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THE EXPRESSION OF INHERITED METABOLIC DISEASE IN CULTURED CELLS*†

ALEXANDER G. BEARN

Department of Medicine,
The New York Hospital–Cornell Medical Center,
New York, New York

I. INTRODUCTION

THE Harvey Society of New York was founded 70 years ago to commemorate the name of one who, with the publication of *De Motu Cordis,* provided definitive evidence for the circulation of blood and established the abiding, but then novel, principle that scientists should not complacently recycle the conventional wisdom of the past. Rather they should, and I now use Harvey's stirring imperative, "Search out and study the secrets of Nature by way of experiment."

Harvey, in addition to being a scientist was a busy medical practitioner, and in the course of his daily practice, he might well have encountered patients suffering from inherited disease. While Harvey is, of course, best known for *De Motu Cordis,* he published in the 73rd year of his life, his second great work *De Generatione Animalium.* Although a scrutiny of *De Generatione* failed to provide the hoped for evidence that Harvey was aware of the existence of inherited disease, there is an extraordinary letter extant, first quoted in full by Garrod in his Harveian Lecture in 1924 (Garrod, 1924), which shows that Harvey was 300 years ahead of his time in appreciating that the study of rare diseases clarifies normal biological and biochemical mechanisms. Harvey's last correspondent was Dr. Jon Vlackfeld, a practitioner living in Haarlem who had consulted him regarding a patient of his that was suffering from stone (Keynes, 1966) (Fig. 1). The paucity of any documentary evidence bearing on Harvey's medical interests can be

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Learned Sir,

There has come to me your very pleasant letter, in which you show both extreme goodwill towards myself and also exceptional industry in the cultivation of our art.

It is indeed so. Nature is nowhere wont to reveal her innermost secrets more openly than where she shows faint traces of herself away from the beaten track. Nor is there any surer route to the proper practice of medicine than if someone gives his mind over to discerning the customary law of Nature through the careful investigation of diseases that are of rare occurrence. Indeed, in practically all things it is apparently arranged that we scarcely perceive what is useful or most serviceable in them unless some are lacking in these features or have a faulty disposition. The case of the plasterer you mention is certainly an unique instance, in the elucidation of which it is possible for much discussion to arise. But it is useless for you to spur me on and for me to gird myself for some new research when I am not only ripe in years but also–let me admit–a little weary. It seems to me, indeed, that I am entitled to ask for an honourable discharge. On the other hand, it will always be a pleasure to me to see distinguished gentlemen such as yourself engaged in such worthy contest. Farewell, elegant Sir, and whatever you do, continue to hold in affection

Yours respectfully,
William Harvey

London, 24 April 1657

FIG. 1. William Harvey's reply to a letter from Dr. Jon Vlackfeld of Haarlem. Harvey died later that year.

laid, at least in part, on governmental interference. With the execution of Charles I, Harvey lost his patient and his friend. He loathed the Parliamentary regime that followed, and they in turn, showed no fondness for him. In a remarkable action, Parliament expressly forbade him to come within 20 miles of the City of London even to those of his patients who needed medical attention (Whitteridge, 1971).

Not content with this infringement of liberty, Parliament in the winter of 1642–1643 plundered Harvey's lodgings at Whitehall and pillaged not only his worldly goods but his entire medical records. And, when in 1666 the Great Fire destroyed the College, all hope of learning more about the medical interests of William Harvey was gone forever (Whitteridge, 1971, 1974).

An interesting historical link between the Harvey Society and genetics arises from the fact that in the same year that the Harvey Society was founded in New York, William Bateson made the suggestion that the word "Genetics" be used to cover the emerging field of

hereditary variation (Bateson, 1928). The ensuing 70 years have seen the science of genetics emerge as the axial thread of biology with great relevance not only to those whose principal activities are rooted in laboratory research of the most fundamental sort, but also to physicians whose primary concern is the alleviation of suffering and the prevention and treatment of disease.

Since 1900 improvements in general hygiene, and the development of antibiotic therapy have enabled many of the epidemic infectious diseases, particularly those of infancy and early childhood, increasingly to be controlled. In contrast, the morbidity and mortality of diseases of genetic origin have been little affected. A recent survey of the patients in the Department at New York Hospital–Cornell Medical Center, disclosed that no less than 12% of patients admitted to the adult medical service suffer from diseases in which a genetic component can be recognized (Childs *et al.*, 1972). It is variously estimated that approximately 3% of the population at birth are afflicted with a genetically determined disease, a figure that would be even more substantial if the base line consisted of all zygotes conceived, rather than infants born. The rapid increase in the number of inherited conditions that are now known is illustrated in Fig. 2.

