

PLASMA PROTEIN COMPOSITION OF HYALINE MEMBRANE IN THE NEWBORN AS STUDIED BY IMMUNOFLUORESCENCE

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Hyaline membrane disease of the newborn has been a subject of ever-growing interest to paediatricians and pathologists as it is the major cause of death in newborn babies, particularly premature ones. Much more is known of the clinical picture and physiological changes in hyaline membrane disease, or respiratory distress syndrome of the newborn (Avery, 1962; Curtis, 1957; James, 1959; Miller, 1962; Rudolph and Smith, 1960), and there is a fair knowledge of the composition of the hyaline membrane (Buckingham and Sommers, 1960). There is, however, no agreement as to its aetiology and pathogenesis, though, according to the majority of investigators, the material constituting the membrane originates from the pulmonary vessels. These authors base their assumptions mainly on the work of Gitlin and Craig, who by means of immunofluorescence detected the presence of fibrin within the hyaline membranes (Gitlin and Craig, 1956). Electron microscope investigations suggest the presence of other plasma proteins as well

(van Breemen, Neustein and Bruns, 1957; Campiche, Prod' hom and Gautier, 1961; Campiche, Jaccottet and Juillard, 1962; Groniowski and Biczyskowa, 1963), but this has not, however, been confirmed by Gitlin and Craig. Histochemical investigations have not established whether there are any other plasma proteins in the membrane (Aronson, 1961; Buckingham and Sommers, 1960; Duran-Jorda, Holzel and Patterson, 1956; Lieberman, 1961; Lynch and Mellor, 1956; Lynch, Mellor and Badgery, 1956).

Differences of opinion as to the exact plasma protein composition of hyaline membranes stimulated us to undertake further investigations on their structure.

Material and Methods

Investigations were undertaken on 29 newborn babies with respiratory distress. Of these, the lungs of 6 children with extensive and well-defined hyaline membrane syndrome constituted the material of the present study (Table 1).

TABLE 1
SIX CHILDREN DYING WITH HYALINE MEMBRANE DISEASE

Case No. and Sex	Gestation (wk.)	Weight (g.)	Length (cm.)	Age (hr.)	Delivery	Autopsy Findings
1 M	29 or 30	1,800	44	10	Normal	Hyaline membrane disease; minute meningeal haemorrhages
2 M	30	2,150	47	36	Normal	Hyaline membrane disease
3 F	31	1,370	41	15	Normal	Hyaline membrane disease; minute ependymal haemorrhage
4 M	33	2,470	47	36	Caesarean section; premature separation of placenta; born in asphyxia	Hyaline membrane disease; intraventricular bleeding
5 M	30	1,580	44	18	Caesarean section; placenta praevia; born in asphyxia	Hyaline membrane disease; small meningeal haemorrhage
6 M	30	1,200	38	14	Normal	Hyaline membrane disease

TABLE 2
CHARACTERISTICS OF REAGENTS

	Protein Content (mg./ml.)	Dye-to-protein Ratios or Dye Content	Quantity of Antigen Reacting with Antisera in Micro-precipitation Test	Optimal Dilution of Anti-sera	Quantity of Tissue Powder Used for Absorption (mg./ml.)
Sera against human fibrin* G γ anti H f. FIT	19.4	15×10^{-3} 0.019 mg./ml.	0.46 γ 0.46 γ	1 : 32 1 : 1	150 100
G γ anti H f. LRB					
Sera against human γ -globulin: G γ anti H γ . FIT	17.2	18×10^{-3}	0.46 γ	1 : 16	150
R γ anti H γ 2. FIT	18.6	8×10^{-3}	0.78 γ	1 : 4	100
Sera against human albumin: R γ anti H a. FIT	22.2	10×10^{-3} 0.014 mg./ml.	0.78 γ 0.23 γ	1 : 4 1 : 1	100 100
R γ anti H a. LRB					

* H = human; G = goat; R = rabbit; a = albumin; γ = γ -globulin; f = fibrin; FIT = fluorescein isothiocyanate; LRB = lissamine rhodamine B 200.

