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Enhanced Proteolytic Activity at pH 2.0 in Lungs of Infants Dying from Hyaline Membrane Disease (HMD).* (28144)

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Hyaline membrane disease of the newborn infant (HMD) represents a fairly circumscribed pathologic entity. A major point of disagreement remaining is whether the "idiopathic respiratory distress syndrome of the newborn" without the typical hyaline membranes on histologic study has the same pathogenetic mechanism(1). It is agreed that the membrane formation is a postnatal event (2) and that the membranes are largely composed of fibrin, thus representing in part at least an exudative phenomenon(3). The etiology of the disease is not understood; however, various investigations indicate an associated deficiency of lung-lining-film substance(4), a pulmonary deficiency of fibrinolytic activity at pH 7.4-8.0(5) and degenerative and regenerative phenomena of the respiratory and bronchiolar epithelium, recently reviewed by Boss and Craig(6).

The clinical course of most of these infants, the presence of hyaline membranes in adults with a history of aspiration of vomitus(7) and the ability to produce similar lesions in animals by tracheal instillation of hydrochloric acid(8) have led one of us (K.B.) to postulate that the disease may be caused by inhalation of acid gastric juice at time of birth. This suggestion is not new(9,10) but no laboratory support has been forthcoming and this etiologic possibility is not seriously considered by most workers. In adults, gastric aspiration is traceable not only by the clinical event, but by histologic identification of food particles in the lung in which a severe chemical and bacterial pneumonitis usually ensues. In the hope of finding evidence for the possible aspiration of gastric content it was conjectured that pepsin, because it is the most active gastric enzyme, might remain active in the lung long enough after death to be detected at autopsy, essentially serving as a tracer.

Two methods of testing this hypothesis are available: a) the immunologic, in which fluorescein-conjugated antibody to pepsin might be used to label any pepsin still contained in the lungs and b) direct enzymological determination of pepsin activity. Because of the costliness of preparing antibody, the uncertainty involved in species crossreaction, and the probable lack of specific localization of the pepsin, it was decided to postpone the immunologic method and attempt the enzyme analysis. It was recognized, however, that finding a proteolytic activity even at pH 2.0 would not constitute conclusive evidence for the presence of pepsin. It has been shown previously that newborn infants of varying gestational ages may have considerable quantities of acid gastric juice at birth(11) and that pepsin secretion is also in progress at this time(12).

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TABLE I. Cases of Fig. 1 Listed Numerically with Weight of Each Infant at Autopsy, Duration of Survival, Method of Delivery and Final Diagnosis.

Case No.	Wt (g)	Age (hr)	Method of delivery	Final diagnosis
1	1700	7	Pelvic	Typical HMD
2	2880	36	C/S	*Idem*
3	1425	92	Pelvic	HMD with resolution; twin 2
4	1800	24	Pelvic	Typical HMD
5	2225	17	C/S	Atypical HMD; widespread aeration; not promptly frozen
6	2523	17	C/S	Congestion, hemorrhage, occasional hyaline membranes; not promptly frozen
7	3310	Stillborn	Pelvic	Macerated postmature; pulmonary hemorrhage
8	3250	328	"	Interstitial pneumonitis; transposition of great vessels
9	1000	5	"	Normal lung; subarachnoid hemorrhage
10	2220	39	Breech	*Idem*
11	580	10	Pelvic	Aspirated amn. fluid; subarachnoid hemorrhage
12	2705	Stillborn	Breech	Normal lung; hydrocephaly
13	440	"	Pelvic	Normal lung; extreme prematurity
14	1360	"	"	*In utero* aspiration pneumonia

Materials and methods. Lungs of infants dying in the neonatal period were collected in the frozen state (–10°C) by colleagues and examined histologically and enzymically at a later date. Usually 2 or 3 separate pieces of the lung were tested. The gastric juice of 2 adults and 2 newborns as well as the gastric mucosa of a newborn infant were also studied.