II. HISTORICAL DEVELOPMENT

Ross Harrison, in his Harvey Lecture delivered in 1908, described experiments that marked the dawn of modern tissue culture (Harrison,

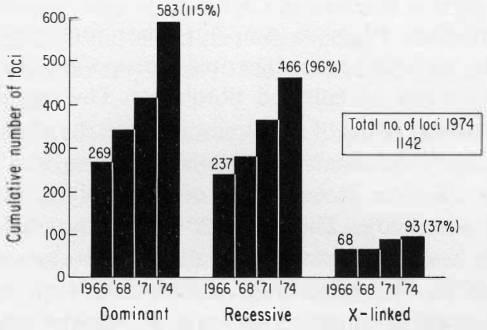

FIG. 2. Recognition of human gene loci (1966–1974). (After McKusick, 1975.)

1908). When the embryonic spinal cord of the frog surrounded by lymph was sealed in a chamber, the cord cells not only remained viable, but from them there developed the outgrowth of nerve axons. These observations, at one and the same time provided the coup de grace to those who were still stubbornly adhering to the view that the nerve axon and the nerve cell were separate and unconnected bodies, and also established the powerful usefulness of cell culture in answering important biological questions. Here at The Rockefeller Institute, Harrison's observations were quickly seized upon by Alexis Carrel. Carrel found, all too quickly, that a major deterrent to the successful cultivation of cells *in vitro* was the frequency with which they became contaminated with bacteria. To combat this difficulty, Carrel devised complicated and bizarrely arcane rituals, including insistence that a black gown and cap were a necessary prerequisite for those who would dare to venture on the treacherous seas of tissue culture. Slowly, however, black magic and alchemy yielded to science and orthodoxy, and tissue culture as a tool in biological research is now firmly established (Carrel, 1964; Bearn, 1972).

The rational use of cell culture as an investigative technique in inherited metabolic disease is rooted in the simple biologic truth that, since a full complement of chromosomes is present in all somatic cells, it should be possible to detect the specific biochemical consequences of gene activity in cultured fibroblasts unless, during the process of differentiation, genes are irreversibly inactivated.

III. THE HURLER AND HUNTER SYNDROMES

Rather more than 10 years ago, our laboratory embarked on an investigation to see whether the inherited mucopolysaccharidoses could be studied profitably in cultured fibroblasts. The mucopolysaccharidoses are rare inborn errors of metabolism, to use the phrase introduced by Garrod in his epoch making Croonian Lectures in 1908 (Garrod, 1908), that result in an accumulation of mucopolysaccharides in various issues of the body. The diseases are characterized by mental deficiency and dwarfism, as well as a variety of other signs and symptoms (McKusick, 1972). The two most common forms of the disease are colloquially known as Hurler's syndrome and Hunter's syndrome, the former being autosomally inherited, the latter X-linked.

An increased hepatic storage of mucopolysaccharides in patients

dying from the disease was first reported by Brante (1952). Later Dorfman and Lorinz (1957), and Meyer and associates (1958) reported an increased urinary excretion of mucopolysaccharides in these diseases. In 1965, Berggård extended these studies in our laboratory, and although he was able to confirm the increased urinary excretion of mucopolysaccharides in patients affected with the disease, he was unable to distinguish clinically unaffected heterozygous carriers from normal subjects (Berggård and Bearn, 1965). He made the interesting prediction that, since both dermatan sulfate and heparan sulfate contained iduronic acid and since both mucopolysaccharides were excreted in excess in the urine, "the mutations might affect enzymes participating in the metabolism of iduronic acid" (Berggård and Bearn, 1965). In an attempt to circumvent the time-consuming chemical determinations necessary to identify specific mucopolysaccharides, we first investigated the possibility that histological methods could be used to identify the presence of mucopolysaccharides in cells grown in tissue culture.