Blocks of lung tissue were rapidly frozen at -70° C. and sectioned at 4 to 6 μ at -20° C. in a cryostat. In order to remove the plasma proteins not closely bound with the tissue, the sections were washed in buffered 0.15 M NaCl for 2, 6 and 25 minutes respectively (buffered saline changed 3-4 times), and fixed in acetone for 15 minutes. Duplicate tissue sections were fixed in acetone without washing.

Specific immuno-histochemical reagents used in this work were rabbit and goat antisera against human fibrin, plasma albumin and γ -globulin, prepared in our laboratory. Antisera were fractionated by precipitation with Na₂SO₄. Immunologically active fractions were conjugated with fluorescein isothiocyanate (FIT), tetramethylrhodamine isothiocyanate (MRIT) according to the method of Coons and Kaplan (1950) in the modification of Riggs (Riggs, Seiwald, Burckhalter, Downs and Metcalf, 1958) and Marshall (Marshall, Eveland and Smith, 1958), and with lissamine rhodamine B 200 (LRB) after Chadwick, McEntegart and Nairn (1958a, b) with minor modifications. The labelled antisera were dialysed against NaCl buffered to pH 7.6, or purified on a DEAE-cellulose chromatographic column.

The reagents thus obtained were examined by the gel double diffusion test and by immunoelectrophoresis, as well as in staining reactions on several control tissue sections from various organs. The reagents that were not monospecific were purified with aliquots of appropriate antigens until free of contaminating antibodies.

The sections were stained directly for 45 minutes with immuno-histochemical reagents, the excess antibody being washed from the sections and the tissues then examined using an ultraviolet light source. Simultaneously, to ensure the specificity of staining, serial sections were treated with unlabelled antisera, then with the labelled ones—similar or different from those unlabelled (blocking procedure). The characteristics of the reagents are given in Table 2.

Duplicate blocks of lung tissue were also fixed in formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin, phosphotungstic acid haematoxylin, Biebrich scarlet, van Gieson, Mallory trichrome, Masson trichrome and by the McManus periodic acid-Schiff method before and after exposure of

sections to diastase. Formalin-fixed frozen sections were stained with Sudan III, Sudan black for lipid and by McManus periodic acid-Schiff method.

Using both light and fluorescent microscopy 198 photomicrographs were made as a permanent record of the observations.

Results

In the standard histological sections of the lungs stained with haematoxylin and eosin, structureless, sometimes fine granular (Figs. 1 and 3), eosinophilic membranes were found in the terminal bronchioles and alveolar ducts. The presence of cell elements could sometimes be discovered within the membranes (Fig. 2). Sometimes also remnants of preserved epithelium were seen covering the membrane (Fig. 3) or underlying it (Fig. 1). Most frequently, however, no epithelial cover could be found. The appearance of membranes was usually accompanied by atelectasis, capillary engorgement and, in two cases, by rather marked oedema.

The results of additional histological stains differed little from those previously described (Buckingham and Sommers, 1960; De and Anderson, 1953; Duran-Jorda *et al.*, 1956).

The results obtained by means of immuno-fluorescence were absolutely uniform in all the six cases investigated. In the unstained sections the membranes showed greyish-blue autofluorescence. An intense yellowish-green (FIT-labelled reagents) or orange-red (LRB-labelled reagents) fluorescence was obtained in the reactions with antisera against fibrin and/or fibrinogen, γ -globulin and albumin (Figs. 4-9). Intense specific fluorescence of the membrane is proof of the presence of all these proteins within the membrane. In sections stained with anti-globulin or anti-albumin reagents, the fluorescence of membranes was uniform. Sections stained with antifibrin reagents showed inconsistent granular and laminar fluorescence of hyaline membranes, especially under higher magnification (Fig. 5). In these

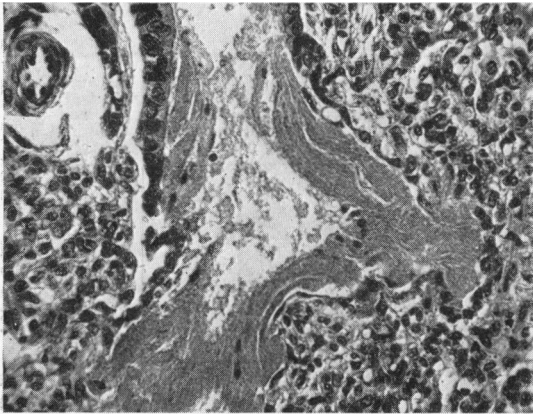


FIG. 1.—Granular hyaline membrane, focally covering the preserved epithelium of terminal bronchiole (Case 2). (H. and E. \times 400.)

