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37. This research was supported by the Medical Research Council of Canada (Grant MRC MA-5473) and by the Weston Foundation.
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39. Received for publication January 10, 1977.
40. Accepted for publication March 28, 1977.

Pediat. Res. *11*: 1202–1207 (1977)

Alveolar macrophage
lysosomal enzymes
lysosomes
oxidative enzymes
phospholipids

Maturation of the Rabbit Alveolar Macrophage during Animal Development. II. Biochemical and Enzymatic Studies

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Summary

Alveolar macrophages (AMs) obtained from 1-day-old rabbits showed high levels of phospholipid, protein, DNA, and RNA compared to those obtained from 7-day-old, 28-day-old, or adult rabbits. The surfactant material released in alveoli during the perinatal period is extensively phagocytosed by AMs, and appears to be primarily responsible for high phospholipid content of these cells. The high protein, DNA, and RNA levels of AMs may result from phagocytosis of cellular debris.

Of the six lysosomal enzymes of AMs studied, acid phosphatase (AP) and lysozyme activities showed a decline in the first postnatal week. In AP this was followed by no significant change. A steady rise in lysozyme activity after the seventh postnatal day was observed. The activities of cathepsin D and DNase did not show significant changes, whereas β-glucuronidase and RNase activities of AMs increased significantly within the first postnatal week.

The activities of glucose-6-phosphate dehydrogenase (G-6-PD), 6-phosphogluconate dehydrogenase (6-PGD), lactate de-

hydrogenase (LDH), and malate dehydrogenase (MDH) in AMs appeared high in AMs of 1-day-old animals compared to other time periods studied. Following a significant decline in these activities in the first postnatal week, a peak was observed in the activities of G-6-PD, 6-PGD, and LDH at 28 days.

Speculation

A high concentration of phospholipid in AMs obtained from newborn animals suggests that AMs play an important role in the removal of excess surfactant from the newborn lung. A relative increase in hexosemonophosphate shunt activities over glycolytic or Krebs cycle activities after birth and an increase in lysozyme activity after the first postnatal week may contribute to the capacity of AMs to kill and degrade the microorganisms.

Alveolar macrophages constitute the major phagocytic defense of the lung. Although considerable research has been directed in the past to the study of the morphology (17, 39, 49, 51), enzymology (16, 18), metabolic behavior (3, 50, 53), and function (10, 14, 36) of AMs in adult animals, little is known about their maturation during animal development.

As described in the previous report (65), a dramatic increase in the number of lavagable AMs of rabbits was observed during the perinatal and postnatal periods. Prominent morphologic changes observed during this period included decreasing glycogen stores, accumulation of lipid droplets, increase in the prominence of the Golgi apparatus, and an increase in the amount of rough endoplasmic reticulum and the number of lysosomes and mitochondria. In the present report several biochemical parameters of the rabbit AM were investigated during animal development with a specific emphasis on the study of the development of lysosomal and oxidative enzyme activities, both of which are known to participate in the metabolic functions of AMs (3).

MATERIALS AND METHODS

ANIMALS

Alveolar macrophages were obtained from New Zealand white rabbits of both sexes (B and H Rabbitry, Rockville, Md.). Groups of 3–22 rabbits of each of the following ages were employed: 1 day (6–20 hr); 7 and 28 days after birth; and 90 or more days after birth (considered adult).

ISOLATION OF MACROPHAGES

AMs were obtained by tracheobronchial lavage as described in the previous report (65). In order to obtain a sufficient quantity of cells, suspensions from littermates of the 1-day-old and 7-day-old animals were pooled. After lavage, the cells were washed and centrifuged at 4° two to three times at 150 × *g* with Krebs-Ringer phosphate buffer, pH 7.2 (62), modified to contain 1.5 mM Ca^{++}. The cell suspension was adjusted to a final concentration of 5–10 × 10^6 cells/ml. The viability of cells in suspension was always more than 95% when assayed by trypan blue exclusion method and alveolar macrophages constituted approximately 95% of the cells in suspension. Aliquots of these suspensions were removed for the measurement of phospholipid, protein, DNA, and RNA and the remainder was sonicated for two 15-sec intervals at 15 kilocycles/sec employing a Biosonik II sonicator (67) for determinations of several enzyme activities.

