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BIOCHEMICAL ASPECTS OF *DIPLOCOCCUS PNEUMONIAE* INFECTIONS IN LABORATORY RATS

It has been known for many years that the capsular polysaccharides of pathogenic pneumococci differ qualitatively and quantitatively in chemical composition. The chemical nature and quantity of capsular material present in any one strain is genetically controlled, but specific environmental conditions can alter its expression.[1] The potential pathogenic properties of pneumococci can be influenced by the alteration of chemical characteristics of the *in vitro* environment.[2] Furthermore, some of the toxicity of pneumococci *in vivo* can be traced directly to metabolic activities of the organisms during the spreading phase of the disease rather than to a specific toxin released as a result of cellular disintegration.[1] In only a few cases have studies been made of the pathogen and the reaction of the host during the progression of infection.[3-5]

In an effort to understand some of the biochemical mechanisms related to bacterial pathogenicity, this investigation was undertaken to study serum biochemical changes in rats during the course of natural and experimentally induced *Diplococcus* pneumonia.

MATERIALS AND METHODS

A shipment of Sprague-Dawley male rats weighing 150-200 grams were obtained from a commercial supplier. Within three days of the animals' arrival two unexplained deaths occurred. In two weeks' time the infection had spread to involve many of the animals. The disease had a sudden onset and was accompanied by respiratory distress. The sick animals were euthanatized and necropsied along with those which died. Direct smears from various sites contained Gram positive diplococci, singly, in pairs and in short chains. Colonies cultured on blood agar for 24 hours were large, raised, mucoid, confluent and resembled droplets of water. They were surrounded by an area of greenish discoloration and a small zone of α-hemolysis. No growth was observed in gelatin after 30 days. Acid was produced in litmus milk within 24 to 48 hours. Acid but not gas was formed in lactose, sucrose and dextrose. The organisms were soluble in 10% deoxycholate and sensitive to optochin. Using Gin's method, the organisms were found to be encapsulated. Serological typing together with the above characteristic were compatible with *D. pneumoniae* type 3.

Naturally infected animals were divided into following groups:
Group 1 (32 animals) all with clinically acute infection.

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* Assistant Professor of Section of Laboratory Animal Sciences and Section of Laboratory Medicine. Supported by Public Health Service Grants GM 16947-02 from the National Institute of General Medical Sciences and FR 05358-07 and FR 00393-02 from the Division of Research Facilities and Resources.

Received for publication 24 November 1970.

Group 2 (30 animals) exposed to *D. pneumonia* infection but not clinically ill. These animals were injected with 10^9 heat-killed *D. pneumoniae* cells.
Group 3 (30 animals) exposed but not clinically ill. Each animal was injected with 5×10^3 viable cells.
Group 4 (32 animals) exposed but not clinically ill. Each animal received 1 ml. cell free extract from *D. pneumoniae* growth cultures.

Experimentally induced *D. pneumoniae* was studied by dividing healthy rats into four groups:
Group 1a (30 animals) injected with 1 ml. of sterile physiological saline solution containing 10^9 *D. pneumoniae* cells.
Group 2a (32 animals) inoculated with 10^6 bacterial cells.
Group 3a (32 animals) inoculated with 10^3 bacterial cells.
Group 4a (32 animals) received 1 ml. of sterile normal saline solution.

All the animals were inoculated intraperitoneally. Blood samples were taken by heart puncture at intervals of 0, 4, 8, 12, 24, 48, 72 and 96 hours; serum was separated by centrifugation. Four animals at each sampling time were killed for biochemical, histopathological and gas chromatographic analyses. The animal tissues from lungs, liver, spleen, heart and kidneys were plated on 5% rabbit blood agar plates. After incubation for 24 hours at 37°C the colonies were counted.