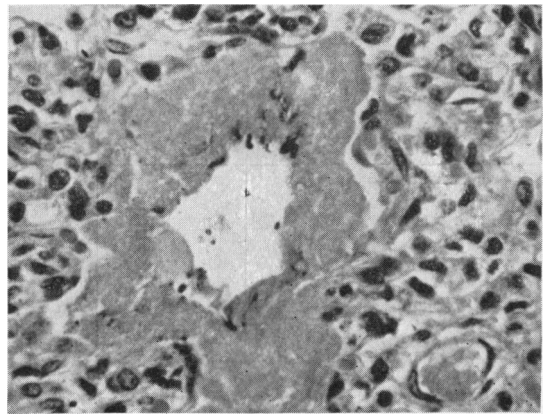


FIG. 2.—Remnants of nuclei incorporated in hyaline membrane (Case 4). (H. and E. \times 800.)

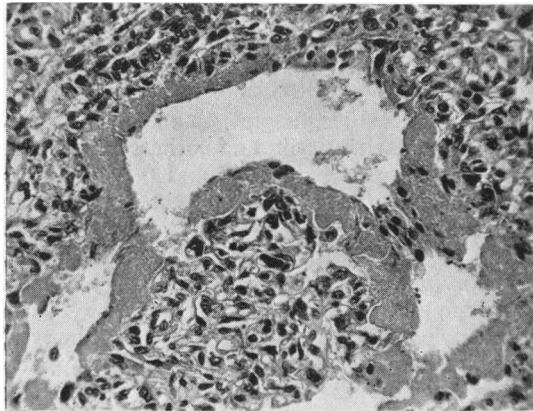


FIG. 3.—Granular hyaline membrane, focally covered with epithelium (Case 4). (H. and E. \times 400.)

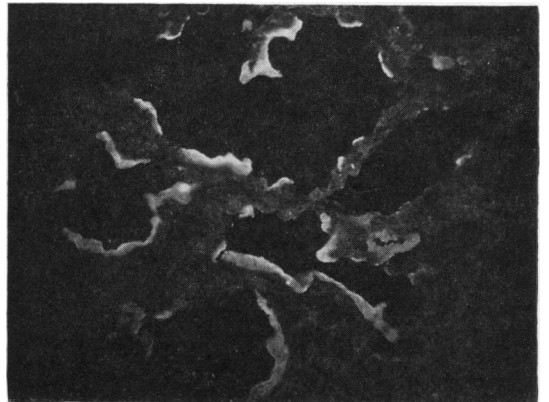


FIG. 4.—Fibrin content of hyaline membranes (Case 4). (Stain. $G \gamma$ anti H f. FIT* \times 320.)

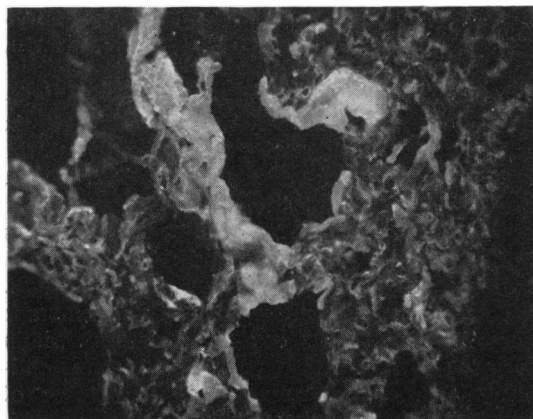


FIG. 5.—Patchy granular and laminar fluorescence of hyaline membranes (Case 4). Note fluorescence of fibrin in septal capillaries of atelectatic portions. (Stain. $G \gamma$ anti H f. FIT \times 320.)