It is well known that the thiazine dye toluidine blue O will form complexes with a variety of negatively charged tissue components. When they do so, there is a shift in the absorption maxima of the dye toward a shorter wavelength. This will result in metachromasia, a change in color from the orthochromatic blue through violet to red. A certain minimum surface charge density is required for metachromasia to become apparent. Thus, with the polysaccharide hyaluronic acid, where the distance between the carboxyl groups is 19–30 Å, metachromasia is usually not evident. However, with the introduction of sulfate ester groups, the intercharge difference is sharply reduced and results in strong and stable red metachromasia. Thus the mucopolysaccharides dermatan sulfate, heparan sulfate and keratan sulfate all stain metachromatically.

Fibroblasts derived from patients with the Hurler and Hunter syndromes show distinct cytoplasmic metachromasia (Danes and Bearn, 1966a), which can be shown by chemical methods to be due to increased cellular uronic acid. The cells derived from the heterozygous parents of the autosomally inherited Hurler syndrome show a similar metachromatic appearance. Although these tinctorial alterations also indicate that the heterozygous carrier can be detected in tissue culture, the degree of staining is similar in both affected individuals and unaffected heterozygotes, and the test cannot be used to distinguish hetero-

zygotes in the general population. In the X-linked Hunter syndrome, the cells derived from the carrier mother also show similar staining characteristics. Cloning experiments performed on cells derived from the X-linked mothers of patients with Hunter syndrome indicate the presence of two distinct tinctorial populations (Danes and Bearn, 1967). In the metachromatic population it appears that the X chromosome bearing the normal gene has been inactivated, whereas in the population of cells which show no metachromasia the chromosome bearing the abnormal gene has been inactivated. These findings provide additional experimental evidence for the validity of the Lyon hypothesis (1962). In contrast, all cells derived from the mothers of patients with the autosomally inherited Hurler's syndrome show metachromasia, confirming that the Lyon hypothesis does not obtain for the autosomes.

The co-cultivation of cells derived from patients with the Hurler syndrome and the Hunter syndrome result in a population of cells which, by their tinctorial characteristics, cannot be distinguished from those derived from normal individuals. The corrective factors have been extensively investigated by Neufeld and her co-workers (Fratantoni *et al.,* 1968; Neufeld, 1973), who showed that the rapid accumulation of sulfated mucopolysaccharides that occurs when the cells from Hurler syndrome and Hunter syndrome are cultivated *in vitro* does not arise if the two cell populations are mixed (Fig. 3). In addition, they also showed that correction can be obtained by the use of culture medium and does not require cell-to-cell contact.

Additional studies by Neufeld have shown the specific enzyme sulfoiduronate sulfatase is deficient in the cells of patients with the Hunter syndrome whereas α-L-iduronidase is deficient in the cells of patients with the Hurler syndrome. It was of particular interest that α-L-iduronidase was also decreased in the Scheie syndrome, as variant of the Hurler syndrome, in which intellectual function is preserved (Fig. 4) (Neufeld, 1974). The observation that the same enzyme was apparently affected in both raised the possibility that the two syndromes are euallelic, in the same way that the genes controlling the synthesis of hemoglobin S and hemoglobin C are euallelic. Euallelism would permit the existence of mixed Hurler/Scheie heterozygotes analogous to hemoglobin S/hemoglobin C heterozygotes. At least eight patients have now been identified in whom the clinical phenotype has features of both the

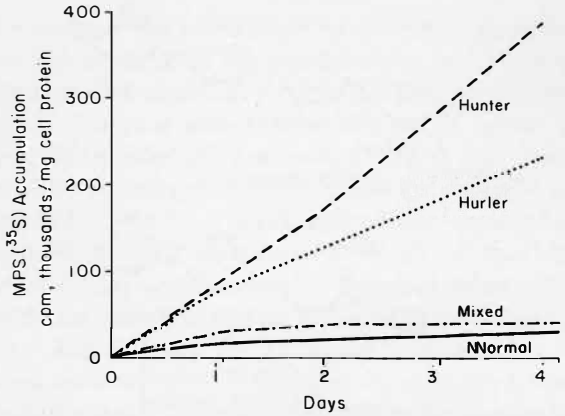

FIG. 3. Accumulation of mucopolysaccharides (MPS) in normal cells (——), in *in vitro*-cultivated Hurler syndrome (. . . .) and Hunter syndrome (– – – –) cells, and in mixed Hurler and Hunter syndromes cells (·—·—). After Neufeld (1973).

Hurler and Hunter syndromes and in whom there is a deficiency of α-L-iduronidase (McKusick *et al.*, 1972). Kinetic and metabolic studies should help to clarify, at the molecular level, possible structural and functional differences between the enzymes defective in the two syndromes.

