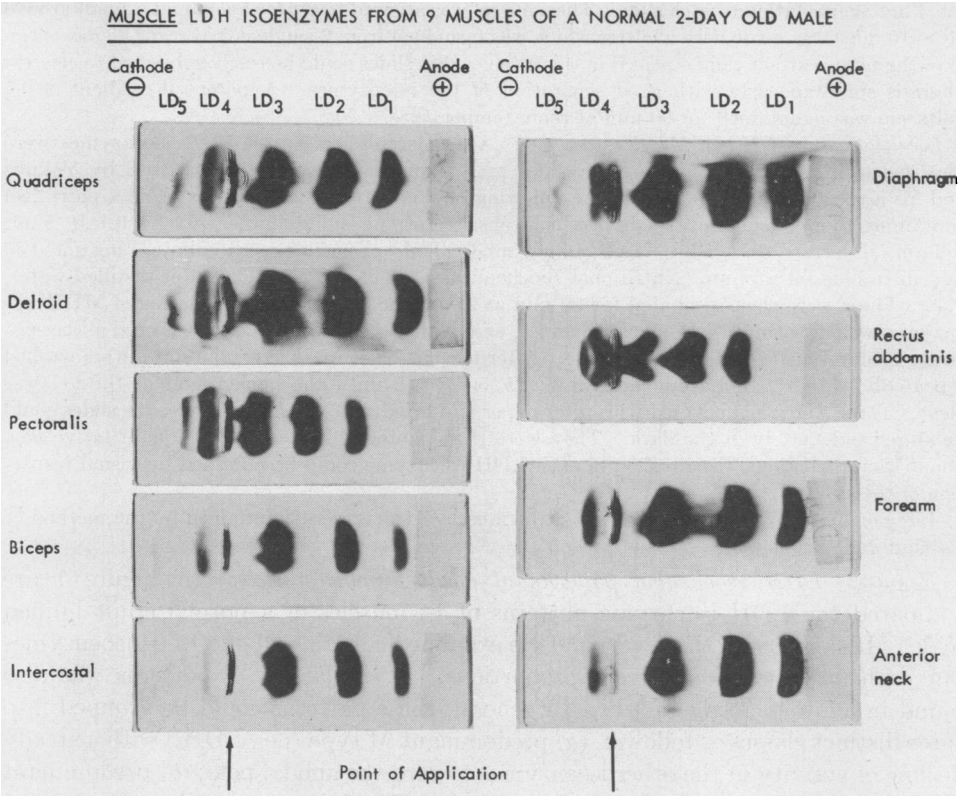

FIG. 2.

MDH isoenzyme patterns of muscles: Contrary to the diverse patterns of the LDH isoenzymes in different muscles, the isoenzymes of MDH show only mild variation in their composition. All the muscles examined contain four MDH isoenzyme bands. The fastest-moving ones (MDH_1 and MDH_2) are very faint in all of them and do not photograph well (Fig. 3). In some of the muscles, as in in group B of Figure 3, the band moving toward the cathode (MDH_4) shows considerable activity. MDH isoenzyme patterns of the muscles from the 2-day-old male child show no characteristic difference when compared to those of the adult, except that MDH_1, MDH_2, and MDH_4 are even fainter in the 2-day-old muscles and only MDH_3 stands out clearly.

Developmental changes of LDH isoenzyme patterns of certain organs: As can be seen in Figure 4, LDH patterns of all of the organs which were examined in a 2-day-old infant show most activity in the fast-moving bands. Only the liver contains more than trace amounts of LDH_5. The greatest changes in isoenzyme patterns during development occur in the liver and some striated muscles. Lesser change takes place in the spleen and none at all in the heart and lung.

Muscle LDH isoenzyme patterns in several myopathies: Biopsy specimens of quadriceps muscle from patients with various myopathies have been analyzed for their LDH isoenzyme compositions. It has been found that in certain forms of myopathy, especially some types of muscular dystrophy, the pattern closely