Serum samples from control animals, naturally infected and *D. pneumoniae* inoculated animals were stored at −20° C for biochemical analyses. Total protein was determined by Biuret reaction, and nucleic acids by absorptions at 280 over 260 mμ ratio determination. The seromucoid, protein-bound hexose (PBH) and hexoseamine were estimated by methods previously reported.[6]

Serum electrophoretic proteins were separated on cellulose acetate at 4°C. Barbital buffer (PH 8.6, ionic strength 0.075) was used and a constant current of 200 V per cell was applied for two hours. A 2 μliter serum sample from *D. pneumoniae* infection (24 hour postinoculation, group 1a) was applied on the paper strips. After the proteins were separated the strips were cleared with 15% acetic acid in methanol. They were scanned with a densitometer. Serum enzymes were determined by the methods described previously.[7]

Gas chromatography of the serum samples of rats infected with *D. pneumoniae* (group 1a) or control animal sera were performed using methods similar to those previously described.[8,9] Immediately before extraction, a 2 ml. sample was thawed and one portion hydrolyzed with 0.2 ml. of 5 N HCl and 1.0 ml. of 0.2 M HCl-KCl buffer (pH 2.0). The liquid was mixed well and centrifuged. The supernatant was lyophilized and then extracted with pyridine. The second portion of serum sample was treated with perchloric acid and centrifuged. The supernatant solution was then treated with phosphotungstic acid to precipitate the perchloric acid soluble glycoprotein components. The precipitate was washed with ethanol and vacuum-dried. The dried material was extracted with pyridine; silyl derivatives were made by using trimethylchlorosilane and hexamethyl disilazane.[8] A 2 μliter sample was analyzed by gas chromatography as previously described.[9]

RESULTS

Pathological and bacteriological findings. Pathological findings at necropsy showed typical acute lobar pneumonia. All the lobes of the lungs of group 1 rats that died were usually affected. The lungs did not collapse when the

thorax was opened and a considerable amount of frothy sero-sanguineous fluid exuded from the cut trachea when pressure was applied to the lungs. Fibrinous pleuritis was present in most cases. When consolidated, the lungs revealed a mosaic pattern of grey or red with dark hemorrhagic regions. In addition several rats had massive focal regions of necrosis within the liver, spleen and kidneys. Several rats sacrificed at different stages of development of infection showed various degrees of lung involvement.

Bacteriological and pathological findings of animals inoculated with 10^9 cells of *D. pneumoniae* were identical to those of naturally infected animals. The colonies of *D. pneumoniae* were counted by plate cultures of lung, liver, spleen, heart and kidney tissues. Rat lungs contained 10^6 *D. pneumoniae* cells per gram of tissue 2-3 hours after inoculation. The concentration of bacteria in lung tissues increased to about 10^8 cells/gram of tissue at 5-6 hours postinoculation (Fig. 1). Spleen and liver tissues showed an increase in viable cells eight hours after inoculations. The cell count reached 5×10^7 cells in these tissues at 72 hours postinoculation time. On the other hand,

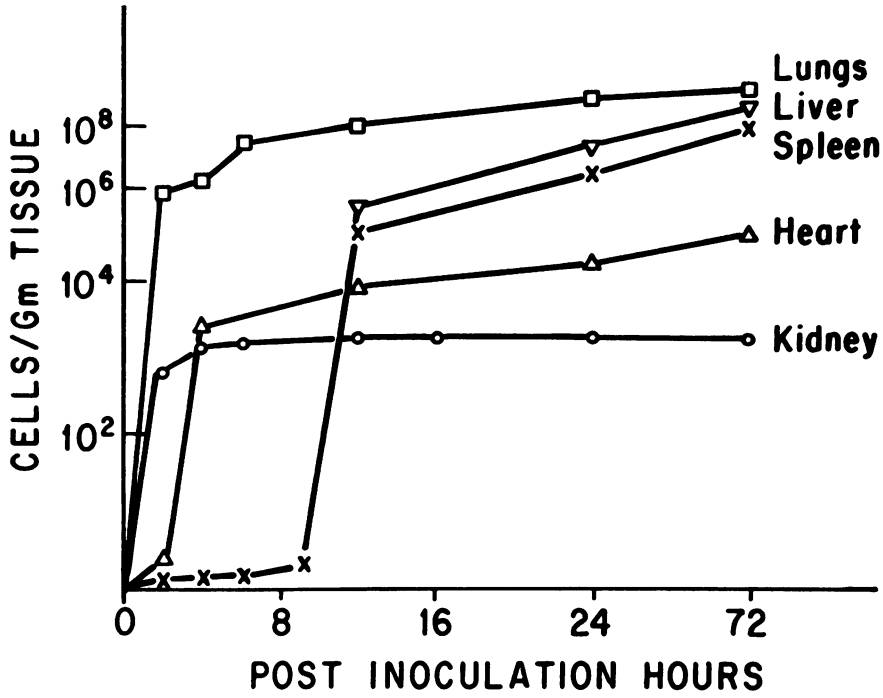

FIG. 1. Distribution of bacteria in rat tissues following an intraperitoneal inoculation of 10^9 *D. pneumoniae* cells. Each point on the graph represents an average value of 4 determinations.

the bacterial concentration in heart and kidney tissues increased steadily at the initial stages of infection followed by a slight increase in number of bacteria 8 hours postinoculation.