The usefulness of tissue culture in investigating inherited metabolic disease does not rest with the biochemical identification of the primary defect. The cells in culture provide a model system of the disease, which can be used to test the possible effectiveness of therapeutic agents. The administration of purified enzyme to patients in whom an enzyme is defective will not be effective unless the enzyme gains access, and in sufficient quantities, to the cell compartment where the enzymic function is required. Although it has not proved to be therapeutically beneficial, it is of interest, in view of its known action on lysosomes, that vitamin A can reverse the tinctorial characteristics of the mutant cells when added *in vitro* (Danes and Bearn, 1966b). Although the elucidation of the biochemical defect in patients with most of the known mucopolysaccharidoses has now been accomplished there are many inherited diseases, far more common than the mucopolysaccharidoses, where the underlying defect remains utterly obscure.

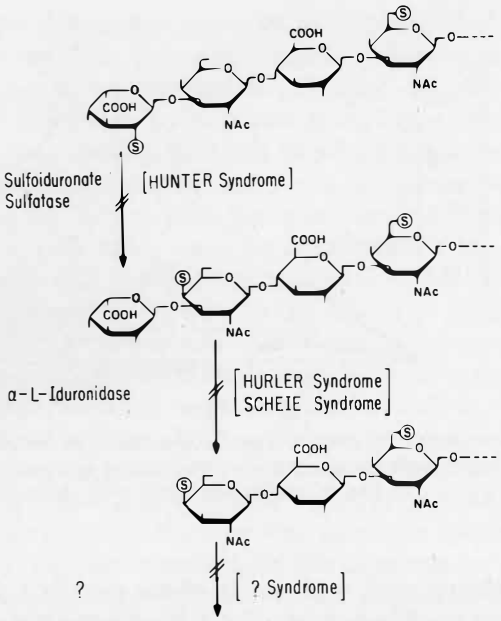

FIG. 4. Normal degradation of dermatan sulfate. (After Neufeld, 1974).

IV. CYSTIC FIBROSIS OF THE PANCREAS

Cystic fibrosis is the most frequently occurring autosomally inherited disease affecting the white population (Table I). It occurs in all populations but appears to be distinctly less common in the Oriental and Black populations. The disease was probably first recognized by Garrod and Hurtley (1912), who believed it was an inherited disorder of fat metabolism in which fats were not absorbed from the alimentary tract in normal quantities. He was led to believe that the disease was autosomally inherited since the normal parents of the affected sibs were first cousins. [It is of some interest that, in fact, an increased consanguinity would not be expected in a homogeneous recessively inherited disease of the frequency of cystic fibrosis.] The first clue that there might be a biochemical defect in cystic fibrosis came from astute

clinical observations. The late summer of 1948 was characterized by a heat wave of short duration but high intensity, and in the space of 4 days, 10 patients were admitted to the Babies Hospital of the College of Physicians and Surgeons with heat stroke, six of whom had cystic fibrosis (Fig. 5) (Kessler and Andersen, 1951). It was quickly appreciated that if the patients with cystic fibrosis lost excessive salt in their sweat compared to the four other patients, an explanation for the circulatory collapse would be at hand. Studies by di Sant' Agnese and his colleagues (1953) demonstrated an increased sweat sodium in the patients with cystic fibrosis, and an increased sweat sodium is now the most reliable diagnostic test for the disease, particularly in childhood. The increased sweat sodium is now recognized to be the consequence of the presence of a factor, known colloquially as the Mangos factor after its discoverer, which is present in the sweat of patients with the disease and inhibits the normal reabsorption of salt (Mangos and McSherry, 1967). The factor occurs in small quantities and, apart from knowing that it is protein in nature, its isolation and characterization has been difficult. As a result of the studies on the defect in salt excretion, the view gained prominence that the disease was primarily a disorder of the exocrine system, a view that gained credence from the known abnormality in pancreatic and bronchial secretions. However, in view of our findings with the mucopolysaccharidoses and because in principle the genetic defect should be present in every cell, we turned to tissue culture, using the same techniques that had previously been useful in investigating the mucopolysaccharidoses.