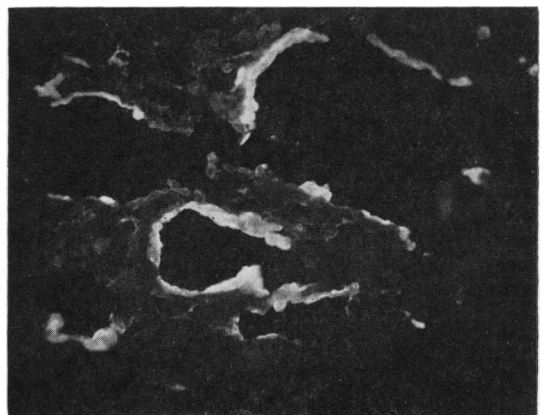


FIG. 6.— γ -Globulin content of hyaline membranes (Case 4). (Stain. $R \gamma$ anti $H\gamma_2$. FIT \times 320.)

* Abbreviations of staining methods in Figs. 4 to 12 are given in Table 2.

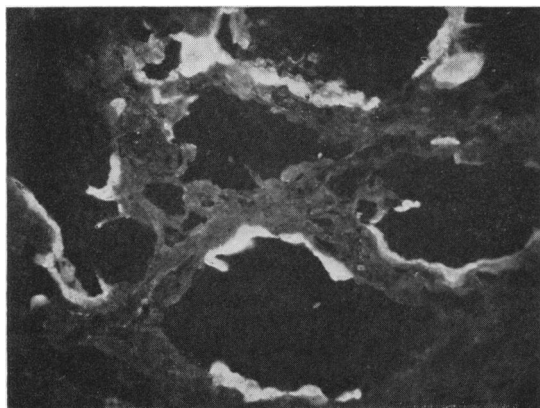


FIG. 7.—Hyaline membrane continuous with alveolar septum impregnated with γ -globulin (Case 4). (Stain. R γ anti H γ_2 . FIT \times 320.)

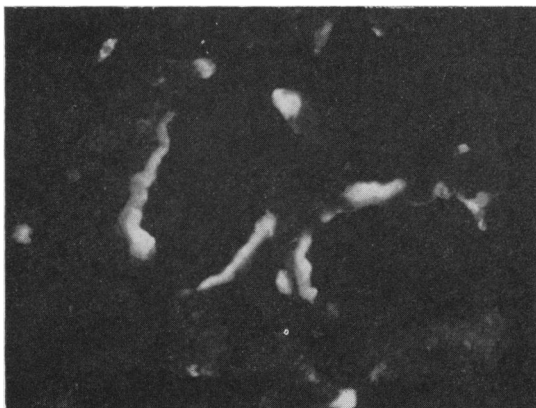


FIG. 8.—Albumin content of hyaline membranes (Case 1). (Stain. R γ anti H a. LRB \times 320.)

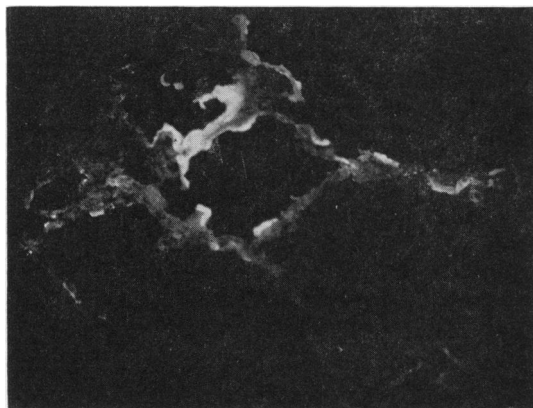


FIG. 9.—Alveolar septum impregnated with albumin (Case 4). (Stain. R γ anti H a. FIT \times 320.)

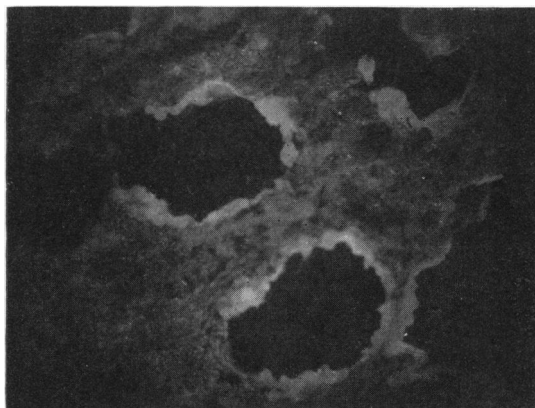


FIG. 10.—Positive effects of blocking procedure—disappearance of specific fluorescence with only autofluorescence preserved (Case 4). (Stain. R γ anti H a + R γ anti H a. FIT \times 320.)