Chemical changes in serum of naturally infected and D. pneumoniae inoculated rats. Chemical changes which were associated with experimentally induced *D. pneumoniae* infections are summarized in Table 1. Total serum proteins increased considerably at 72 hours postinoculation, although the total nucleic acids had increased significantly at 24 hours. Forty to fifty percent elevations in serum, protein bound hexose (PBH) and seromucoid were observed at 4 hours postinoculation, whereas hexoseamine changes were more pronounced when the animals were acutely ill (72 hours postinoculation).

Results of the serum electrophoresis for total proteins are shown in Fig. 2. Albumin content decreased about 50% in the serum of naturally infected animals, whereas α_1 and α_2-globulins were increased from 20 to 30%. β-globulin increased more than 80% while gamma globulin was slightly decreased. Changes in electrophoretic proteins were less pronounced in serum samples obtained from rats inoculated with 10^9 cells of *D. pneumoniae*.

It is apparent from Fig. 3 that *D. pneumoniae* infection in rats is associated with increases in the activity of specific serum enzymes. The magnitude and the time at which these changes in enzyme levels occurred were directly related to the size of infective dose. Administration of high concentrations of *D. pneumoniae* resulted in significant alterations of serum enzymes within 6 to 12 hours, whereas similar changes following infection with progressively smaller dosages were not observed until 24 to 48 hours. There was a slight initial increase in the activity of transaminases, dehydrogenases and phosphohexose isomerase (PHI) after an inoculation with 10^3 cells of *D. pneumoniae* in rats. No significant changes in enzyme levels in the serum of animals inoculated with 10^9 heat-killed *D. pneumoniae* cells were noted.

TABLE 1. CHEMICAL CHANGES IN SERUM OF RATS INFECTED WITH D. PNEUMONIAE TYPE 3

Chemical	*Hours after inoculation** 0	4	24	72
Total protein (g%)	7.4 (± 0.4)	7.6	7.8	9.8
Nucleic acid (%)	0.95 (± 1.0)	1.3	4.5	4.7
Protein bound hexose (mg%)	92. (± 7.2)	153.7	182.0	377.0
Seromucoid (mg%)	11.2 (± 1.2)	15.5	18.9	31.8
Hexose amine (mg%)	11.0 (± 0.9)	11.2	11.3	12.9

* Values are averages of 10 determinations. Standard deviations are given in parentheses.

Serum enzymes of naturally-infected animals were elevated to levels similar to those observed in rats with induced infections (Fig. 4). Injections of 5×10^3 cells or one ml. extract of *D. pneumoniae* culture in rats, that were previously exposed to infections but had no clinical signs of illness, showed a rapid rise in enzyme levels, whereas the same group of animals when inoculated with 10^9 heat-killed *D. pneumoniae* organisms showed an initial rapid rise in enzyme levels followed shortly by a return to normal levels.

Gas chromatographic analyses of serum.

Further chemical analysis of serum samples of animals with *D. pneumoniae* infections was carried out by ultrasensitive gas chromatographic (GC) methods for detection of characteristic metabolic changes. The elution patterns in chromatograms prepared from serum of *D. pneumoniae* natural infections and induced infections were similar (Fig. 5). There were several peaks present in control, uninoculated serums. Also there were several peaks found in serums of naturally infected animals which were absent in serum samples from animals inoculated with 10^6 or 10^9 *D. pneumoniae* cells. However, there were at least five metabolites which were characteristically present in serums of all animals infected with *D. pneumoniae*. These metabolites