The initial studies indicated that, as in the mucopolysaccharidoses, the cells derived from patients with cystic fibrosis showed metachromasia (Danes and Bearn, 1969a). This metachromasia, which also

TABLE I

CYSTIC FIBROSIS OF THE PANCREAS

Incidence	1/2500 Live births
Inheritance	Autosomal recessive
Population variation	Rare in Orientals and Blacks
Frequency heterozygote	1/25

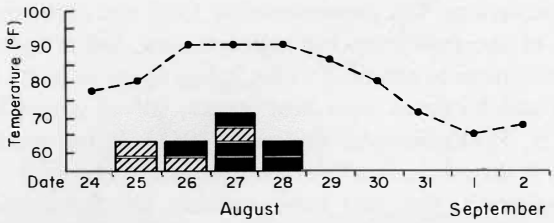

FIG. 5. Relation of ambient temperature to patients admitted to Babies Hospital, New York City, with circulatory collapse (August–September 1948). ■, Cystic fibrosis; ▨, non-cystic fibrosis. 1 rectangle represents 1 patient. After Kessler and Andersen (1951).

occurred in the heterozygotes, could not be distinguished from that occurring in the mucopolysaccharidoses and certain other diseases. However, the increased mucopolysaccharide content so characteristic of the mucopolysaccharidoses was not regularly observed in the cells from patients with cystic fibrosis (Danes and Bearn, 1969b). Moreover, whereas the cells from patients with the mucopolysaccharidoses stained with alcian blue at high electrolyte concentrations, the cells from the cystic fibrosis patients did not (Danes *et al.*, 1970). As the study progressed, it was apparent that not all patients with cystic fibrosis had metachromatically stained fibroblasts and, further, that the proportion of patients showing metachromasia differed in different populations (Table II). Although there does not appear to be a clear-cut correlation between the presence or the absence of metachromasia and the clinical findings, recent studies by Danes *et al.* (1975), in which they studied an adult population of patients with cystic fibrosis at the Brompton Hospital, the frequency of metachromasia was found to be over 90% (Table II). It cannot be sufficiently emphasized that, although metachromasia is usually observed in the heterozygous parents of patients in whom metachromasia is present, the test is difficult, at times unpredictable, and cannot be used to detect heterozygotes with the disease. This is further emphasized by those families in whom this cell marker cannot be detected even in those clinically affected (Bearn and Danes, 1969).

In 1956, Alexander Spock made the curious but important observation that serum from patients with cystic fibrosis contained a substance

TABLE II

PROPORTION OF PATIENTS SHOWING METACHROMASIA IN DIFFERENT POPULATIONS

Population	Number of patients	Metachromatic	Ametachromatic
New York City	30	26	4 (13%)
Minneapolis	27	22	5 (19%)
Denmark	87	56	31 (35%)
England (adults)	42	39	3 (7%)

that had the property of disorganizing the synchronous movements of oyster cilia derived from rabbit tracheal explants (Spock *et al.*, 1967). This factor, now known as the Spock factor, bears some similarity to the Mangos factor in that it is protein in nature. As with the Mangos factor, isolation and detailed characterization of the factor has been difficult.

A systematic attempt to investigate other ciliated organisms, in an effort to find a simpler biological system, led Bowman and her colleagues to investigate oyster cilia (Bowman *et al.*, 1969, 1970, 1973). Although many investigators prefer the rabbit tracheal system (Conover *et al.*, 1973), we have used the oyster (*Crassostrea virginica*) in our studies in cystic fibrosis.

Although the addition of normal serum had no effect on the synchronous beating of oyster cilia, the addition of serum from patients with cystic fibrosis, as well as the serum of patients who are heterozygous carriers, usually caused the cessation of movement within 60 seconds. The test, like nearly all biological tests, is highly variable and very subjective. Duplicate samples must always be obtained and the tests run in a double-blind fashion. Even when these precautions are taken, there are many occasions when the test is unsatisfactory. In the summer months, as Bowman has shown, the excessive production of mucus by the oyster makes the test essentially useless (Lockhart and Bowman, 1973). Despite these limitations, the test is providing concrete evidence of the presence of a substance in the serum of most patients with cystic fibrosis which inhibits the cilia. It is also of interest

that serum from heterozygous as well as homozygous individuals, which consistently does not inhibit the cilia, is usually derived from patients in whom metachromasia is not apparent in their cultured cells.

