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COMMISSION ON EPIDEMIOLOGICAL SURVEY

Armed Forces Epidemiological Board
Washington, D. C.

December 1972

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COMMISSION ON EPIDEMIOLOGICAL SURVEY

ANNUAL REPORT

TO THE

ARMED FORCES EPIDEMIOLOGICAL BOARD

FISCAL YEAR 1972

Washington, D.C.

DECEMBER 1972

23

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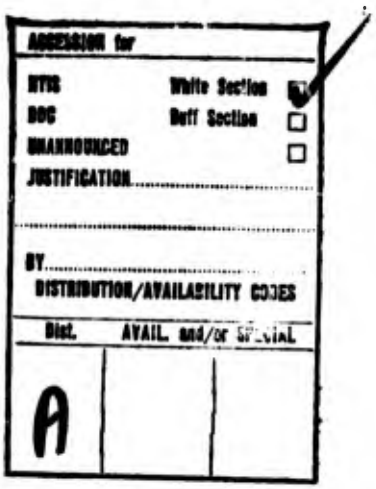

COMMISSION ON EPIDEMIOLOGICAL SURVEY

ANNUAL REPORT TO THE ARMED FORCES EPIDEMIOLOGICAL BOARD

FISCAL YEAR 1972

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DECEMBER 1972

SUMMARY

This is the fifth annual report of the Commission on Epidemiological Survey since its consolidation with the Commission on Radiation and Infection. Annual reports of two contractors, Trudeau Institute, Saranac Lake, N. Y. (DADA 17-68-C-8124), dated September 1972, and the University of Maryland School of Medicine, Baltimore, Md. (DA-49-193-MD-2867) dated August 1972, are included in this last official report to the Armed Forces Epidemiological Board. These reports are available to others from Defense Documentation Center, Cameron Station, Alexandria, Va. 22314

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13. ABSTRACT

Progress is reported in selected areas of research in medical defense aspects of biological agents by the U. S. Army Medical Research Institute of Infectious Diseases and two contractors.

KEYWORDS:
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Hemorrhagic fever
Staphylococcal enterotoxin B
Bacterial diseases
Metabolism
Amino acids
Rickettsial diseases
Biochemistry

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully acredited by the American Association of Accreditation of Laboratory Animal Care.

Studies employing volunteers in research tests were governed by the principles, policies and rules for medical volunteers as established by Army Regulation 70-25 and the Declaration of Helsinki.

COMMISSION ON EPIDEMIOLOGICAL SURVEY

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Greisman, Sheldon E., M.D.

Hornick, Richard B., M.D.

Wisseman, Charles L., Jr., M.D.

* Deceased, 12 February 1972.

** Absent.

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THE DIRECTOR'S SUMMARY REPORT

The Commission on Epidemiological Survey (CES) held its annual meeting at Walter Reed Army Institute of Research (WRAIR) on 21-22 September 1972. In his introductory remarks, the Chairman welcomed LTC Norman E. Wilks, MSC, USA, and acknowledged with gratitude the contributions of his office and the help of Mrs. Betty Gilbert who has made it a pleasure to conduct the work of the Commission. Equal gratitude was extended to Mrs. Louise Kline, secretary to COL Dan Crozier, and to Mrs. Phebe Summers and Mrs. Pearl Flohr for their support and administrative assistance.

Dr. Gustave J. Dammin, President, Armed Forces Epidemiological Board (AFEB) attended the meeting and made helpful scientific contributions regarding the work presented. Dr. Dammin and the Chairman discussed the contemplated new organizational changes relative to the AFEB and its Commissions which will become effective on or about 1 January 1973. Throughout its functional years, the CES has been coordinating its activities closely with various intramural programs particularly in relation to the activities of the U. S. Army Medical Research Institute (USAMRIID), Fort Detrick. The nature of the change which will occur within the CES is unclear.

Those representatives of the Departments of Army, Navy and Air Force, personnel of USAMRIID and guests who attended one or both sessions exclusive of the speakers were:

Army
COL E. L. Buescher, WRAIR
COL F. C. Cadigan, Jr., USAMRDC
COL T. R. Cutting, OTSG
COL T. H. Lamson, WRAIR
COL P. K. Russell, WRAIR
COL D. W. Sample, USAMRDC
LTC J. C. Quake, WRAIR
H. E. Noyes, WRAIR

Navy
CAPT R. D. Comer, BuMed

Air Force
LTC O. W. Jones, HQ, SGPP

Guest
A. S. Benenson, Commission on Immunization

USAMRIID
COL H. G. Dangerfield
COL J. E. Hill, Jr.
COL J. F. Metzger
LTC G. A. Eddy
LTC R. O. Spertzel
MAJ D. E. Hilmas
MAJ D. S. Mosher
CPT R. A. Proctor
1LT D. T. George
W. R. Beisel
J. V. Jemski
R. H. Kenyon
E. W. Larson
H. A. Neufeld
W. C. Patrick, III
G. H. Scott

The agenda of the two day meeting was devoted to reviewing the latest work completed or in progress by contractors and investigators of USAMRIID.

REVIEW OF THE USAMRIID RESEARCH PROGRAM

Current Status of Administrative Structure at Fort Detrick

Colonel Dan Crozier reported that the future of Fort Detrick is extremely bright. Following the Presidential Proclamation on offensive biological warfare, significant reorganization of the Biological Laboratories of the Army Materiel Command occurred. The National Cancer Institute negotiated a major research contract with Litton Bionetics, Inc. This contractor is in the process of expanding into the majority of the permanent buildings in the southern area of the post. In addition, four Army Medical Department units moved to Fort Detrick during the summer: the Medical Biomechanical Research Laboratory, the Medical Equipment Research and Development Laboratory, the Medical Historical Unit, and the Health Service Data Systems Agency. Fort Detrick is now a medical installation under the direct command of the Commanding General, U. S. Army Medical Research and Development Command. The mission and staff of USAMRIID have been expanded. Phase II of the laboratory construction program is now complete; these unique laboratories are in full operation. Some "shakedown" problems were encountered but were not major or insurmountable.

There is no classified research being conducted anywhere at Fort Detrick. The inner security fence, which once isolated the classified area has been removed and control of visitors is minimal.

Mission and Research Approaches, William R. Beisel, M.D.

With the completion of the new building this year and the closing of the Biological Laboratories, USAMRIID has been assigned a broader, more complex research mission: "Conducts studies on the pathogenesis, diagnosis, prophylaxis, treatment and epidemiology of infectious diseases with particular emphasis on problems associated with medical defense against biological warfare, on naturally occuring diseases of peculiar military importance, and on microorganisms, the study of which requires special containment facilities."

The entire Institute functions as a single unit. A new research division has been added to the administrative structure for conduct of studies requiring aerobiological capability. Also, there is a new Animal Resources support division. To assist in communication among all research divisions, wide periodic use is made of peer review committees and in-house technical progress evaluation.

Major areas of research include: (a) study of pathogenesis, employing animal models when possible and microscopic, biochemical, physiological, aerobiological and radiobiological techniques; (b) development of new immunologic and nonimmunologic techniques leading to rapid diagnosis; and (c) prophylaxis and treatment, including vaccine development, study of cellular and humoral immunogenic mechanisms, viral chemotherapy and general supportive care. Emphasis has been reduced on studies and characterization of specific

microorganisms. Instead, projects seek information applicable to broader classes of infections, and continue to avoid overlapping within those areas of infectious diseases which might be under intensive and effective appraisal in other military, Federal or University laboratories.

Research in Bacteriology Division, COL Harry G. Dangerfield, MC

Research activities are directed toward host response to, interaction with, and protection against microorganisms and/or their products.

Studies of subcellular organelles in an infected or immunized host should assist in clarifying mechanisms of pathogenesis of, and defense against, microbial diseases. Enzymatic and physical properties of liver subcellular organelles differed in rats infected with Diplococcus pneumoniae or the living vaccine strain of Pasteurella tularensis. In pneumococcal infection, there was loss of specific enzyme activities normally associated with smooth endoplasmic reticulum, and a decrease in peroxisomal particles. The tularemia vaccine model showed similar changes in endoplasmic reticulum but the decrease in peroxisomal particles was moderate and transient.

Microtubular proteins isolated from the brain of rhesus monkey are employed as immunogens for production of specific antibody. Staphylococcal enterotoxin B (SEB) has been found to affect rabbit liver mitochondrial membranes and to accumulate in lysosomes of kidney cells. There are related studies describing the role of lysosomes in degradation of SEB. Another aspect of the program is directed toward cellular responses to antigenic stimulation based upon the hypothesis that activation of lymphocytes by antigen is triggered by a conformational change of surface membrane receptors. Techniques utilizing electron paramagnetic resonance and spin labeled compounds were developed to detect changes in the conformation of membrane constituents.

Humoral antibody remains the best indicator for host recognition of microbial antigens, but often cannot be related to protection. Humoral antibody per se does not participate in protection against alimentary intoxication but does indicate a potential for development of resistance. The earliest detectable responses to infection involve modification of the metabolic activity of a host. The generally nonspecific nature of these responses suggests that supplementary studies will be required to define the etiology of an infection, such as techniques for recovery and identification of specific antigenic moieties in clinical specimens rather than recovery of infectious microorganisms. Investigations will be initiated to regulate immune response by differential manipulation of thymic-dependent and bone marrow-derived lymphocytes.

Research in Virology Division, LTC Gerald A. Eddy, VC

This Division has oriented its program toward the development of killed and attenuated vaccines. Unquestionably, the development of the attenuated Venezuelan equine encephalomyelitis (VEE) vaccine has been responsible for

averting a public health calamity that might well have rivaled the 1918 flu epidemic as a cause of serious widespread human illness. Killed vaccines have been developed against Eastern and Western encephalitis (WEE) viruses and more recently against VEE using the attenuated TC-83 vaccine strain. Such a vaccine produced from an attenuated virus will negate many objections to the use of a live VEE vaccine within countries free of the disease. Current projects are directly related to vaccine development and include the biochemical and antigenic characterization of VEE virus subunits that constitute the immunogenic component of the virion; they include efforts to obtain high titers of St. Louis and California viruses for vaccine and the development of new cell culture techniques leading to high titer vaccines. The development of a Mayaro vaccine grown in WI-38 cells is nearing completion. The study of Q fever rickettsia continues with a view to preparing a killed vaccine, free of the hypersensitizing component, and to obtain more information on an attenuated Q vaccine. A Rocky Mountain spotted fever vaccine grown in chick embryo cells is near completion. Appropriately, there will be a close appraisal of response to such vaccines.

Mechanisms of virulence and the role of immune complexes in the pathogenesis of certain Group A arbovirus infections will be examined. More information must be developed relative to cell mediated immunity and its application to killed and live vaccines, including the degree of cross protection provided. A program to study Bolivian hemorrhagic fever is being initiated. It will include a vaccine development effort in addition to various clinical and immunological studies in the rhesus monkey.

Research in Animal Assessment Division, LTC Richard O. Spertzel, VC

Research is concerned primarily with study of the pathogenesis and pathophysiology of infectious diseases, interaction of irradiation and infection on host response, physiology and pharmacophysiology of infectious disease processes in animal models, and the testing of candidate vaccines in non-human primates. In some instances, these latter two objectives require development of an appropriate animal model. The areas of interest are diverse, and specific work units are often multidisciplinary, requiring joint effort between this and one or more other divisions.

Virus-host interrelationships have been appraised. In mice, inoculation of the dam with attenuated VEE virus from the 10th-13th days of gestation resulted in decreased litter size, increased stillbirths and decreased survival in the birth-to-weanling age. Inoculation of the dams later in gestation affected only survival of the young. Other investigators using a similar mouse-virus model for St. Louis encephalitis virus have observed fetal deaths and malformations throughout the gestation period. Other arboviruses are being investigated using the mouse-virus model; attenuated WEE virus appears to behave like TC-83 in affecting fetal development. The Asibi strain of yellow fever virus, a Group B virus, appears to be innocuous (unlike St. Louis encephalits, also a Group B arbovirus).

Animal models will be used for investigation of trace metal analysis, free amino acids, renal and hepatic function, tissue damage localization and effects on various organ systems. Attenuated VEE vaccine is safe and effective for mice exposed to sublethal and low doses of radiation. In essence, antibody formation and protection is merely delayed, not inhibited.

Research in physiology and pathophysiology of infectious disease processes requires development of animal models, as well as standardized techniques and criteria for measurement of the important changes in the biological processes of these model systems. For example, techniques have been devised for long-term implantation of pressure transducers in the ventricular musculature of the rhesus monkey in order to obtain reproducible, quantitative estimates of myocardial contractility. Following accumulation of baseline data, monkeys offer a unique opportunity to study infectious agents, either individually or sequentially.

Research in Physical Sciences Division, Robert W. Wannemacher, Jr., Ph.D.

This Division is responsible for basic research of infection-related alterations in host metabolism. The long-range objectives are to define infection-related pathogenic and metabolic alterations in the host, develop a biochemical profile for the early detection of the infectious process, and define possible therapeutic techniques. Research is underway in three basic areas: (a) energy utilization of the host during infectious disease, (b) protein synthesis during infectious disease, and (c) regulation of various metabolic functions in the host.

The infectious process involves an increased expenditure of energy, as demonstrated by elevated body temperature, increased loss of body nutrients, and mobilization of the host defense mechanisms. In infected rats, liver glycogen is rapidly decreased and exogenous glucose is not converted as rapidly into hepatic glycogen. These studies support the concept of an increased rate of utilization of glucose as an energy source during an infectious process.

Certain specific proteins within the body play an important role in host defense. Early in an infectious process there is rapid movement of amino acids from peripheral tissues, such as skeletal muscle, to liver where there is an increased rate of synthesis of specific serum proteins. Effects of infection are studied on circulating concentrations and rates of utilization of many hormones. A mediator protein appears in the circulation which stimulates many of the early infection-related alterations in liver metabolism and serum protein synthesis. This mediator is released from stimulated phagocytizing cells and can influence the flux of amino acids and trace metals and synthesis of RNA and protein in the liver of the infected animal.

The possible role of the infectious process is being studied for changes in membrane conformation or structure. D. pneumoniae infection results in marked biochemical lesions in hepatocellular membranes which are not detected by electron microscopy.

Research in Aerobiology Division, Mr. Edgar W. Larson

With the disestablishment of the Biological Laboratories, USAMRIID acquired one complex of the highly unique aerobiology facilities. Specialized capability is provided by two 6,200-liter, gas-tight, brine-jacketed, environmentally controlled aerosol chambers capable of temperature control within the range of -20 to 120 F and relative humidity control from approximately 20% to saturation. Microbial suspensions are aerosolized, samples collected, and experimental animals exposed in cabinets surrounding each chamber. These cabinets are continuous with other cabinets designed as animal holding suites and necropsy hoods.

Research is focused on the medical aspects of airborne infections, concentrating on most aspects of respiratory disease, including epidemiology, pathogenesis, prophylaxis, immunity and therapy. Future studies will include airborne particle size effects, since the depth of penetration and site of deposition in the respiratory tract is largely dependent on particle size. Comparative studies to evaluate aerogenic immunization will be considered. The relationships between humoral and secretory antibodies will be examined as a function of the route of administration.

Research has been initiated using influenza virus in the rhesus monkey as a model system. Three types of investigation are in progress: (a) pathogenesis of sequential respiratory infections including influenza and bacterial pneumonia, (b) relationships between humoral and secretory antibody responses as a function of the route of administration of the antigen, and (c) protective effects that might be achieved with aerogenic immunization.

Research in Pathology Division, COL Joseph F. Metzger, MC

The mission of this Division is divided into service, training and research. A clinical laboratory offers supporting services to the Institute and Troop Medical Clinic. Volunteer studies are supported by division personnel. The training program in veterinary pathology, headed by LTC Stookey, is in its fourth year. Two fully trained Army veterinary pathologists are about to take their qualifying Board examination.

Research focuses in several areas: (a) histopathological studies dealing with the pathogenesis of rickettsial diseases, yellow fever and other viral infections. This unit offers service to other investigators within the Institute providing them histopathologic correlation with physiological and other experimental parameters. (b) Examination of ultrastructural alterations in viral and rickettsial diseases. (c) Immunological investigations utilizing radioimmunoassays and measurement of extremely small amounts of physiologic, pharmacologic and toxic substances. (d) Study of microbial toxins including their production, purification, chemistry and pathogenesis. A 50-liter, automated fermenter permits the production of toxin under rigorously controlled conditions. The chemistry and mechanisms of action are under study in man and animals. (e) The rapid identification of bacteria using chromatographic techniques to characterize volatile acids produced during their growth.

Following the presentations by Colonel Crozier, Dr. Beisel and research Division Chiefs, specific papers were presented on the following subjects: vaccines, metabolism, staphylococcal enterotoxin B, animal models for infectious diseases and the contractual work at Johns Hopkins University and the University of Maryland.

Administrative Session

During the Executive Session, plans for adapting the CES to reorganization of the AFEB and its Commissions were discussed. Throughout its existence under the Chairmanship of Doctor Richard Shope and Doctor Woodward and the executive chairmanships of Colonels Tigertt and Crozier, there has been close integration between intramural (in-house) and extramural research programs. This has permitted coordinated research, avoidance of repetition and opportunities to take advantage of military and civilian competence. New and expanded facilities at Fort Detrick now permit an even broader study of pathogenesis of infectious diseases and development of better means of control.

An advisory group consisting of knowledgeable experts will be appointed to serve USAMRIID much as the CES has functioned. More consultative advice by advisors visiting Fort Detrick will be sought and an ad hoc group of experts will be appointed for specific projects or problems.

The advisory group is scheduled to meet in Frederick on 27-28 September 1973, possibly in collaboration with consultants in the fields of rickettsial and enteric diseases.

Grateful appreciation is expressed to members of the Commission who through many years have given much to the development of new leads useful for control of infectious diseases and who through their unstinting contribution of time and effort have helped keep the military service abreast of where the problems are and how they might be solved. The intellectual stimulus and counsel provided by the members are immeasurable in terms of benefit to the Armed Forces.

Theodore E. Woodward, M.D.

INTENSIVE IMMUNIZATION OF HUMANS

Charles S. White, III, Captain, MC*

Intensive immunization of animals with far larger proportionate doses of antigen than used in humans has been shown to produce adverse effects. Amyloidosis has been induced in animals by a variety of antigens;[1] it was first described in horses hyperimmunized to diphtheria toxin.[2] Hypersensitivity reactions have been seen in both men and animals.[3] Multiple myeloma has been produced in several strains of mice,[4,5] again with a variety of antigens. Three cases of human multiple myeloma associated with allergy hyposensitization have been reported.[6,7]

That other adverse reactions to immunization have not been observed in man, may be related to the relatively low doses of antigen that are given. However, at Fort Detrick, there was an opportunity to study a human population prophylactically immunized with a variety of antigens in large quantities. In 1956,[8] Peeler, Cluff and Trever selected a group of 99 immunized subjects for study and long term follow-up. This group was restudied in 1962;[9] on both occasions each person had a complete history, physical examination, electrocardiogram (EKG), chest x-ray, and laboratory tests on blood and urine.

The data from these two studies are summarized in Table I. While 24 had relative lymphocytosis in 1956 and 24, again in 1962, it is noteworthy that only 6 qualified on both occasions. This may indicate that the relative lymphocytosis is either a transient phenomenon or is not really an abnormality. Proteinuria was measured on a single voided specimen as trace to 2+ (on a scale of 4+); 6 had proteinuria on both occasions. In liver function tests, although all alkaline phosphatase and cholesterol values were said to be normal, some bilirubins and bromsulphalein (BSP) retentions were abnormal. However, several investigators have documented the rise in BSP retention with age.[10] Hexosamine, a glycoprotein, has been found in high concentration in tissue amyloid deposits, and elevated serum hexosamine levels occur in some humans with amyloidosis and in the experimental condition in animals.[1,11] When examined in 1962 serum hexosamines were significantly elevated when compared to a control group. An early measure of anti-gammaglobulin factors was also studied only in 1962.

Twenty-three persons in 1956 and 19, in 1962 showed qualitative alterations in serum protein electrophoresis (Figure 1). Including specimens drawn in 1958, 46 persons demonstrated the pattern on the right at one time or another but only 12 persons, on every occasion.

* U. S. Army Medical Research Institute of Infectious Diseases.

TABLE I. FINDINGS OF STUDIES OF 1956[8] AND 1962[9]

PARAMETER	1956 (89 persons)	1962 (76 persons)
Lymphocytosis - relative	24	24
Proteinuria	15	8
Elevated liver function tests - BSP	7	22
Bilirubin	1	3
Serum hexosamines	ND	Elevated ($p < 0.001$)
Anti-γ globulin factors - latex fixation +	ND	22
zinc turbidity +	ND	10
Qualitative alteration in serum protein electrophoresis	23	19

There were no quanitative abnormalities; that is the serum concentrations of α-, β- and γ-globulins were the same as controls. The alteration consisted of a merging of the α_2 and β globulins. This pattern has been observed in persons hyperimmune to diphtheria toxin[12] and in persons with familial amyloidosis.[13] The group of persons with this abnormality did not differ from the rest of the immunized persons with respect to age, duration of immunizations, or reactions to immunization. In addition, at Johns Hopkins Hospital the pattern was observed in 0 of 102 blood bank donors and in only 6 of 1,000 consecutively hospitalized patients.

By 1962, the tissues from 3 postmortem examinations, 7 gum biopsies and 3 kidney biopsies were available for study. Hematoxylin-eosin, thioflavine T, and Congo red staining studies were negative for amyloid on all tissues examined.

In both studies, it was concluded that no clinical illness was observed that might be attributed to intensive immunization. It was postulated that the laboratory abnormalities discussed might be due to intensive immunization.

Several reasons led us to restudy this population: (1) 10 years had elapsed since the 1962 study without follow-up of these individuals or of their laboratory data; (2) more sophisticated techniques were now available with which to study these persons; and (3) our interest in the possible adverse effects of intensive immunization is certainly keener as the list of antigens recommended for routine immunization grows.

In 1956, about 700 Fort Detrick employees were receiving a full schedule of immunizations; from this group the original 99 white males immunized the longest were selected.

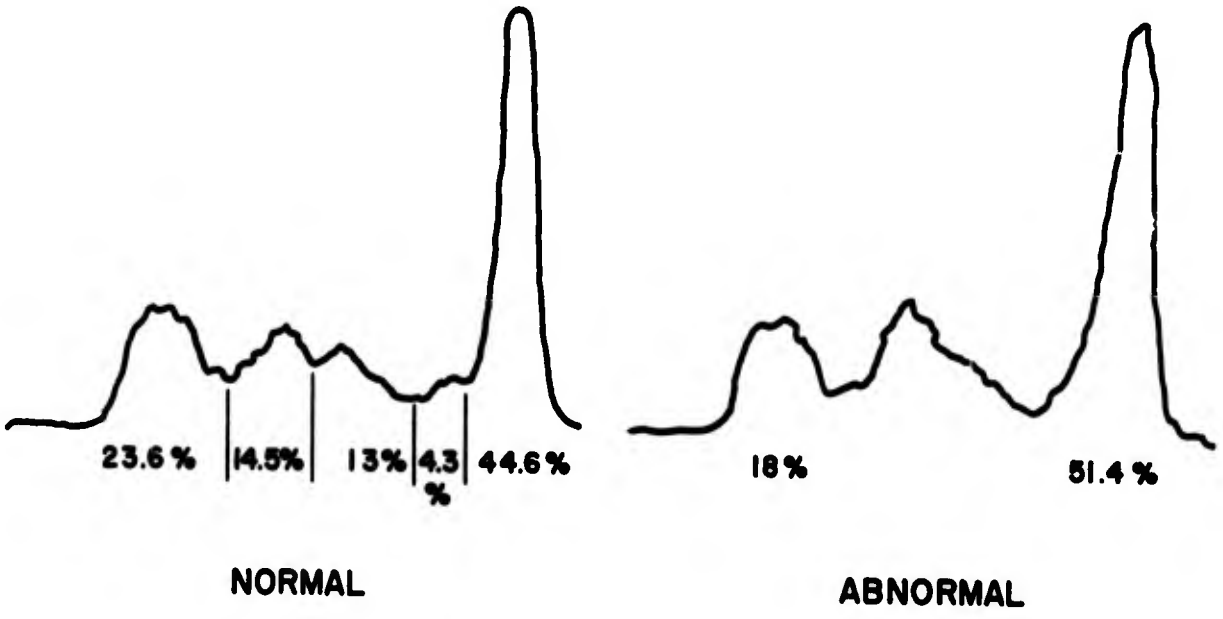

FIGURE 1. TYPICAL NORMAL AND ABNORMAL SERUM ELECTROPHORETIC PATTERNS.

These personnel were immunized against the diseases listed in Table II. Table III lists some other parameters of description of the persons involved. In addition there were averages of 8 inoculations/year, 2 skin tests/year, and 21 different antigens. Table IV lists the present status of the original 99 participants.

TABLE II. DISEASES OF CONCERN

Anthrax
Botulism
Brucellosis
Diphtheria
Eastern, western and venezuelan equine encephalomyelitis
Influenza
Plague
Poliomyelitis
Psittacosis
Q fever
Rift Valley fever
Rocky Mountain spotted fever
Smallpox
Tetanus
Tularemia
Typhus
Yellow fever

The 77 subjects followed up in 1971 have each had a complete medical history and physical examination, employing a check list for completeness. EKG, chest x-ray and laboratory tests on blood and urine were obtained on each. Outpatient and immunization record and data from the 2 previous studies have all been obtained; each person's entire record has been reviewed in detail. The 11 deaths have been followed up.

Table V shows the possible occupational illnesses of the 77 individuals studied. Examining nonoccupational illnesses, only 2 diseases occur with greater frequency than expected, hypertension in 21 and peptic ulcer disease in 10. Also noted were 3 cases of cancer, 2 of which were carcinoma of the skin and one, of the colon with apparently successful resection. In short, no clinical illness was found that might be attributable to immunization.

TABLE III. PARAMETERS EXAMINED

ITEM	1956	1962	1971
Mean duration of immunization (years)	10.4	15.3	24
Mean age (years)	40.1	46.3	54-55
Mean antigen received (ml)	52.8	73.5	97
Mean number of skin tests	20.1	30.1	55
Number with complete history, physical examination, and chart review	93	76	77
Number with blood tests, EKG, x-ray	89	76	77

TABLE IV. 1971 STATUS OF THE ORIGINAL 99 SUBJECTS

PARTICIPANTS	NO.
Studied	77
Not studied	11
Refused	4
Unable to come to Fort Detrick	5
Unable to be reached	2
TOTAL	99

TABLE V. OCCUPATION-CONNECTED ILLNESSES

ILLNESS	NO.
Tularemia	2
Brucellosis	2
Paratyphoid B enteritis	1
Undiagnosed fever	14
TOTAL	19

Of the 11 deaths, shown in Table VI, postmortem or biopsy tissue reports were available for 5. Review of the hospital records or letters from the attending physician were available for 4 and minimal information on 2. Those tissues still available were restudied by Dr. John Warren, Pathology Division; no pathological findings were attributable to immunization.

TABLE VI. CAUSES OF 11 DEATHS

Arteriosclerotic heart disease	4
Cancer	3
Oat cell carcinoma of lung (1)	
Questionable brain tumor (1)	
Colon carcinoma (1)	
Chronic lung disease	2
Sudden death	2

Two persons, not in the original group of 99, but who received special immunizations, have been found to have lymphoid neoplasma. One person, immunized over a 12-year period, has lymphosarcoma. One, a 34-year old black female, developed an aggressive multiple myeloma and died within 3 months of the diagnosis of pneumonia and hypercalcemia. Plasma cells were easily found in smears of her peripheral blood.

Controls for all the laboratory data were 26 age-matched Fort Detrick employees who had received no special immunizations.

Looking at the results of those tests that were repeated because of positive findings on the previous occasions, the first category was relative lymphocytosis. Hematologic data are summarized in Table VII. When one

TABLE VII. HEMATOLOGIC DATA

ITEM	1956	1962	1971	CONTROLS
Hemoglobin (%)	--	--	15.7	15.8
Hematocrit (%)	--	--	47.7	48.5
White blood cells (No.)	--	--	7400	7460
Number with > 40% lymphocytes	24/89	24/76	16/74	12/26
%	(27)	(31.6)	(21.6)	(46)
> 3% eosinophils	17/89	23/76	19/74	6/26
Increased prothrombin time (> 1½ sec > control)	--	--	11/74	2/26
Increased partial thromboplastin time (sec)	--	--	19	0
Erythrocyte sedimentation rate	--	--	12.6	6.7 ($p < 0.001$)

examines the lymphocyte data in a manner utilized by the previous studies, one sees that the percentage of persons with > 40% lymphocytes is not high with respect to controls. Individual absolute lymphocyte counts, performed by Dr. Adler of Pathology Division, were all normal. It is also seen that there were no abnormal individual hemoglobulin and hematocrit values and that the total white and differential counts were virtually identical. The difference in prothrombin times is not significant; however, the difference in partial thromboplastin times and in erythrocyte sedimentation rates are both significant and unexplained. Neither α- nor γ-globulin concentrations showed significant differences. Finally, lupus erythematosis cell preparations and rheumatoid arthritis screening tests were negative in the study population, although one control was positive for rheumatoid arthritis.

The second category restudied was renal function. Study of Table VIII emphasizes the extent of the present findings. We feel that these data represent better the status of renal function than urine "dipstick" readings on a single voided specimen.