The following reagents employed in the present studies were obtained from Sigma Chemical Company, St. Louis, Mo.: calf thymus DNA (type I); yeast RNA (type XI); *p*-nitrophenyl phosphate; *p*-nitrophenyl-β-D-glucuronide; bovine hemoglobin (type I); lyophilized *Micrococcus lysodeikticus*; crystalline egg white lysozyme; glucose-6-phosphate monosodium salt; NADH (grade V) and oxalacetate (grade I).

BIOCHEMICAL ASSAYS

Phospholipid Determination. Phospholipids were extracted as described by Folch *et al.* (28) and phosphorus was quantitated by the method of Bartlett (7). Approximately 1 × 10^6 cells were used for the assay.

Protein Determination. Protein was determined by the method of Lowry *et al.* (44) using bovine serum albumin as standard.

Nucleic Acid Determination. DNA was extracted from cells by the method of Leyva and Kelley (41) and measured by the method of Burton (13) with overnight color development at 37°. Purified calf thymus DNA was used as standard. RNA was extracted and measured as described by Schneider (54), employing yeast RNA as a standard. Approximately 1 × 10^6 cells were used for each of these assays.

Enzyme Assays. The following enzyme activities were measured: acid phosphatase (AP), β-D-glucuronidase (β-G), RNase, DNase, lysozyme, cathepsin D, G-6PD, 6-PGD, LDH and MDH. Reaction rates of all enzyme assays were linear throughout the specified incubation periods. The substrate concentrations were optimal. Enzyme activities were proportional to the protein concentration used within the specified range and were expressed as units/2.5 × 10^6 cells.

Acid Phosphatase (EC 3.1.3.2). The assay mixture for AP contained a final concentration of 140 mM sodium acetate buffer (pH 5.0), 8 mM *p*-nitrophenyl phosphate, and 0.1–0.2 mg macrophage protein (6). Incubations were performed in total volume of 2.0 ml for 15 min at 37°. The reaction was stopped by the addition of ice-cold 1 M Tris-HCl buffer (pH 8.5) containing 0.4 M K_2HPO_4 as suggested by Torriani (61) and the extinction measured at 420 nm.

β-D-Glucuronidase (EC 3.2.1.31). β-G was measured using *p*-nitrophenyl-β-D-glucuronide as substrate (6). The assay mixture contained at a final concentration 100 mM Na acetate buffer (pH 5.0), 6.8 mM glucuronide, 0.1–0.2 mg macrophage protein in a total volume of 1.1 ml. Incubations were carried out for 15–30 min at 37° and the reaction was stopped by addition of 1.5 ml 0.5 M sodium carbonate-bicarbonate buffer as described by Barrett (6); the absorbance was measured at 420 nm.

Cathepsin D (EC 3.4.4.23). The assay for cathepsin D was based on the method described by Anson (2). Purified bovine hemoglobin in 0.025 N HCl at a final concentration of 2% was incubated with 0.1–0.2 mg macrophage protein for 30 min at 37° in an assay volume of 2.0 ml. The reaction was stopped by the addition of 2.0 ml ice-cold 5% trichloroacetic acid. Solubilized protein degradation products were measured at 280 nm after proper dilution of the filtrate.

Ribonuclease (EC 2.7.7.16). The assay for RNase consisted (6) of 3.0 mg yeast ribonucleic acid, 200 mM sodium acetate buffer (pH 5.0), and 0.1–0.2 mg macrophage protein in a final volume of 2.0 ml. After incubation for 30 min at 37° the reaction was terminated by the addition of 2.0 ml perchloric acid-uranyl acetate reagent (10 g $HClO_4$ + 0.25 g uranyl acetate brought to 100 ml with water); after standing for 60 min at 4° the insoluble precipitate was discarded by centrifugation, the supernatant was diluted, and the absorbance was measured at 260 nm. The nanomoles of solubilized RNA products were calculated as described by deDuve *et al.* (19).