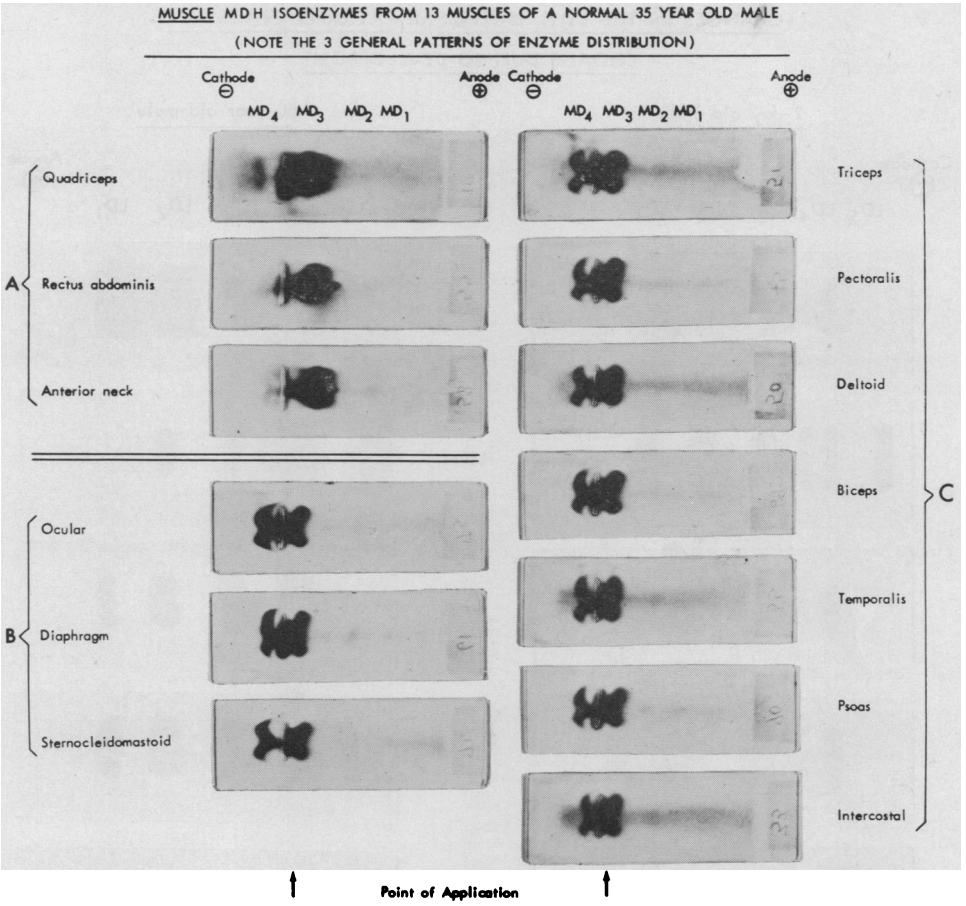

FIG. 3.

resembles that of the fetal quadriceps muscle. Others, however, show no abnormality in LDH composition. Details of this study will be presented in a separate paper.

Discussion.—The data presented in this paper show that in the human subject the enzyme LDH exists in several molecular forms in many body tissues and that the proportions of these forms differ from one tissue to another. Moreover, as previously found in the chicken,[13] the composition of LDH isoenzyme forms varies even in the same tissue, namely, striated skeletal muscle. Important lessons to be learned from these observations are that: (*a*) care must be taken to sample the same muscle each time during a serial investigative study, (*b*) the muscle which has been analyzed should be clearly defined in each report, and (*c*) when analyzing diseased muscle, control samples should be obtained from the *same* muscle of a normal subject.

It is probable that the different isoenzyme patterns which are found in various tissues and organs are reflections of the various metabolic requirements within the specific tissues. If this is the case, then it implies that differing physiological and metabolic roles are being played by various segments of the body musculature.