Results of liver function tests are shown in Table IX. Abnormalities have persisted and are increasing in incidence. However, all the elevations are very mild and are not significantly different from the controls.

TABLE VIII. RENAL FUNCTION TESTS

PARAMETER	NO. ABNORMAL BY YEAR			
	1956	1962	1971	Control
Elevated blood urea nitrogen (20-30)	0	3	11	0
Creatinine > 1.5	ND	ND	0	0
24-hr urine protein > 200 mg	ND	ND	4	0

TABLE IX. LIVER FUNCTION TESTS

PARAMETER	NO. BY YEAR					
	1956 (89)	1962 (76)	1971 (77)	%	Control (26)	%
Alkaline phosphatase	0	3	12	16.2	3	11.4
Bilirubin	1	3	8	10.8	2	7.0
SGOT	--	0	5	6.8	3	11.4

For serum hexosamine, the study group differed significantly ($p < 0.001$). However, more is known about hexosamine and its relationship to amyloid than in 1962. Glycoproteins, such as hexosamine, are in most cases not a constituent of amyloid fibril nor closely related to fibril formation or deposition. It apparently accumulates as a secondary phenomenon.[14] Hence, while the serum hexosamines in the 1971 immunized group remain significantly elevated, the relationship of the finding to amyloidosis or to immunization is unclear.

The last of the follow-up studies is serum protein electrophoresis. The alteration in the protein electrophoretic pattern shown in Figure 1 was not observed in any of the immunized or control subjects on this occasion. There are two explanations: possibly newer methods have effected better separation of the α_2 and β components, but, more probably, none of the subjects had been immunized at all for a period of at least 9 months.

Several additional investigational approaches were utilized to examine the effects of intensive immunization. At the Institute, changes in serum

trace elements[15] and glycoproteins (personal communication, G. L. Cockerell) have been seen in acute infection. So far as the present data have been analyzed, no differences exist between the 2 groups with regard to uric acid, Ca, P, Zn, Cu, and Fe. Glycoproteins, other than hexosamine, were also studied; no apparent differences were found. Serum lipoproteins and serum lipids (cholesterol, triglyceride and free fatty acid), which were examined partly as a service to these cooperative individuals, showed no differences between the immunized and control groups. Figure 2 shows the mean percentages for the immunized group, 26 controls, and another control group of Project Whitecoat individuals (i.e., normal young males).

Qualitative and quantitative determinations of serum and urine immunoglobulins were made; the data were compiled by Dr. Virginia McGann of Bacteriology Division (Figure 3). No monoclonal or Bence-Jones proteins were observed. There were no significant differences for IgG, IgA, IgM and complement, as measured by the C'3 component. IgD levels had no significant differences between mean values for the 2 groups. However, there are differences in the number of negative responses. Figure 4 shows the immunoglobulin values in the more usual units of mg/100 ml.

Lymphocyte studies were performed by Dr. William Adler, Pathology Division. Fresh lymphocytes were obtained by mixing 2 ml of venous blood with 1 ml of 6% dextran. The mixture was incubated for 1 hr at 37 C. The supernatant fluid of white blood cells and plasma was aspirated, centrifuged and washed. Total and differential counts were obtained on the pellet. They were then subjected to phytohemagglutinin (PHA) and staphylococcal enterotoxin B (SEB) stimulation with appropriate controls. PHA is a mitogen capable of stimulating that population of lymphocytes known as T-cells or thymus-derived lymphocytes.[16] SEB is a mitogen that stimulates B-cells or bone marrow derived lymphocytes.[17] Any cells remaining were cultured in an attempt to derive continuous cell lines. Control and immunized groups showed no differences in the responses to PHA or SEB stimulation (Figure 5). Culture results showed that the lymphocytes of 16 immunized persons and 4 controls established cell lines although the difference was not significant.

In summary, at 24 years, a follow-up was conducted of 99 intensely immunized individuals and a small series of age-matched controls. Earlier findings have been reexamined and the scope of the survey expanded considerably. No clinical illness can be attributed to intensive immunization. Follow-up of the laboratory abnormalities found in 1956 and 1962 demonstrates that few of the original findings persisted when age-matched controls were studied. The significance of the few remaining differences between groups remains unclear.

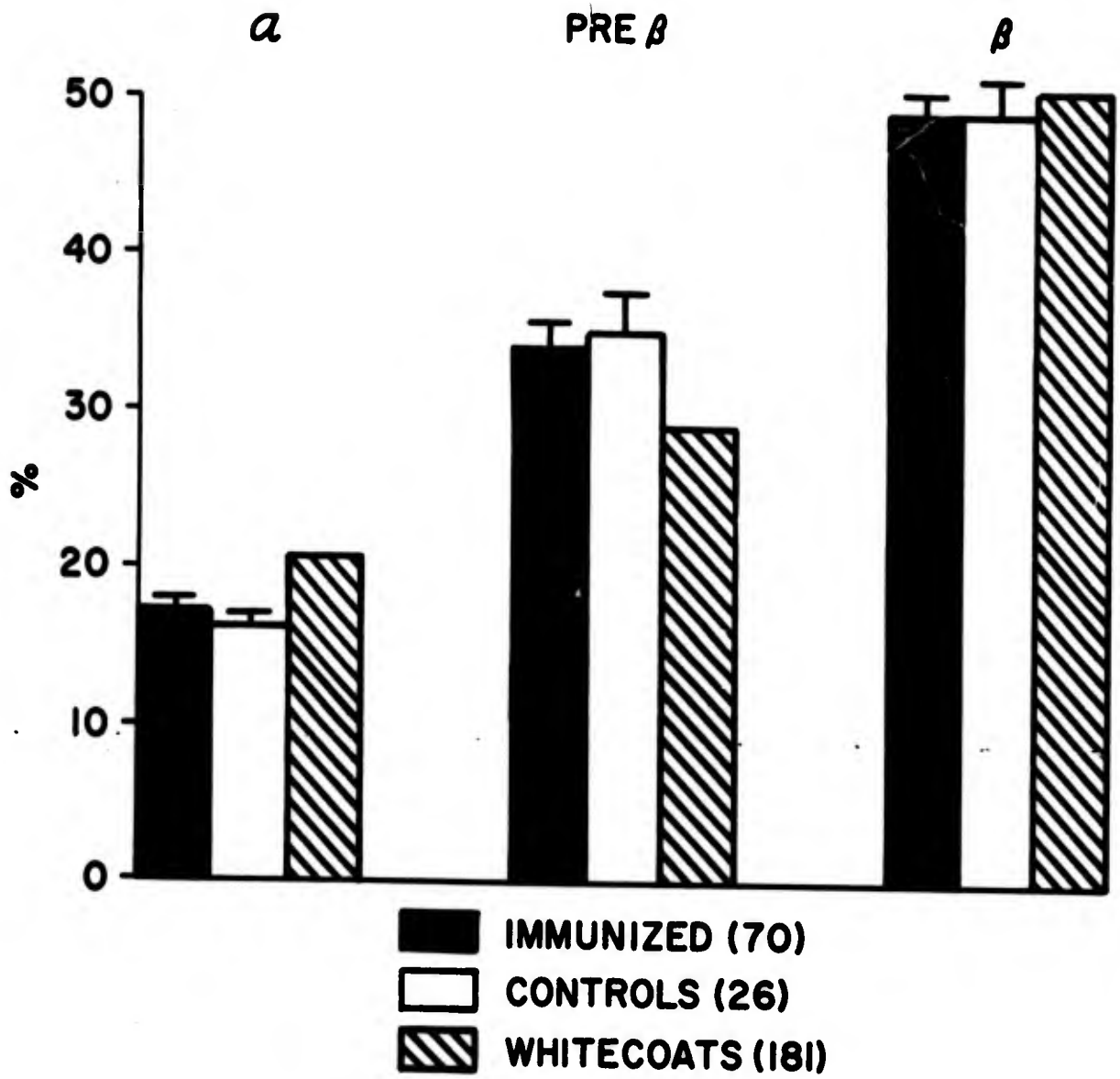

FIGURE 2. LIPOPROTEIN DISTRIBUTIONS IN HYPERIMMUNIZED MEN, PAIRED CONTROLS, AND PROJECT WHITECOAT VOLUNTEERS.

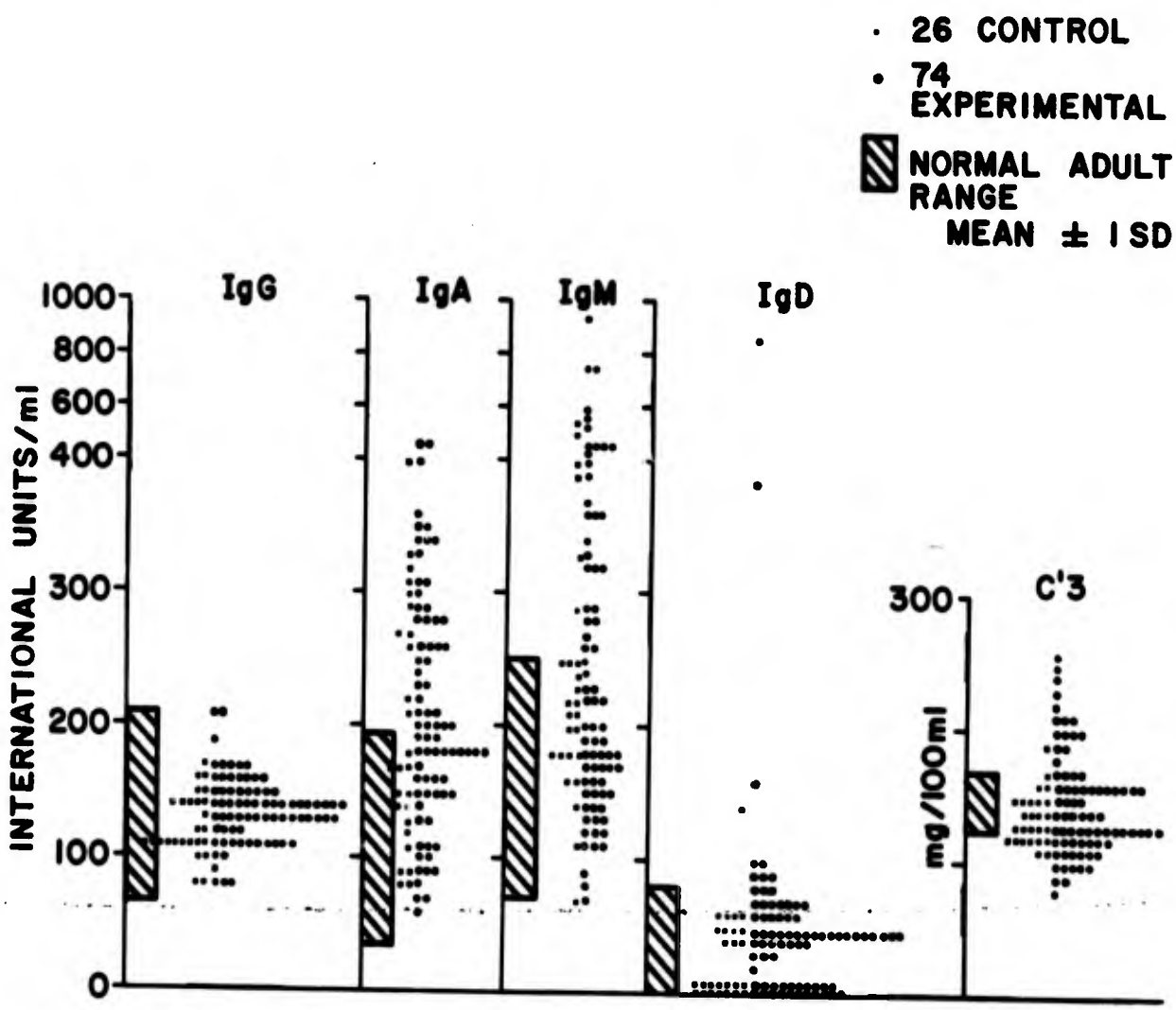

FIGURE 3. DISTRIBUTION OF INDIVIDUAL VALUES OF IMMUNOGLOBULINS AND COMPLEMENT IN THE 1971 STUDY.

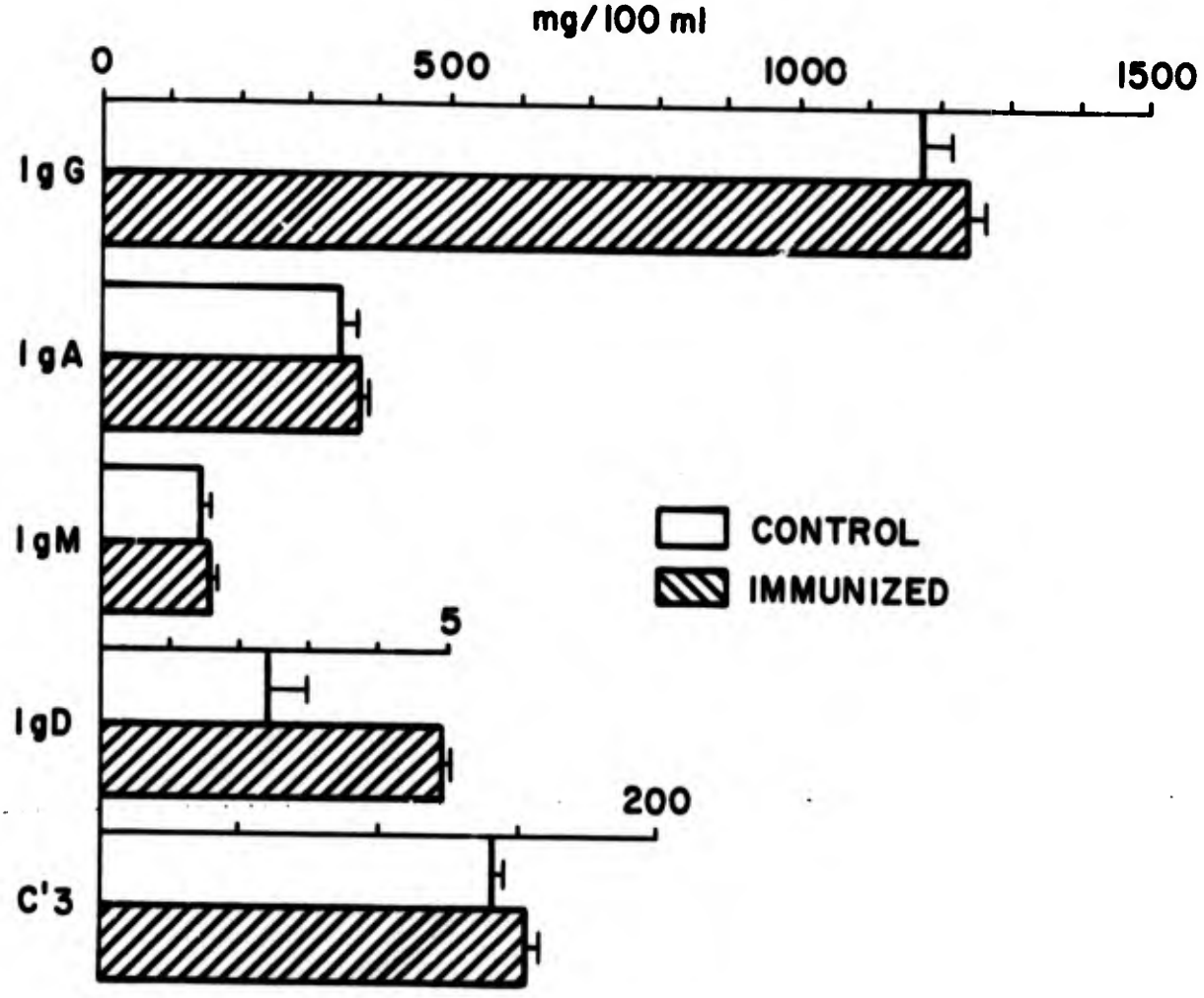

FIGURE 4. MEAN DISTRIBUTION OF IMMUNOGLOBULINS AND COMPLEMENT IN THE 1971 STUDY.

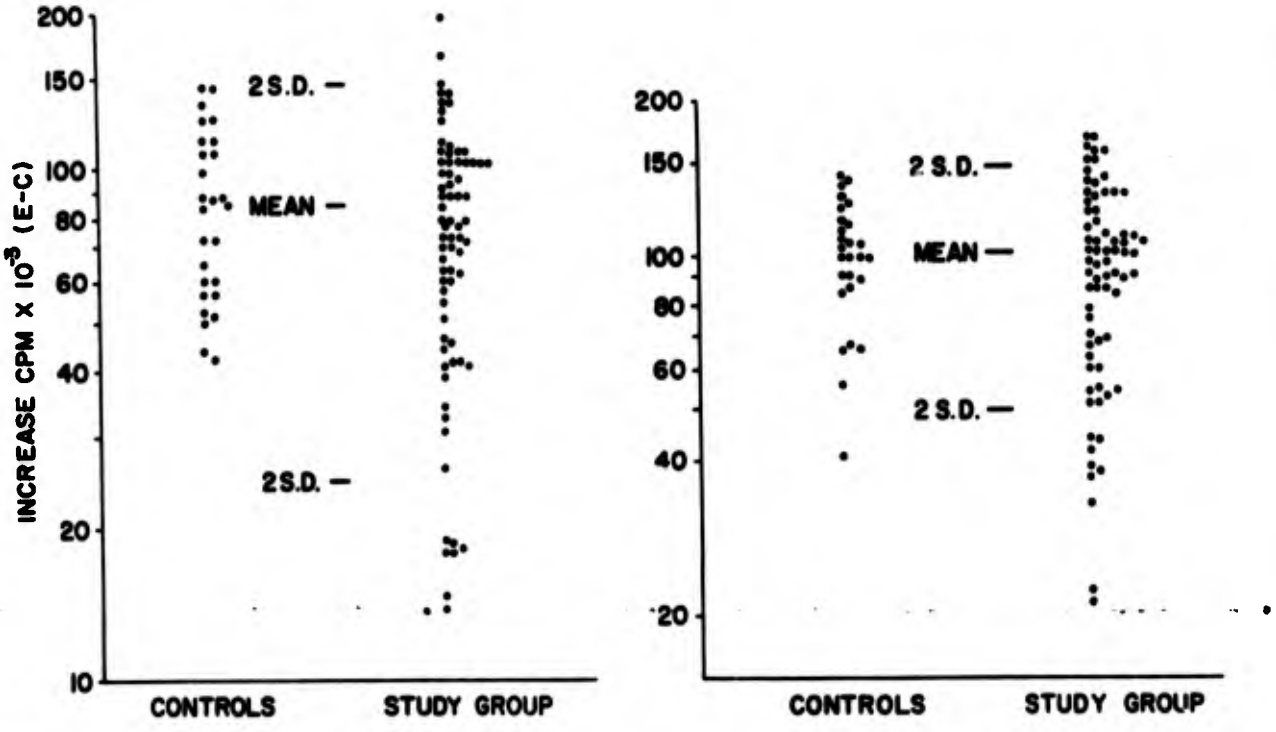

FIGURE 5. ^{3}H-THYMIDINE INCORPORATION IN SHORT-TERM LYMPHOCYTE CULTURES STIMULATED WITH PHA AND SEB.

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VENEZUELAN EQUINE ENCEPHALOMYELITIS PLAQUE VARIANTS

Carl E. Pedersen, Jr., Major, MSC*

In light of the Venezuelan equine encephalomyelitis (VEE) epizootic in the United States during the Summer of 1971, there has been a renewed interest in the disease potential of VEE. The Venezuelan complex represents a large number of serologically related viruses distinguished primarily by geographic distribution, antigenic properties, epidemiologic characteristics, and virulence in various mammalian hosts. A degree of cross-protection and similar biologic behavior offer evidence of interrelatedness between members of the complex;[1,2] however antigenic variation, possibly due to geographic distribution or evolutions of the viruses, serves to distinguish the strains.[3]

The current vaccine used to prevent infection with VEE is the attenuated TC-83 strain derived from Trinidad virus isolated from the brain of a donkey.[4] Objections concerning the use of this vaccine have been based on the possibility of reversion of TC-83 to the virulent parental form.[5,6] It has therefore been suggested that the Florida strain, Fe 3-7c, might be considered as a replacement for TC-83, since it has not shown virulence for equines.[5]

This report describes a 2-part study: (1) investigation of the differential adsorptive capacity of calcium phosphate columns for several subtypes and variants of the VEE complex, and (2) use of this technique and other markers to separate a Florida strain into 2 distinct plaque size variants.

Columns of $Ca_3(PO_4)_2$, employed initially as a simple and useful chromatographic tool for the purification of influenza virus,[7] has recently been used to demonstrate different elution profiles among members of the Group B arboviruses,[8] vaccine and progeny strains of polioviruses,[9] and plaque variants of Mengo,[10] encephalomyocarditis,[11] and Sindbis viruses.[12] The viruses tested were representative of the 4 Venezuelan virus antigenic subtypes defined by Young and Johnson[3] employing the kinetic hemagglutination inhibition (HI) technique (Table I). Stock virus preparations, propagated in primary duck or chick embryo cells (DEC or CEC) were concentrated and partially purified by ion exchange chromatography on diethylaminoethyl (DEAE) cellulose. Peak viral fractions were pooled, banded on sucrose by centrifugation, and dialyzed prior to chromatography on calcium phosphate.

The brushite form of calcium phosphate was prepared as described by Burness.[11] One milliliter of the concentrated virus containing approximately $10^{10.5}$ plaque forming units (PFU)/ml was added to the gel; a linear elution gradient consisting of 0.1-0.6 M phosphate buffer was applied. Fractions (5 ml) were collected; the infectious titer of each was determined by plaque assay.

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TABLE I. ANTIGENIC SUBGROUPS OF VEE VIRUS

ANTIGENIC GROUP[a]	REPRESENTATIVE STRAINS	YEAR ISOLATED	COUNTRY
1a	Trinidad donkey	1943	Trinidad
1a	TC-83 (vaccine)	1961	USA
1b	Ica	1946	Peru
1b	9859	1971	USA
1c	P676	1963	Venezuela
1d	3880	1961	Panama
1e	Mena II	1962	Panama
II	Florida	1963	USA
III	Mucambo	1954	Brazil
IV	Pixuna	1961	Brazil

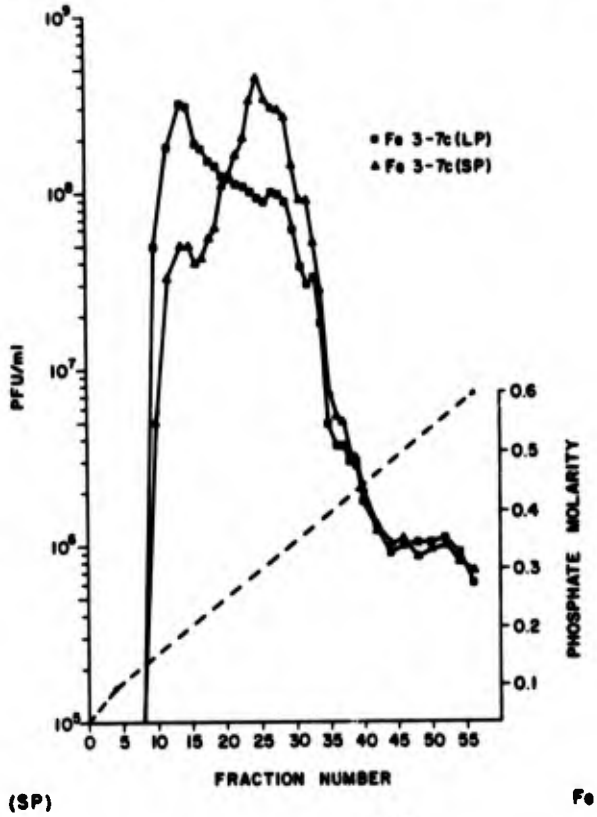
A reproducible elution profile was obtained with routine recovery of at least 90% of the viral infectivity. Figure 1 illustrates the results of chromatography with the TC-83 vaccine strain of VEE virus. The pattern is representative of the viruses examined in that both viral infectivity and hemagglutinating activity eluted in a sharp peak which was associated with a minor peak of optical density.

The phosphate molarity which was found to correspond with the maximal infectivity of VEE viruses studied is shown in Table II. Subtype I viruses

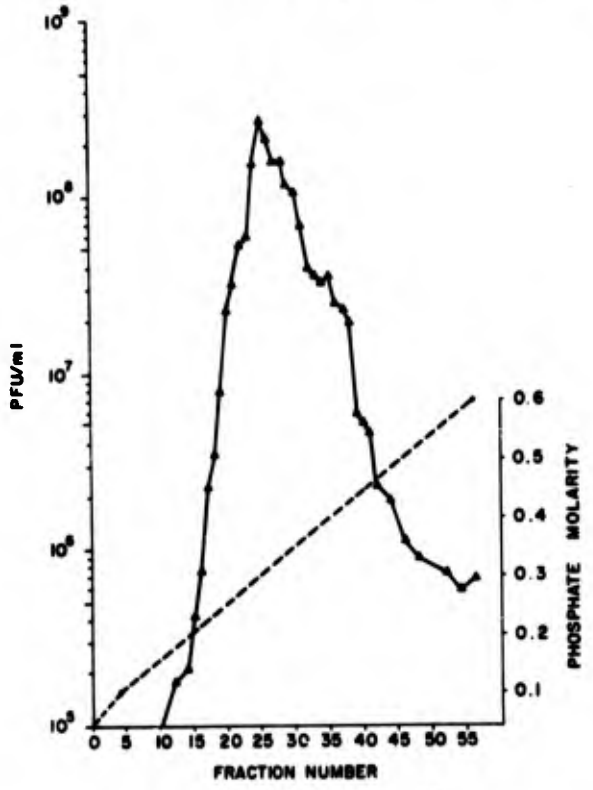
TABLE II. PHOSPHATE MOLARITY OF PEAK VIRUS INFECTIVITY

ANTIGENIC GROUP[a]	VIRUS STRAIN	FRACTION NO.	PHOSPHATE MOLARITY
1a	Trinidad	20	0.25
1a	TC-83	18	0.23
1b	Ica	20	0.25
1b	9859	20	0.25
1c	P676	19	0.24
1d	3880	17	0.22
1e	Mena II	18	0.23
II[a/]	Fe 3-7c	14, 25	0.19, 0.30
III	Mucambo	31	0.36
IV	Pixuna	27	0.32

a. 2 peaks of virus infectivity observed.



Fe 3-7c (SP)



Fe 3-7c (LP)

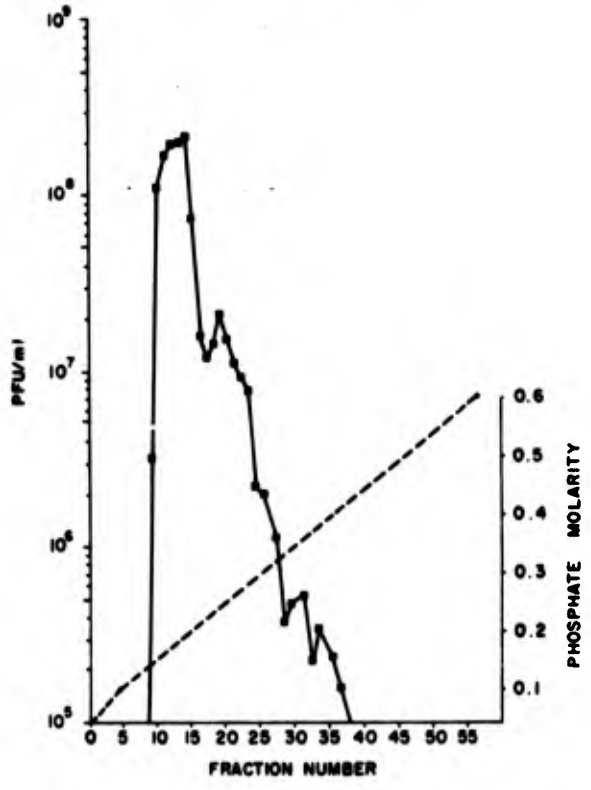


FIGURE 1. ELUTION PROFILES OF Fe 3-7c VIRUSES FROM $(Ca_3PO_4)_2$ COLUMN

gave elution profiles within a very narrow range which suggested relatively similar biochemical characteristics of the virus membrane. Members of Subtypes II, III, and IV had profiles distinctly different from that of Subtype I. Young and Johnson[2] showed that Mucambo (Subtype III) and Pixuna (Subtype IV) do not confer complete protection to rodents challenged with a strain of antigenic Subtype I. Available evidence suggests that the enzootic habitats of Mucambo and particularly Pixuna viruses, differ from other members of the complex.

Just as others have observed differing elution patterns of viruses with distinct characteristics, our results clearly show that Mucambo and Pixuna differ substantially not only from the viruses of antigenic Subtype I, but from each other. The exact nature of the differences expressed on calcium phosphate remain to be elucidated but confirm the unique properties possessed by members of the Venezuelan virus group.

Endemic VEE in the United States was first isolated by Chamberlain, et al in 1963[13] from Culex mosquitoes collected in the Florida Everglades. The prototype Florida strain (Fe 3-7c) has been placed in antigenic Subtype II on the basis of kinetic HI serology.[3] This virus was suggested as a candidate for development of a live vaccine[5] as it was considered avirulent for horses and appeared to confer protection equivalent to that elicited by the TC-83 strain.[6]

The elution profile of the Florida (Subtype II) virus is illustrated in Figure 2. Two distinct peaks were obtained. Plaques were individually scored as large, i.e. > 2 mm (LP) or small, < 2 mm, (SP). The first peak eluted in the region of low ionic strength, contained predominantly LP virus, while the second peak contained predominantly SP virus.