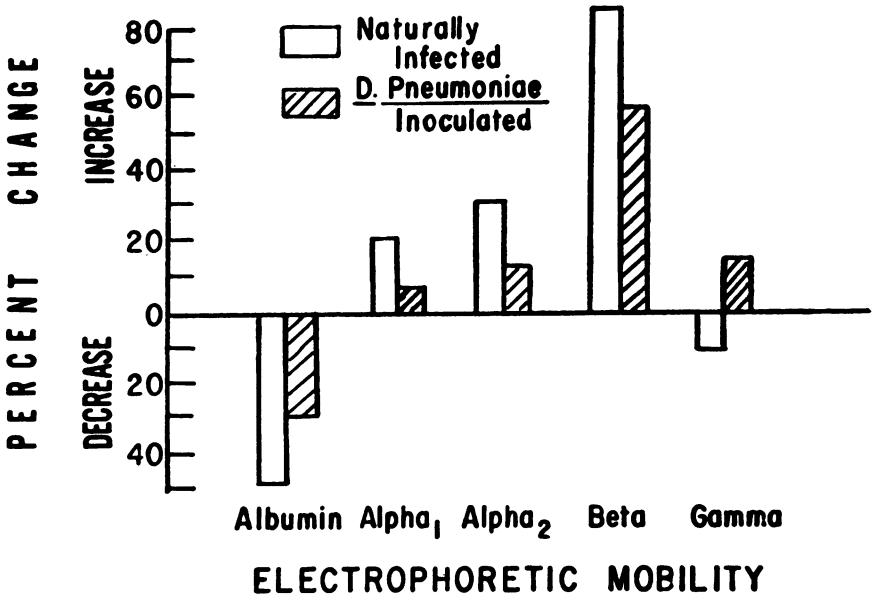

FIG. 2. Changes in serum electrophoretic protein in rats with natural *D. pneumoniae* infections (N.I.) (group 1) and induced infections (I.I.) (group 1a). The samples were taken at 24 hours postinfection. The values represent an average of 12 determinations. The normal control is indicated as zero and the percent changes represent the percent of mean.

were not found in uninoculated controls or other microbial infected serums analyzed by similar methods (Mitruka and Jonas, *unpublished data*).

Gas chromatography of PAS soluble fraction of serums from pneumococcal infected rats showed characteristic peaks representing pentoses, hexose, hexoseamines and polysaccharides as early as four hours postinoculation