Deoxyribonuclease (EC 3.1.4.6). DNase activity was measured (6) as follows: 1.5 mg calf thymus DNA; 200 mM sodium acetate buffer (pH 5.0) containing 200 mM potassium chloride; and 0.1–0.2 mg macrophage protein were incubated for 30 min at 37° in a final volume of 2 ml. The reaction was then stopped by addition of 2 ml ice-cold 10% (w/v) perchloric acid. After allowing to stand for 20 min at 4°, the insoluble precipitate was removed by centrifugation and the absorbance of the supernatant was measured at 260 nm. The nanomoles of nucleotides liberated were calculated as described by deDuve *et al.* (19).

Lysozyme (EC 3.2.1.17). Lysozyme activity was determined according to the method of Shugar *et al.* (55) and Litwack (42) with minor modifications. To a 2.5-ml suspension of lyophilized

Micrococcus lysodeikticus (10 mg/100 ml) in 66 mM potassium phosphate buffer, pH 6.2, 0.1 ml lysozyme solution (4–25 units egg white lysozyme) or 0.1 ml macrophage homogenate containing 0.05–0.2 mg protein were added. The change in absorbance at 450 nm was measured immediately and was followed up to 1–2 min. A unit of lysozyme was defined as that amount of enzyme which caused a reduction in the absorbance of a Micrococcus suspension by 0.001 at 450 nm in 1 min at pH 6.2 in 2.6 ml reaction mixture and using a light path of 1 cm.

Glucose-6-phosphate Dehydrogenase (EC 1.1.1.49). G-6-PD was measured by the method of Marks (45). The assay mixture contained 50 mM glycylglycine buffer (pH 7.5), 20 mM $MgCl_2$, 0.1 mM NADP+, 2 mM glucose-6-phosphate (monosodium salt), and 0.1–0.2 mg macrophage protein in a final volume of 2.5 ml. The increase in absorbance was measured at 340 nm (molar extinction coefficient of NADPH at 340 nm = 6.22×10^6 cm^2 mol^{-1} (33)). The unit of activity was described as that amount of enzyme which will oxidize 1 nmol glucose-6-phosphate to 6-phosphogluconate/min at pH 7.5 at 25° in the presenceof NADP+.

6-Phosphogluconate Dehydrogenase (EC 1.1.1.43). The method employed for the assay of 6-PGD was identical to that of G-6-PD except that 6-phosphogluconate was used as substrate (46).

Lactate Dehydrogenase (EC 1.1.1.27). The method employed for the assay of LDH was similar to that of Bernstein and Everse (9). The assay mixture contained 100 mM sodium phosphate buffer (pH 7.5), 0.09 mM NADH, 0.67 mM sodium pyruvate and 0.1 and 0.2 mg macrophage protein in a final volume of 3.0 ml. The decrease in absorbance was measured at 340 nm against a reference cuvette containing no enzyme protein (molar extinction coefficient of NADH at 340 nm = 6.22×10^6 cm^2 mol^{-1} (33)). A unit of activity was defined as that amount of enzyme which will reduce 1 nmol pyruvate to lactate/min at 25° in the presence of NADH.

Malate Dehydrogenase (EC 1.1.1.37). The method of Dupourque and Kun (23) was used for the assay of MDH with some modifications. The assay system consisted of 100 mM sodium phosphate buffer (pH 7.5), 0.09 mM NADH, 7.5 mM oxalacetate, and 0.1–0.2 mg enzyme protein in a final volume of 3.0 ml. The reaction was followed by measuring the decrease in absorbance at 340 nm. The unit of activity was defined as that amount of enzyme which will reduce 1 nmol oxalacetate to malate/min at 25° in the presence of NADH.