We subsequently cloned and chromatographed the variants separately and found that the peak of LP variant activity was eluted from the column by 0.19 M phosphate while the peak of SP variant infectivity was eluted by 0.30 M phosphate buffer. These results were consistent with the experimental chromatography of the mixed parental population (PV). Plaques of the SP variants ranged from 1.4-2.0 mm after 48 hr on DEC. Individual plaques did not have well defined margins, but were generally very clear throughout the plaque. The LP variant plaques ranged from 4.0-5.2 mm after 48 hr. Individual plaques had very clear centers with slightly more opaque, irregular margins.

Table III shows the results obtained when weanling mice, hamsters and guinea pigs were inoculated with PV, LP and SP variants of the Florida subtype of VEE virus. The LP variant was the most virulent of the viruses studied and elicited a virulence profile very similar to the PV. The SP variant was avirulent for guinea pigs and hamsters, but some weanling mice were killed. Immunization with the PV, LP or SP variants conferred protection to the survivors, however this protection was not absolute. Suckling mice were uniformly susceptible to intracerebral (IC) injection with either the LP or SP variants of the Florida virus. Although this test host

TC 83

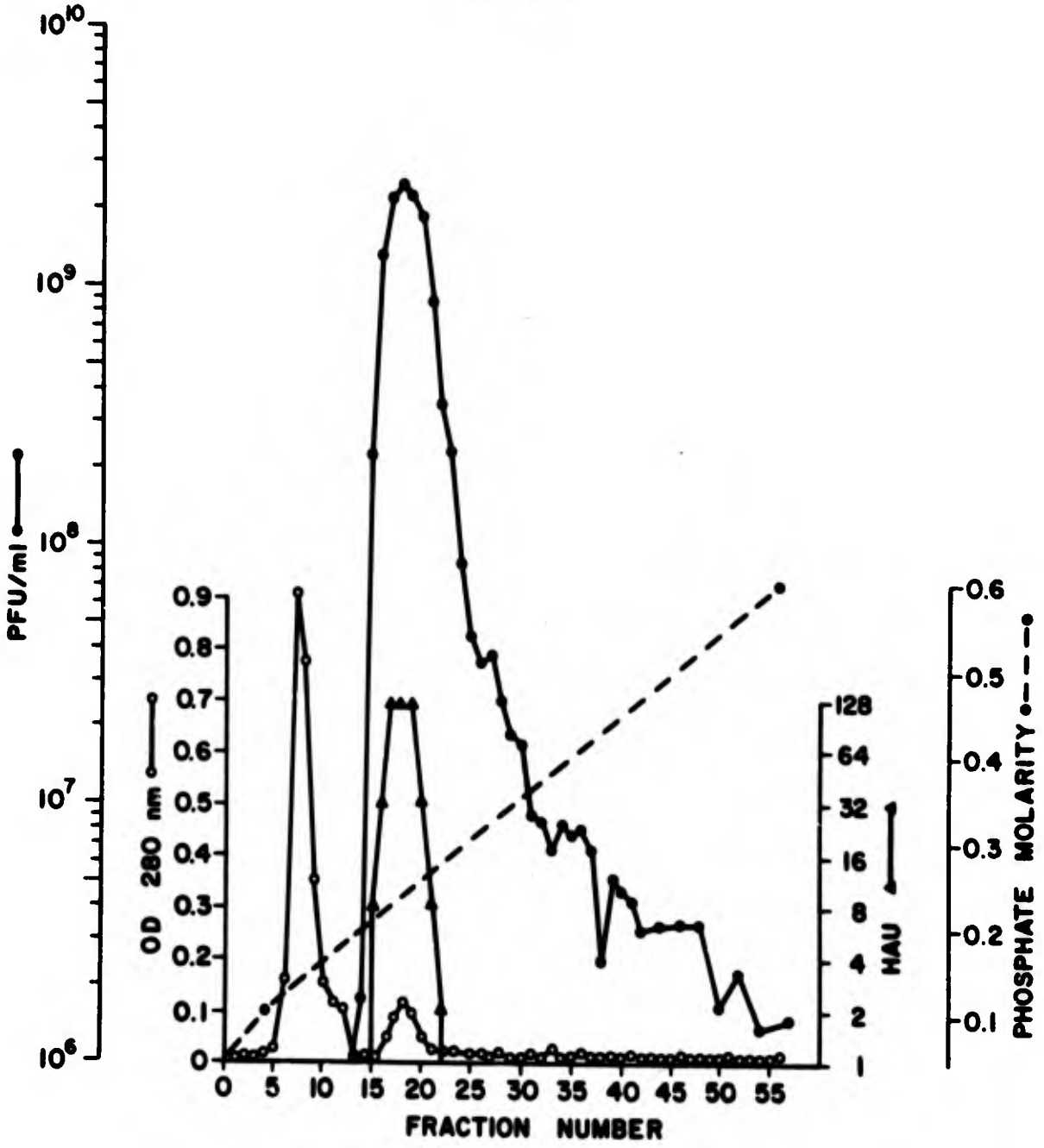


FIGURE 2. ELUTION PROFILE OF TC-83 VIRUS FROM A $(Ca_3PO_4)_2$ COLUMN.

TABLE III. PROTECTION AFFORDED BY Fe 3-7c VEE AGAINST TRINIDAD VEE CHALLENGE (10^3 $SMICLD_{50}$ AT 21 DAYS)

Fe 3-7c VIRUS CEC PFU[a]		WEANLING MICE (n=8,5)		HAMSTERS (n=5)		GUINEA PIGS (n=5)	
		1[b]	2[c]	1	2	1	2
PV	2×10^5	0		20	1/1	100	5/5
	2×10^4	0		20	1/1	100	4/5
	2×10^3	0		20	1/1	100	4/5
	2×10^2	0		40	2/2	100	5/5
	0	100	0/8	100	0/5	100	0/5
LP	2×10^5	0		0		0	
	2×10^4	0		0		0	
	2×10^3	0		20	1/1	20	1/1
	2×10^2	0		40	2/2	80	2/4
	0	100	0/5	100	0/5	100	0/5
SP	2×10^5	20	1/1	100	4/5	100	4/5
	2×10^4	60	3/3	100	4/5	100	4/4
	2×10^3	60	3/3	100	4/5	100	4/5
	2×10^2	40	2/2	100	4/5	100	2/5
	0	100	0/5	100	0/5	100	0/5

a. Weanling mice and guinea pigs 0.5 ml IP; hamsters 0.5 ml SC.
b. Column 1 - % survivors.
c. Column 2 - Protected as No./Survivors.

was approximately 10-fold more sensitive than the plaque assay of infective viruses, no differences were observed between the lethality of the LP and SP variants by the IC route.

Using guinea pig sera we performed kinetic cross-neutralization tests with PV, LP, and SP variants of Fe 3-7c. The results showed that although the LP and SP variants were related antigenically, the SP variant was consistently neutralized to a greater extent that the PV or the LP variant. Gel diffusion tests failed to show any differences between the plaque variants.

Although the Venezuelan virus complex consists of a serologically related group of viruses representing a spectrum of biological characteristics, distinct antigenic variation among the various strains has been observed. In an attempt to extend the tools available for the study of these agents, we found that Venezuelan viruses were amenable to elution from calcium phosphate gels by a linear gradient of phosphate buffer. Adsorption to, and subsequent differential elution of virus populations from these gels probably involves properties unique to each prototype virus, which may be a reflection of the charge on the viral membrane.

Cloned plaque variants derived from the Florida strain exhibited distinct biological and chemical characteristics. These differences may be expressed by the charge on the viral membrane thus accounting for the individual elution profiles of the variants on calcium phosphate columns.

The LP variant was uniformly lethal for weanling mice and highly virulent for hamsters, guinea pigs, and adult mice. In constrast, the SP variant was avirulent for hamsters, guinea pigs, and adult mice, and less virulent for weanling mice than either the PV or the LP variant. Although our studies indicate that the SP variant of Florida virus offers no immunological advantage over the TC-83 vaccine strain currently in use, we feel further studies might be warranted to determine its relative pathogenicity. In the report which follows comparative pathology of VEE is presented.

In summary, subtypes of VEE viruses were shown to have individual elution profiles on calcium phosphate columns. Subtype I VEE viruses were eluted within a narrow range of phosphate buffer molarity while Subtypes III and IV were shown to differ substantially not only from the viruses of Subtype I but from each other. The endemic Subtype II virus (Fe 3-7c) was shown to exhibit 2 peaks on calcium phosphate which could be separated into large (LP) and small plaque (SP) variants. The LP and SP variants were shown to have different chemical and biological properties, the LP variant being the more virulent.

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A COMPARISON OF THE HISTOPATHOLOGY PRODUCED IN HAMSTERS BY TWO STRAINS OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS

Garrett S. Dill, Jr., Captain, VC*

During Major Pederson's investigations of a Florida strain (Fe 3-7c) and the TC-83 vaccine strain of Venezuelan equine encephalomyelitis (VEE) virus, he noticed that infected hamsters underwent a marked change in behavior after infection, becoming more aggressive and difficult to handle than the sham inoculated controls. Necropsies were done on 10 hamsters that had been used in his experiments, 2 controls and 8 that had been infected with TC-83 some weeks before. Lesions of old hemorrhage and encephalitis were found in the olfactory bulbs of 5 of 8 of the infected animals.

Austin and Scherer[1] recently reported that they found TC-83 virus produced no central nervous system (CNS) lesions in hamsters. To resolve this discrepancy with our findings, a project was initiated with two purposes in mind: (1) to determine if TC-83 does produce a brain lesion in the Syrian golden hamster, and (2) since an enzootic strain of VEE, the Florida isolate (Fe 3-7c) has been suggested for vaccine use, to compare the lesions, if any, produced by the 2 strains in this highly susceptible animal.

Ninety hamsters were divided into 3 groups: Group I, 35 hamsters, was inoculated subcutaneously with 750 plaque forming units (PFU) of the small plaque variant of Fe 3-7c suspended in 0.5 ml of phosphate buffered saline (PBS). Group II, 35 hamsters, was inoculated subcutaneously with 1215 PFU of TC-83 prepared by Merrill-National Laboratories, suspended in 0.5 ml of PBS. Group III, 20 hamsters, was sham inoculated subcutaneously with 0.5 ml of PBS.

At 3-day intervals after inoculation, 4 hamsters from each experimental group and 2 controls were killed, representative tissues were fixed in formalin and prepared for histopathological examination.

All of the significant lesions seen were confined to 3 organ systems, hematopoietic, lymphoid, and nervous.

The first lesion observed in any tissue was a mild to moderate, myeloid necrosis in the TC-83 hamsters killed on day 3. By day 6 this necrosis was severe in 3 of the 4 animals examined. None of the Fe 3-7c hamsters had bone marrow lesions at the times they were examined.

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As compared to the controls there were minimal to mild lymphoid depletions of the germinal centers of the spleen in some animals, infected with both strains, in the groups killed on days 3 and 6. Two hamsters, one FE 3-7c and one TC-83, had mild lymphoid necrosis of the germinal centers of their spleens. All lesions in hematopoietic and lymphoid tissues were transient. The animals examined 9 days postinoculation had normal bone marrows and lymphoid tissues; there were no lesions in these organs after that.

Other than those described, the only consistent, significant lesions seen were in the brain and affected the olfactory bulbs, the pyriform lobes and the myelinated lateral olfactory tracts.

In the primitive brain of rodents the olfactory bulbs are prominent structures extending upward and forward toward the nares from the pyriform lobe of the cerebral cortex. They are connected with the sensory nerve endings in the nasal mucosa by the olfactory nerves which run back and penetrate the cribiform plate. Layers of neurons and their interconnections run through the bulb to nuclei in the pyriform lobes from whence connections are made with other parts of the brain. There are several myelinated tracts connecting the olfactory bulbs and the pyriform lobes.

The first lesion seen in the brain was in one Fe 3-7c hamster on day 3 and consisted of a mild to severe, multifocal, acute vasculitis, with perivascular hemorrhage in the olfactory bulbs and pyriform lobes. This lesion on the later days became more chronic, endothelial swelling was not so noticable, and the perivascular space of affected vessels became filled with mononuclear cells. There were vasculitis and/or perivascular cuffing of lymphocytes present in all of the Fe 3-7c and most of the TC-83 hamsters killed on day 6 and later. The lesion was more severe and distributed more caudally in the brains of those infected with Fe 3-7c.

The lateral olfactory tracts were infiltrated with glial cells and demyelinated in most hamsters infected with both strains after the 9th day (Table I).

By the 9th day postinoculation, necrosis of neurons and a glial response were marked in the olfactory bulbs of the Fe 3-7c animals. This same lesion was present in a few of the TC-83 animals but most were not affected (Table I). The end result of this necrotizing inflammatory process in the olfactory bulbs, when it occurred, was the partial to almost total destruction of their normal architecture. A few of the Fe 3-7c hamsters had lesions outside the pyriform lobes and olfactory bulbs such as focal demyelination in the medulla oblongata or a non-suppurative encephalitis, meningitis and vasculitis in other areas of the cerebral cortex.

TABLE I. DISTRIBUTION OF CNS LESIONS IN HAMSTERS INOCULATED WITH TC-83 AND Fe 3-7c SMALL PLAQUE VARIANT

LESION	TIME	TC-83		Fe 3-7c	
		No./Total	%	No./Total	%
Demyelination of olfactory tracts	< Day 9	0/8	0	1/8	12
	≥ Day 9	13/20	65	17/20	85
Neuronal and glial necrosis of olfactory bulbs	< Day 9	0/8	0	2/8	25
	≥ Day 9	3/20	15	17/20	85
Vasculitis and/or perivascular cuffing	< Day 9	4/8	50	6/8	75
	≥ Day 9	15/20	75	20/20	100

In summary, when hamsters were infected with TC-83 and the small plaque variant of the Florida (Fe 3-7c) strains of VEE virus and the histopathology compared it was found that consistent, significant lesions were produced by both strains in the olfactory bulbs, lateral olfactory tracts, and pyriform lobes of the cerebral cortex. Although they were similar in type they varied markedly in time of appearance, degree of severity, and amount of extension toward more vital centers of the brain. The Fe 3-7c strain produced more severe lesions in more animals; the lesions appeared earlier in the course of infection and extended deeper into the brain.

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MULTIPLE VACCINES IN COMBINATION

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During the past decade, evidence has become available which suggests that the major, if not only, tool to implement effective prophylactic control of an infectious disease will be vaccination. Programs, initiated with such high hopes in the years following World War II, for eradication of vectors or intermediate hosts associated with yellow fever, malaria, plague and others have not only failed but have introduced additional problems. Not only have vectors developed resistance to specific insecticides but area wide application of insecticide may have contributed to the resistance developing in vectors associated with other diseases. Although this disadvantage has been temporarily overcome by development of new compounds, the current political climate characterized by social stigma, increased liability risks and legislative requirements for testing and licensing of insecticides, indicate that their use will be curtailed, if not ended. Consequently, increasing emphasis must necessarily be given to the use of vaccines as the basic measure for prophylaxis against an infectious disease.

Although 45 vaccine preparations were licensed for use in the United States as January 1972,[1] with few exceptions they are employed for protection against only one or more of the infections shown in Table I. It

TABLE I. DISEASES FOR WHICH U. S. POPULATION IS MOST COMMONLY VACCINATED

1.	Diphtheria	10.	Typhoid fever
2.	Pertussis	11.	Salmonellosis
3.	Tetanus	12.	Cholera
4.	Smallpox	13.	Plague
5.	Poliomyelitis	14.	Yellow fever
6.	Rubeola	15.	Typhus
7.	Mumps	16.	Rocky Mountain spotted fever
8.	Rubella	17.	Rabies
9.	Influenza	18.	Acute respiratory disease (Adenovirus)

would be safe to assume that most persons at this meeting have some state of immunity against every disease listed except rabies, and that, in most instances, immunity was achieved by vaccination rather than infection.

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Many of us have also received one or more, usually more, investigational vaccines such as the attenuated TC-83 strain of Venezuelan equine encephalomyelitis (VEE), Q fever, Rift Valley fever, the living vaccine strain of tularemia, etc. In addition, efforts are underway to develop an impressive number of vaccines against other diseases. It immediately becomes obvious that a formidable number of inoculations could be required to evoke a primary or anamnestic response. This situation apparently has been anticipated by the Army as seen in this quotation from TB-MED 114 on immunization: "Service policies vary; for Army personnel only, any immunization required to protect the individual, and to assure that military units can perform their missions anywhere in the world without the series danger of disease, will be administered to an individual with or without his consent. In accomplishing this, medical personnel are expected to use only that amount of force necessary to administer the immunization."[2]

Currently, at this Institute, investigational vaccines developed for defense against biological weapons are routinely administered to at-risk personnel in dosages and schedules independent for each vaccine. We reasoned that combination of these antigens into a single multivalent vaccine might afford an adjuvant effect as well as the obvious advantage of reducing the number of inoculations and the attendant logistical requirements.

We postulated that such a product should meet the following criteria: (1) vaccine should be contained in one vial; (2) a minimum number of injections should be required; (3) each component in the combination must evoke a response equivalent to that of the monovalent product; and (4) safety and acceptability must meet standards established by the U. S. Public Health Service.

We elected to employ investigational vaccines that have been approved by the Army Investigational Drug Review Board for use in man. Selection was thereby limited to 9 candidate products; 3 live vaccines, 1 toxoid and 5 inactivated particulate vaccines. It was arbitrarily decided to combine the latter group, i.e. vaccines for western equine encephalitis (WEE), eastern equine encephalitis (EEE), Rift Valley fever (RVF), Chikungunya (CHIK) and Q fever (Q).

Before initiating the study, it was of primary importance to determine the physical compatibility of the combination. With an antigenic mass equivalent to 1 human dose assigned a value of 1, each lyophilized vaccine was suspended in diluent to concentrations of 1, 2, 3 or 4. When mixtures of corresponding concentrations of the 5 vaccines were prepared, appearance of the combined product was similar to reconstituted monovalent preparations; there was no visible precipitate or change in turbidity. All combinations met USPHS criteria for safety when tested in mice and guinea pigs.

Having established that the 5 components were compatible, we determined median effective doses (ED_{50}) for each vaccine as a measure of its efficacy against a standard challenge with the homologous virulent strain. Initially we hoped to conduct this evaluation in a single animal species; however,

titrations of intraperitoneal (IP) median lethal doses (LD_{50}) or, in the case of Q fever, the IP median fever dose (FD_{50}) with standard challenge strains indicated that no single species was susceptible to all 5 diseases. Therefore, WEE, EEE and RVF vaccines were evaluated in golden Syrian hamsters, Q fever in Hartley strain guinea pigs and CHIK vaccine in weanling Swiss CD-1 mice. Groups of animals were immunized with a series of dilutions of each monovalent preparation (Table II). The standard schedule for immunization of hamsters and guinea pigs consisted of 2 doses administered on days 0 and 28; animals were challenged 14 days later with the respective homologous challenge strains. Because of increasing variability in the resistance of adult mice to CHIK virus, a modified schedule was employed; immunization consisted of 2 IP doses administered on days 0 and 7 and intracerebral (IC) challenge on day 14. The results of these assays are shown in Table III. Median effective doses were calculated by the method of Reed and Muench[3] and represent that volume of vaccine which protected 50% of animals against death or fever. The criterion for a febrile response was rectal temperature $\geq$ 40 C for at least 2 consecutive days. Studies with EEE and RVF indicated that ED_{50} values remained essentially the same for different levels of challenge. Initially, we intended to combine 50 ED_{50} of each component in a 1-ml volume; however, these results indicated that the volume for 1 immunizing dose of such a combination would be 3.1 ml, of which 1.5 ml were RVF vaccine. By reducing the RVF dose to 13 ED_{50} or 0.5 ml, the volume could be reduced to 2.2 ml, an amount still considered excessive for administration to experimental animals or man. Further reduction in volume to 0.85 ml per dose was achieved by resuspending the first 4 lyophilized vaccines in fluid CHIK vaccine. Characteristics of the experimental pentavalent product are shown in Table IV. In consideration of the reconstitution procedure, it was not unexpected that the mixture was hypertonic to body fluids. Despite hypertonicity, the pentavalent combination met USPHS criteria for safety when tested in mice and guinea pigs. This combination was employed for immunizing hamsters and guinea pigs but the volume was still excessive for weanling mice. Consequently, further modification was required. Volume and composition of the immunizing dose for each species are shown in Table V. Median effective doses for all components, but particularly for CHIK vaccine, were decreased for studies in mice. Schedules for immunization and challenge were the same as described for assay of the monovalent products.

Serological responses, as measured by hemagglutination-inhibition (HI) or complement fixation (CF) tests, were evaluated for comparable groups of immunized animals immediately prior to challenge. Results are shown in Table VI. Because of technical difficulties, serological evaluation of responses to CHIK antigen was not performed but antigenicity of the other components was essentially unaffected by combination. Although not shown in this table, the immunization schedule employed for WEE, EEE and RVF monovalent vaccines produced essentially no cross-reacting antibodies. There was no statistical difference between antibody responses to WEE, EEE, or RVF antigens between pentavalent and monovalent vaccines.

TABLE II. STANDARD SCHEDULES FOR VACCINE EVALUATION

SPECIES	IMMUNIZATION		CHALLENGE		
	Vaccine	Schedule (Day)	Dose LD_{50}	Route	Day
Hamsters	WEE	0, 28	10^3	IP	42
	EEE	0, 28	10^3	IP	42
	RVF	0, 28	10^3	IP	42
Guinea pig	Q	0, 28	10^3 [a/]	IP	42
Weanling mouse	CHIK	0, 7	10^3	IC	14

a. Median fever dose (FD_{50}).

TABLE III. ED_{50} FOR COMPONENTS OF A PENTAVALENT VACCINE

ASSAY ANIMAL	MONOVALENT VACCINE	CHALLENGE DOSE LD_{50}	ED_{50} µl
Hamster	WEE	630	3
	EEE	40,000	11
		200	9
	RVF	1,000	38
		31,000	31
Guinea pig	Q	500 [a/]	2
Weanling mouse	CHIK	1,600	17

a. FD_{50}.

TABLE IV. CHARACTERISTICS OF THE PENTAVALENT VACCINE

pH	7.65
Specific gravity	1.023
Total protein	2.4 gm/100 ml
Osmolality	736 mosmol
Na	358 mEq/L
K	13 mEq/L
USPHS SAFETY TEST:	
Mouse	Passed
Guinea pig	Passed

TABLE V. VOLUME AND COMPOSITION OF PENTAVALENT IMMUNIZING DOSE

SPECIES	VOLUME ml	ED_{50} (ml)				
		WEE	EEE	RVF	CHIK	Q
Hamsters and guinea pigs	0.85	50	50	13	50	50
Weanling mice	0.25	25	25	10	10	25

TABLE VI. SEROLOGICAL TITERS FOLLOWING IMMUNIZATION

TEST	TEST ANTIGEN	GEOMETRIC MEAN TITER		
		Pentavalent	Monovalent	Control
Hemagglutination-Inhibition	WEE	240	123	5
	EEE	63	30	11
Complement fixation	RVF	4	4	1
	Q	237[a]	447	ND[b]

a. Significant difference between groups immunized with pentavalent or monovalent vaccines ($p < 0.05$, t-test).
b. Not tested.

However, the pentavalent preparation was somewhat less effective than a monovalent vaccine for production of antibodies against Q fever ($p < 0.05$). The significance of the difference and of the apparently poor serological response to RVF vaccine is difficult to evaluate in view of the data shown in Table VII. It can be seen that there was no difference in protection afforded by pentavalent and monovalent vaccines against challenge with WEE,

TABLE VII. EFFICACY OF AN EXPERIMENTAL PENTAVALENT VACCINE

CHALLENGE		PROTECTED/TOTAL (%)		
Organism	LD_{50}	Pentavalent Vaccine	Monovalent Vaccine	Unimmunized Controls
WEE	11,130	10/10 (100)	10/10 (100)	0/8 (0)
EEE	100	10/10 (100)	10/10 (100)	0/8 (0)
RVF	1,000	10/10 (100)	10/10 (100)	0/8 (0)
Q	1,160[a]	8/10 (80)	10/10 (100)	1/10 (10)
CHIK	1,800	23/24 (96)	21/24 (88)	2/20 (10)

a. Median fever dose.

EEE or RVF. Although 2 animals given pentavalent vaccine became febrile following challenge with Q, Chi square analysis indicated that there was no significant difference between response to pentavalent and monovalent vaccines. The same may be said for responses to CHIK challenge.

A comparison of ED_{50} for 4 components in the pentavalent preparation and their corresponding monovalent vaccines is presented in Table VIII.

TABLE VIII. ED_{50} VALUES FOR ANTIGENS USED SINGLY AND IN COMBINATION

CHALLENGE ORGANISM	STATE OF HOMOLOGOUS VACCINE	ED_{50} µl
WEE	Monovalent	3.4
	Pentavalent	4.5
EEE	Monovalent	10.8
	Pentavalent	6.4
Q	Monovalent	2.1
	Pentavalent	2.8
CHIK	Monovalent	17.0
	Pentavalent	8.5

RVF antigen was not evaluated because facilities for challenge were no longer available. Variation between the monovalent antigen and its homologous analogue in the pentavalent product is within the limits of accuracy for the bioassay technique, indicating that the combination effected neither enhancement nor suppression of immunogenicity.

In summary, utilizing 5 formalin-treated investigational vaccines, currently being administered to at-risk personnel, we have prepared a pentavalent vaccine and tested it in appropriate animal models. All components were physically compatible, passed safety tests and retained immunogenicity as determined by challenge with virulent homologous strains.

If we are to progress to studies in man with this penavalent product, or any other combination of antigens, a number of factors must be considered. Obviously consideration must be given to decreasing volume of the immunizing dose while maintaining isotonicity of the product. Although this can be accomplished by inclusion of a dialysis step, purification and concentration of investigational antigens by continuous flow, density gradient, zonal ultracentrifugation, controlled-pore glass bead chromatography or other methods appear to be preferable. There is evidence to suggest that purification and concentration of vaccine results not only in increased potency but also in increased stability of all components in a combined vaccine.[4,5]

For any combination of antigens, studies such as we have reported would have to be performed to ensure immunological balance of components

and thereby avoid the possibility of mutual competition or interference. Furthermore, as von Magnus has advocated, components selected for inclusion in a polyvalent product preferably would be limited to those that are without significant side effects.[6]

Basic to any of these considerations is the question of desirability of combined vaccines for military personnel. As alluded to previously, certain advantages may be accrued: improved acceptance by vaccinees, increased efficiency in terms of logistics and use of medical or paramedical personnel, simplification of immunization schedules and records, and the potential for rapidly developing broad immunogenic coverage.

In assessing the pentavalent vaccine under study, it is immediately apparent that the limited requirement for use of this product to at-risk personnel would tend to minimize all advantages except that of more rapid development of broad protection.

If these investigations are to be continued, additional areas to be considered could be: (1) development of one or more combined vaccines for military required routine immunizations and/or (2) application and extension of the Cole-McKinney concept of specific geographical coverage for arboviral diseases to protection against other infections.[7] Although considerable thought has been given to the desirability and advisability of combined vaccines for military personnel, a final conclusion has not been reached. Therefore, we would appreciate comments and guidance from the members of this Commission with regard both to significance and future direction of this work.

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MICRONUTRIENT METABOLISM AND THE INFECTION-INFLAMMATORY PROCESS

Robert S. Pekarek, Ph.D.*

Acute and chronic infectious diseases produce significant alterations in trace metal metabolism. Although concentrations of serum iron, copper and more recently zinc, have been measured in many clinical studies, relatively little was known concerning (1) the timing of onset of these alterations in relation to the stages of the infectious process; (2) the pathophysiologic mechanisms involved in altered trace metal metabolism; or (3) the functional roles of these metals during infection or inflammation.

In a recent series of prospective clinical studies in man, we have demonstrated that alterations in serum trace metal concentrations occur early in the incubation period after the initiation of either acute bacterial or viral infections.[1-5]

As shown in Figure 1, alterations in serum Zn and Fe concentrations were examined serially in groups of volunteers receiving either sandfly fever virus (Medical Division Protocol FY 70-1), live attenuated Venezuelan equine encephalomyelitis (VEE) virus vaccine (MDP FY 69-1), Salmonella typhi (University of Maryland Contract No. DA-49-193-MD-2867), or Francisella tularensis (MDP FY 68-4). Daily serum Zn and Fe values were determined by atomic absorption spectrophotometric technique and compared to the subjects baseline values ± 1 SE, as illustrated by the shaded horizontal bands. Serum Zn and Fe concentrations fell precipitously early after exposure to the respective microorganisms and just prior to the onset of any detectable febrile illness. These serum metal changes were significantly different from those that could be explained on the basis of day-to-day variability or differences among individuals.

Figure 2 illustrates in somewhat more detail, the trace metal profile in man during experimentally induced sandfly fever, a benign self-limited viral infection. Not only were the early significant decreases in serum Zn and Fe concentrations observed, but a concomitant rise in serum Cu concentrations, also, were observed. Further, it may be noted, that in the case of an illness of short duration, such as sandfly fever, both the serum Zn and Fe concentrations returned quickly to preexposure baseline values with the remission of the illness in these, normally, healthy volunteers. The delay in the return of Cu to control values is most likely due to the half-life of its carrier protein, ceruloplasmin, which also increases significantly during acute infections.[6] As shown at the bottom, urinary Zn and Cu excretions also decreased significantly just prior to, or with the onset of symptomatic illness. Although not significant, there was a tendency for urinary Zn excretion to increase during the convalescent period following sandfly fever.