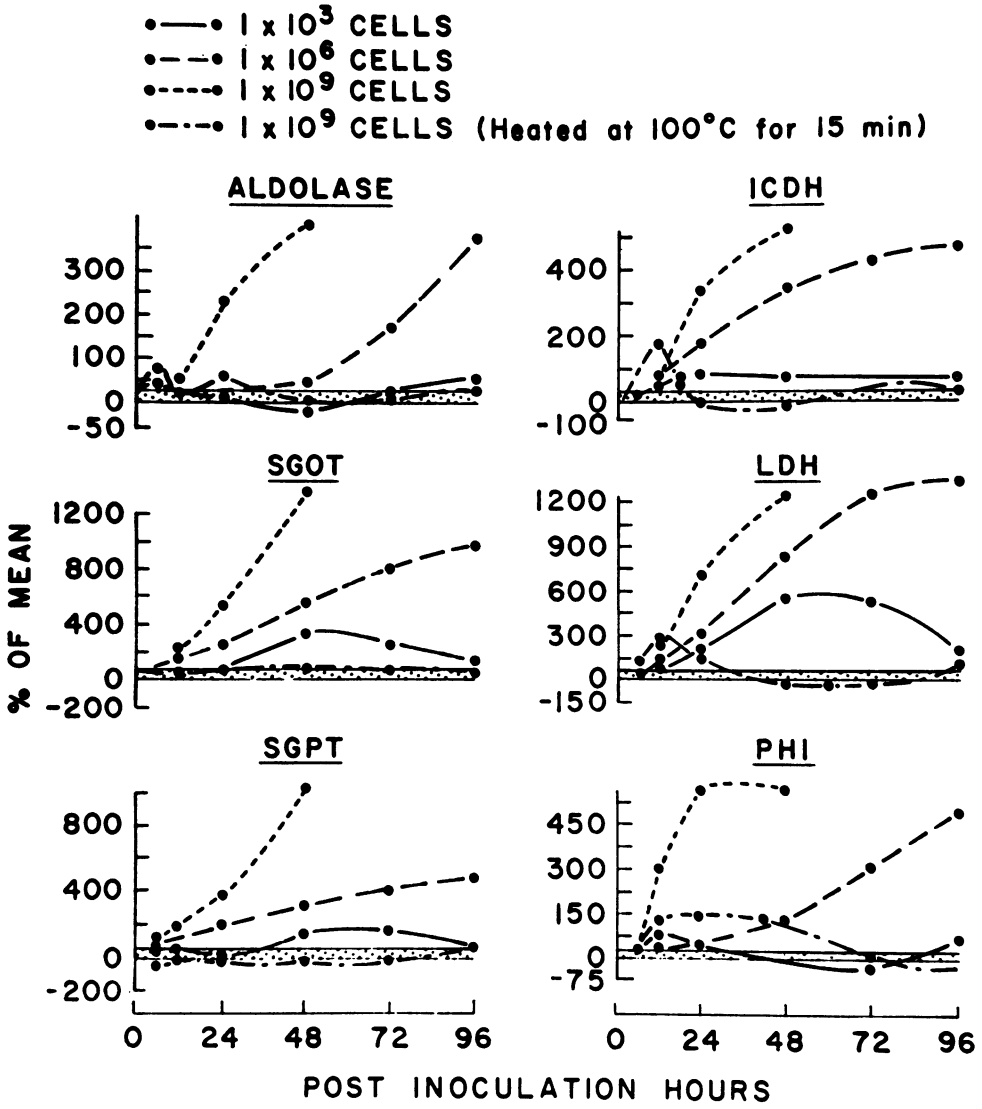

FIG. 3. Serum enzyme activity after infection with various concentrations of *D. pneumoniae* and after administration of killed cells. Shaded areas indicate normal ranges.

(Fig. 6). The peaks of retention times 750, 900, 1125, 1950 and 3488 seconds were present at the early stages of *D. pneumoniae* infections. Glycoprotein components with retention times of 2438, 2737 and 3375 seconds appeared in the serums after 24 hours of inoculations. The uninoculated control rat serums did not contain these metabolites.