Although the isolation and characterization of cystic fibrosis from cystic fibrosis serum has not been accomplished, a number of properties have been described (Bowman *et al.*, 1973; Danes *et al.*, 1973; Bowman, 1973). Prominent among these has been the finding that the factor is associated with the γ-globulin fraction of serum (Fig. 6). However, the possibility that the factor was itself an antibody was excluded by the immunological studies of Bowman and her colleagues (Herzberg *et al.*, 1973). Synthesis of the factor by cultured fibroblasts was established by growing cells derived from patients with cystic fibrosis in a synthetic protein-free medium. When purified IgG was added to the protein-free cultured fluid, the cystic fibrosis factor emerged associated with IgG in the void volume (Fig. 7). The properties of cystic fibrosis compiled from several sources are summarized in Table III.

Although the appearance of the "factor" in the serum of patients is of extreme interest, its exact nature and its relevance to the primary defect are obscure. Presumably the defect, in conformity with other

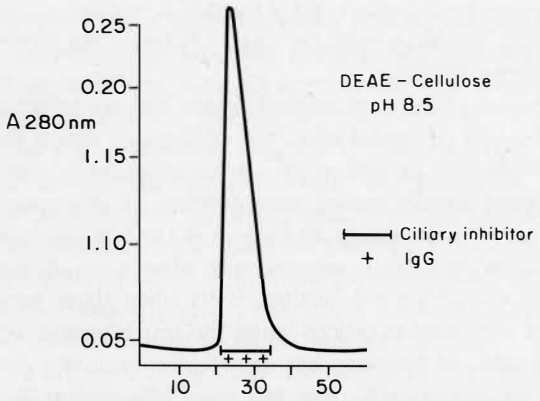

FIG. 6. Serum protein fraction derived from patient with cystic fibrosis separated by column chromatography. The ciliary inhibitor is eluted with the IgG fraction.

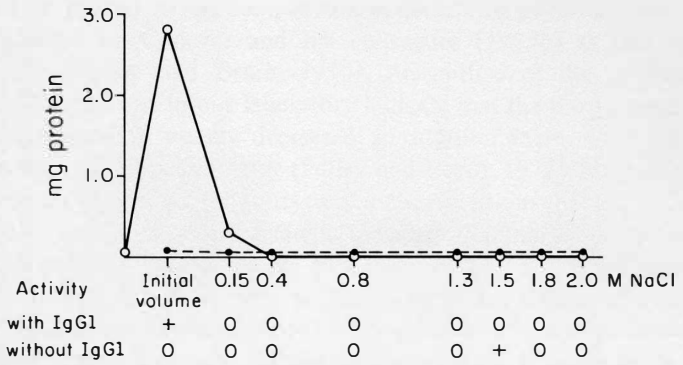

FIG. 7. Cystic fibrosis factor activity in serum-free culture medium. Dowex 1-2X, chloride (0.9 X 44 cm). o——o, Addition of 3 mg/ml of IgG1 •– – –•, no IgG1 added.

inborn errors of metabolism, is one that affects a specific enzyme. It is possible that the presence of the factor in the serum represents the accumulation of a substance behind an enzymatic metabolic block. The cystic fibrosis factor, according to this hypothesis, might be a normal serum component that occurs in increased concentration in patients with cystic fibrosis. The small molecular weight of the cystic fibrosis factor raised the possibility, among the many others, that the factor

TABLE III

PROPERTIES OF CYSTIC FIBROSIS FACTOR

Molecular weight	4,500–10,000 Daltons (Gel filtration Amicon filters)
Isoelectric focusing	pH 9.10–9.30
Inactivation	Pepsin pH 3.8
	Papain pH 8.5
	Heat 50°C for 15 min
Reaction with serum proteins	
Binds:	IgG (IgGl, IgG2) β_2 M, heavy chains
No binding:	IgA, IgM, IgD, Fab, Fc, light chains
	Haptoglobin
	Group-specific component