STATISTICAL ANALYSIS

The results of the enzyme assays are presented as mean activity (units/2.5 × 10^6 cells) ± SEM. Statistical analyses were performed by employing Student's *t* test (22).

RESULTS

The results of phospholipid-P, protein, DNA, and RNA determinations of AMs obtained from animals at different postnatal ages are shown in Table 1. It may be seen that the highest concentrations of each of these constituents were present in the AMs of newborn animals, after which a gradual decline was observed in later age groups. No significant variations in any of these constituents in AMs were observed after 7 days. An approximately 20-fold excess in the amount of phospholipid-P was observed in AMs from newborn animals when compared to AMs from adult animals. This correlated with the large numbers of phospholipid inclusions seen at this time (65). The protein concentration of AMs from newborn animals was about 1.6-fold higher than that obtained at other periods. The DNA concentration measured in AMs from newborn animals was approximately 2-fold higher than that obtained at later time periods. The RNA content of AMs showed similar changes with levels approximately 5-fold higher at birth than at later periods.

Table 1. *Postnatal changes in concentrations of phospholipid-P, protein, DNA, and RNA of rabbit alveolar macrophages*

Age, days	Phospholipid-P, $\mu g/10^6$ cells	Protein, $\mu g/10^6$ cells	DNA, $\mu g/10^6$ cells	RNA, $\mu g/10^6$ cells
1	21.73 ± 8.8[1] (4)[2]	395 ± 44 (11)	24.8 ± 4.0 (4)	127.2 ± 44.6 (3)
7	3.66 ± 0.62 (6)	237 ± 21 (12)	11.1 ± 1.6 (8)	29.8 ± 7.4 (8)
28	1.83 ± 0.34 (4)	232 ± 15 (17)	11.6 ± 0.9 (4)	24.0 ± 5.0 (4)
~90	1.15 ± 0.09 (8)	250 ± 14 (22)	10.8 ± 0.3 (4)	23.3 ± 3.0 (8)

[1] mean ± SEM.

[2] Figures in parentheses indicate the number of experiments performed.

The postnatal changes in activities of lysosomal hydrolases of AMs are illustrated in Figure 1. A significant decline in activity of AP ($P < 0.05$) of AMs was observed during the first postnatal week after which no significant changes were seen. A similar decline in the initial activity was seen with lysozyme ($P < 0.05$), which was followed by a significant steady increase ($P < 0.05$) up to the adult state. No significant changes in cathepsin D and DNase activities were seen during all the time periods studied, whereas the β-G and RNase activities of AMs showed a significant initial increase ($P < 0.05$) in the first postnatal week followed by no change up to the adult period.

The postnatal changes in oxidative enzyme activities of AMs are illustrated in Figure 2. After a significant initial decline in activities of G-6-PD, 6-PGD, LDH, and MDH ($P < 0.05$) during the first postnatal week, a peak in activities of G-6-PD, 6-PGD, and LDH was observed at 28 days. No further significant changes were observed in the activities of G-6-PD and MDH but significant declines in the activities of 6-PGD and LDH were observed between 28 days and the adult period ($P < 0.05$).

DISCUSSION

The results of the present investigations reveal that AMs undergo profound changes in certain biochemical constituents and enzyme activities during postnatal periods. The mammalian lung exhibits significant biochemical (4, 27, 34, 38) and structural (11, 12, 60) changes during the perinatal period in response to environmental changes and these changes may be reflected in those exhibited by AMs.

During later periods of gestation a rather marked increase in production of multilamellar bodies occurs in lung, which is followed by release of these bodies into the alveoli at the onset of breathing (34). These bodies form the source of surfactant material, which consists primarily of dipalmitoyl lecithin, along with several other phospholipids, some proteins, and minor components such as carbohydrates and nucleic acids (35). In preliminary studies in our laboratory, the acellular fraction of the lung lavage has been shown to consist mainly of lamellar and other surfactant-related myelin structures derived from type II penumocytes (64); similar structures have been shown to be extensively phagocytosed by AMs obtained in the perinatal period (65). In the present study a 20-fold excess of phospholipid material was observed in AMs from 1-day-old compared to adult animals, further supporting the suggestion that AMs play a major role in clearance of surfactant material (48, 49, 65). In addition, a portion of the phospholipid of AMs of newborn animals may also arise from *de novo* membrane synthesis by AMs during phagocytosis (25, 26).