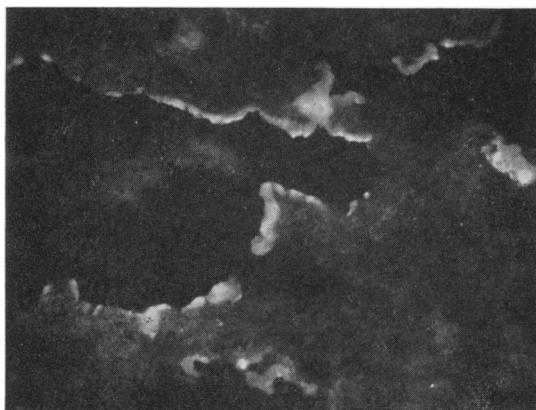


FIG. 11.—Negative effects of blocking procedure—fluorescence preserved (Case 4). (Stain. R γ anti H a \times R γ anti H γ_2 . FIT \times 320.)

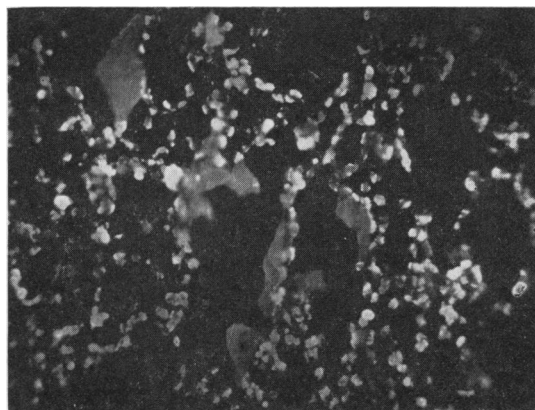


FIG. 12.—Almost complete abolition of fluorescence in hyaline membranes where only few fluorescent nuclei can be seen; intensive fluorescence of nuclei in alveolar septa (Case 4). (Stain. LE-serum + R γ anti H γ_2 . FIT \times 320.)

sections, a thin fibrin layer was clearly fluorescent in septal vessels. The membranes were sometimes fused with alveolar septa impregnated with fibrin, γ -globulin and albumin (Figs. 5, 7 and 9).

A prolonged (25 minutes) rinsing of sections in buffered saline before fixing them resulted in a decrease in the number of membranes, whereas in the persisting membranes the intensity of fluorescence was either not diminished or only minimally so. This was true not only of the fibrin but also of albumin and γ -globulin, proving that all these proteins help to form the membrane.

By treating the sections with homogeneous unlabelled reagents, before treating them with the same labelled reagents, fluorescence was completely abolished (Fig. 10). Moreover similar treatment with unlabelled sera against other fractions of human serum was without any influence on the intensity of specific fluorescence (Fig. 11). On the one hand this provides proof of the staining specificity, on the other hand proof of the antigen monospecificity of the histochemical reagents used.

In sections covered with lupus erythematosus-serum (LE-serum, containing the antinuclear factor) and then stained with the reagent against human γ_2 -globulin, a considerable decrease, and even focal disappearance, of fluorescence of hyaline membranes was observed, whereas the nuclei of alveolar septa and nuclei or their fragments incorporated in the membranes showed intense fluorescence (Fig. 12). Each of several stainings gave the same result (always with duplicate sections stained only with reagent against human γ_2 -globulin as control). The reaction made it possible to detect cell nuclei in hyaline membranes.

Unfortunately the attempts to give more exact data as to the quantitative relations and location of individual plasma proteins within the membrane, by means of mixtures of labelled reagents in contrasting colours, failed. A uniform brownish fluorescence of the membranes could be seen. In some individual instances, albumin seemed to prevail slightly.

Fats possessing autofluorescence could also be observed within some of the membranes. This method of fat detection does not, however, seem to be more sensitive than routine histological stainings.