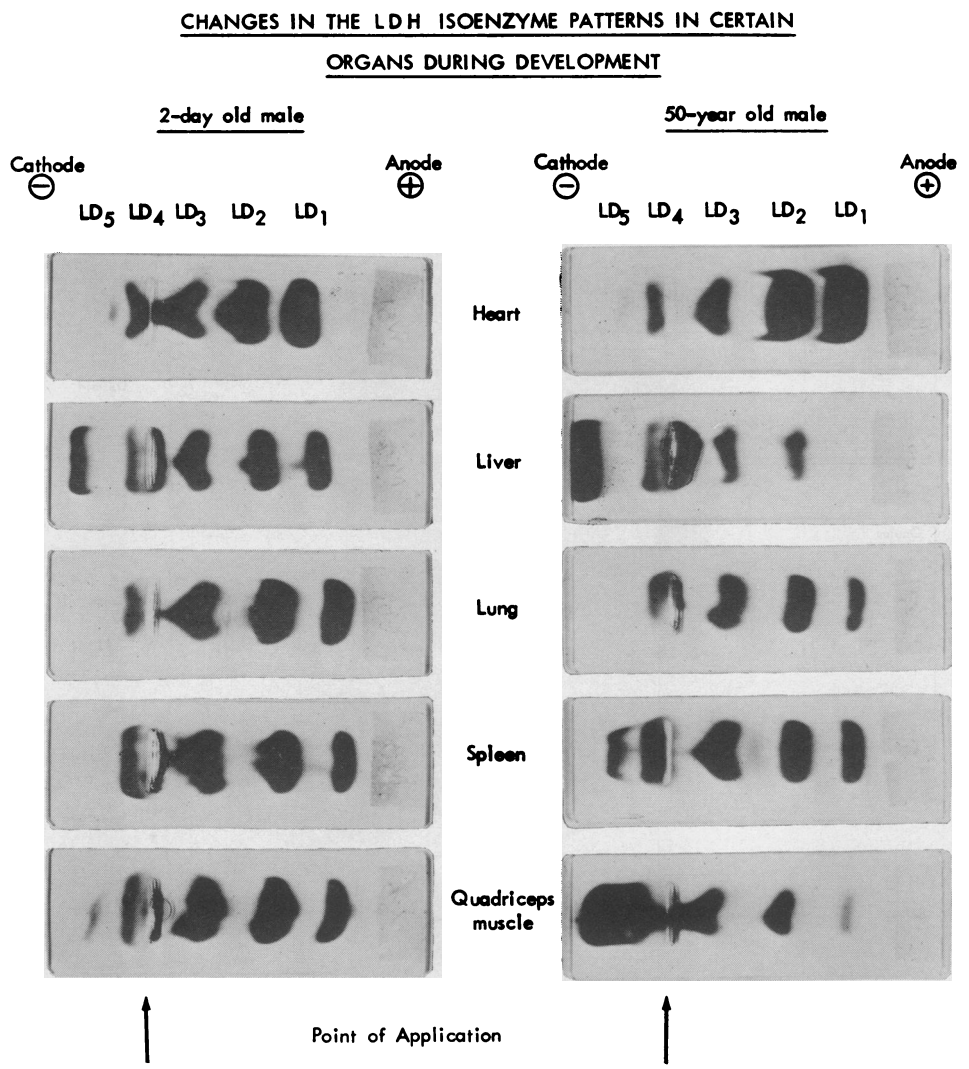

FIG. 4.

Blanchaer and van Wijhe[14] have looked into this matter by examining both red and white portions in several muscles from guinea pigs, rabbits, and mice. They found that red muscle contained all five bands of LDH in nearly equal proportion, whereas in white muscle only LDH_4 and LDH_5 were consistently present. Man does not clearly have red and white muscles as such, and all muscles probably contain a mixture of both red and white fibers. Muscles do, however, vary somewhat in the intensity of their coloration so that the patterns of LDH isoenzymes may reflect the proportional complement of each of these two types of fibers. In addition to the variable intensity of coloration of muscle, which is due to its myoglobin and cytochrome content, red and white fibers also have been shown to have other histochemical[18] and functional[19] differences.

As already shown in other animal species, during embryonic development a

progressive transition occurs in the relative abundance of different isoenzymes when these are viewed after electrophoretic separation.[11] In many mammalian species in early embryonic life the greatest concentration of LDH enzyme activity is localized in the negatively migrating fractions of LDH_4 and LDH_5 (M type) with progressive shifts of isoenzyme concentration in the various tissues as they differentiate and mature. Only one brief mention has been made in the literature to LDH isoenzyme studies in the human fetus.[20] Our studies in one newborn infant have confirmed the finding that in the human fetus the greatest concentration of isoenzymes is localized in the positive more rapidly migrating fractions of the H type (Fig. 4). This implies that in man the H type of subunits predominate early and that only later in development does the M type make its appearance in sizable proportion. Further studies on human fetuses of various ages must be done before these conclusions can be fully verified.

The specific physiological roles of the two separate types of LDH are not yet very clear. Several authors[11, 12] recently presented evidence which suggested different functions for the two types of LDH. The M type (i.e., LDH_5) has been found to be less sensitive to changes in pyruvate concentration than is the H type (i.e., LDH_1). Hence the M type, which is tolerant of high pyruvate concentration, is found in the tissues where glycolysis is the primary source of energy, e.g., in most of the skeletal muscles. Conversely, H type is present in the tissues which carry on mostly aerobic metabolism such as in the heart. Very recently, evidence has been presented in support of the hypothesis that these two different types of LDH are each controlled by a separate gene.[21]

The reasons for the diverse LDH isoenzyme patterns in different muscles in the adult are not fully understood. The predominance of LDH_5 in the thigh, psoas, deltoid, and pectoralis muscles may be due to the sudden participation of these muscles in maximum contraction and hence to their greater complement of white fibers. Since most of the muscles possess a mixture of all five types of LDH, the inherent heterogeneity, and yet specificity, of the individual muscles of the body is thus emphasized.

Summary.—The LDH isoenzyme patterns of normal human skeletal muscles and visceral organs have been found to vary greatly, ranging from predominant M type to H type. The human fetal type of LDH has been shown to be of the electrophoretically rapid migrating H type. During maturation and development after birth there occurs a shift toward the M type in most muscles like quadriceps, psoas, deltoid, and pectoralis, and in organs like the liver. MDH does not show any very characteristic pattern in different muscles and organs. In future studies on LDH isoenzymes in skeletal muscle it will be necessary to recognize both the specificity and the heterogeneity of that tissue.