Two possibilities exist to explain the high protein content in the AMs of newborns: (*1*) phagocytosis of surfactant material and cellular debris, or (*2*) *de novo* synthesis similar to that

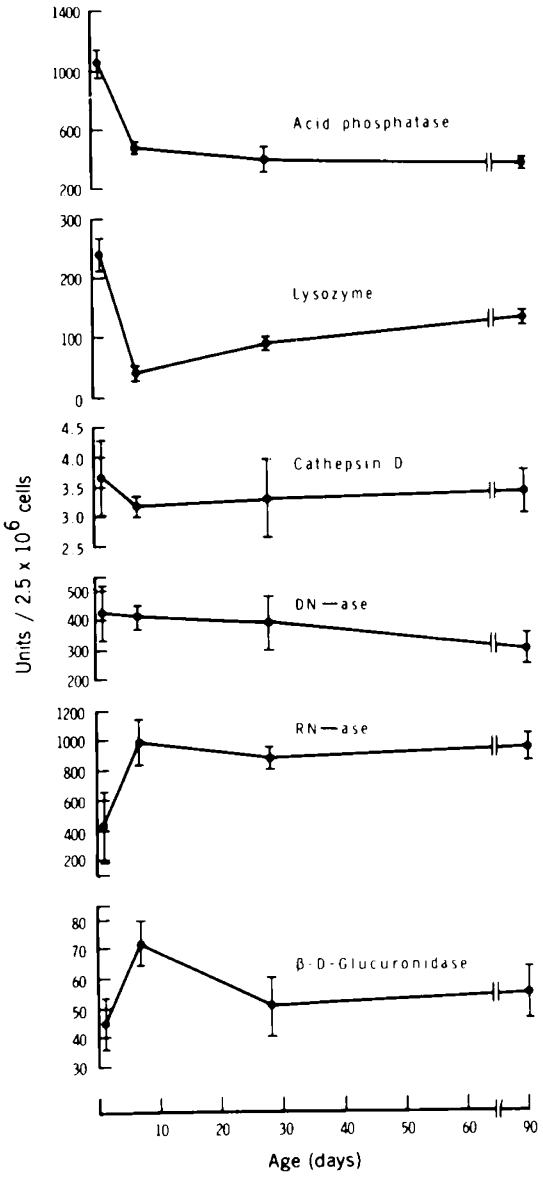

Fig. 1. Activities of lysosomal enzymes of alveolar macrophages obtained from rabbits of varying ages.

following inflammatory stimuli (24). The finding of a large number of phagosomes seen in the newborn AMs (65), which decrease in size within the first postnatal week, supports the first of these possibilities.

The DNA concentration of AMs from 1-day-old animals was similarily elevated. A large amount of phagocytosed material, epithelial cells, and other debris could in part account for this increase. The acellular fraction prepared from the lung lavage from 1-day-old animals showed a significant amount of DNA, unlike a similar fraction obtained from 28-day-old animals in which no DNA was detected. This finding lends further support to the possibility that a portion of DNA of AMs from 1-day-old animals was derived from their surrounding debris. Alternatively, proliferation of AM precursors in the lung interstitium appears to occur before birth (1) and it is possible that cells in G_2 phase with diploid content of DNA may enter the alveoli during the perinatal influx of cells (65).