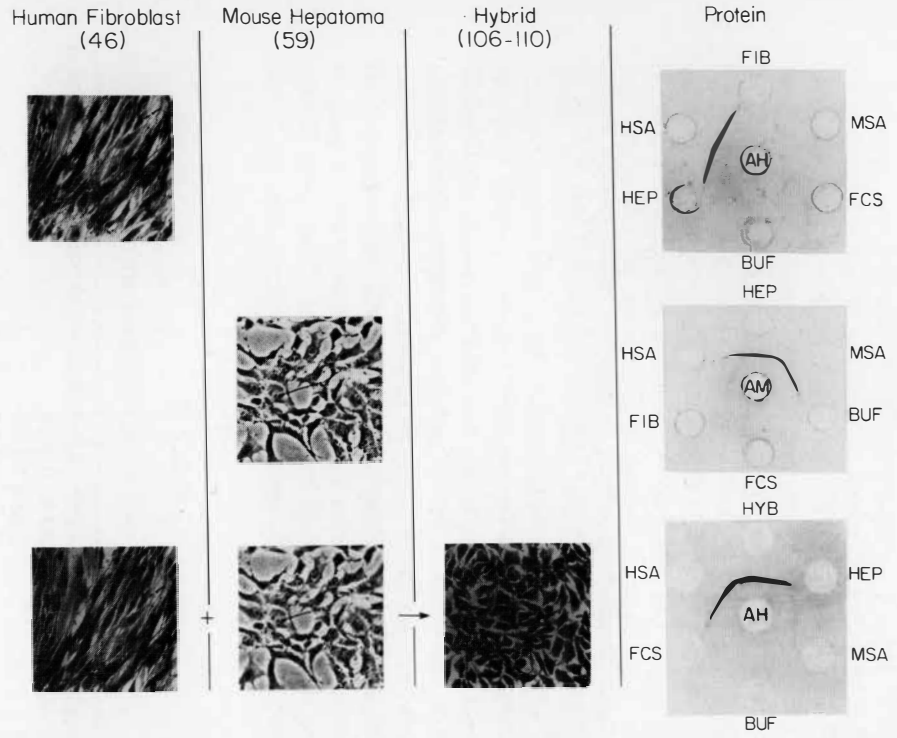

FIG. 8. Fusion of human fibroblasts and mouse hepatoma cells yield a hybrid that synthesizes immunologically detectable human serum albumin. Control studies indicate the specificity of the albumin synthesized. *Abbreviations:* FIB, Fibroblasts; HSA, Human serum albumin; MSA, Mouse serum albumin; HEP, Mouse hepatoma; FCS, Fetal calf serum; BUF, Buffer; AH, Antihuman albumin; AM, Antimouse albumin; HYB, Hybrids.

might be related to the complement system. This possibility has been investigated by Conover and his colleagues (1973b) as well as by ourselves (Polley and Bearn, 1974). An outline of the preliminary studies carried out in our laboratory indicate that the third component of complement is usually decreased; in addition there is a decreased conversion of C3 proactivator (Polley and Bearn, 1974). Moreover, the incubation of purified C3 by trypsin under conditions that split C3 into C3a and smaller tryptic fragments indicates that the small-molecular-weight peptides inhibit oyster cilia whereas C3, as well as the split products C3b and C3a, have no inhibitory effect (Polley and Bearn, 1975). Although further studies on the possible relevance of the complement system to cystic fibrosis are in progress, it seems likely that any abnormality in this system is of secondary importance.

Although the application of cell culture to investigating inherited metabolic disease has been restricted, in this presentation, to two main groups of diseases, more than 70 inherited diseases have been investigated using these techniques, and their number continues to increase. Already, many of these diseases can be detected prenatally by the cultivation of fetal cells obtained by amniocentesis, and in those instances where prenatal detection can be reliably performed the possibility of selective abortion can be entertained.

The recent use of mouse hepatoma human hybrids as developed by Darlington and her colleagues (1974) has raised the possibility that under appropriate experimental conditions liver-specific proteins, such as serum albumin, can be synthesized by normal human fibroblasts. The synthesis of human serum albumin by cultured fibroblasts following the fusion of mouse hepatoma cells with normal human fibroblasts is illustrated in Fig. 8. If, as seems likely, the findings obtained thus far can be extended to other organ-specific proteins, the possibility arises that inherited diseases in which the metabolic abnormality appears to be restricted to a specific differentiated organ may be approached by the use of cell hybridization techniques. It is clear that the usefulness of cell culture in the study of inherited disease is still in its infancy.

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ADDENDUM (July 1976)

In the 18 months that have elapsed since the delivery of this lecture, the primary metabolic defects in most of the mucopolysaccharidoses have been determined. The number of inherited diseases detectable in tissue culture continues to increase and the number of instances where accurate prenatal diagnosis can be made is also increasing rapidly (McKusick, 1975). Although the number of reported metabolic aberrations in cystic fibrosis shows no signs of abatement, the primary enzymatic defect remains elusive.