The enzyme assay is based on Herriott's (13) modification of the procedure originally reported by Anson(14), in which acid denatured hemoglobin is used as a substrate for pepsin. Proteolysis is followed with time, the undigested hemoglobin being precipitated by trichloroacetic acid (TCA) and removed by centrifugation. Folin's phenol reagent then gives a blue color with the supernatant, indicating the tyrosine and tryptophane content of the soluble peptides. To differentiate between the hypothesized pepsin and *normal* tissue cathepsins, the proteolysis is assayed simultaneously at pH 2 and 4.

Crystalline human hemoglobin is diluted to 2 g% with a) .06 M HCl and b) .05 M sodium acetate-acetic acid mixture, and the pH adjusted to 1.5 and 4.0 respectively. One gram of freshly thawed lung tissue is ground in a Potter-Elvehjem homogenizer with 5.0 ml of cold 1% saline solution and then centrifuged at 0°C at 6600 g for 20 minutes. The pH of the supernatant is uniformly 5.8. Five tubes each at pH 1.5 and 4.0 are prepared with .3 ml of hemoglobin (a or b) plus .7 ml of distilled water and equilibrated to 30°C in a water bath. To these tubes is added .2 ml of the lung supernatant and the reaction is allowed to proceed at 5 minute intervals to 20 minutes. The addition of lung supernatant raises the pH of the solution to 2.1 and 4.3 respectively. To stop reaction 2.0 ml of 5% TCA is added and the precipitate is centrifuged. Of this supernatant 2.0 ml is pipetted from each tube into a corresponding series of tubes containing 2.0 ml of .5 NaOH. The blue color that forms upon addition of .25 ml of Folin's phenol reagent diluted 1:3 is allowed to develop for 10 minutes and read at 660 mμ on a Zeiss or Beckman spectrophotometer. This reaction yields a linear plot of optical density against time at each pH. A series was run on 14 neonatal lungs (Table I).

Several supplementary experiments were designed to ascertain whether the enzyme found to be active at pH 2.0 had other known properties of pepsin. Taking advantage of the well documented irreversible denaturation of pepsin above pH 8(15,16), several samples of the clear supernatant from the homogenate of HMD lungs were brought to pH 8.5 with NaOH for 5 minutes and then back to pH 5.8 for the assay. This procedure was also carried out on one sample of neonatal gastric juice and one sample of neonatal stomach, several samples of commercial hog pepsin and 2 samples of adult gastric juice, all in the presence of normal lung homogenate which

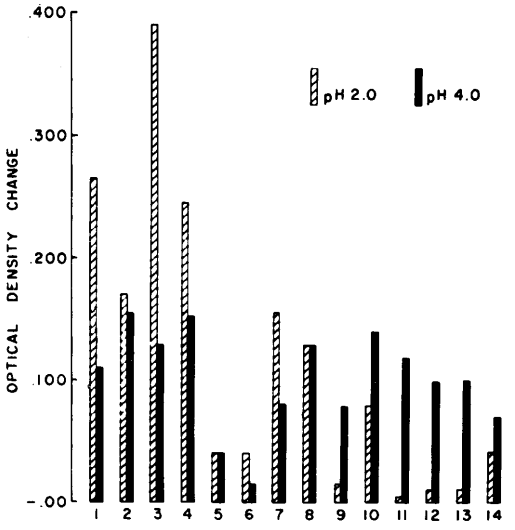

FIG. 1. Histogram of proteolytic activity of neonatal lung extracts at pH 2 (left) and pH 4 (right) in each case. Ordinate is optical density increase after 20 min; abscissa cases 1-14 as described in Table I.

had very low pH 2 proteolytic activity. The chemical analyses were performed without knowledge of the histologic findings. Later, the results of both were correlated.