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[1] Vessel, E. S., and A. G. Bearn, *Proc. Soc. Expt. Biol. Med.*, **94**, 96 (1957).
[2] Vessel, E. S., and A. G. Bearn, *J. Clin. Invest.*, **40**, 586 (1961).
[3] Wieland, T., and G. Pfleiderer, *Biochem. Z.*, **329**, 112 (1957).
[4] Hess, B., *Ann. N. Y. Acad. Sci.*, **75**, 292 (1958).
[5] Wieme, R. J., *Clin. Chim. Acta*, **4**, 46 (1959).
[6] Blanchaer, M. C., *Clin. Chim. Acta*, **6**, 272 (1961).

[7] Plagemann, P. G. W., K. F. Gregory, and F. Wroblewski, *J. Biol. Chem.*, **235**, 2282 (1960).
[8] Markert, C. L., and F. Moller, these PROCEEDINGS, **45**, 753 (1959).
[9] Hess, B., and S. I. Walter, *Ann. N. Y. Acad. Sci.*, **94**, 890 (1961).
[10] Kaplan, N. O., and M. M. Ciotti, *Ann. N. Y. Acad. Sci.*, **94**, 701 (1961).
[11] Markert, C. L., and H. Ursprung, *Develop. Biol.*, **5**, 363 (1962).
[12] Cahn, R. D., N. O. Kaplan, L. Levine, and E. Zwilling, *Science*, **136**, 962 (1962).
[13] Kaplan, N. O., and R. D. Cahn, these PROCEEDINGS, **48**, 2123 (1962).
[14] Blanchaer, M. C., and M. van Wijhe, *Am. J. Physiol.*, **202**, 827 (1962).
[15] Wroblewski, F., C. Ross, and K. Gregory, *New Engl. J. Med.*, **263**, 531 (1960).
[16] Yakulis, V. J., C. W. Gibson, and P. Heller, *Am. J. Clin. Path.*, **38**, 378 (1962).
[17] Cabaud, P. G., and F. Wroblewski, *Am. J. Clin. Path.*, **30**, 234 (1958).
[18] Engel, W. K., *Neurology*, **12**, 778 (1962).
[19] Denny-Brown, D., *Proc. Roy. Soc.* (*London*), **B104**, 371 (1929).
[20] Fine, I. H., N. O. Kaplan, and D. Kuftinec, *Biochemistry*, **2**, 116 (1963).
[21] Shaw, C. R., and E. Barto, these PROCEEDINGS, **50**, 211 (1963).
[22] Wroblewski, F., and J. S. La Due, *Proc. Soc. Exp. Biol. Med.*, **90**, 210 (1955).

STUDIES ON THE PROTEIN COMPONENTS OF CILIA FROM TETRAHYMENA PYRIFORMIS

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Numerous studies with the electron microscope have established that all cilia and protozoan flagella possess essentially the same fine structure.[1, 2] However, interpretation of the function of this structure has been hindered by the fact that little is known about the chemistry of the constituents. ATP can induce motility in glycerinated cilia and flagella,[3, 4] and the isolated organelles possess ATPase activity,[4, 5] so that the energy for movement presumably comes from dephosphorylation of ATP. Work on the nature of ciliary proteins has been handicapped by their apparent insolubility at neutral pH.[5, 6] The experiments reported in this paper demonstrate that the apparent insolubility reported by previous workers is due to the membrane around the cilium, and that after removal of the membrane the remaining protein is soluble in salt solutions at neutral pH. Differential extraction and reconstitution of the ciliary structure, combined with electron microscopy, have made it possible to locate the probable site of ATPase activity. A partial fractionation of the protein components has been accomplished.

Materials and Methods.—Tetrahymena pyriformis, strain W, was grown at room temperature in a medium containing 1% peptone, 0.1% yeast extract, and phosphate buffer pH 6.5. The cilia were isolated by a procedure slightly modified from that of Watson and Hopkins,[6] and were stored in a small volume of tris-Mg solution. (Tris denotes tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate. Other abbreviations are those accepted as standard by the *Journal of Biological Chemistry*.)

Except where otherwise stated, all operations were carried out at 0–4°C. Two solutions which were used repeatedly will for convenience be designated as *tris-Mg solution* (2.5 mM $MgSO_4$, 0.03 *M* tris-HCl, pH 8.3 at 0°C), and *tris-EDTA solution* (0.1 mM EDTA, 1 mM tris-HCl, pH 8.3 at 0°C). Routine assays for ATPase activity were carried out by incubating at 20°C in a medium containing 1 mM ATP, 1.2 mM $MgSO_4$, and 0.03 *M* tris-HCl, pH 7.8. Subsequent assay for inorganic phosphate was by the method of Fiske and Subba-Row.[7] Protein assays were