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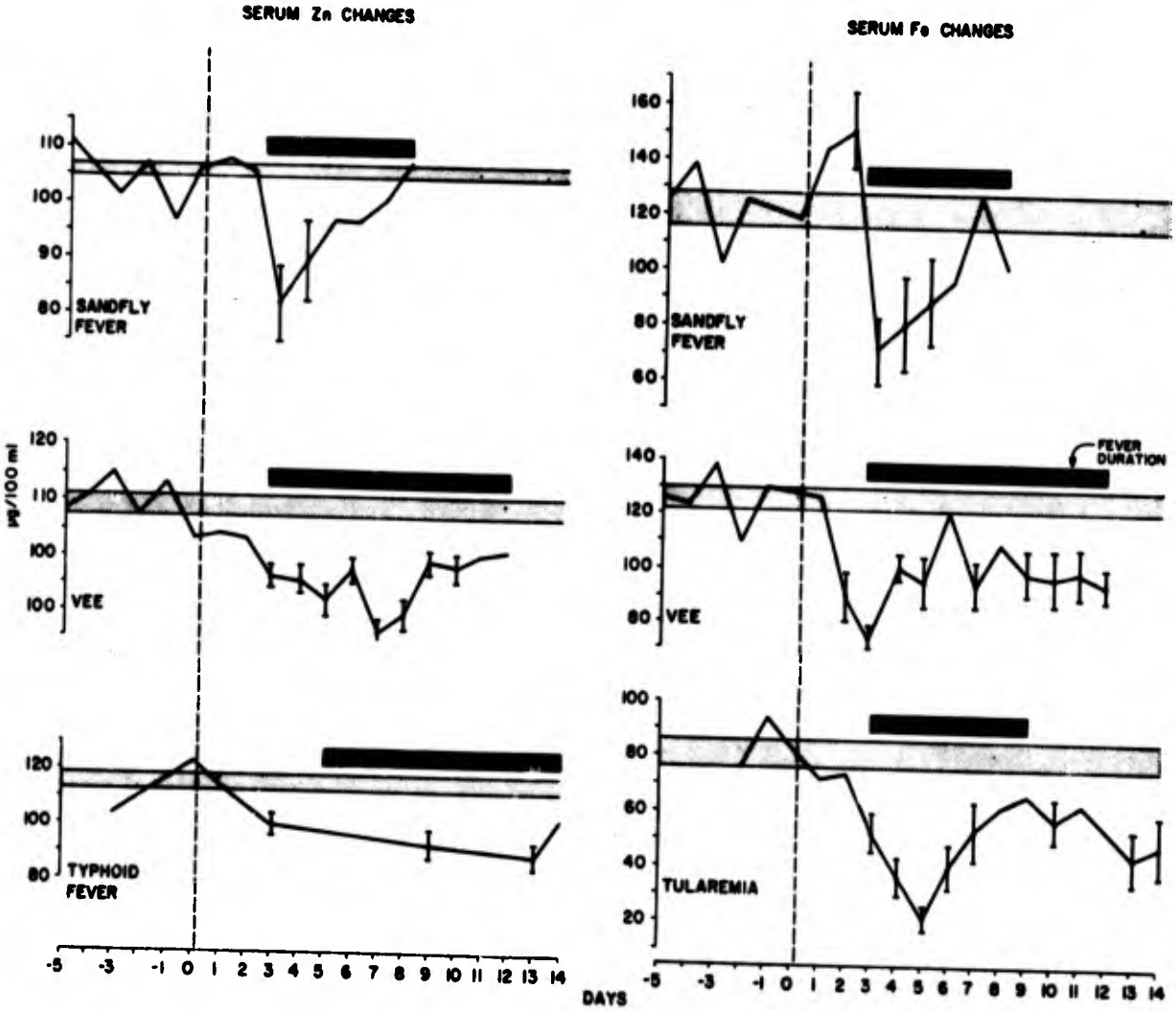

FIGURE 1. EFFECT OF ACUTE BACTERIAL AND VIRAL INFECTIONS ON SERUM Zn AND FE CONCENTRATIONS IN MAN.

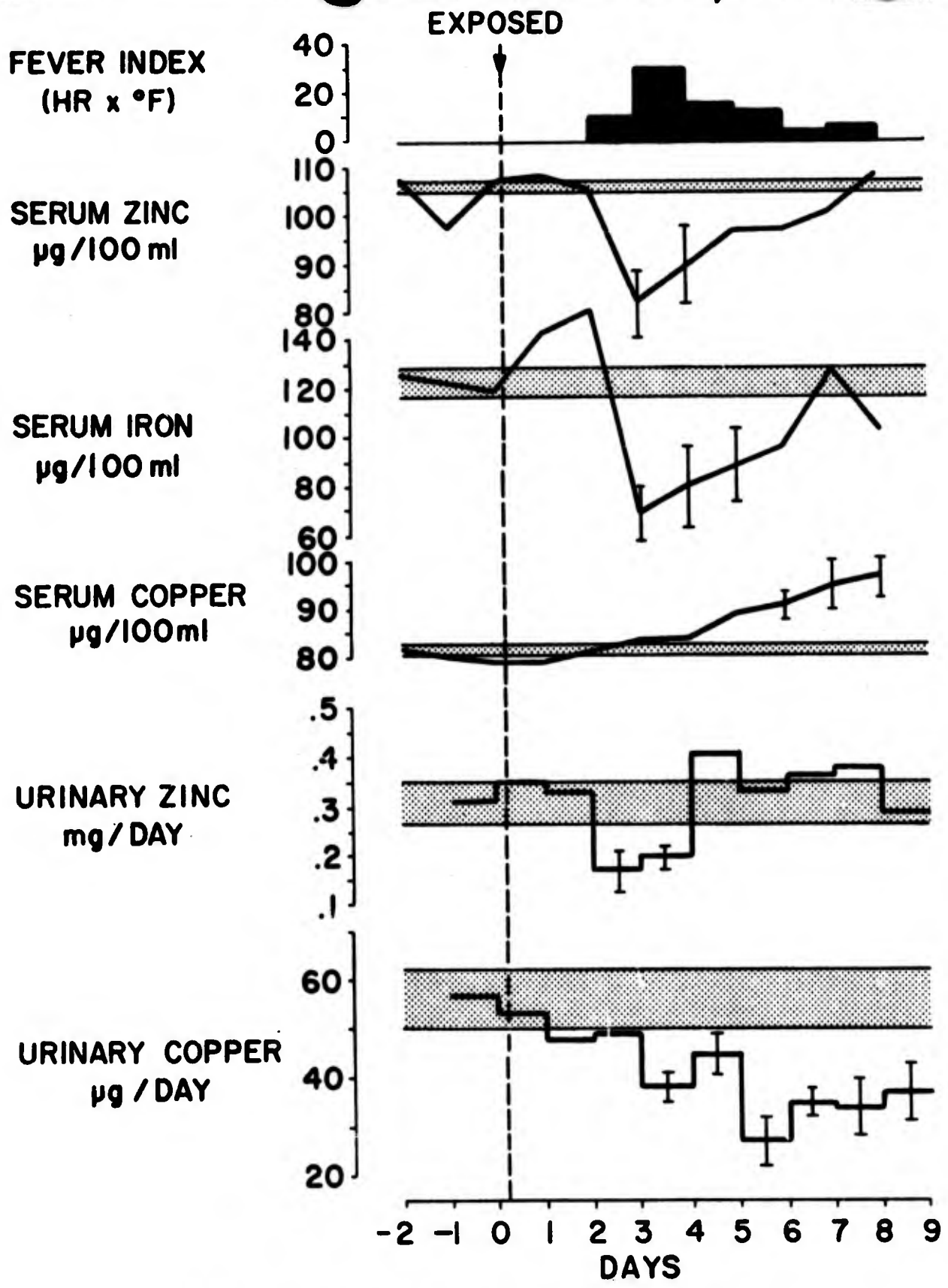

FIGURE 2. EFFECT OF SANDFLY FEVER VIRUS ON SERUM Zn, Fe AND Cu AND URINARY Zn AND Cu CONCENTRATIONS IN MAN.

While anorexia may occur during febrile or symptomatic illness, the previously described studies indicated that the early decreases in serum Zn and Fe concentrations, were not the result of increased excretion or decreased gastrointestinal absorption, but rather, were due to a rapid redistribution of the two metals within host tissues.

To demonstrate this, rats were infected subcutaneously (SC) with approximately 1 x 10^7 Diplococcus pneumoniae organisms. Serum, muscle and perfused liver samples were obtained 24 hr later and analyzed for their Zn content. The results were compared to data obtained from control rats receiving sterile normal pyrogen-free saline and are shown in Figure 3. As expected, serum Zn concentrations were significantly depressed in the infected rats as compared to the sham-inoculated controls. However, the pneumococcal infection also induced a rather marked hepatic uptake of Zn, as well as producing a significant increase in the Zn content of muscle.

It now appears evident that the observed alterations in trace metal me abolism are activated as part of the host's response to infection and possibly other inflammatory stresses. However, until recently, the pathophysiologic mechanisms leading to these alterations in trace metal metabolism have remained unknown. There is now evidence that most microorganisms, if not all, induce fever indirectly by stimulating the release of a circulating endogenous pyrogen from phagocytizing cells.[7] Therefore, we theorized that the observed alterations in trace metal metabolism also might be mediated via some similar type of endogenous humoral mechanism during the infection.[3] In attempting to evaluate this mechanistic concept further in a variety of animal models, we found and reported[8] that an endogenous mediator of altered serum Zn and Fe metabolism could be recovered from the serum of animals within 2 hr after infection or endotoxin intoxication, and in sufficient concentration such that its serum Zn- and Fe-depressing effect could be transferred to normal recipient animals of the same or different species.

Since a cross-species susceptibility to the endogenous mediating factor appeared to exist, we attempted to demonstrate its presence in the serum of humans with an acute infection. Last year we reported that endogenous mediator could be detected by a bioassay in the serum on the first day of febrile illness from a group of volunteers infected with Salmonella typhi.[3,5] This work has been extended further. Serum samples from febrile patients with documented D. pneumoniae infection and patients with mixed bacterial infections were obtained from Dr. Klainer (Ohio State University Contract No. DADA-17-68-C-8080). Normal serum, as well as serum from the groups of infected subjects, were millipore-filtered and injected intraperitoneally (IP) into respective groups of rats. The shaded horizontal band in Figure 4 represents the mean serum Zn concentration in normal rats ± 1 SE. The previously reported data on the volunteers with typhoid fever are also included in Figure 4. As shown normal human serum elicits no significant effects on serum Zn concentrations in the rat. By contrast, the filtered serum from infected individuals produced significant depressions in serum Zn concentrations within 4 hr. The same postinfection serum, if heated at 90 C for 30 min, produced no significant changes. Thus, a heat-labile

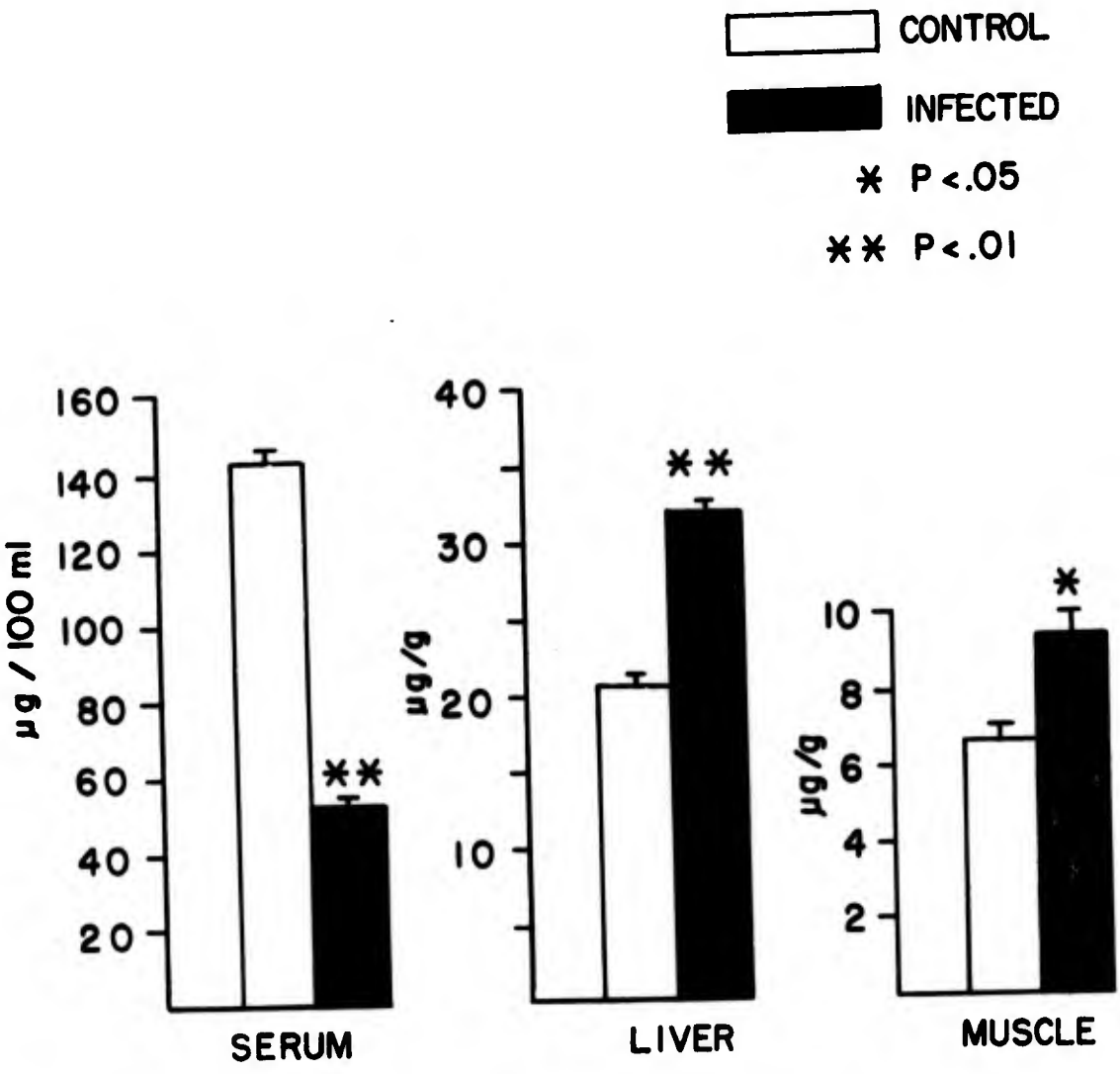

FIGURE 3. EFFECT OF *D. PNEUMONIAE* INFECTION OR THE DISTRIBUTION OF Zn IN THE RAT.

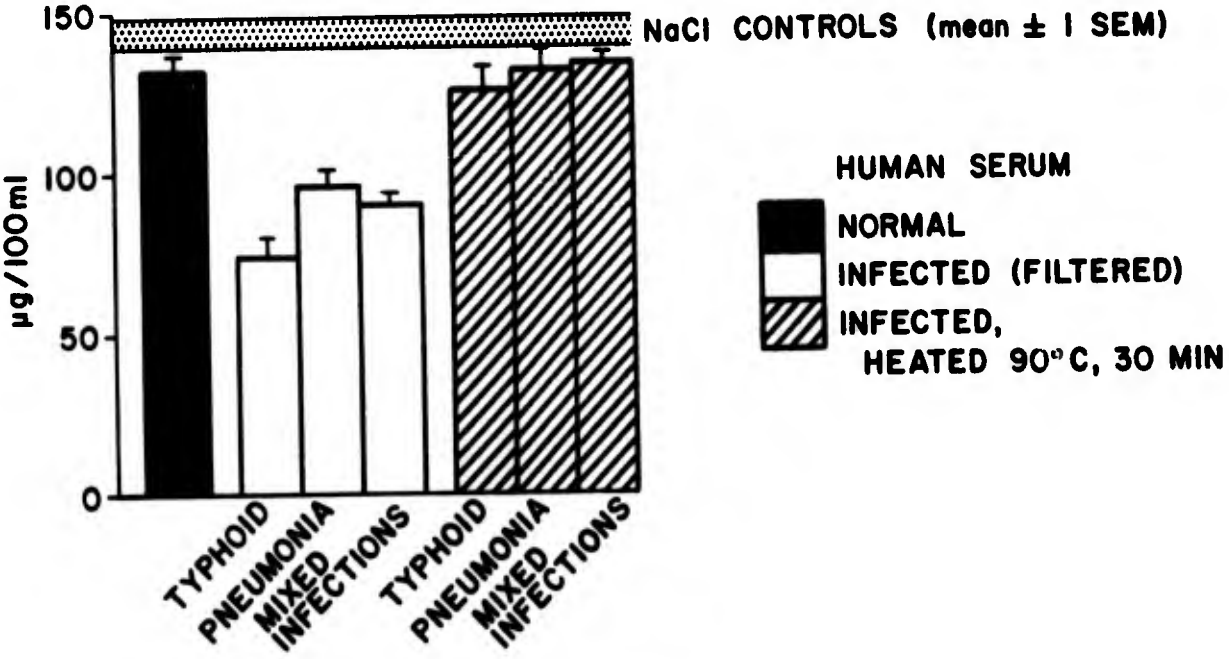

FIGURE 4. HUMAN MEDIATOR EFFECTS ON SERUM Zn OF RATS GIVEN 1 ml HUMAN SERUM IP.

endogenous mediator of altered trace metal metabolism appears to be present in the serum of a febrile individual; the fact that the effect of this mediator can be transferred to a laboratory animal may prove to have potential diagnostic value.

Further studies in our laboratory have shown that this endogenous mediator is released, in part, by polymorphonuclear (PMN) leukocytes. We have initially characterized leukocytic endogenous mediator or LEM as a heat-labile, non-dialyzable, low molecular weight protein, which is soluble in some organic solvents and has a certain amount of cross-species susceptibility.[8]

Since the observed alterations in Zn and Fe metabolism during infection and other inflammatory stresses appear to represent a redistribution and sequestering of these substances within the host, then leukocytic endogenous mediator or LEM, if truly an intermediate, should stimulate a redistribution of both metals as suggested by transfer studies. To test the effect of LEM on the tissue distribution of Zn and Fe, LEM was first prepared from PMN leukocytes of rat peritoneal exudates. Test and control rats were pulse labeled with either ^{65}Zn or ^{59}Fe. After 18 hr, the test rats were administered 1 ml of LEM, while controls received sterile normal pyrogen-free saline. After an additional 6 hr, control and test rats were killed and the distribution of the ^{65}Zn and ^{59}Fe in the various tissues, organs and plasma of the test and control groups were determined.[9]

As shown in Figure 5 the LEM, indeed, produced a significant redistribution of the metals. The liver appeared to be one of the main target organs, showing a significant uptake of both metals. The role of functioning liver in taking up Zn and Fe during most infections is further supported by observations that with acute liver cell dysfunction, as in acute infectious hepatitis, serum values for both metals tend to rise rather than to fall.

The metabolism of Cu also has been shown to be significantly altered during infection. The increase in serum Cu in our studies was shown to be slightly delayed when compared to the lowering of serum Zn and Fe concentrations.[2-5] The delay in the release of Cu into the serum is most likely due to the time necessary to produce increased amounts of ceruloplasmin. Ceruloplasmin synthesis has been shown to be necessary for removal of Cu from its storage pool in the liver;[10] Kleinbaum[5] has reported that infection-induced increases in serum Cu concentrations were in the indirect-reacting Cu fraction or ceruloplasmin-bound Cu and were related to the stimulation of apoceruloplasmin synthesis. In order to examine this, we designed an experiment to see if LEM also would induce or mediate a change in Cu metabolism. Rats were administered 1 ml IP of a rabbit LEM preparation. An equal portion of LEM was heat-inactivated. This was done as a control to insure that observed changes were not due to any contaminating heat-stable endotoxins. A third group of rats were inoculated with an equal volume of pyrogen free normal saline and served as an added set of controls.

Rats from each group were bled 6, 12, and 24 hr after administration. Serum Cu and ceruloplasmin concentrations were determined. As shown in Figure 6, both were significantly increased by 12 hr and remained so at 24 hr.

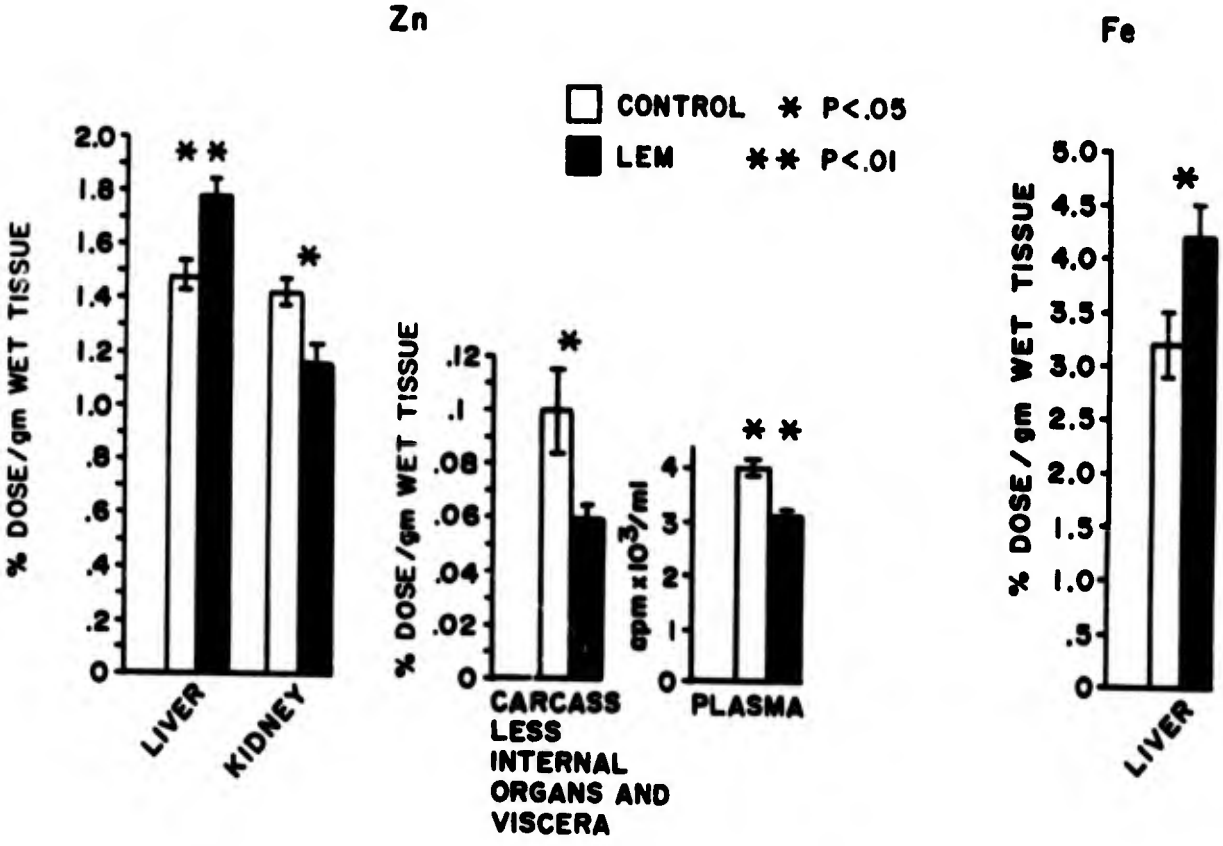

FIGURE 5. EFFECT OF LEM ON THE TISSUE DISTRIBUTION OF ^{65}Zn AND ^{59}Fe IN THE RAT.

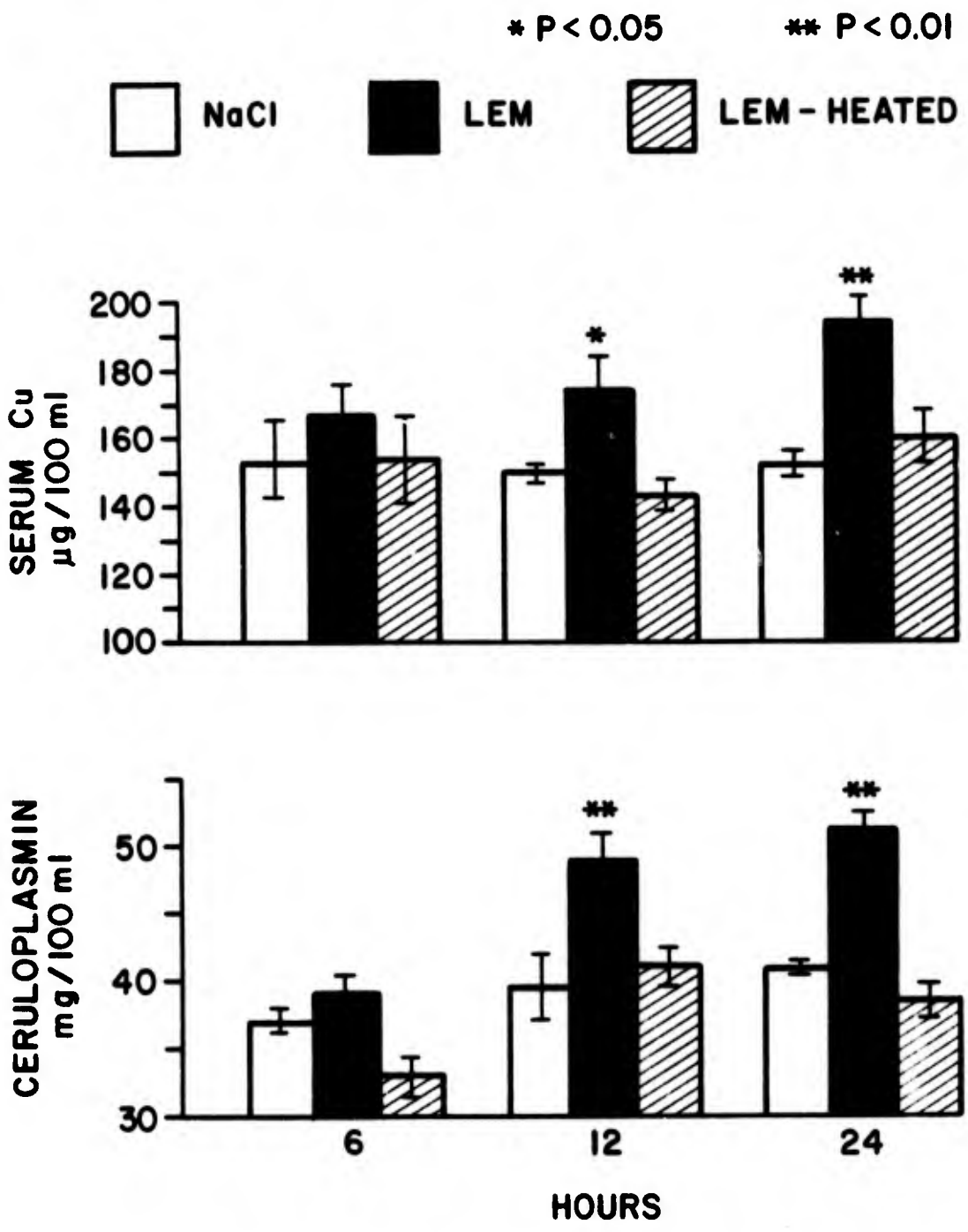

FIGURE 6. THE EFFECT OF LEM ON SERUM Cu AND CERULOPLASMIN CONCENTRATION IN THE RAT 6, 12, AND 24 HR POSTADMINISTRATION.