Results. The proteolytic activities of the extracts of these lungs at pH 2 and pH 4 are shown in Fig. 1. In the first 4 cases, representing lungs with typical HMD, there was an excess of proteolysis at pH 2 over that observed at pH 4, while considerably less activity existed in the extracts of lungs from those infants not exhibiting the typical picture of HMD. The individual cases are listed in Table I which correlates histologic findings, age and weight of each infant. Sex has been omitted since it was not found to correlate with any other finding. It is evident from Fig. 1 that considerable proteolytic activity at an acid pH was detected in most lung extracts. It is the relative rates of proteolysis at the 2 pH's which appear to differ most between the cases with HMD and the controls, there being considerable variability in the absolute proteolytic activities from lung to lung and, to some extent, from section to section of the same lung.

Marked proteolysis at pH 2 was found in HMD lungs of premature and mature infants whether delivered by Cesarean section or naturally. The activity was present as early as 7 hours after birth and the highest level was in case 3, an infant who lived almost 4 days. It may be significant that histologic examination of this lung showed extensive HMD with evidence of resolution as described by Boss and Craig(6) with the attending macrophage infiltration. The low values obtained in cases 5 and 6 are presumably due to inadequate freezing of the lungs after autopsy. All other infants died from other causes and no hyaline membranes were present at autopsy although other pulmonary pathology was found in cases 8 and 14 (Table I). Of interest is the degree of proteolysis from extracts of the lung of case 7, an early macerated, stillborn postmature fetus whose lung was hemorrhagic. Whether only cathepsins were responsible for this enzymatic activity and whether the baby would have developed HMD if it had been liveborn cannot be determined.

Extracts of HMD lungs with the described pH 2 proteolytic activity did not lose this activity after having been brought to pH 8.5 as described in *Methods*. Similarly, the results of preliminary experiments with gastric juice indicate that neonatal pepsin is more resistant to alkaline denaturation than adult human or commercial swine pepsin. A larger series of such materials must be investigated, however, before one may suggest that pepsin has an embryonic form as has been shown for hemoglobin(17), adenylic acid deaminase (18) and muscle lactic dehydrogenase(19).

Discussion. Although our results may be interpreted as showing the presence of pepsin in the lungs, other interpretations are tenable. The assay employed detects as little as .2 μg of swine pepsin (a slope of .300 O.D. units/20 minutes is equivalent to 2 μg of pepsin or .002 ml of adult gastric juice in one case analyzed); however, neonatal gastric juice appears to contain much less pepsin activity so that a much larger quantity would have to be inhaled if this is in fact the source of the measured proteolytic activity. Furthermore, the enzyme would have to be much more resistant to inactivation at the normal body pH than adult pepsin for its maintained presence as a tracer, as seems to be suggested by our preliminary experiments.

In view of the multiple experimental and clinical causes of HMD (oxygen poisoning, acid instillation or inhalation, vagal section with pulmonary edema etc.) it might be better to regard the proteolytic activity of these lung extracts as a secondary concomitant of the disease. That is, the proteolytic activity at pH 2 might be an expression of cathepsin activity, liberated from the lysosomes of the lung by the degenerative changes which take place in this organ. In this regard, Dingle (20) has already shown that cathepsin with a maximal activity at pH 2 can be obtained by treatment of liver lysosomes with vit. A. Cell death liberates hydrolytic and proteolytic enzymes from the lysosomes or "suicide bags"(21). In support of the presence of cathepsin proteolysis one may note the variable amounts of pH 4 activity present in all cases and the larger variations of the proteolysis at pH 2 of the "control" lungs. It varies from negligible amounts to levels equal to the pH 4 activity and probably represents enzymes liberated by the autolytic process in the lung. In this respect it is interesting that Lieberman(5) noted a reduced plasminogen or fibrinolytic activity (measured by clotting at pH 8) in HMD lungs. It is possible then to conjecture that during destructive or autolytic events in the lung, normally present cathepsins have been converted to proteolytic activities with a lower pH optimum. Such an explanation would readily fit case 7, the partially autolyzed stillborn with considerable pH 2 activity.