Discussion

Investigations were carried out by means of a very specific and sensitive method of immunofluorescence. The results were consistent in all the six cases investigated, and we conclude that all the three elementary serum proteins (fibrin, γ -globulin and albumin) are integral parts of hyaline membrane.

Washing tissue sections before fixing them, accord-

ing to Gitlin and Craig, removes all the proteins not closely bound with the tissue (Gitlin and Craig, 1956). Prolonged washing of completely unfixed sections resulted in the disappearance of albumin and γ -globulin in the vessels of alveolar septa, and of the oedema fluid in the alveoli; it also completely removed a part of the hyaline membranes. However, in the remaining membranes, a large amount of albumin and γ -globulin was still to be found. Taking the intensity of fluorescence as criterion of the quantity of protein in washed sections stained with the same immuno-histochemical reagent, the amount of albumin and γ -globulin in hyaline membranes remained unchanged or changed only minimally.

The detection of fibrin, albumin and γ -globulin as integral components of the hyaline membrane makes it seem likely that the other plasma proteins (α -globulin and β -globulin) are equally present. In other words, the hyaline membrane consists of a plasma clot, in which some cell remnants and fats can also be found.

The existence of cell nuclei within the hyaline membrane has been confirmed by means of immunofluorescence with the use of antinuclear serum (LE-serum). A considerable decrease or complete disappearance of fluorescence of the whole membrane may be interpreted as follows. The antibodies contained in a drop of the reagent covering the section are absorbed by the cell nuclei, previously subjected to LE-serum. In view of the character of the fluorescence, it may be presumed that γ -globulin and albumin impregnate the whole membrane homogeneously, whereas the fibrin preserves a granular, slightly laminar structure. The method applied is a qualitative one and allows conclusions to be drawn as to the relative amount of antigen only when the same immuno-histochemical reagent is used.

The fusion of hyaline membranes and of alveolar walls focally impregnated with albumin, γ -globulin and fibrin (Fig. 7) seems to imply capillary damage.

The results of the present study confirm the observations made with the electron microscope. They support the pathogenetic theories that assume the endogenous origin of hyaline membranes. Gitlin and Craig who represent the same view do not mention explicitly why fibrin should be the only component of the hyaline membrane.

Lungs of newborn babies only were studied. The detection of several plasma components in the hyaline membranes of the newborn establishes a strong relation between hyaline membrane disease and other conditions of children and adults in which the same membranes can be observed in a light microscope. A temporary increase of capillary

permeability is the feature common to these conditions, and for this reason fibrin and other serum proteins escape from the vessels, as has also been observed in experimental studies on hyaline membrane (Buckingham and Sommers, 1960; De and Anderson, 1954; Farber, 1937).

The lack of a consistent occurrence of general oedema in hyaline membrane disease prompts us to search for the causes of this seemingly selective injury of the pulmonary vessels. An increased surface tension in the alveoli may account for the increased permeability (Buckingham and Sommers, 1960; Pattle, Claireaux, Davies and Cameron, 1962). An inflammatory origin of the exudate, on the other hand, cannot be entirely excluded; Groniowski and co-workers support the inflammatory origin of hyaline membrane disease (Groniowski, Gabryel and Grembowicz, 1957; Groniowski and Biczyskova, 1963), while Laughton (1963) claims to have isolated a specific organism from a case of hyaline membrane disease.

Summary

The lungs of six newborns with well-defined hyaline membrane disease were studied by means of immunofluorescence. It was found that the hyaline membranes were composed of fibrin, γ -globulin and albumin, which also focally impregnated the alveolar septa. Moreover, cell nuclei were found within the membranes by means of fluorescent antibody procedure.

It was concluded that the hyaline membrane may be formed in the plasma-clotting process resulting from capillary wall damage and increased capillary permeability.