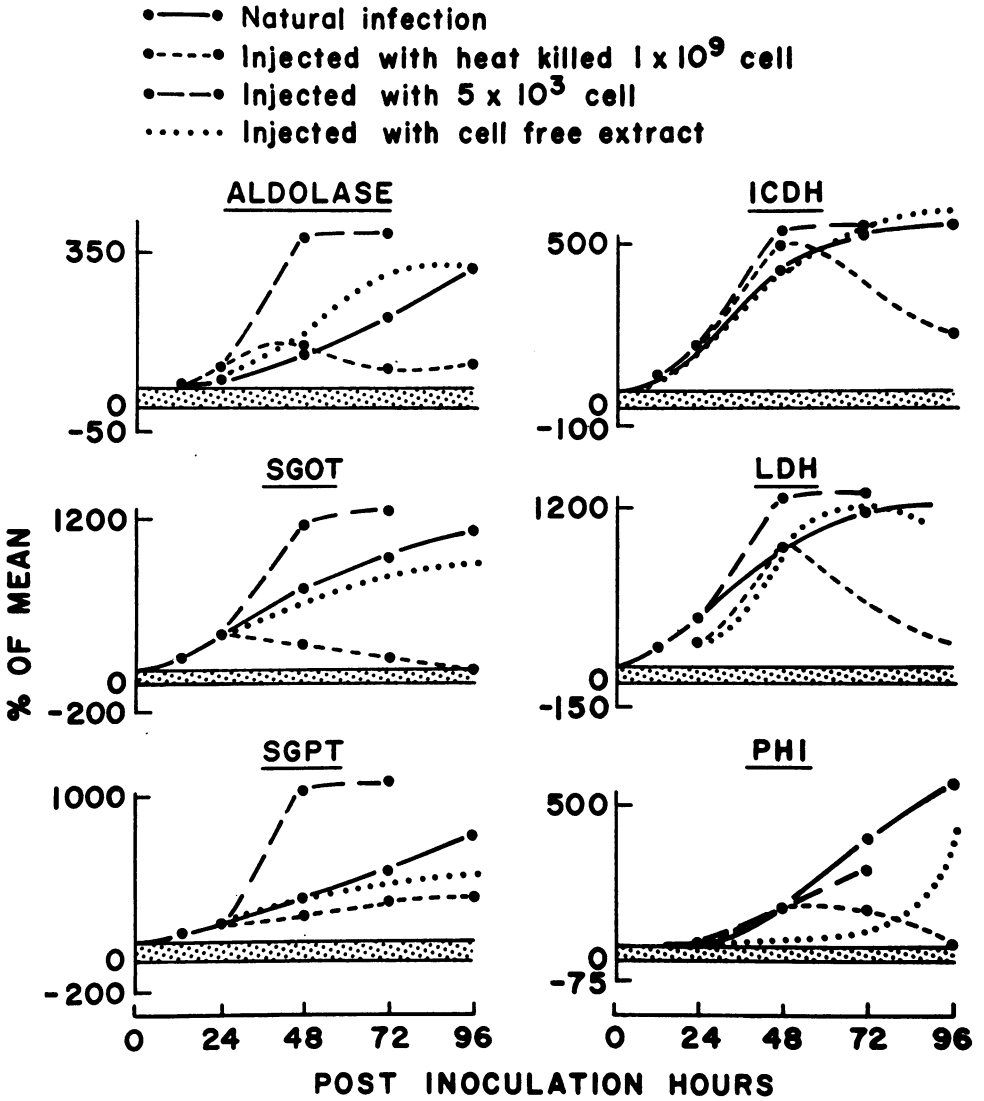

FIG. 4. Serum enzyme activity after natural infection with *D. Pneumoniae* (group 1) and after administrations of killed or viable cells or cell free extract. Shaded areas indicate normal ranges.

DISCUSSION

These studies demonstrated that rats infected with *D. pneumoniae* had a marked increase in the levels of serum enzymes, proteins, nucleic acids, and glycoproteins. These metabolic changes occurring *in vivo* may not necessarily be specific for *D. pneumoniae,* since some of these changes have been also reported in human as well as in experimental infections.[10] Alternatively, some characteristic bacterial metabolites may be produced which relate to a