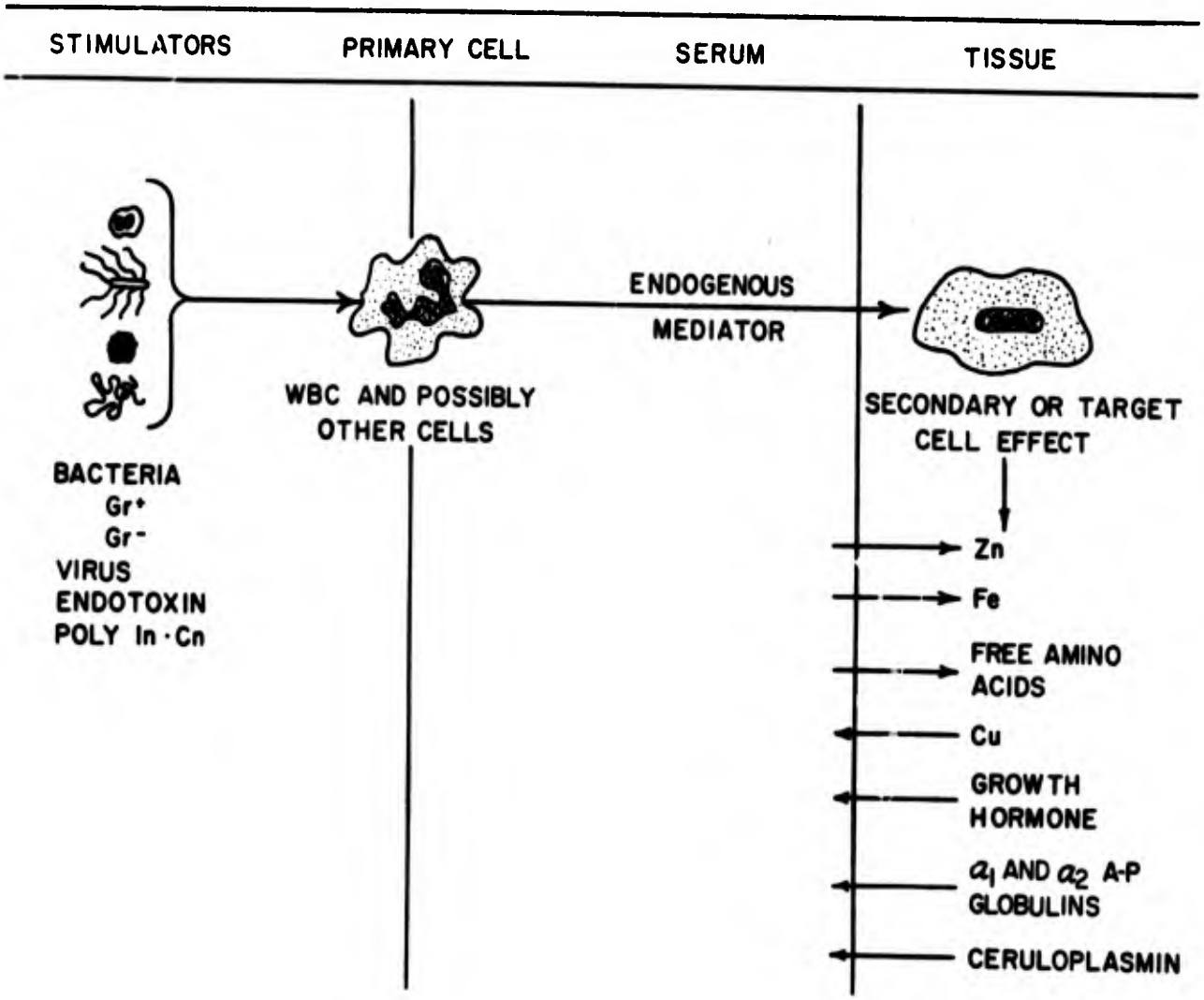

FIGURE 7. CURRENT CONCEPTS OF THE ROLE OF ENDOGENOUS MEDIATOR(S).

The rise in Cu and ceruloplasmin was slightly delayed in timing as compared to the changes in Zn and Fe. However, the significant increase in ceruloplasmin suggests that LEM stimulated either an increased synthesis or release of this metal-carrying protein.

Figure 7 shows the current mechanistic concepts on how the inflammatory process stimulates certain nonfebrile host responses. As shown to the left, a variety of inflammation-inducing stimulators have been shown to be able to activate or react with both PMN leukocytes, macrophages and possibly other cells. These cells, in turn, release into the circulation an endogenous humoral factor or factors, which can act either directly or indirectly on certain target cells, resulting in a variety of metabolic responses. The present report has shown that LEM induces significant alterations in Zn, Fe and Cu metabolism. However, LEM has more far reaching effects of host metabolism. As shown, LEM stimulates a flux of amino acids into the liver,[11] the synthesis and release of α_1 and α_2 acute phase globulins into the serum,[12] and the release of growth hormone.[13] The effects of this hormone-like substance or substances on these parameters, as well as on hepatic RNA synthesis, are the topics of discussion in the reports which follow.

The reason why alterations in the metabolism of Zn, Fe, Cu, and other trace metals take place during infection is not fully understood nor are the functional roles of the redistributed metals during the diseased state. However, it is known that Fe is diverted to, and held in, reticuloendothelial system tissue during infection, thus making it unavailable to the bone marrow for erythropoiesis.[14] In instances of repeated or chronic infections, sequestration of Fe, can lead, to anemia of infection, which resembles iron deficiency anemia. On the other hand, both Zn and Fe have been shown to be essential co-factors for many metalloenzymes and to be important for ribosomal integrity and for protein synthesis. The possible roles of Zn and Fe as either essential co-factors or precursors for the early anabolic responses to infection will become more evident in the succeeding reports and in the overall concept report by Dr. Wannemacher.

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EFFECTS OF INFLAMMATORY PROCESSES ON SERUM PROTEIN-BOUND SUGARS AND GLYCOPROTEIN CONCENTRATION

Gary L. Cockerell, Captain, VC*

All proteins isolated from plasma, with the notable exception of albumin, have been shown to contain covalently linked carbohydrate, and are accordingly termed glycoproteins.[1] The exact significance of this protein-bound carbohydrate is not known in all cases and it is for this reason that we have for some time been involved in an evaluation of plasma glycoproteins in the early diagnosis of infectious illness.

An appreciation of the amount of carbohydrate covalently attached to protein in normal serum is gained from Table I. The major protein-bound

TABLE I. SERUM PROTEIN-BOUND CARBOHYDRATE (Mean ± SEM)

CARBOHYDRATE	MAN		MONKEY		RAT	
	mg/100 ml	%	mg/100 ml	%	mg/100 ml	%
Hexose	93.5 ± 2.5	42.5	92.9 ± 7.0	40.1	146.0 ± 6.3	37.2
Hexosamine	77.2 ± 2.4	35.0	81.7 ± 0.7	35.3	118.3 ± 4.7	30.1
Sialic acid	40.0 ± 1.2	18.2	49.7 ± 1.0	21.4	119.1 ± 6.3	30.5
Methylpentose	9.3 ± 0.3	4.3	7.2 ± 0.1	3.2	10.2 ± 0.1	2.2
TOTAL		100.0		100.0		100.0

carbohydrate moieties are hexose, hexosamine, sialic acid and methylpentose. In the species shown here, man, rhesus monkey and albino rat, protein-bound hexose is found in greatest concentrations followed in generally decreasing order by hexosamine, sialic acid and methylpentose. The last one contributes only a minor percentage to total concentration. It can be seen that concentrations of each sugar moiety differ among the species. In general, quantities in monkey serum are similar to those in serum from man whereas much higher concentrations are present in rat serum. The total amount of carbohydrate covalently linked to serum proteins is twice, and in some species such as the rat, nearly 3 times as great as the concentration of blood glucose in normal physiological states.

Another method of illustrating this protein-bound carbohydrate among the serum proteins is cellulose acetate electrophoresis of serum and subsequent staining with periodic acid - Schiff (PAS) reagent which selectively

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reacts with the carbohydrate portion of the glycoprotein molecule.[2] Examples of stained cellulose acetate electropherograms and the resulting densitometric scans are shown in Figure 1. The small albumin peaks for all 3 species reflect the small amount of covalently bound carbohydrate in this fraction. Each species, it should be noted, is characterized by its own particular glycoprotein electrophoretic pattern reflecting the amount of carbohydrate attached to the proteins migrating with this fraction. Again, monkey appears similar to man, peaks for man, however, being more distinct and separated. The rat pattern shows a characteristic spike in the α_1 fraction corresponding to an intensely staining α_1 band on the strip.

A great number of studies, particularly during the past 20 years, has indicated that the concentration of serum glycoproteins may be markedly increased in humans or experimental animals during a wide variety of pathological conditions, including neoplastic disease, tuberculosis, diabetes, pregnancy, rheumatic fever, and fractures or thermal injury.[3] Several investigators at our Institute have been able to characterize changes in glycoproteins during various infectious processes.[4,5] One such study is illustrated in Figure 2. In this example, serum glycoprotein and protein alterations were evaluated in naturally occurring cases of falciparum malaria in soldiers in Vietnam.[6] Glycoprotein patterns are shown on the left and protein electrophoretic patterns on the right. Normal patterns appear at the top. Serum taken from patients during the acute stage of malaria revealed a tremendous increase in the α_1 glycoglobulin fraction. Moreover, patients developing secondary bacterial complications showed an increase in the α_2 fraction as well as α_1, whereas in persons with hepatic involvement the β fraction increased concomitantly. These glycoprotein changes occurred despite a relatively normal serum protein profile, indicating a greater sensitivity to change in the glycoproteins. The α_1 glycoglobulin spike seen in acute malaria is unique in that this particular pattern has not been seen in other diseases. This however, tends to be an exception to the rule since in most cases increases in glycoglobulins occur nonspecifically with regard to the eliciting agent.[7]

In an effort to elucidate patterns and possible mechanisms of these elevated glycoglobulin concentrations we compared localized inflammation and acute generalized infection in the rat. Inflammation was induced by the subcutaneous (SC) administration of 1 ml steam-distilled turpentine and infection, by the SC injection of 10^7 Diplococcus pneumoniae. Controls in both cases received 1 ml sterile normal saline given similarly. Turpentine-inflamed rats and their controls were killed at 48 hr and pneumococcal infected rats and their controls 36 hr after inoculation. Rats used for determination of survival time did not succumb to the turpentine inoculation whereas those infected with pneumococci died at 48-72 hr. This experimental protocol permits one to observe host responses to 2 different types of stress-producing agents, one a physical irritant and the other an infectious microorganism.

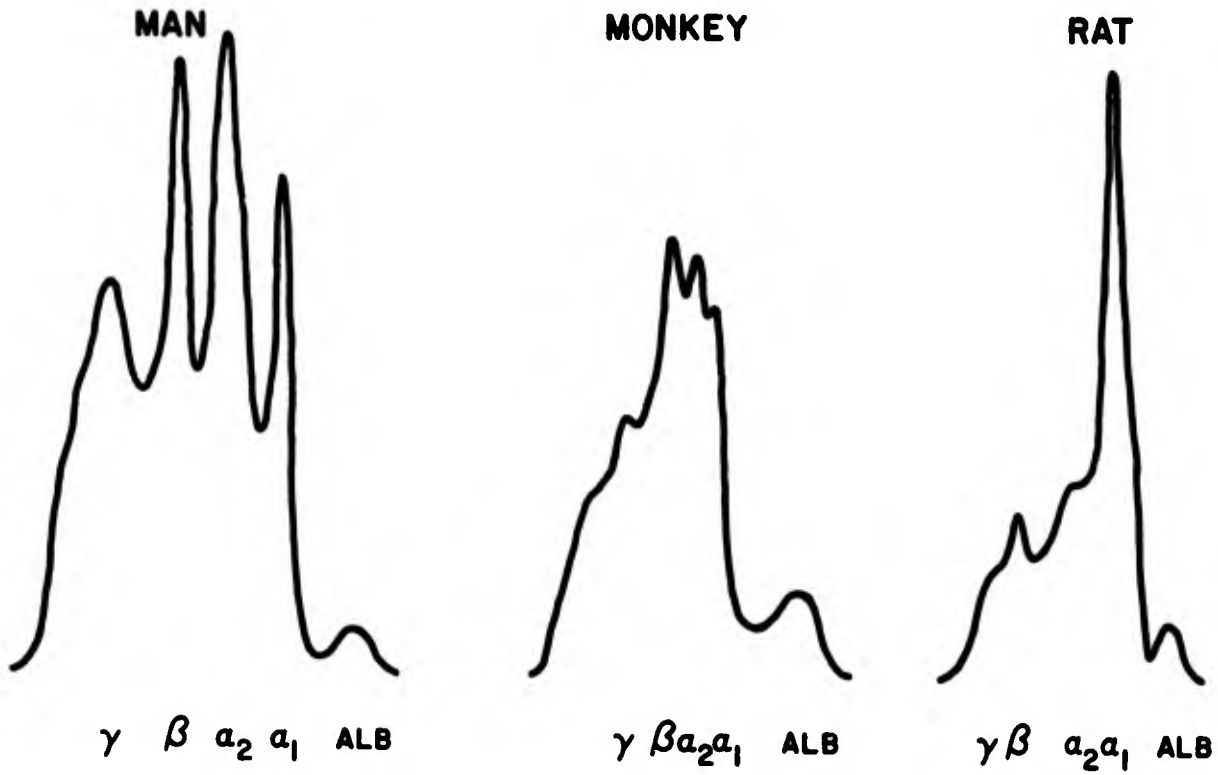

FIGURE 1. SERUM GLYCOPROTEIN DISTRIBUTION IN 3 MAN, RHESUS MONKEY AND ALBINO RAT.

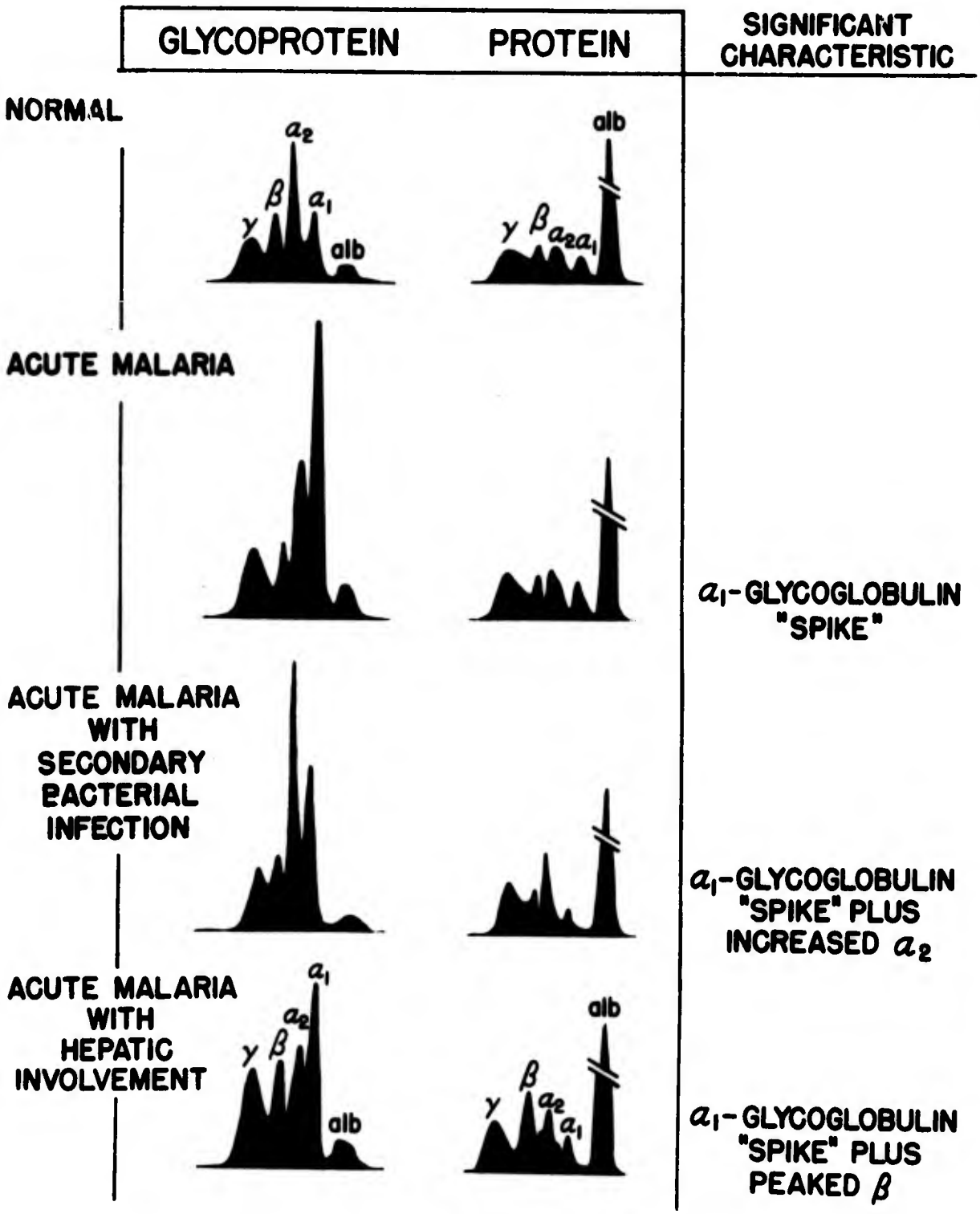

FIGURE 2. ELECTROPHORETIC PATTERNS IN MALARIA.[6]

An indication of host reaction to the 2 stresses is shown in Figure 3. Experimental rats are denoted by solid bars, controls by the slashed bars. Asterisks represent levels of significance. In contrast to the febrile state produced by pneumococci in infected rats turpentine-inflamed rats are not significantly febrile at the time they are killed. Differences in control values between the pneumococcus and turpentine models in this and subsequent parameters are most likely due to the fact that these two experiments were not run simultaneously.

Results of determinations of protein-bound carbohydrates for pneumococcal infected rats are depicted in Figure 4. The carbohydrate moieties are abbreviated as H, HA, SA and MP for hexose, hexosamine, sialic acid and methylpentose respectively. With the exception of methylpentose which contributes very little to total protein-bound carbohydrate concentration, all components developed significant increases in infected rats over controls. By comparison, turpentine inflammation produced the same pattern of alterations in protein-bound sugars.

The summation of the concentration of each of these protein-bound carbohydrate moieties in the plasma of a particular rat gives us an index of total plasma glycoprotein (TPGP) concentration. This figure together with a determination of total plasma protein (TPP) is shown in Figure 5. Particularly striking for the pneumococcal infected rats is the highly significant increase in total glycoprotein concentration in infected rats over controls. Turpentine-stressed rats reveal identical alterations in their total glycoprotein concentrations. The variable changes in total plasma protein as compared to dramatic increases in carbohydrate attached thereto may be interpreted as a preferential increase in plasma of carbohydrate-rich glycoproteins.

Cellulose acetate electrophoresis of plasma and subsequent reaction with PAS reagent was performed and this in combination with total glycoprotein concentration gave us plasma glycoprotein distribution in mg % as illustrated in Figure 6. It can be seen that for pneumococcal infected rats significant increases are seen in the α_1, α_2, and β glycoglobulin fractions. By comparison turpentine-inflamed rats reveal the same pattern of alteration in the α_2 and β fractions. No increase could be demonstrated for the α_1 fraction but this seems to be due to an unusually elevated value in the controls.

Occurrences of similar patterns of change to two diverse phlogogenic agents, one producing a systemic febrile infection and the other a localized afebrile inflammatory reaction, suggest that the alterations caused by each agent may have come about through similar mechanisms. Despite the apparent diversity in these agents, polymorphonuclear cell response were prominent in both the diplococcus infection[8] and the turpentine abscess.[9] Therefore, it seemed plausible that the common mechanism involved may be similar to the mediator released from stimulated leukocytes

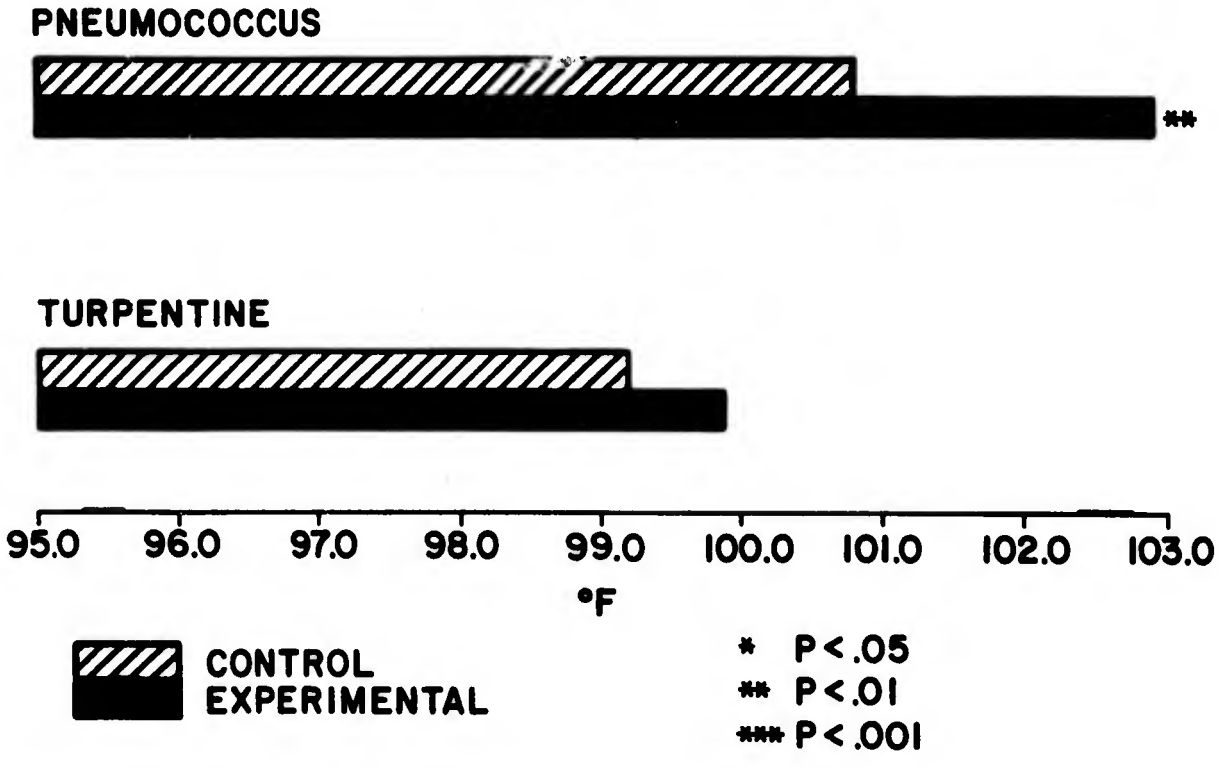

FIGURE 3. BODY TEMPERATURE IN RATS STRESSED WITH DIPLOCOCCI AND TURPENTINE.

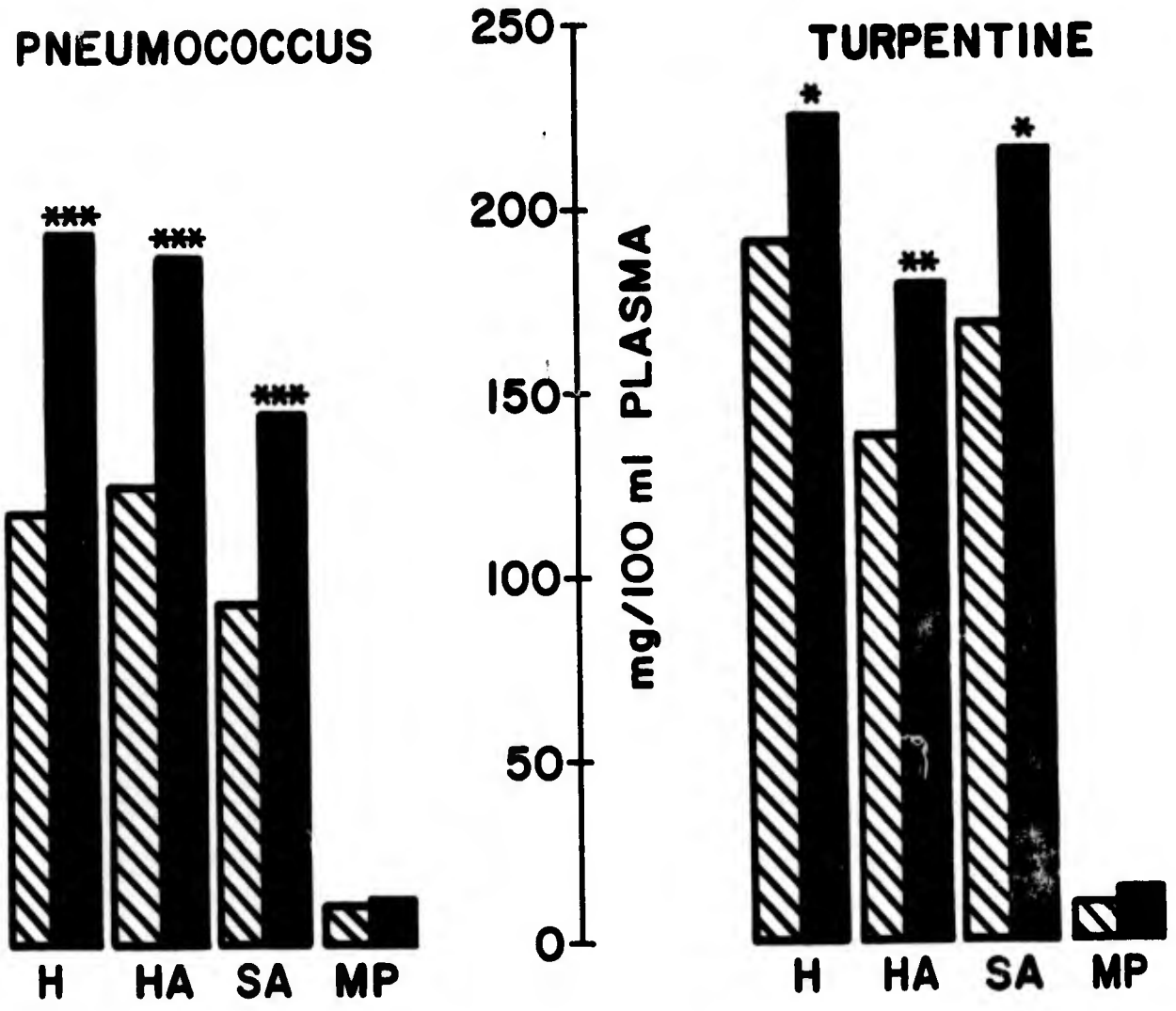

FIGURE 4. PROTEIN-BOUND CARBOHYDRATES DURING 2 STRESSES IN RATS. (See figure 3 for legend).

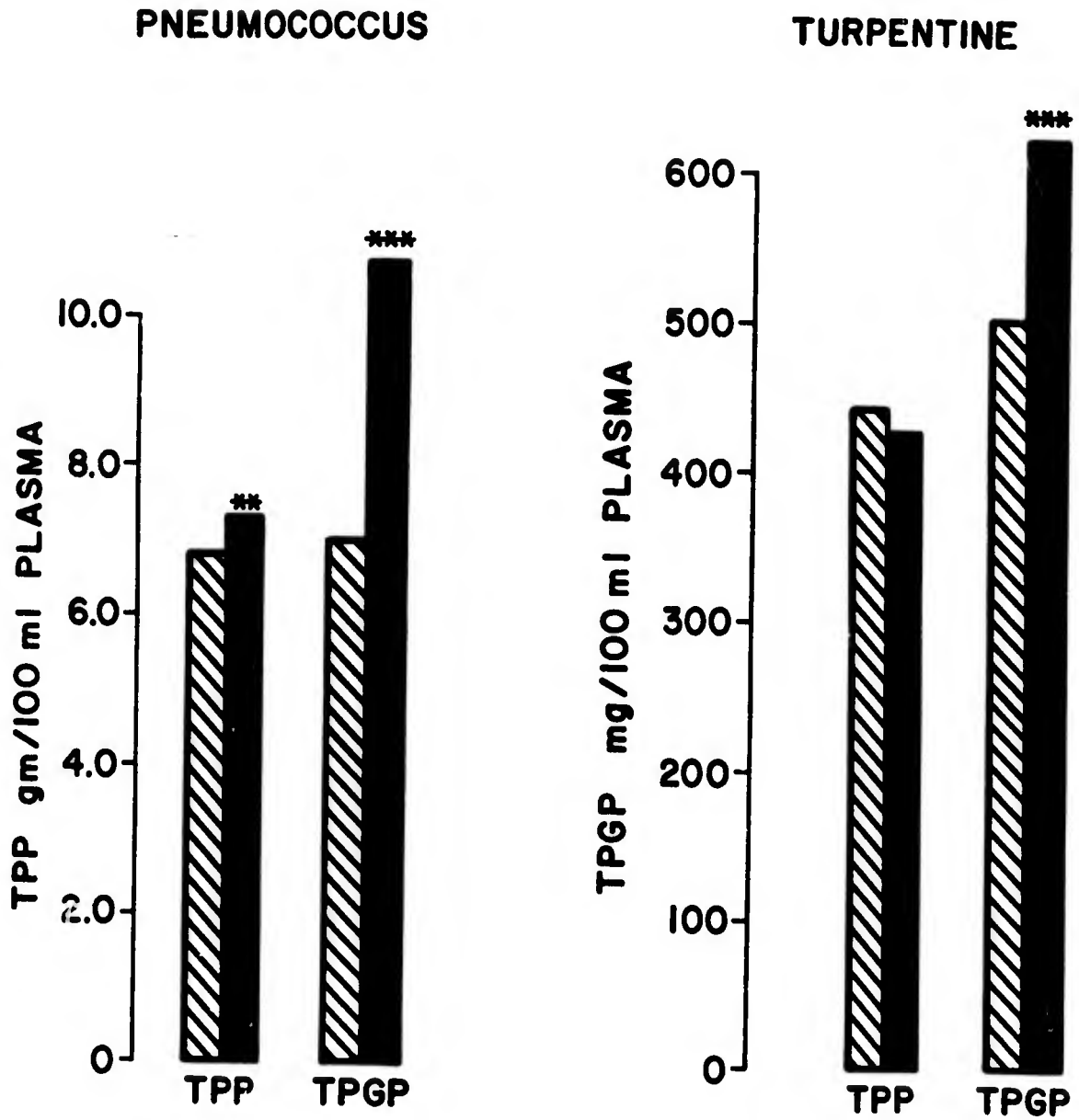

FIGURE 5. TOTAL PLASMA PROTEIN (TPP) AND GLYCOPROTEIN (TPGP) CONCENTRATIONS DURING 2 STRESSES IN RATS. (See figure 3 for legend).