The similarity between patterns of changes in content of RNA and other constituents of AMs studied suggests that the high level of RNA of AMs from 1-day-old animals may be also derived from phagocytosed debris. The elevated level of RNA at this time may also indicate a preparatory step for the formation of RER which was seen to be increased ultrastructurally (65) during first postnatal week and thus would equip for and facilitate protein synthesis. Monocytes and macrophages have been shown to be capable of active RNA (14, 56, 57) and protein synthesis (15, 40, 43).

Of the six lysosomal enzymes studies in AMs, AP, and lysozyme showed a significant decrease in their activities between the first and seventh postnatal days. The change in AP activity may be more apparent than real, since this activity has been shown to be present in the lamellar inclusions (5, 21, 29) which are phagocytosed in great abundance by AMs at birth (65). In a separate set of experiments we have demonstrated the presence of AP in the acellular fraction from lung lavages from 1-day-old animals. In contrast to the other lysosomal enzymes, the lysozyme activity of AMs appears to change in a unique manner. A decline was observed in this activity between the first and seventh postnatal day which might be due to accelerated release of this enzyme. In support of this possibility, AMs or peritoneal macrophages from adult animals have been shown to secrete large amounts of lysozyme, keeping only moderate amount of cell-associated enzyme (30, 32) and lysozyme activity shows almost parallel changes in serum in the first

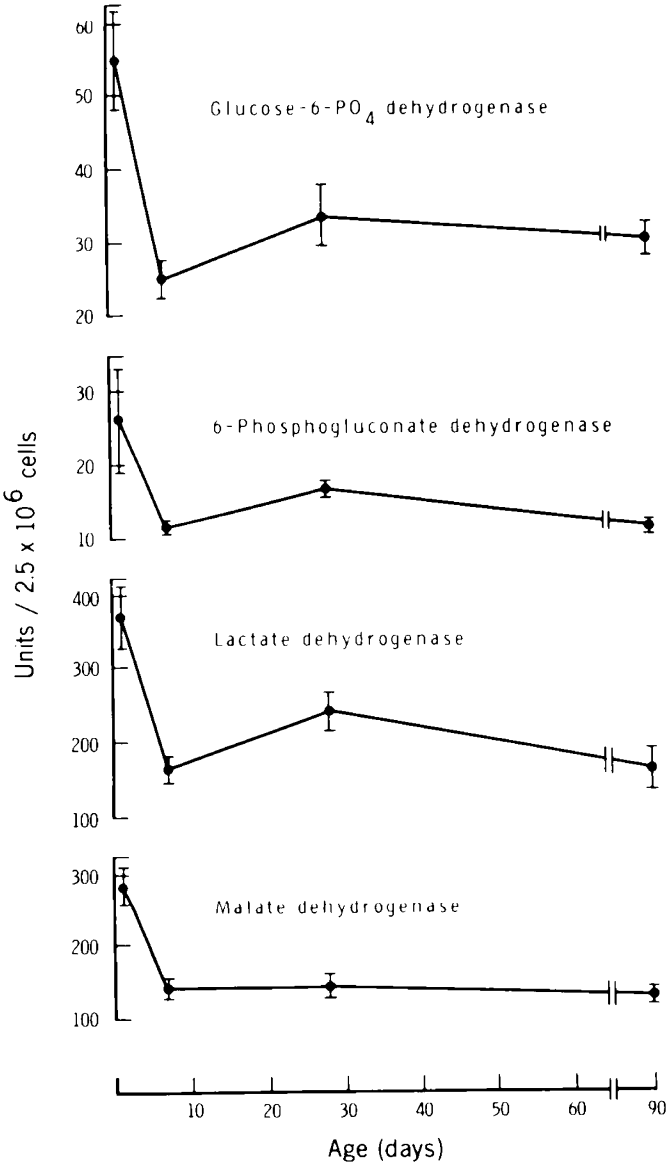

Fig. 2. Activities of oxidative enzymes of alveolar macrophages obtained from rabbits of varying ages.