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REFERENCES

- Aronson, N. (1961). Studies on hyaline membranes. *Pediatrics*, 27, 567.
- Avery, M. E. (1962). Cardiopulmonary changes in respiratory distress. Clinical observation and physiological exploration. *ibid.*, 30, 859.
- van Breemen, V. L., Neustein, H. B. and Bruns, P. D. (1957). Pulmonary hyaline membranes studied with the electron microscope. *Amer. J. Path.*, 33, 769.
- Buckingham, S. and Sommers, S. C. (1960). Pulmonary hyaline membranes. A study of the infant disease and experimental hyaline membranes induced pharmacologically. *A.M.A. J. Dis. Child.*, 99, 216.
- Campiche, M., Jaccottet, M. and Juillard, E. (1962). La pneumonose a membranes hyalines. Observations au microscope electronique. *Ann. Paediat. (Basel)*, 199, 74.
- , Prod' hom, S. and Gautier, A. (1961). Etude au microscope électronique du poumon de prématurés morts en détresse respiratoire. *ibid.*, 196, 81.
- Chadwick, C. S., McEntegart, M. G. and Nairn, R. C. (1958a). Fluorescent protein tracers. A simple alternative to fluorescein. *Lancet*, 1, 412.
- (1958b). Fluorescent protein tracers; a trial of new fluorochromes and the development of an alternative to fluorescein. *Immunology*, 1, 315.
- Coons, A. H. and Kaplan, M. H. (1950). Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. exp. Med.*, 91, 1.
- Curtis, P. (1957). Hyaline membrane disease. *J. Pediat.*, 51, 726.
- De, T. D. and Anderson, G. W. (1953). Hyaline-like membranes associated with diseases of the newborn lungs: A review of the literature. *Obstet. gynec. Surv.*, 8, 1.
- (1954). The experimental production of pulmonary hyaline-like membranes with atelectasis. *Amer. J. Obstet. Gynec.*, 68, 1557.
- Duran-Jorda, F., Holzel, A. and Patterson, W. H. (1956). A histochemical study of pulmonary hyaline membrane. *Arch. Dis. Childh.*, 31, 113.
- Farber, S. (1937). Studies on pulmonary edema I. The consequences of bilateral cervical vagotomy in the rabbit. *J. exp. Med.*, 66, 397.
- Gitlin, D. and Craig, J. M. (1956). The nature of the hyaline membrane in asphyxia of the newborn. *Pediatrics*, 17, 64.
- Groniowski, J. and Biczyskova, W. (1963). The fine structure of the lungs in the course of hyaline membrane disease of the newborn infant. *Biol. Neonat. (Basel)*, 5, 114.
- , Gabryel, P. and Grembowicz, L. (1957). Badania zespołu błon szklistych płuc noworodka, I. Badania morfologiczne [Investigations on the hyaline membrane syndrome in the lungs of newborns. I. Morphological study]. *Pat. pol.*, 8, 13.
- James, L. S. (1959). Physiology of respiration in newborn infants and in the respiratory distress syndrome. *Pediatrics*, 24, 1069.
- Laughton, N. (1963). Pneumonitis due to a pleuropneumonia-like organism and a corynebacterium associated with hyaline membrane disease. *J. Path. Bact.*, 85, 413.
- Lieberman, J. (1961). The nature of the fibrinolytic-enzyme defect in hyaline-membrane disease. *New Engl. J. Med.*, 265, 363.
- Lynch, M. J. G. and Mellor, L. D. (1956). Hyaline membrane disease of lungs. *J. Pediat.*, 48, 165.
- and Badgery, A. R. (1956). Hyaline membrane disease. Its nature and etiology. The poisonous metabolic effects of excess oxygen. Neural control of electrolytes. *ibid.*, 48, 602.
- Marshall, J. D., Eveland, W. C. and Smith, C. W. (1958). Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technic with a modification of its application. *Proc. Soc. exp. Biol. (N.Y.)*, 98, 898.
- Miller, H. C. (1962). Respiratory distress syndrome of newborn infants. II. Clinical study of pathogenesis. *J. Pediat.*, 61, 9.
- Pattle, R. E., Claireaux, A. E., Davies, P. A. and Cameron, A. H. (1962). Inability to form a lung-lining film as a cause of the respiratory-distress syndrome in the newborn. *Lancet*, 2, 469.
- Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M. and Metcalf, T. G. (1958). Isothiocyanate compounds as fluorescent labeling agents for immune serum. *Amer. J. Path.*, 34, 1081.
- Rudolph, A. J. and Smith, C. A. (1960). Idiopathic respiratory distress syndrome of the newborn. An international exploration. *J. Pediat.*, 57, 905.