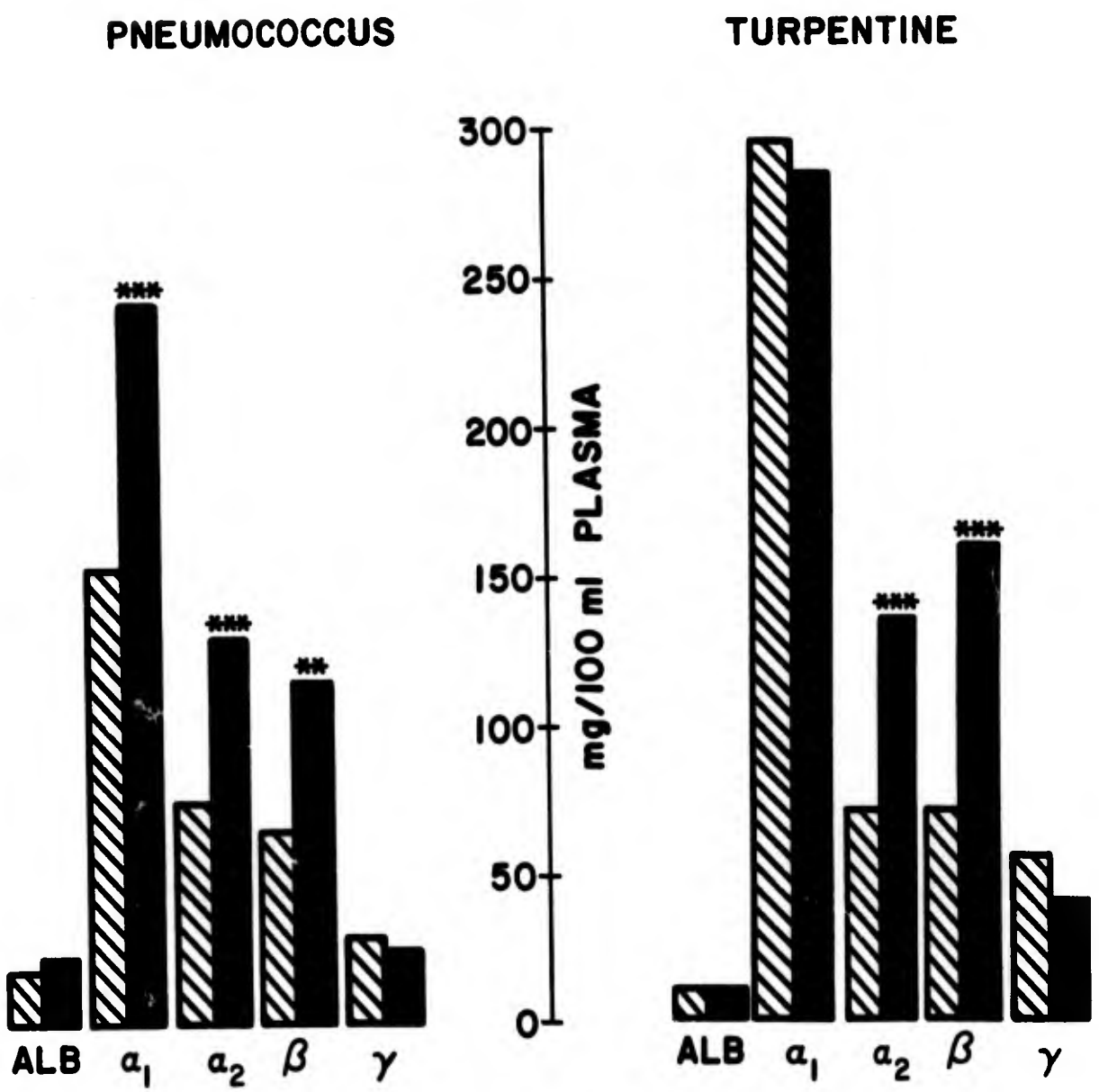

FIGURE 6. PLASMA GLYCOPROTEIN DISTRIBUTION DURING 2 STRESSES IN RATS. (See figure 3 for legend).

as described by Pekarek.[10] When 1 ml of this leukocytic endogenous mediator (LEM) was injected intraperitoneally (IP) into normal rats changes were seen as depicted in Figure 7. Patterns and directions of alterations are identical with those seen in pneumococcal infected or turpentine-inflamed rats. Protein-bound hexose, hexosamine and sialic acid were all increased, though not significantly, in rats receiving mediator over those receiving heat inactivated mediator. Since methylypentose contributes such a small percentage to total protein-bound carbohydrate its determination was not included. Total plasma glycoprotein, represented as the sum of the foregoing protein-bound carbohydrates, showed a significant increase over controls. In the percentage distribution of glycoproteins, increases were seen in α_1, α_2 and β globulin fractions, although only the β increase showed a significant difference from control. That several of these parameters are increased but fail to reach significant differences from controls may be explained by the single exposure to an exogenous source of mediator in this experiment as contrasted to a continued release of endogenously produced mediator in the turpentine- or pneumococcal stressed rats.

In summary, several conclusions can be drawn. The host response, as monitored through plasma protein-bound carbohydrates and glycoprotein patterns, is similar whether caused by a localized, afebrile, nonfatal inflammatory reaction or a systemic, febrile, fatal infection. This fact supports the conclusion that concentrations of plasma glycoproteins are elevated nonspecifically with regard to the inciting agent. Secondly, when taken in concert with evidence presented by Dr. Powanda in the following paper, demonstrating a similar movement of amino acids into the liver for pneumococcus, turpentine and mediator-injected rats, these glycoglobulin alterations appear to be at least in part triggered or mediated by a humoral factor.

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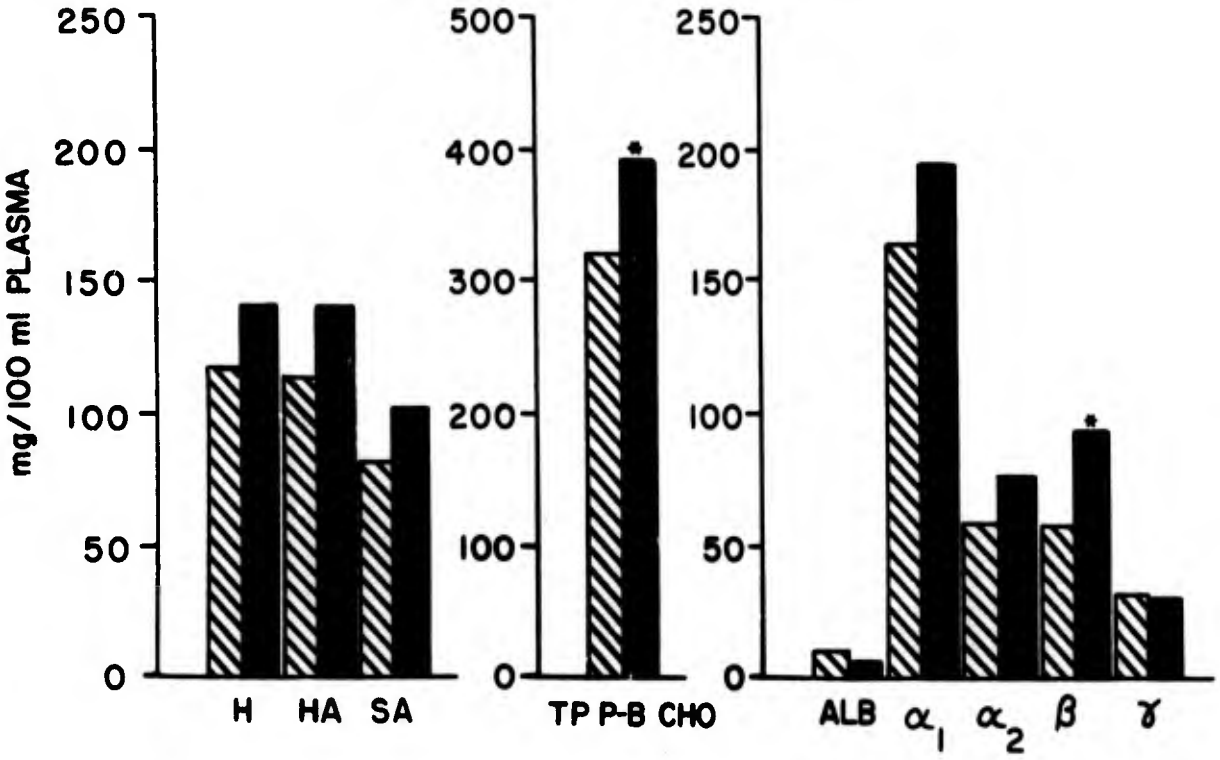

FIGURE 7. EFFECT OF 1ml OF LEM GIVEN IP ON PLASMA PROTEIN BOUND CARBOHYDRATES AND GLYCOPROTEINS. (See figure 3 for legend).

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INFLUENCE OF BACTEREMIA, ENDOGENOUS MEDIATOR OR TURPENTINE ABSCESS ON AMINO ACID MOVEMENT AND SERUM PROTEIN SYNTHESIS

Michael C. Powanda, Ph.D., Captain, MSC*

Profound alterations and/or increases in amino acid and protein metabolism occur in many infectious illnesses as evidenced by negative nitrogen balance and modified serum protein patterns. Therefore, as part of a systematic examination of nonspecific host responses, undertaken in the expectation of aiding diagnosis, prophylaxis and/or treatment, we have focused on the changes in host protein synthesis which occur early in infection, long before the production of specific immunoglobulins. As a model system for a systemic infectious illness we have studied the rat injected subcutaneously (SC) with a large inoculum of Diplococcus pneumoniae.

Given a dose of 10^8 D. pneumoniae, bacteremia and fever become apparent in rats about 12-14 hr after exposure and maximal at 24-28 hr. Death generally occurs between 48 and 72 hr. For comparison we have employed the SC injection of turpentine to produce a localized inflammatory reaction. Prior to inoculation with either agent, the animals were administered ^{14}C-cycloleucine, a nonmetabolizable amino acid analogue used successfully by Akedo and Christensen as an indicator for the transport of neutral amino acids.[1] At various times after exposure, the animals were given a pulse dose of tritiated leucine to assess protein synthesis.

Figure 1 shows that within 12-18 hr after the SC administration of either 10^8 D. pneumoniae or 1 ml of steam distilled turpentine, there is a significant increase in the rate of incorporation of ^{3}H-leucine into total serum proteins. This increase in synthesis of proteins which are produced almost exclusively in the liver, parallels the accelerated uptake of ^{14}C-cycloleucine by that organ. Concomitantly and in contrast, there is a marked diminution in the rate of ^{3}H-leucine uptake into muscle protein.

The decrement in uptake of radioactivity into muscle protein during pneumococcal sepsis is consistent with the increased excretion of creatinine[2] by, and lowered muscle ^{14}C-cycloleucine content[3] in these animals. Small but significant decreases in muscle cycloleucine content have also been observed at various times during turpentine-induced inflammation.

In order to gauge the relative necessity of increased serum protein synthesis in the overall host response to infection, weanling rats were fed an agar gel diet containing either 6 or 18% protein for 28 days prior to their exposure to D. pneumoniae. The 18% diet has been found to be optimal for growth.

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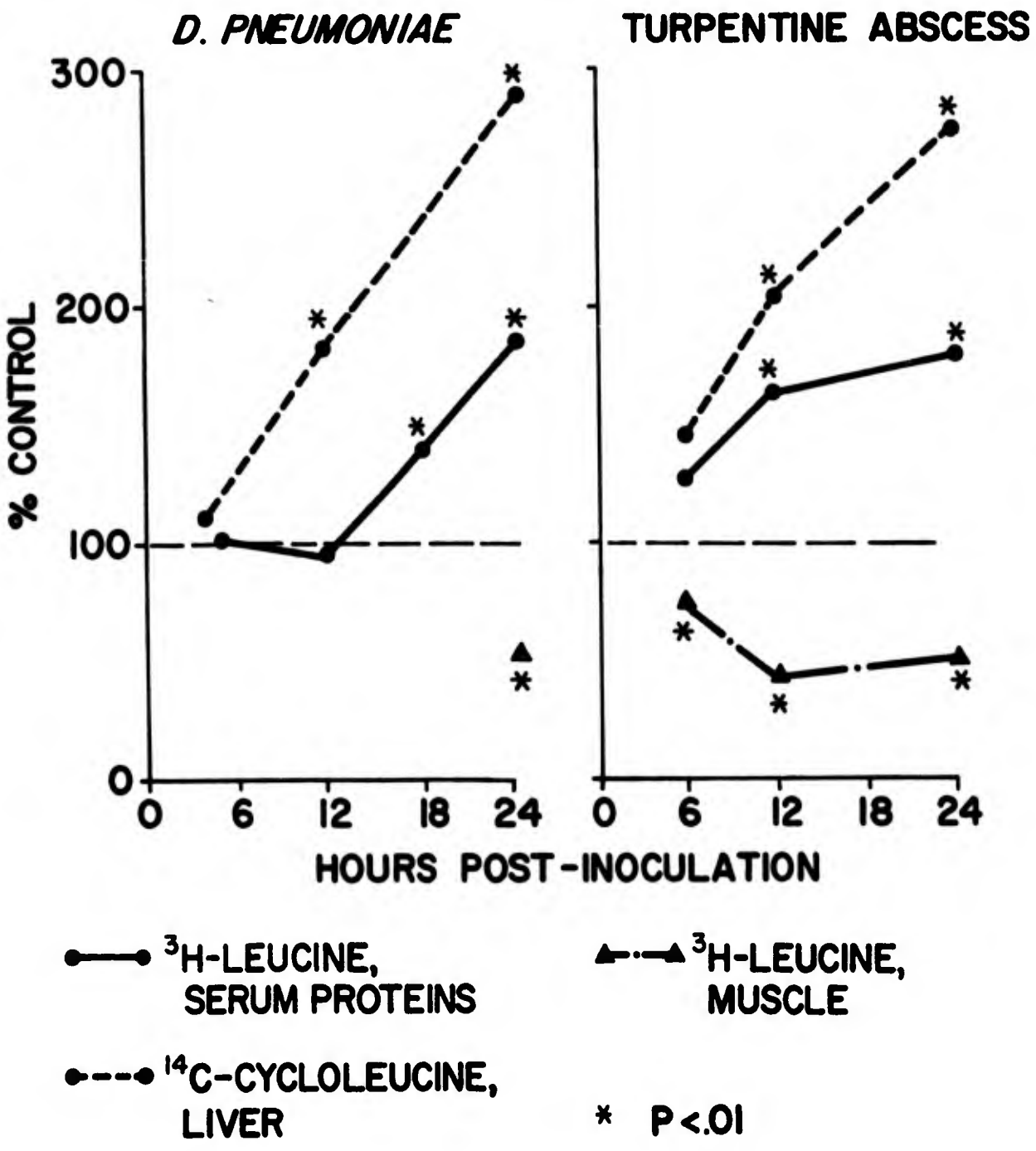

FIGURE 1. AMINO MOVEMENT AND PROTEIN SYNTHESIS EARLY IN INFECTION AND INFLAMMATION.

At 24 hr following initiation of infection and 2 hr after the injection of ^{3}H-leucine, the animals were killed. Even those animals on a diet lacking adequate protein for optimal growth, i.e., the 6% diet, still demonstrated a highly significant increase in serum protein synthesis equal to or perhaps even greater than that exhibited by those animals on the 18% diet (Table I). Despite the increase in serum protein synthesis, no differences in hepatic protein synthesis between control and infected animals were noted for either diet group.

TABLE I. EFFECT OF DIET ON PROTEIN SYNTHESIS DURING INFECTION

DIET % Protein		DPM/μg PROTEIN ± 1 SEM (8/GROUP)		
		Serum	Liver	Muscle
6	Control	4.2 ± 0.5	3.7 ± 0.4	0.44 ± 0.03
	Infected	10.3 ± 0.7[a]	3.8 ± 0.2	0.15 ± 0.01[a]
	Change	+ 147%[a]	+ 3%	- 65%[a]
18	Control	4.7 ± 0.2	3.0 ± 0.1	0.43 ± 0.01
	Infected	9.6 ± 0.5[a]	3.2 ± 0.2	0.20 ± 0.02[a]
	Change	+ 104%[a]	+ 7%	- 54%[a]

a. $p < 0.001$.

Both groups of animals displayed highly significant decreases in muscle protein synthesis. This decrease, consistent with the evidence of amino acid movement from muscle to liver, would be an example of how that synergism of infection and malnourishment,[4] so well documented in humans, may occur. It might be expected that recurrent infections would further complicate an inadequate diet by mobilizing essential nitrogen from muscle mass to the liver for the synthesis of acute phase globulins.

In the last few years studies have shown that material secreted by stimulated leukocytes elicits (1) a decrease in the Zn and Fe concentration of serum;[5,6,7] (2) an increase in these same metals within the liver;[8] (3) a flux of amino acids to the liver;[9] (4) an increase in serum α_1 and α_2 acute phase globulins;[10] and (5) an increase in circulating ceruloplasmin.[11] We were thus curious to see to what extent this leukocytic material, designated leukocytic endogenous mediator (LEM), would induce alterations in host protein synthesis akin to those observed in infection and inflammation. In order to approximate infection and inflammation, in which it might be supposed that the mediator or mediators are chronically being released,

6 injections of LEM were given intraperitoneally, one every 4 hr. Two hours after the last LEM injection and 2 hr prior to necropsy, the animals were administered a pulse dose of tritiated leucine. A portion of each of the sera was subjected to cellulose acetate strip electrophoresis which were then stained to ascertain protein distribution. The radioactivity associated with each of the fractions was assessed by liquid scintillation techniques. Another aliquot of serum was analyzed for seromucoid content according to the procedure of Neuhaus et al.[12] The specific activity of muscle tissue protein was also determined.

Table II summarized the data of this experiment and allows comparison of these data with those from animals exposed to pneumococci or turpentine. A plus sign indicates an increase and a minus sign, a decrease. These changes are significant ($p < 0.01$). The serum protein data are expressed as % total protein. The changes depicted would still be evident even if

TABLE II. EFFECT OF INFECTION, INFLAMMATION AND LEM ON PROTEIN SYNTHESIS

	STRESS	CHANGE IN %				
		Albumin	α_1	α_2	β	γ
Protein	D. pneumoniae	-	0	+	+	-
	Turpentine	-	0	+	+	0
	LEM	-	0	+		0
^{3}H-Leucine Incorporation	D. pneumoniae	-	±	+	+	0
	Turpentine	-	0	+	+	0
	LEM	-	-	+	+	-

STRESS	CHANGE		
	SEROMUCOIDS		MUSCLE
	mg%	Specific activity	Specific activity
D. pneumoniae	+	+	-
Turpentine	+	+	-
LEM	+	+	-

the data were formulated in absolute values, since we have detected little or no increases in total serum protein concentration within the first 24 hr of infection or inflammation. All 3 stimuli induce highly significant decreases in albumin content and the amount of radioactivity associated with this fraction. All 3 also provoke like increases in the α_2 and β fractions. The seromucoid fraction of serum, defined as those proteins soluble in 0.6 M percholoric acid and which contains many of the acute phase globulins such as orosomucoid, α_1 antitrypsin and haptoglobulin[13] is elevated both for concentration and specific activity. LEM also causes the specific activity of muscle protein to decrease as does infection and inflammation. There appear to be some differences among the 3 stimuli when one looks at the α_1 and γ fractions. Whether these differences are significant and of diagnostic use remains to be ascertained by using more sensitive protein fraction techniques such as acrylamide gel electrophoresis.

The concentration of any given protein in the serum is a function both of its synthesis and degradation. Thus, we cannot discount the effect of an altered pattern of catabolism being in part responsible for the quantitative changes seen to occur during infection in those proteins normally present in serum, such as albumin. Certainly there is evidence of increased degradation of orosomucoid in dogs during inflammation.[14] But there is also the synthesis of new proteins such as C-reactive protein[15] and macrofetoprotein[16] as well as the increased synthesis of those already present such as orosomucoid. The RNA studies are another indication of increased hepatic synthesis of proteins for export. Moreover, the unpublished findings of Dr. Canonico of this Institute suggest that the liver is in fact synthesizing serum proteins at the expense of its own intracellular protein as evidenced by the decrease of β-glucuronidase, a marker for smooth endoplasmic reticulum, along with a loss of peroxisomal particles which are derived from smooth endoplasmic reticulum.

There is substantial documentation that protein synthesis by the liver varies in proportion to amino acid availability.[17,18] The infection-enhanced flux of amino acids to the liver might thus explain the quantitative aspects of the increased rate of serum protein formation by the liver. It remains to be determined how the qualitative changes noted above are brought about.

As is well known, infections often lead to negative balances of nitrogen and minerals. Since these elements are lost from the body one must construe their loss as wastage. But might there also be wastage within the body? For example, the increased expenditure of metabolizable fuels during fever would fall into this category until fever is proven to be beneficial to host defense. The lowering of serum iron concentrations and the sequestration of iron in reticuloendothelial tissue making it unavailable for incorporation into hemoglobin may be another example of functional wastage. Might not the flux of amino acids from muscle to liver and their subsequent incorporation into serum proteins be yet another instance, since the function of these new and increased proteins is yet unknown.

In summary, many of the alterations in protein metabolism which are part of the nonspecific response of an animal to infection and inflammation can be induced by the multiple injection of a substance or substances secreted by stimulated leukocytes. Whether the movement of amino acids from muscle to liver and the subsequent increased synthesis of serum proteins by the liver during infection and inflammation are a form of wastage or a necessary aspect of host defense remains to be determined.

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INFECTION-RELATED CHANGES IN HEPATIC RNA SYNTHESIS AND UTILIZATION

William L. Thompson, B.S.*

Several years ago, in a presidential address at the Clinical Research Meetings in Atlantic City, Dr. Barry Wood emphasized the need for clinical investigators to familiarize themselves with the new techniques of molecular biology. We have used these techniques in the studies of infection that will be described in this paper.

The regulation of protein synthesis in mammalian cells takes place for the most part at two different levels, the transcriptional and the translational. Figure 1 illustrates both the transcriptional and translational mechanisms which involve the majority of the normal cellular processes leading toward the synthesis of new protein. The transcriptional process involves the replication of information from the DNA, containing the genetic information, onto a complementary strand of messenger or ribosomal RNA. These RNA's are then transported through the nuclear membrane to the site of protein synthesis in the cytoplasm of the cell. The conversion of information contained on the messenger RNA into a specific protein, at this site, is known as the translational process.

Previous investigators at this Institute had been able to show an increased rate of *in vitro* amino acid incorporation in terms of the amount of protein present in microsomes isolated from infected rats as compared to controls during the earlier stages of infection.[1] These data suggested some sort of translational regulation which caused the stimulation or activation of the ribosomes from infected animals to produce more protein.

However, when an attempt was made to verify these results in a purer *in vitro* system,[2,3] by isolating ribosomes from control and infected rats, and expressing the results in terms of the amount of RNA present, it was impossible to show any increased activity in amino acid incorporation or in the number of active ribosomes in the total population. In addition, no significant change was found in the polyribosomal pattern in response to infection using sucrose density gradient procedures.[4]

This led to further investigation of previous work done by Lust and Kehoe[5] that showed increased *in vivo* RNA production in addition to protein synthesis in the livers of infected animals. The method we used involved giving an *in vivo* pulse of ^{14}C-orotic acid (which is incorporated only into RNA) at various times after a subcutaneous inoculation of *Diplococcus pneumoniae*.[6,7] We then subfractionated the liver into total homogenate, nuclear, soluble, free ribosome and total ribosome fractions.

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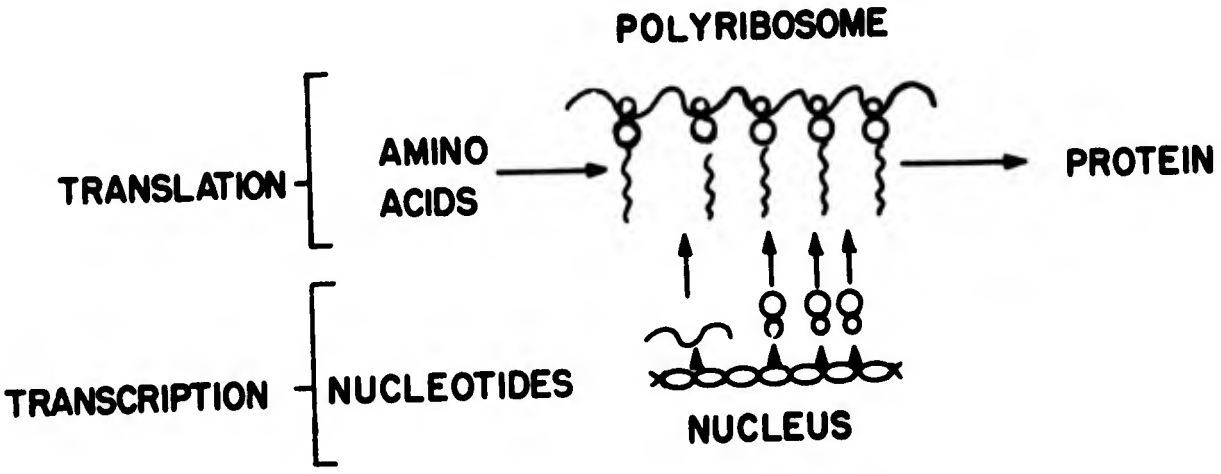

FIGURE 1. DIAGRAMMATIC REPRESENTATION OF TRANSCRIPTIONAL AND TRANSLATIONAL PROCESSES.

The 2 ribosome fractions were separated since there is strong evidence that the free ribosomes are involved in the production of intracellular proteins while the ribosomes bound to the endoplasmic reticulum supply the extracellular or plasma proteins.[8] By separating these fractions we would then be able to tell if any response that was observed to an infection had an effect on protein production or was directed toward the making of either extracellular and intracellular proteins or a specific type of protein messenger. Since free and bound make up the total ribosomes of the cell, we could determine the values for bound ribosomes by subtracting free from the total. Each of these subfractions of the liver were then assayed for amount of orotic acid incorporated into RNA as well as concentration of RNA in the particular cellular fraction.

Our first experiments were done on rats that had been infected 16 hr before receiving a pulse dose of labeled orotic acid. The rats were killed at 0.5, 1, 2, and 4 hr after injection of the acid. By using this method, we were able to verify the flow of messenger and ribosomal RNA production starting at the nucleus and migrating to the cytoplasm as well as a check for a response to infection. Subsequent experiments were then done at various times after infection using a 4-hr pulse of orotic acid for each time period, since most of the labeled RNA is in the cytoplasm by that time.

Figure 2 shows the specific activity of the total homogenate from rats raised on a normal (18% casein) diet and rats raised on a low (6%) protein diet. The latter group were used to determine the ability of hepatic cells having reduced amino acid pool to respond to an infection.[9] The curves on the left are from 16-hr infected and control rats at varying times after a pulse dose of labeled orotic acid. In the infected rats the overall specific activity of liver RNA was significantly increased at 2 hr in both normal and low protein animals as compared to controls. Increases to even greater extents occurred at 4 hr. If we look at the specific activities during the course of an infection in both groups using the 4-hr pulse of orotic acid at each time period, we see a significant increase in both diet groups in the specific activity of hepatic RNA from infected rats as compared to controls. Increased rates of hepatic RNA synthesis continued through 20 hr and were back to normal by 28 hr.

Figure 3 shows the activity of the 4 subfractions of the liver on a cellular basis by expressing the results with respect to DNA, since DNA should remain constant in the liver. As is shown, there is a much greater increase in bound ribosomal activity in the 16 hr infected animals as compared to controls than in the other 3 fractions in both normal and low protein diet rats. The fact that no significant change was noted in the RNA incorporation of orotic acid into the nuclear fraction would suggest that infection does not alter the RNA precursor pool size.

Figure 4 shows the change in hepatic RNA:DNA ratios during the course of the infection in the total homogenate as well as in the various fractions

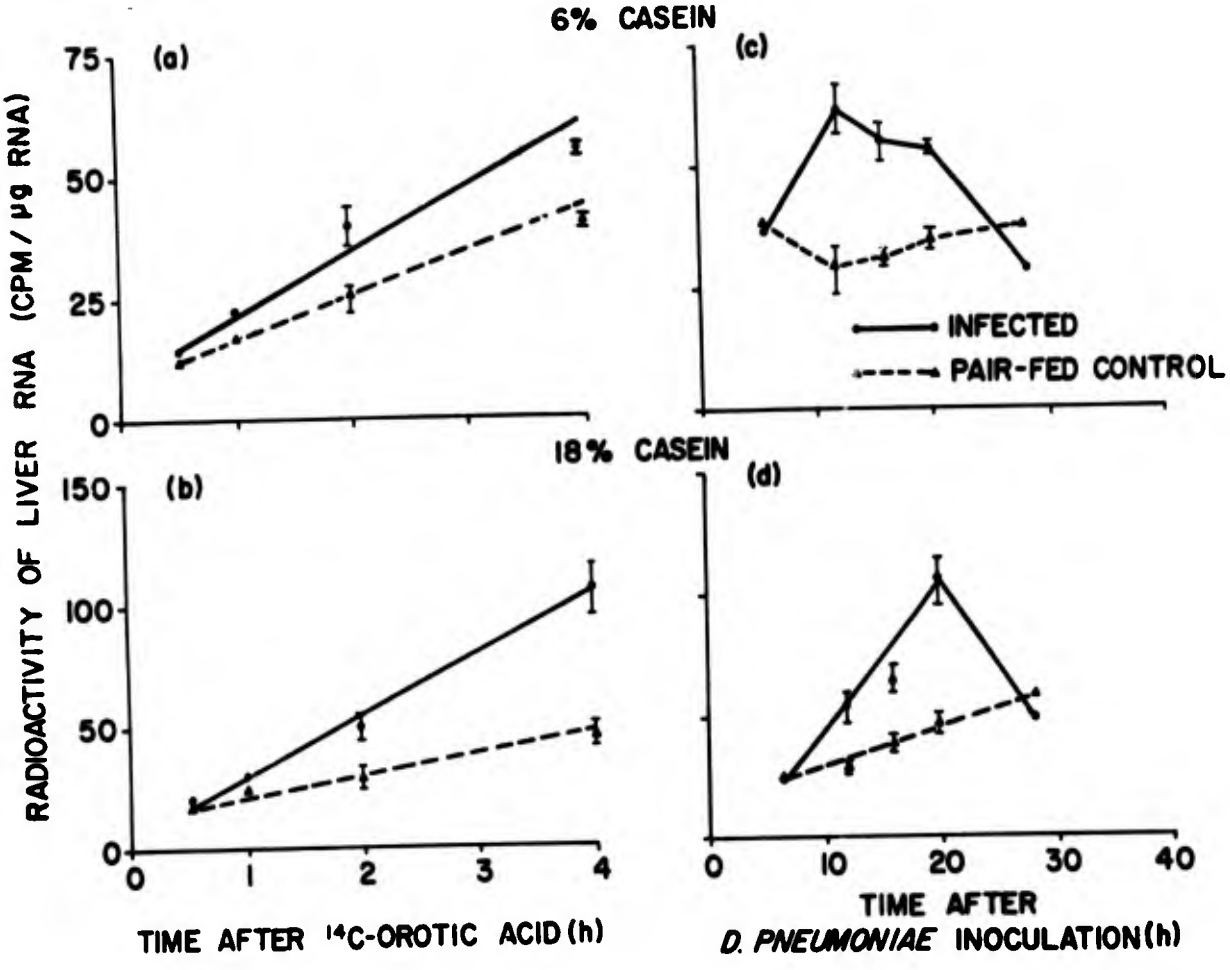

FIGURE 2. EFFECT OF INFECTION AND DIET ON UP TAKE OF ^{14}C-OROTIC ACID INTO HEPATIC RNA OF RATS.