postnatal week (37). In the present study, the steady increase in lysozyme in AMs after the seventh postnatal day may be related to the formation of new lysosomes (65). Unlike the activities of cathepsin D and DNase, which did not show significant changes, β-G and RNase activities of AMs increased significantly within the first postnatal week; this may be related to the appearance of lysosomal granules during this period (65). Previous work from other laboratories has suggested the existence of subpopulations of lysosomes in AMs (58, 59) and may explain the selective increase in the activities of only some of the lysosomal enzymes measured in the present studies. Although an increased number of lysosomes was observed morphologically in the subsequent time periods (between the 7th and 28th postnatal days), there were no statistically significant increases in the activities of lysosomal enzymes except in the case of lysozyme (Fig. 1). This may suggest redistribution of lysosomes through the process of division and fusion rather than increased synthesis of lysosomal enzymes (20). Finally, a full spectrum of lysosomal enzymes may not be present in granules visualized by electron microscopy.

The activities of four oxidative enzymes studied, (G-6-PD, 6-PGD, LDH, and MDH) of AMs from 1-day-old animals appeared to be high compared to the other time periods studied and may be related to the available substrates and the metabolic and phagocytic activities of these cells. The high glycogen utilization, characteristic of the energy metabolism of the tissues of the newborn animals, was also seen in AMs of the newborns; a depletion of prenatally accumulated glycogen stores and simultaneous change from glycolytic carbohydrate utilization, presumably to fat oxidation, was observed (65). We have also reported previously the appearance of lipid droplets in AMs during the first postnatal week together with an increase in the number of mitochondria (65), a major site for fat oxidation. It thus appears that glycogen provides an endogenous substrate pool for glycolytic and hexosemonophosphate shunt (HMPS) activities. The higher activities of LDH, G-6-PD, and 6-PGD in AMs at birth might be a result of the high concentration of the substrate for these enzymes and may in turn facilitate the utilization of the substrate for the generation of energy. Moreover, the high phagocytic load at birth (65) may also contribute to the heightened activity of these enzymes, and as a consequence generate additional energy and NADPH required during the phagocytic event. Finally, a nonspecific activation of HMPS of macrophages by surface-active agents, presumably related to the perturbation of plasma membrane, has been reported (31). Heightened activities of G-6-PD and 6-PGD of newborn AMs residing in surfactant-enriched environment in the lung may be related to similar phenomena.

Although the major pathways of carbohydrate metabolism are functional in AMs at different time periods studied, their relative contribution to energy metabolism appears to differ at varying ages. After adjusting for glucose equivalents metabolized ratios of activities of LDH/G-6-PD and MDH/G-6-PD were 3.4 and 1.7, respectively, at birth and decreased to 2.7 and 1.4, respectively, in the adult period (Fig. 2), thus indicating a relative increase in the HMPS activity in the adult period. This relative increase in activity of HMPS may be in response to the environmental challenges these cells encounter. Increased metabolism via HMPS during active phagocytosis and killing of bacteria have been described previously by Myrvik and Evans (47) and Rossi and coworkers (52, 53). Although the significance of the present findings is yet unclear, these appear similar to the changes in HMPS enzymes activities reported in human neutrophils (8, 63). It is tempting to speculate that these changes may be correlated with the development of bactericidal activity of the AMs during maturation (66).

CONCLUSION

AMs obtained from rabbits of varying ages were studied for their phospholipid, protein, DNA, and RNA content. A great excess of phospholipid in AMs at birth indicates the role of AM in removal of surfactant from the lung. The relative increase in HMPS activities over other pathways of carbohydrate utilization observed between the neonatal and adult periods and lysozyme activity after the first postnatal week may be in response to the stimuli the AMs encounter and may contribute to their capacity to destroy microorganisms. The variability in changes in lysosomal enzymes of AMs between the neonatal and adult periods may be indicative of subpopulations in the lysosomal granules.

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68. The authors wish to thank Katherine C. Hill for her assistance.
69. This research was supported by National Institutes of Health Grant HL-16748.
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71. Received for publication January 26, 1977.
72. Accepted for publication April 14, 1977.

Printed in U.S.A.