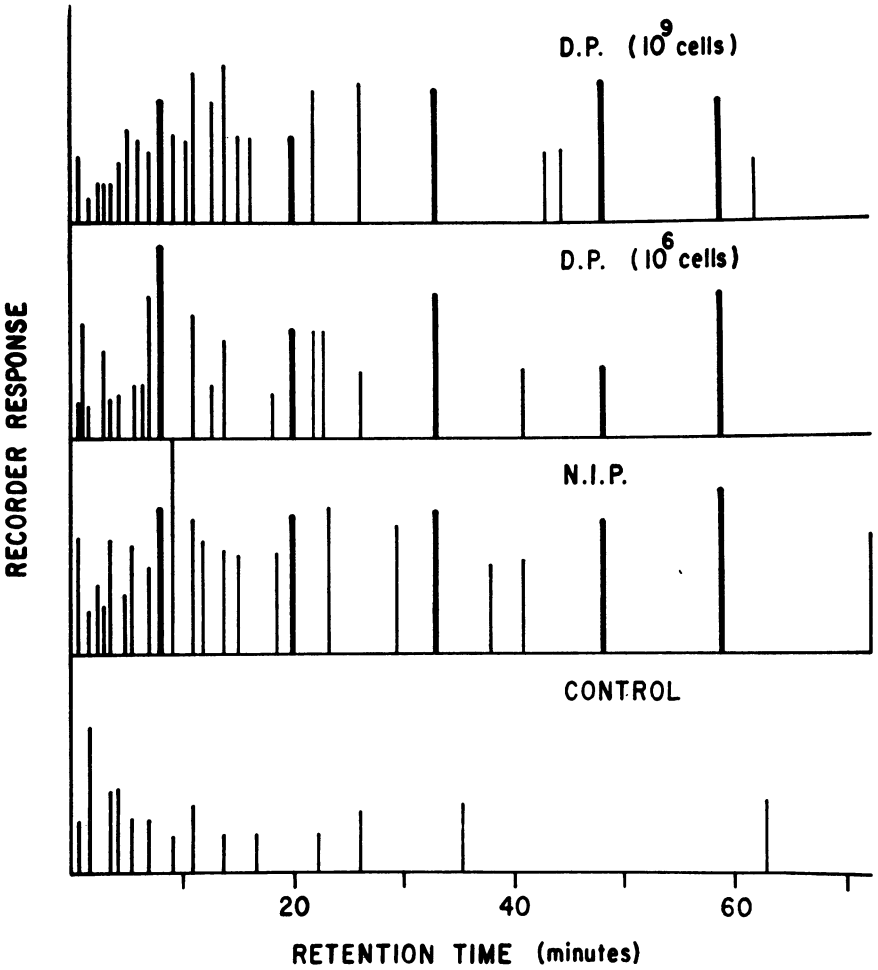

FIG. 5. Gas chromatographic elution patterns of serum of rats naturally infected with *D. pneumoniae* (N.I.P.) or inoculated with 10^6 or 10^9 cells of *D. pneumoniae* (D.P.). The samples were taken at 24 hours postinoculation. Each bar in the graph represents a peak in the chromatogram.

biochemical basis of pneumococcal pathogenicity in rats. In order to understand the mechanisms of microbial pathogenicity, some detailed knowledge is required of the pathology and histochemistry of the appropriate infection and disease. In only a few cases are correlative biochemical data on pathogens and the reaction of the host to the disease available.[11] Studies of this

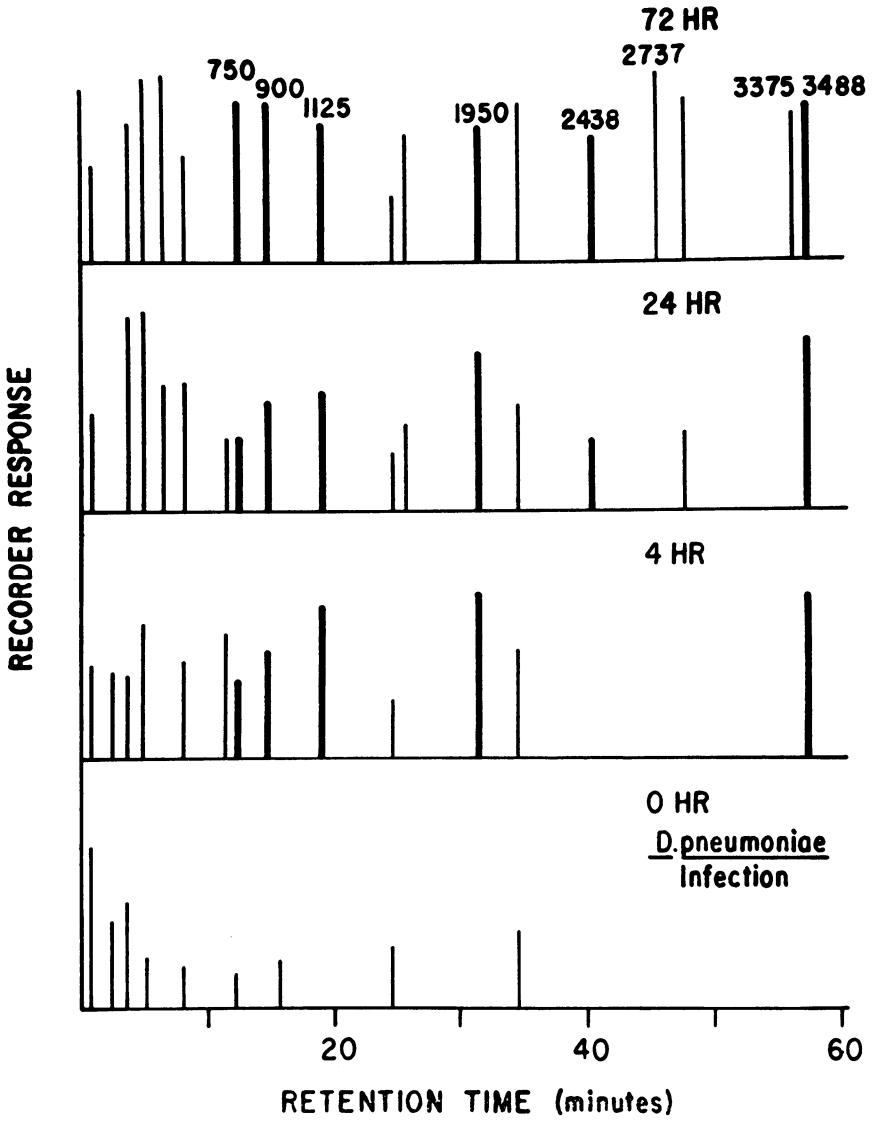

FIG. 6. Gas chromatographic analysis of perchloric acid soluble (PAS) fraction of rat serum taken at various times after experimentally induced *D. pneumoniae* infection (group 1a). Each bar in graph represents a peak in the chromatogram.

sort can indicate important aspects of the aggressive action and the type of compounds (e.g. antiphagocytic and bactericidal) produced *in vivo*. Such factors undoubtedly play important roles in the outcome of disease and thereby influence the metabolic expression of disease in animal tissues and body fluids.