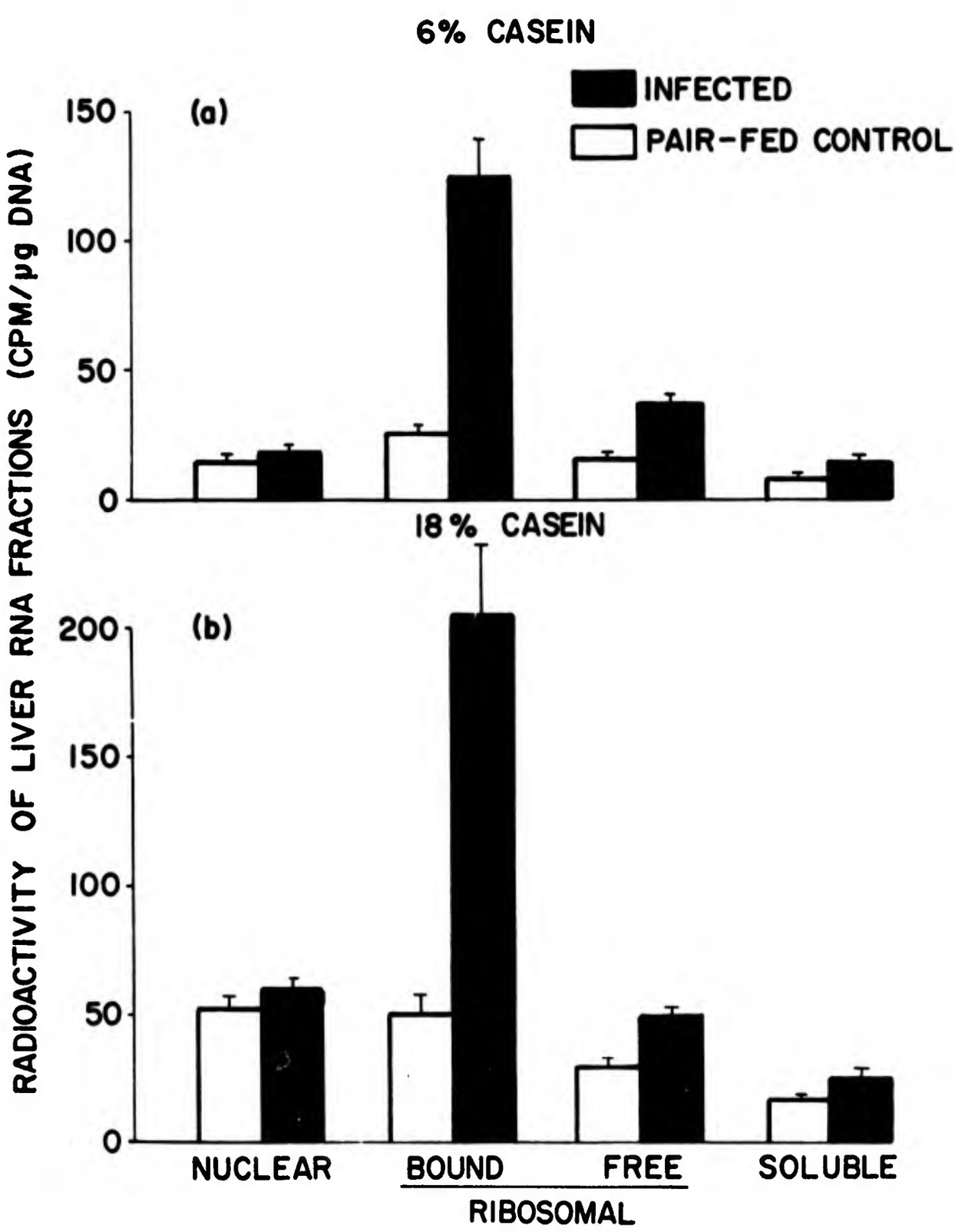

FIGURE 3. EFFECT OF INFECTION AND DIET ON CELLULAR UPTAKE OF LABELED ORTIC ACID INTO HEPATIC RNA FRACTIONS.

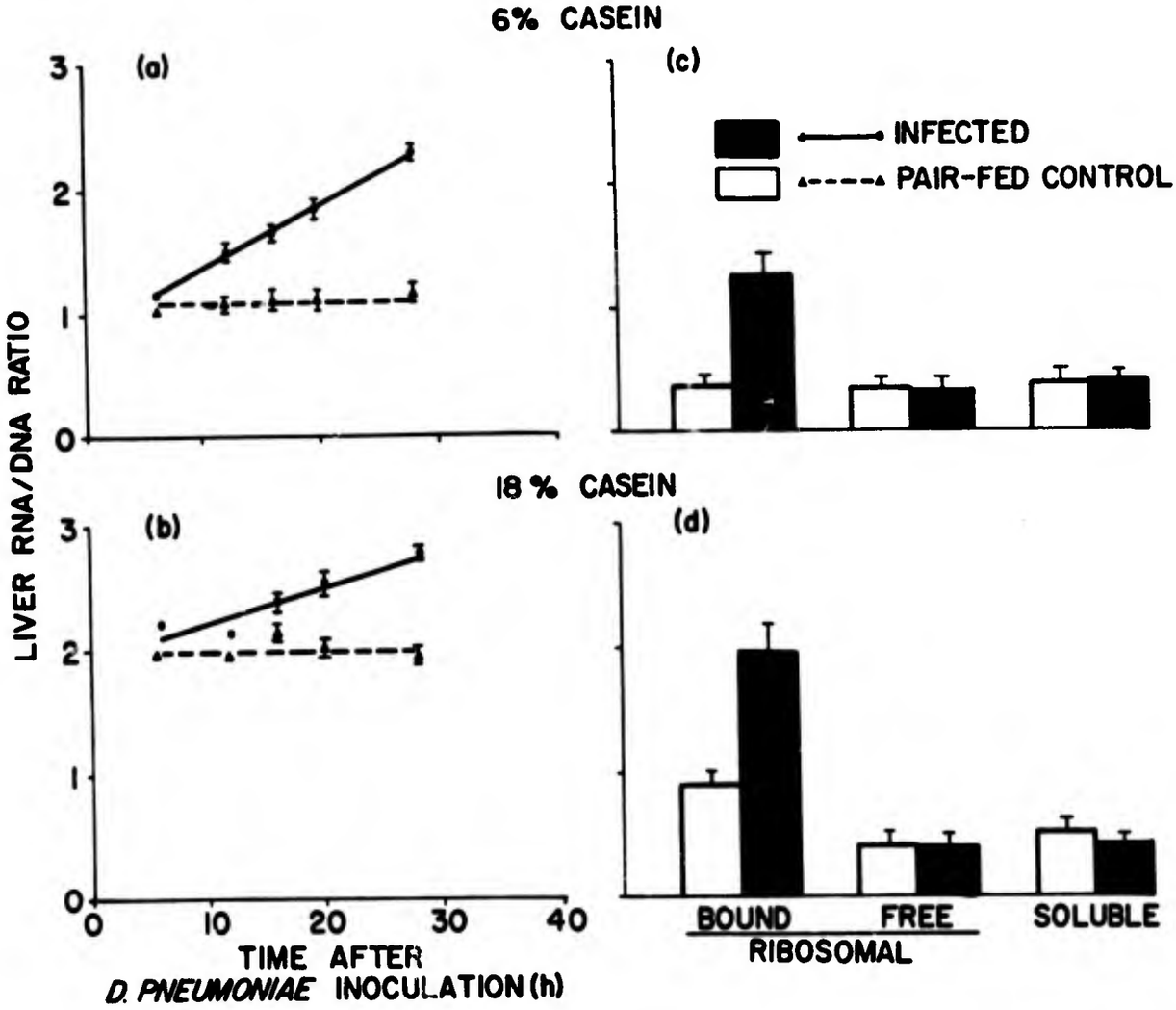

FIGURE 4. EFFECT OF INFECTION AND DIET ON RNA : DNA RATIOS IN TOTAL LIVER AND CELLULAR FRACTIONS.

during the later stages of the infection in normal and low protein diet rats. As shown on the left, there is an overall increase in liver RNA in both dietetic groups of rats as the infection progresses. If this is broken down into the fractions (on the right) we see that practically all of this additional RNA from infected animals is going into the bound ribosomal fraction. Thus, the elevated rate of synthesis of bound ribosomal RNA results in a measurable increase in RNA concentration of this fraction in the liver of infected rats. This then indicates that the previously reported increase in in vivo rates of serum protein synthesis is not due to a translational effect by inducing the ribosomes to produce proteins at a more rapid rate, but to a transcriptional effect whereby the DNA is somehow induced or derepressed into the production of more messenger and/or ribosomal RNA directed toward the production of extracellular protein from the bound ribosomes.

Since leukocytic endogenous mediator (LEM) has been shown to stimulate increased rates of serum protein synthesis (page 63, this report) we were interested in determining also whether LEM would induce elevated rates of bound ribosomal RNA production in the liver of treated rats.

The in vivo labeling experiment was repeated using earlier time periods than before (4-14 hr), since we would be bypassing the earlier stages of the animals response to an infection. In addition, the rats were given a 2-hr pulse of orotic acid before killing.

Figure 5 shows the specific activity and cellular concentration of label in hepatic RNA at varying times after the injection of LEM or in saline controls. Regardless of the method of expressing the data, incorporation of ^{14}C-orotic acid into total liver RNA of rats injected with LEM was increased when compared to controls, with maximal response at 10 hr. This is approximately 6-8 hr before the peak response associated with pneumococcal infection.

Looking at the specific activity, expressed in terms of the RNA present, in each of the fractions at various times after the addition of LEM (Figure 6) little change in the nuclear and soluble fractions is seen. However, a significant increase in the activity in free and bound ribosomes reached a peak at 10 hr, much like the curve seen with the total homogenate (Figure 5).

Figure 7 shows these same fractions expressed on a cellular basis in terms of DNA. Here again little change in nuclear or soluble fractions was observed. However, there is a very marked increase in incorporation into the bound ribosomal fraction with little change in radioactivity of the free ribosomal fraction.

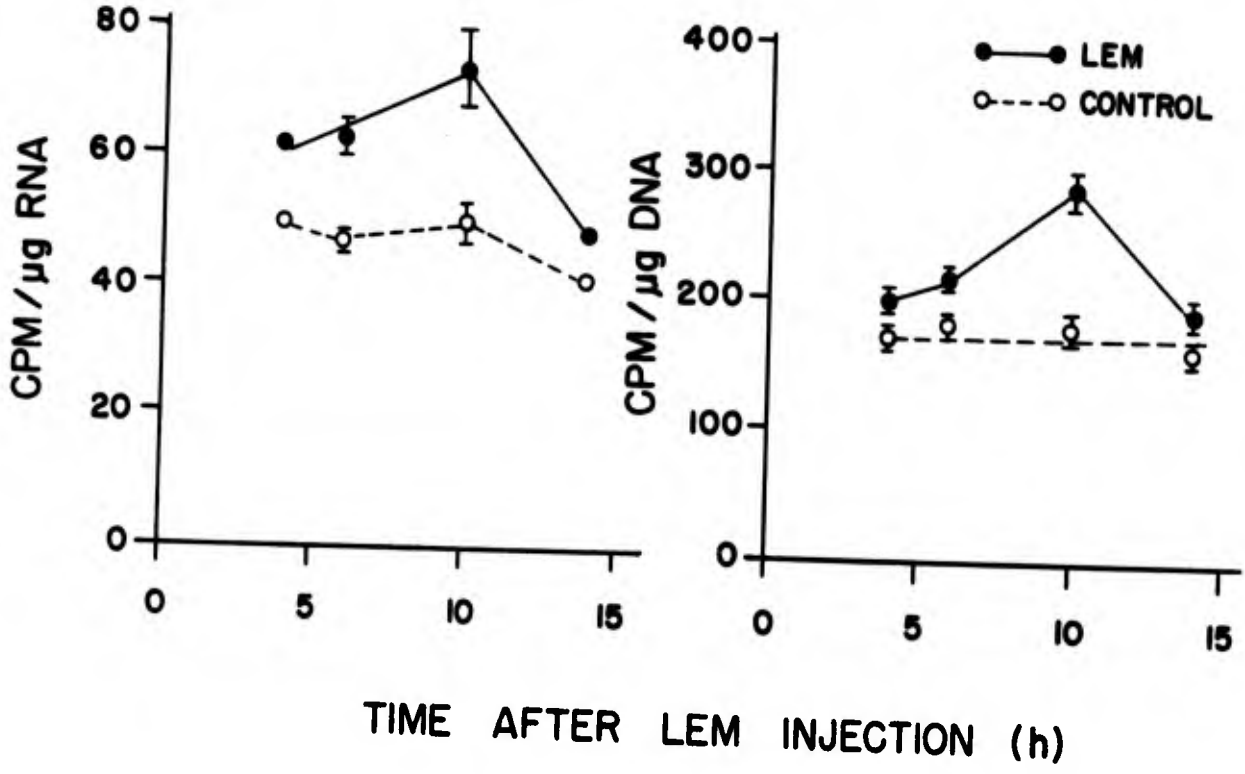

FIGURE 5. EFFECT OF LEM ON UPTAKE OF LABELED OROTIC ACID INTO HEPATIC RNA OF RATS.

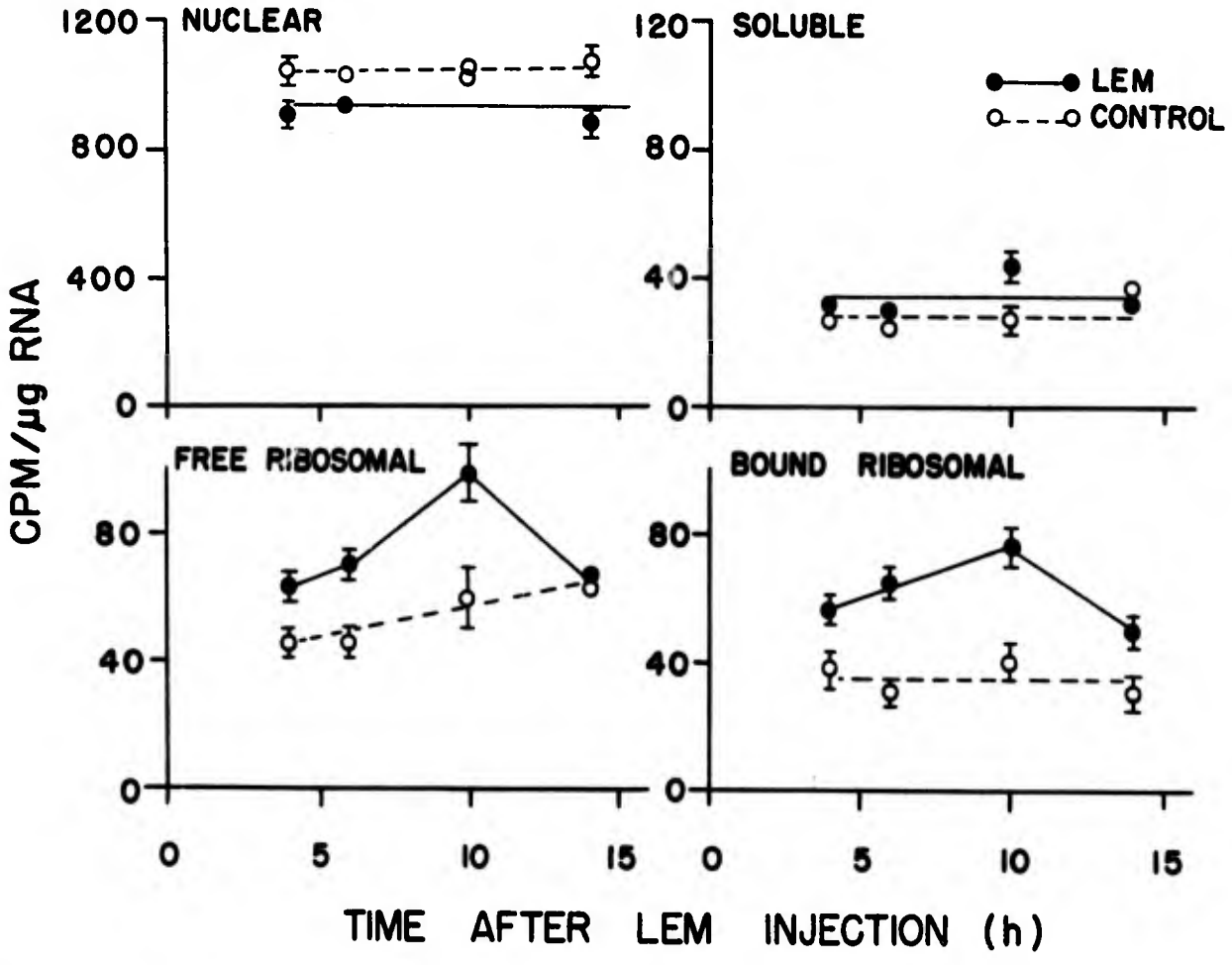

FIGURE 6. EFFECT OF LEM ON SPECIFIC ACTIVITY OF VARIOUS RNA FRACTIONS OF LIVER.

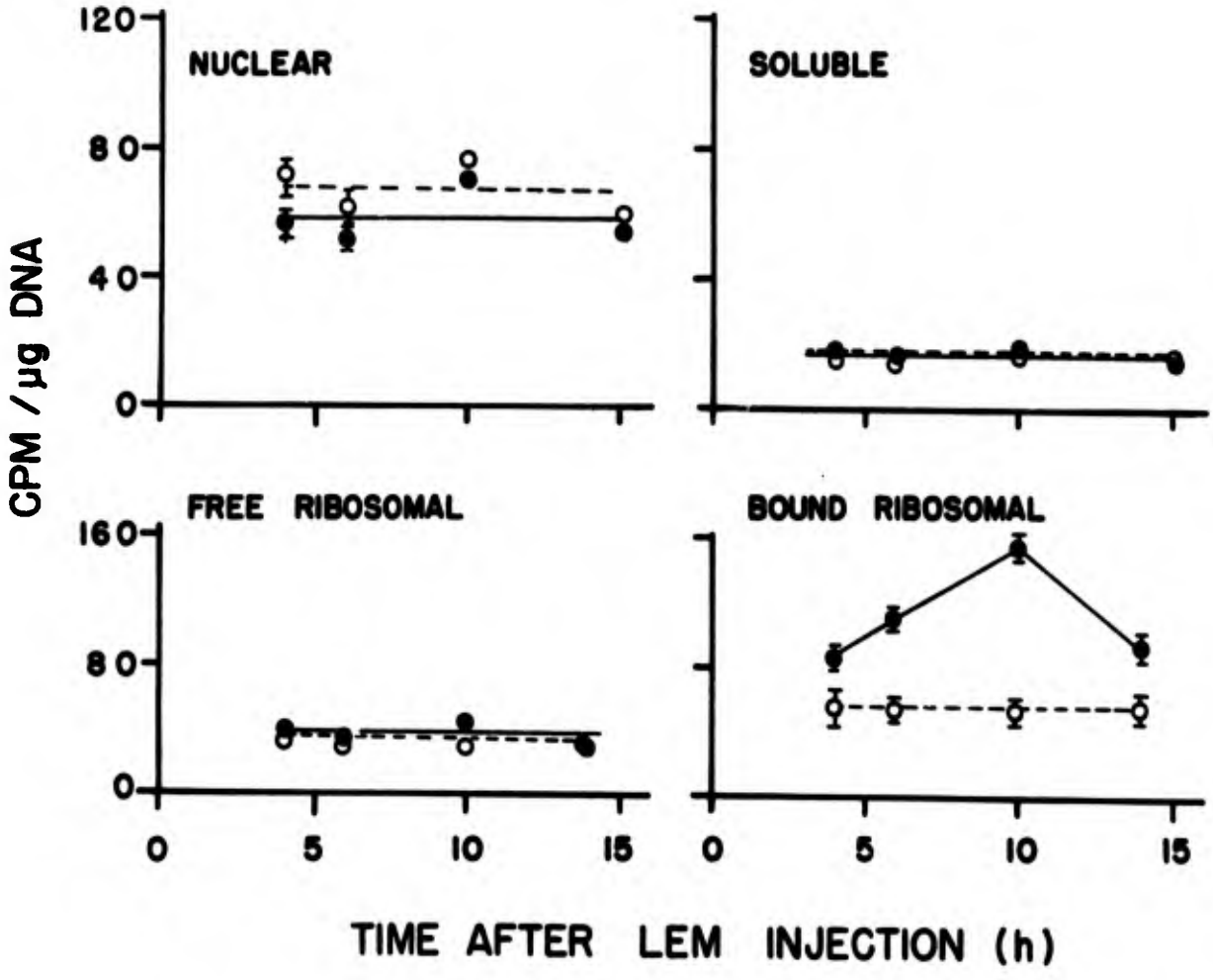

FIGURE 7. EFFECT OF LEM ON CELLULAR UPTAKE OF ^{14}C-OROTIC ACID INTO VARIOUS RNA FRACTIONS OF LIVER.

We can therefore conclude (1) the response to LEM in hepatic cells is very similar to that of an infectious agent except that it occurs 6-8 hr earlier, which also agrees well with the proposed mechanism of response to infection; (2) the theory is substantiated further that response to LEM, as with the infection, results in increased production of RNA directed toward the synthesis of extracellular proteins on the bound ribosomes, therefore is transcriptionally rather than translationally regulated; and (3) this response to an infection in hepatic cells is not prevented by the presence of a reduced cellular amino acid pool as is the case with the rats raised on a low protein diet.

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EFFECTS OF INFECTION ON GLYCOGEN AND GLUCOSE METABOLISM

Randall T. Curnow, Major, MC*

Infectious diseases have long been known to promote prompt and often profound alterations in host carbohydrate homeostasis.[1,2] These changes are thought to represent fundamental alterations in metabolic fuel production and utilization. It has been suggested that these alterations might be significant factors in morbidity and lethality of infectious disease.[3] However, very little is known concerning infection-related alterations in carbohydrate metabolism at the molecular level. In one aspect of carbohydrate metabolism, glycogen metabolism, dramatic increases in knowledge concerning the events and control mechanisms at the molecular level have occurred in recent years. Scattered reports have suggested that septic processes accelerated glycogenolysis and inhibited glycogenesis.[4,5] However, no systemic studies on the influence of infectious diseases on glycogen metabolism have been reported. We have studied the sequential changes in liver glycogen enzymic machinery during both Diplococcus pneumoniae and Salmonella typhimurium sepsis in rats.

The enzyme systems primarilly controlling liver glycogen synthesis and degradation are shown in Figure 1, with the major control sites and factors involved.

Glycogen synthetase (GS) activity is a major control site and is rate limiting in glycogen synthesis. There are 2 interconvertible forms of synthetase: TS-I is physiologically active while GS-D is relatively inactive. The I to D conversion is catalyzed by synthetase kinase - one form of which is identical to protein kinase. The D to I conversion is catalyzed by synthetase D phosphatase.

Phosphorylase (PHOS) is the major control site for glycogen degradation. There are also 2 interconvertible forms of phosphorylase. Phosphophosphorylase, the physiologically active form (PHOSa) and dephosphophosphorylase (PHOSb). The conversion of PHOSb to PHOSa is catalyzed by kinase. The reciprocal conversion is catalyzed by phosphorylase phosphatase. Only phosphophosphorylase activity can be measured in liver.

An increase in intracellular cyclic AMP concentration initiates an enzyme cascade beginning with activation of protein kinase. This increase causes a rapid simultaneous conversion of synthetase from the I to D form and phosphophosphorylase from the dephospho- to phosphophosphorylase. This concomitant activation of phosphorylase and inactivation of synthetase prevents needless recycling of glucosyl units during glycogenolysis.

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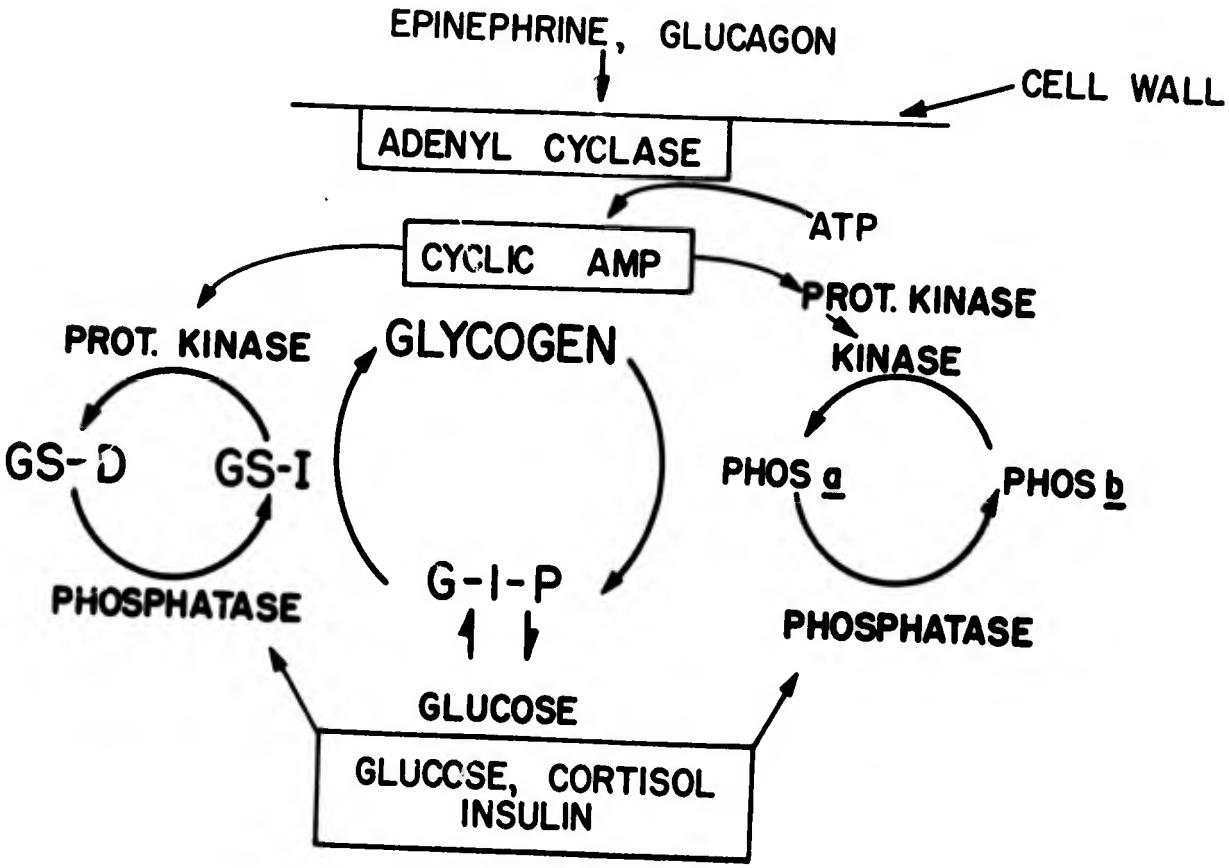

FIGURE 1. MAJOR CONTROL SITES AND FACTORS IN LIVER GLYCOGEN METABOLISM.

Another potential control site for these enzymes is the phosphatase enzymes. Much less is known concerning factors that influence the state of activities of these enzymes. Glucose and glycogen concentration, as well as hormonal stimulation, are important factors in control of these enzymes.

All rats used in the pneumococcal infection experiment were allowed access to food until inoculated subcutaneously (SC) with 10^7 D. pneumoniae organisms after which time all food was removed. Similarly fasted controls were given saline SC. Sepsis was confirmed by blood culture.

Rats were anesthetized with intraperitoneal (IP) sodium pentabarbital 20 min before being killed.