At this time it is impossible to identify the reported acid proteolytic activity of these lungs as being due to either pepsin or cathepsin. If indeed it did reflect in part pepsin in the lungs of HMD, one will have to rule out in future studies the possibility of converted pepsinogen originating from inhaled amniotic fluid and fetal blood. Moreover, if pepsin had served as tracer of gastric juice aspirated at birth in these studies, it is not suggested that the pepsin is the injurious substance. Experimental evidence with tracheal instillation suggests that the acidity is the noxious agent(22). This would presumably be neutralized very quickly, possibly sooner than therapeutic application of alkaline chemicals. The differentiation of cathepsin from pepsin in these lungs would appear to be an urgent problem as it is conceivable that fetal gastric secretions could be influenced pharmacologically before birth were one able to confirm gastric aspiration as an etiology of HMD.

Summary. Extracts of lung tissues from newborn infants contain variable amounts of proteolytic activity. At pH 2 lungs with hyaline membrane disease of the newborn exhibit more activity than control lungs without the disease. Exposure to pH 8.5 does not abolish this activity. Likewise, preliminary studies indicate that neonatal gastric juice is not inactivated by pH 8.5 in contrast to adult gastric juice and hog pepsin. The nature of this proteolytic activity is discussed with reference to lysosomal cathepsin and fetal pepsin from aspirated gastric juice and their possible rôles in the genesis of hyaline membrane disease of the newborn.

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Inhibition of Enzymatic DNA Synthesis by Certain Pathological Human Sera.* (28145)

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Sera from patients with systemic lupus erythematosus and certain other "collagen diseases" contain antibodies reacting with cell nuclei, nucleoprotein, and DNA‡ as demonstrated by complement fixation and precipitin reactions(1). At least two factors could be differentially adsorbed: one reacted with cell nuclei and was responsible for the "LE phenomenon"; another, present only in certain sera, reacted with purified DNA from different sources and species. Using a fluorescent antibody technic, Calabresi *et al.*(2) detected antinuclear globulins in the sera of all patients studied with SLE or with Felty's syndrome of rheumatoid arthritis. Williams and Schilling(3), however, did not find any marked effects of SLE and FS sera, compared to normal sera, on incorporation of adenine or thymidine into DNA of human blood leukocytes *in vitro*. Holman *et al.*(1), Lachman (4), and Rapp(5) found that the antinuclear factors present in SLE sera did not interfere with the growth of normal or tumor cells in tissue culture, and, from these and other data, concluded that the factors could not penetrate viable cells.

Inability of DNA-reactive antibody to penetrate to the nucleus of viable cells might explain the negligible effect of the antinuclear factors on incorporation or growth of whole cells *in vitro*. However, the possibility that anti-DNA factors present might affect the biosynthesis of DNA in cell free systems remained to be tested. The enzymatic incorporation of deoxyribonucleoside triphosphates into DNA in presence of a small amount of heat-denatured primer DNA, catalyzed by a purified DNA polymerase from calf thymus gland(6), provides a system to test this hypothesis.

In the present study, we measured the effect of pathological and normal sera on synthesis of DNA with purified calf thymus polymerase. Certain pathological sera do contain DNA-reactive material as demonstrated by inhibition of DNA synthesis. The method is sensitive, rapid, and provides a means of obtaining information on the combining sites of serum factors with DNA.

Materials and methods. Agar tube precipitin tests. Calf thymus DNA (Worthington) was dissolved in .01 tris buffer at pH 8.1, heated at 100°C for 2 min, and cooled immediately by immersion in an ice bath. The DNA was added to agar in .02 M sodium phosphate buffer, pH 7, held at 50°C. Final concentrations were 10 and 50 μg DNA/ml and 0.5% agar. Fifteen-mm columns of DNA- agar were pipetted into 5-mm ID tubes

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‡ The following abbreviations are used: DNA, deoxyribonucleic acid; SLE, systemic lupus erythematosus; FS, Felty's syndrome of rheumatoid arthritis; dCMP, the monophosphate of deoxycytidine; tris, tris-(hydroxymethyl)-aminomethane; dCTP, TTP, dATP, dGTP, and ATP, the triphosphates of deoxycytidine, thymidine, deoxyadenosine, deoxyguanosine, and adenosine; RA, rheumatoid arthritis.