Although the detailed pathological findings are not reported in this study, the bacteriological and biochemical changes supplement the knowledge of clinical manifestations of the disease syndrome. Serum enzyme levels, which are indicative of altered metabolic activity, increased in relation to the number of cells inoculated whereas heat-killed cells did not effect the enzyme activities (Fig. 3). These data suggest that the active growth of the organism is responsible for the changes in enzyme levels by either tissue destruction or increased biosyntheses of proteins. The enzyme activities increased rather rapidly when the previously exposed animals were inoculated with a small dose of cells or cell free extracts (Fig. 4), indicating an increased cellular response to either the presence of foreign material or the effect of endotoxin on the external gut wall. However, a rapid return to normal activity at 24 hours indicated more effective host resistance in these preexposed animals. The data from these studies also indicate that the quantitative and qualitative changes in serum proteins and glycoproteins for naturally infected and induced *D. pneumoniae* show many significant differences. The most common pattern was that albumin fraction was decreased and $alpha_1$, $alpha_2$ and Beta fractions were increased, which agrees with our previous findings with other bacterial infections in rats.[7] However, a more than 80% increase in beta globulin due to *D. pneumoniae* infections can not be explained at this time. There was generally a good correlation in the proteins and enzyme changes between the groups of animals which were naturally infected and those inoculated with 10^9 *D. pneumoniae* cells thus demonstrating that the rat is an excellent laboratory model for acute bacterial infections.

Our results show that highly sensitive gas chromatographic methods can be employed for the characterization of pneumococcal metabolites *in vivo* and for host responses to these infectious agents. Such methods may prove useful for rapid detection of bacteremia and identification of the causal organism. Identification would be facilitated if the early bacterial product detected were a unique metabolite. The present findings with *D. pneumoniae* show that at least five metabolites representing pentoses, hexoses, hexoseamines and polysaccharides were associated with the *in vivo* activity of the bacteria. These distinctive products were elaborated by the bacteria as early as four hours postinoculation and were also found in the serums of naturally infected animals and in *in vitro* cultures of *D. pneumoniae*. These

results correspond to our previous studies on mice[9] and on bacterial infections of man (Mitruka and Jonas, *unpublished data*).

SUMMARY

In view of the severe metabolic disturbances occurring in animal hosts during invasion by pathogens, this study was undertaken to determine biochemical changes in the serum of rats due to *D. pneumoniae* type 3 at various stages of development of infections. The inoculation of Sprague-Dawley rats with 10^3 to 10^9 cells of *D. pneumoniae* or cell free extracts stimulated significant increases in several enzymes including aldolase, glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, lactic dehydrogenase, isocitric dehydrogenase and phosphohexose isomerase. The rates of changes in enzymic activity of naturally infected animals were similar to those of animals inoculated with 10^9 *D. pneumonia* cells. Bacteria were isolated from lung, kidney and heart tissue at two to six hours post-infection and in blood, liver, and spleen after eight hours. The total proteins, nucleic acids, and glycoprotein changes followed the patterns of enzyme activities. Serum electrophoretic proteins showed a more than 80% increase in beta globulin fractions and a 10 to 40% increase in alpha globulins of *D. pneumoniae* infected rats. Gas chromatographic analysis of serum samples showed characteristic changes in mucopolysaccharides and glycoprotein components at the early stages of infection. The results suggest that specific chemical alterations in serum, when present, may be useful indicators of *in vivo* metabolic processes affected by bacterial infections.

ACKNOWLEDGMENTS

The author wishes to thank Dr. D. H. Percy for performing animal necropsies and Drs. A. M. Jonas and E. Gralla for many helpful suggestions in the preparation of this manuscript.

The technical assistance of M. Carolina Marino is gratefully acknowledged.

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