Figure 2 demonstrates the sequential changes in liver glycogen concentration, as a function of time following inoculation with pneumococci compared to control animals given a single SC bolus of sterile physiological saline. The points and vertical lines represent the mean and standard error of the mean respectively. Six animals were killed at each point in time in this and subsequent experiments. Asterisks indicate points statistically significantly different from controls ($p < 0.05$). As can be seen, there was an accelerated rate of liver glycogen depletion in infected animals, the concentration being approximately half that of fasted controls at 8 hr.

Pneumococcal sepsis had a distinct effect on the synthetase system. As can be seen in Figure 1, there was a decrease in the active form of synthetase in the I form after 8 hr which became statistically significant by 16 hr and persisted thereafter.

The influence of pneumococcal sepsis on the liver phosphorylase system was less dramatic. Liver phosphophorylase activity was higher than controls only at 16 hr (Figure 2).

These experiments indicate that pneumococcal sepsis evoked significant alterations in glycogen metabolism in the rat liver. Figure 3 demonstrates the net effect of pneumococcal sepsis on the liver glycogen enzymic machinery. These studies demonstrated that this septic process not only promoted an increase in the rate of glycogenolysis as compared to noninfected fasted controls but that this phenomenon seemed more closely related to decrease in the activity of the enzyme system responsible for glycogen synthesis - that is glycogen synthetase - than to an increase in the activity of the enzyme system controlling glycogen degradation, i.e., glycogen phosphorylase.

Next a gram negative infection was studied. Figure 4 demonstrates the effect of S. typhimurium sepsis on liver glycogen concentration. All rats used in this study were allowed free access to food following IP

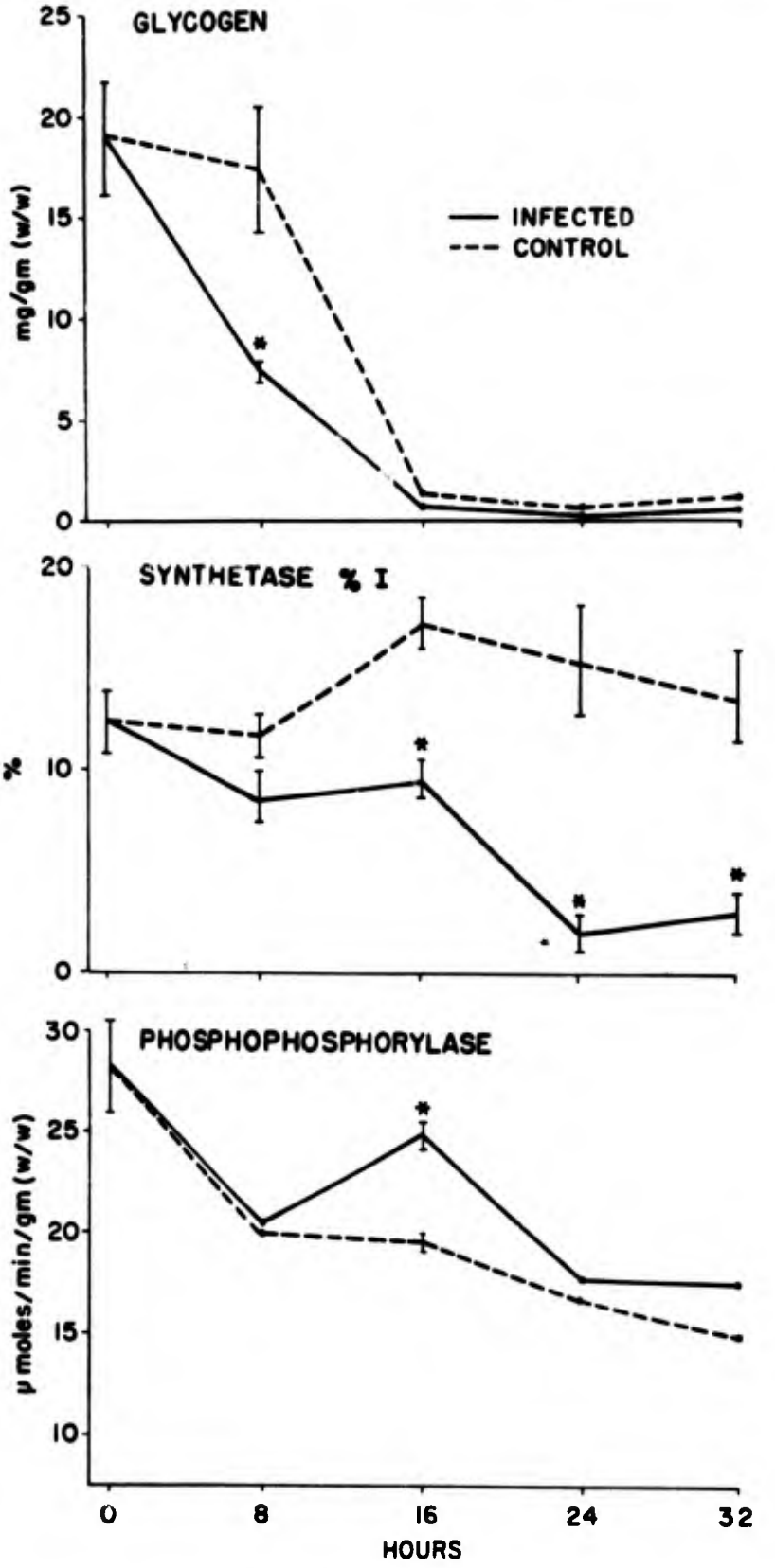

FIGURE 2. EFFECT OF PNEUMOCOCCAL SEPSIS ON GLYCOGEN CONCENTRATION, SYNTHETASE I, AND PHOSPHORYLASE ACTIVITY IN RAT LIVER.

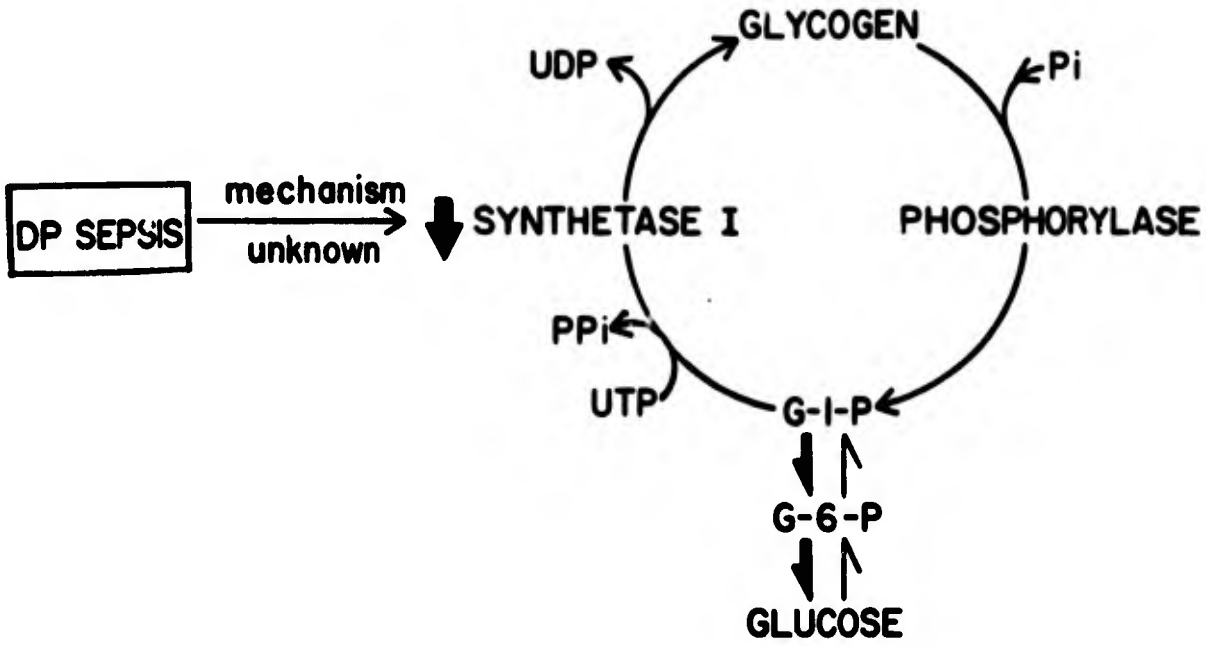

FIGURE 3. EFFECT OF PNEUMOCOCCAL SEPSIS ON LIVER GLYCOGEN ENZYMIC MACHINERY.

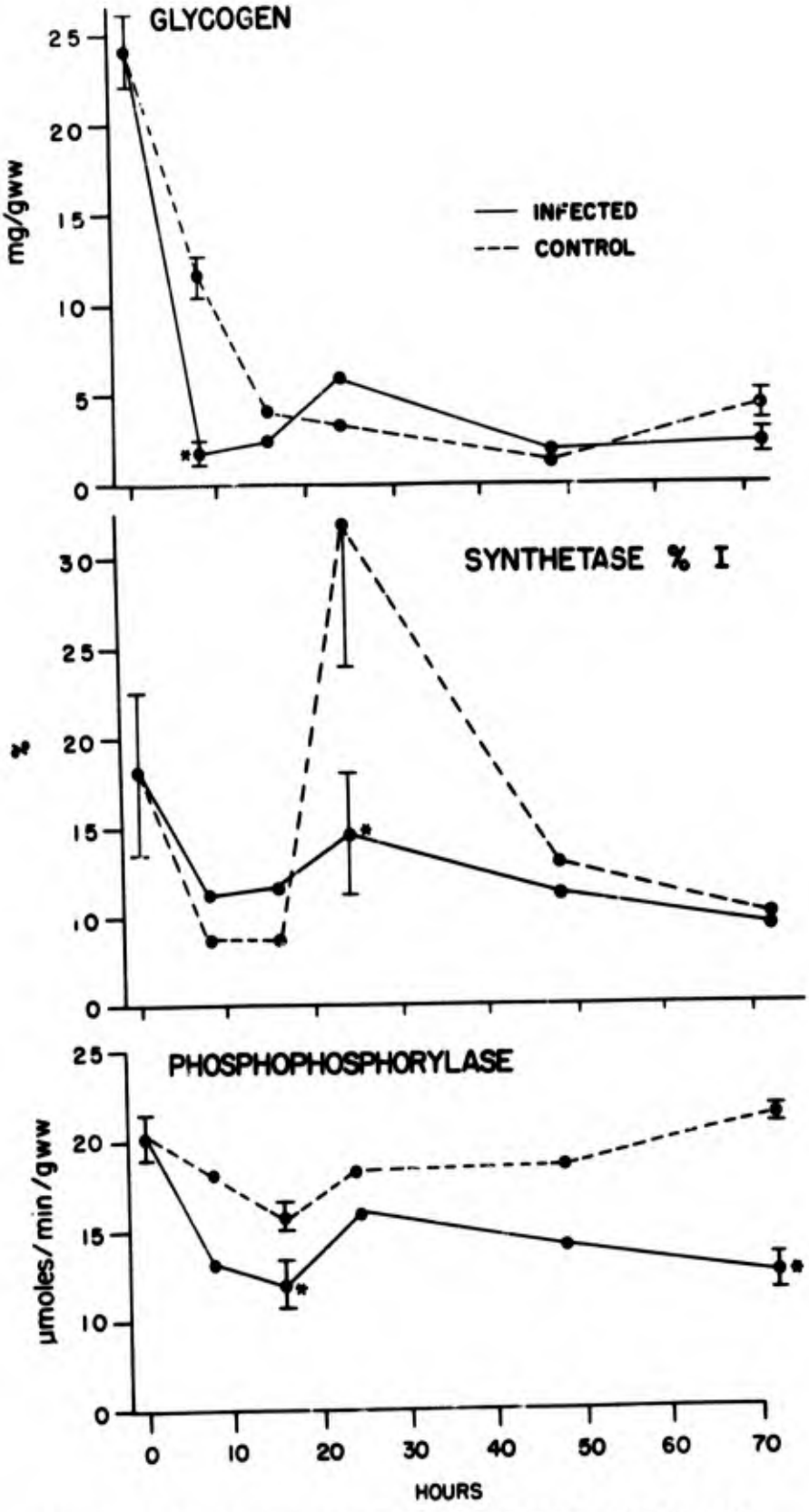

FIGURE 4. THE EFFECT OF *S. TYPHIMURIUM* SEPSIS ON GLYCOGEN CONCENTRATION, SYNTHETASE I, AND PHOSPHORYLASE ACTIVITY IN RAT LIVER.

inoculation with 10^6 viable S. typhimurium organisms. Pair-fed controls were given sterile physiological saline as before. As with pneumococcal infection there was an accelerated rate of liver glycogen depletion in infected rats compared to controls. The livers of infected rats had < 1/4 as much glycogen as controls at 8 hr.

The influence of this infectious process on liver glycogen synthetase and phosphorylase activity was more complex than that seen in pneumococcal sepsis. There was little difference in the percent synthetase (Figure 4) in the active form between infected and control rats until 24 hr when a dramatic increase in the percent I occurred in controls that did not occur in infected animals.

Fasting is known to promote an increase in the amount of synthetase in the active form in normal rats.[6] Because infected rats did not eat between the 4th and 24 hr of infection, controls were also fasted and the normal expected increase in the percent I occurred. Thus, sepsis inhibited the expected activation of synthetase that normally occurs with fasting.

Unlike the late modest increase in phosphorylase activity that occurred during pneumococcal sepsis, a paradoxical decrease in liver phosphorylase activity was noted at 8 hr becoming significant by 16 hr (Figure 4). The significance of this finding is unknown and is currently under investigation.

Having found significant alterations in liver glycogen metabolism in rats infected with both pneumococcus and Salmonella, further studies were carried out to test the responsiveness of this system during sepsis to glucose, a known physiologic modifier.

Acute glucose administration is known to promote liver glycogen deposition in fasted rats associated with an activation of glycogen synthetase and inactivation of phosphorylase activity.[7] We administered 1 gm/kg of glucose IP (GTT) to rats fasted for 41 hr and infected for 24 hr with S. typhimurium as in the previous study. Controls were similarly fasted and given saline IP. Figure 5 shows the plasma glucose response in animals killed sequentially over the 2-hr period following glucose administration. Little difference is noted between the noninfected control rats and infected rats either in the maximum plasma glucose concentration reached or the rate of glucose disappearance. Thus, in fasted rats Salmonella sepsis did not promote a further impairment of glucose tolerance.

Sepsis promoted a significant diminution of glycogen concentration compared to controls as seen at 0 time (Figure 5). Furthermore, it can be seen that while a prompt significant increase in glycogen concentration was promoted by glucose administration in controls, glucose produced no significant increase in this parameter in infected rats. Infection inhibited glycogenesis.

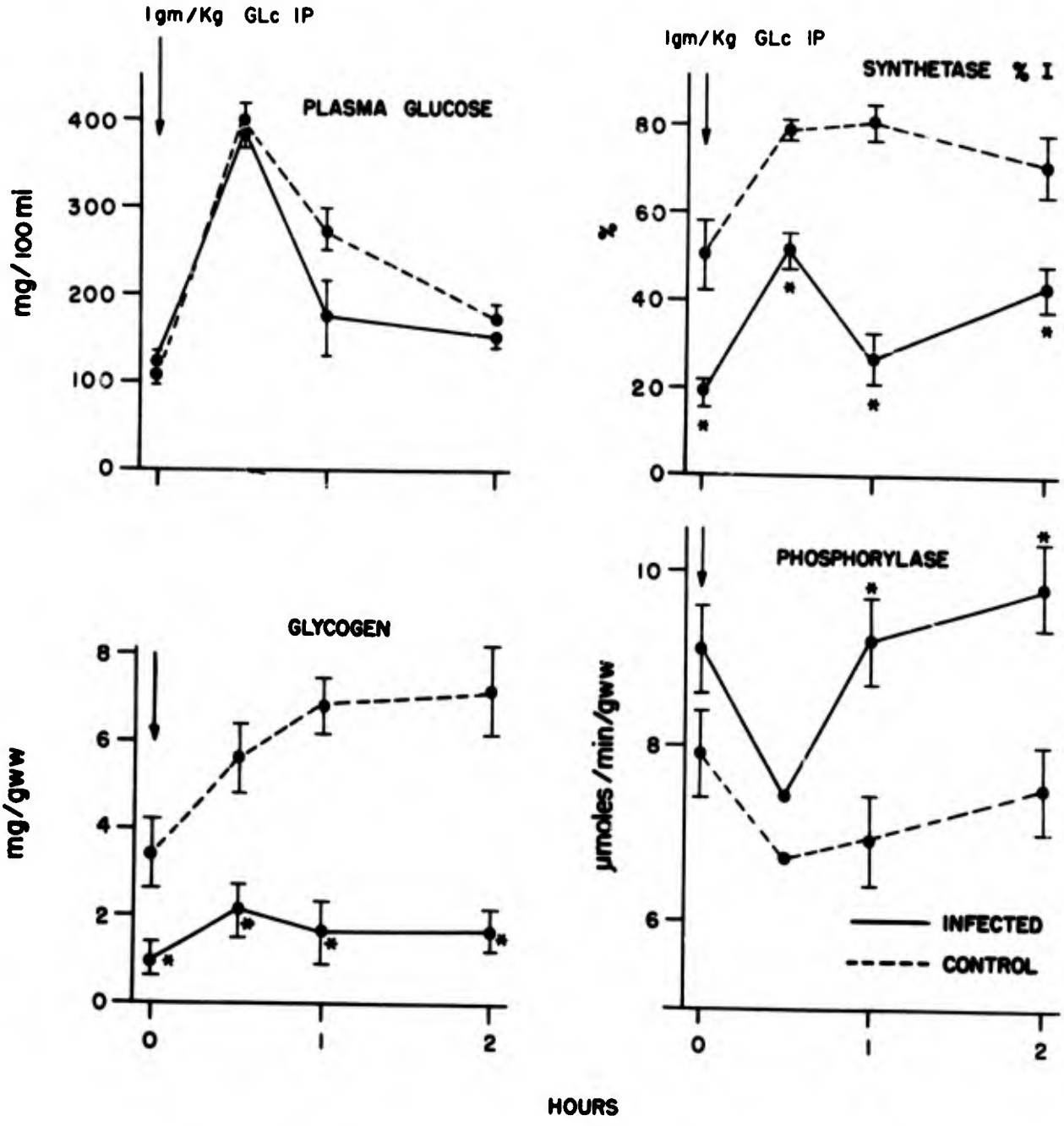

FIGURE 5. EFFECT OF A SINGLE 1gm/kg IP BOLUS OF GLUCOSE ON PLASMA GLUCOSE AND GLYCOGEN CONCENTRATIONS, SYNTHETASE I, AND PHOSPHORYLASE ACTIVITY IN *S. TYPHIMURIUM*-INFECTED RATS.

Response of the liver synthetase system to glucose administration is also shown in Figure 5. As in previous studies the percent synthetase in the I form was significantly lower in infected animals than controls at 0 time. In both groups glucose promoted a rapid increase in the active form of synthetase. However, the height of the maximal increase reached in infected rats was less and the increase less persistently sustained throughout the period of study than controls.

In both groups there was an initial depression of phosphorylase activity (Figure 5). This depression was sustained in the noninfected animals. In infected rats however, this depression was not sustained and levels returned to baseline by 1 hr and were above it at 2 hr.

Thus, infection-related inhibition of liver glycogenesis following carbohydrate loading is possibly related: (1) to a diminished degree of activation of the glycogen synthetase system and (2) to a diminished degree of inactivation of the liver phosphorylase system.

Thus, the results of these experiments show that certain infectious processes promote acceleration of glycogenolysis and inhibition of glycogenesis, the net effect possibly being to redirect available carbohydrate from storage to other metabolic fates of higher priority to the host during infectious illness. Of particular interest, however, are the findings that the responses of rats to differing septic processes at the molecular level are not stereotypes and are in fact quite complex.

Work is ongoing to correlate the changes in glycogen metabolism during infectious illness to concomitant changes in other aspects of carbohydrate homeostasis, to determine the mechanisms responsible for these effects, and to establish the significance of these alterations in relationship to the morbidity and lethality of infectious disease.

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EFFECTS OF INFECTION ON HEPATIC SECOND MESSENGER (ADENYL CYCLASE/cAMP) SYSTEM

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Recent investigations have led to the development of the second messenger role of cAMP in the mediation of hormone action (Figure 1). According to this concept, a hormone (the first messenger) must interact with the adenyl cyclase enzyme located in the cell membrane of the target tissue.[1] This causes an alteration in the intracellular level of the second messenger, cAMP, producing the particular physiological response which depends upon the enzyme profile of the cell type involved.

cAMP once formed can then either react with cPDEase to form AMP which is inactive or stimulate protein kinases which alter certain proteins and thereby produce a specific physiological response. A particular adenyl cyclase may also contain distinct receptors for more than one hormone. For example, epinephrine and glucagon are both thought to activate the same adenyl cyclase enzyme in fat cells.

The second messenger concept envisions adenyl cyclase as being composed of two basic components.[2] The hormonal receptor which is located on the outside of the cell membrane and the catalytic unit which is located on the innersurface of the cell membrane. While NaF has been shown to interact only with the catalytic unit and to stimulate adenyl cyclase, hormones must interact with the hormonal receptors on the outside of the cell to stimulate adenyl cyclase. This has been proven by the fact that exposure of intact cells to proteolytic enzymes, e.g., trypsin, can completely destroy the hormonal responsiveness of adenyl cyclase but have no effect on the NaF activation.[3]

Sutherland and his associates[1] have established certain criteria which should be satisfied before one can conclude that the action of a particular hormone is mediated by cAMP: first, hormones should be capable of stimulating adenyl cyclase in broken cell preparations from the appropriate target cells; second a physiological dose of hormone should increase cellular cAMP and the increase should precede the physiological response; third, drugs which inhibit cyclic phosphodiesterase, such as caffeine, should act synergistically with hormones that act by stimulating adenyl cyclase; and finally, it should be possible to mimic the hormone by application of exogenous cAMP. The mechanism of action of cAMP in the positive inotropic response of the heart to epinephrine is an example. Tissue cAMP levels were measured simultaneously monitoring contractile force and phosphorylase activity. cAMP increased about 4-fold within 3 sec after a single injection of epinephrine and clearly before the increase in contractile force or phosphorylase activity.[4]

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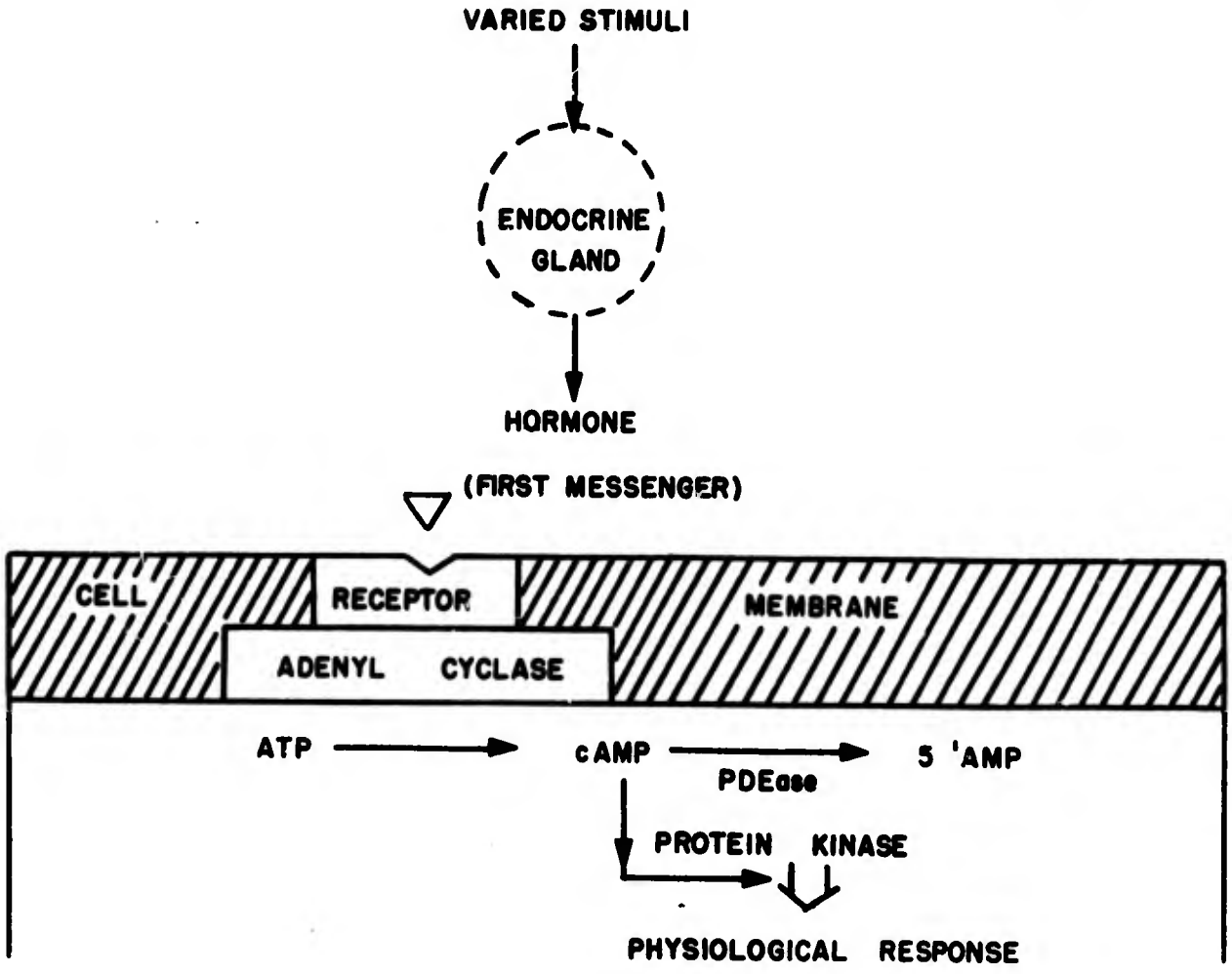

FIGURE 1. SECOND MESSENGER SYSTEM INVOLVING ADENYL CYCLASE.

cAMP has been found to produce a large number of effects in animals and microorganisms, some of which are shown in Table I. Recently cAMP has been

TABLE I. KNOWN EFFECTS OF CYCLIC AMP
(After Robison, et al[4])

ENZYME OR PROCESS AFFECTED	TISSUE	CHANGE IN ACTIVITY OR RATE
Phosphorylase kinase activation	Muscle	
Glycogen synthetase inactivation	Muscle	
Tyrosine transaminase induction	Liver	
PEP carboxykinase induction	Liver	
Glucose-6-phosphatase induction	Liver	
Serine dehydratase induction	Liver	
β-Galactosidase induction	Escherichia coli	
Glycogenolysis	Liver	
Gluconeogenesis	Liver	
Urea formation	Liver	
Permeability	Kidney tubules	Increased
Amino acid uptake	Liver	
Lipolysis	Adipose	
Insulin release	Pancreatic islets	
ACTH release	Anterior pituitary	
TSH release	Anterior pituitary	
Thyroid hormone release	Thyroid	
Force of contraction	Cardiac muscle	
Membrane potential	Smooth muscle	
DNA synthesis	Thymocytes	
Aggregation	Platelets	
Histamine release	Leukocytes	Decreased

found to play a role in the mechanisms of action of cholera. Cholera toxin has been found to increase the level of cAMP in isolated intestinal loops[5] and to simulate glycogenolysis in liver.[6] Since collaborative data from this Institute had implied that the liver played an important part in the

host response to infection,[7] it was decided to monitor *in vitro* hepatic adenyl cyclase after injection of rats with *Diplococcus pneumoniae*. The rats were fed high and low protein diets (18 and 6% protein respectively).

As previously stated, proteolytic enzymes have been shown to destroy selectively the hormonal responsiveness of adenyl cyclase, but not change the catalytic unit's responsiveness to NaF. It was decided to monitor both the catalytic unit and the hormonal receptor of hepatic adenyl cyclase, using NaF and glucagon as respective stimuli. To determine whether anticipated changes in adenyl cyclase were specific for the plasma membrane, another enzyme found there, 5'-nucleotidase,[8] was also monitored.

In vitro hepatic adenyl cyclase was measured in Fisher Dunning rats 18-hr after subcutaneous injection with 4×10^8 cells of virulent or heat-killed *D. pneumoniae*. There were 8 rats used in the control and infected groups for each diet. Table II summarizes the findings for the 2 groups.

TABLE II. 18-HR CHANGES IN VARIOUS PARAMETERS IN RATS INFECTED WITH *D. PNEUMONIAE*

PARAMETER	CHANGE	
	18% Protein Diet	6% Protein Diet
Temperature	+ 2.8°	None
SLDH	None	+ 278%
SGOT	None	+ 74%
SGPT	+ 25%	+ 70%
Death occurred at	45 hr	27 hr

The change in SGPT was significantly elevated 25% in the 18%-diet rats, indicating slight changes in membrane permeability, or damage. In 6%-diet rats serum LDH, SGOT, and SGPT were greatly elevated above the controls thus indicating more significant liver damage. No changes in the structure of the liver plasma membranes of the 18% group were apparent when they were examined by light or electron microscopy.

However, as shown at the top of Figure 2, the basal and glucagon-stimulated adenyl cyclase activities in the infected 18% rats were only 57 and 77%, respectively, of the control rats, while the NaF-stimulated adenyl cyclase activity was unchanged. This indicated that, although the adenyl cyclase receptor for glucagon on the outside of the cell membrane

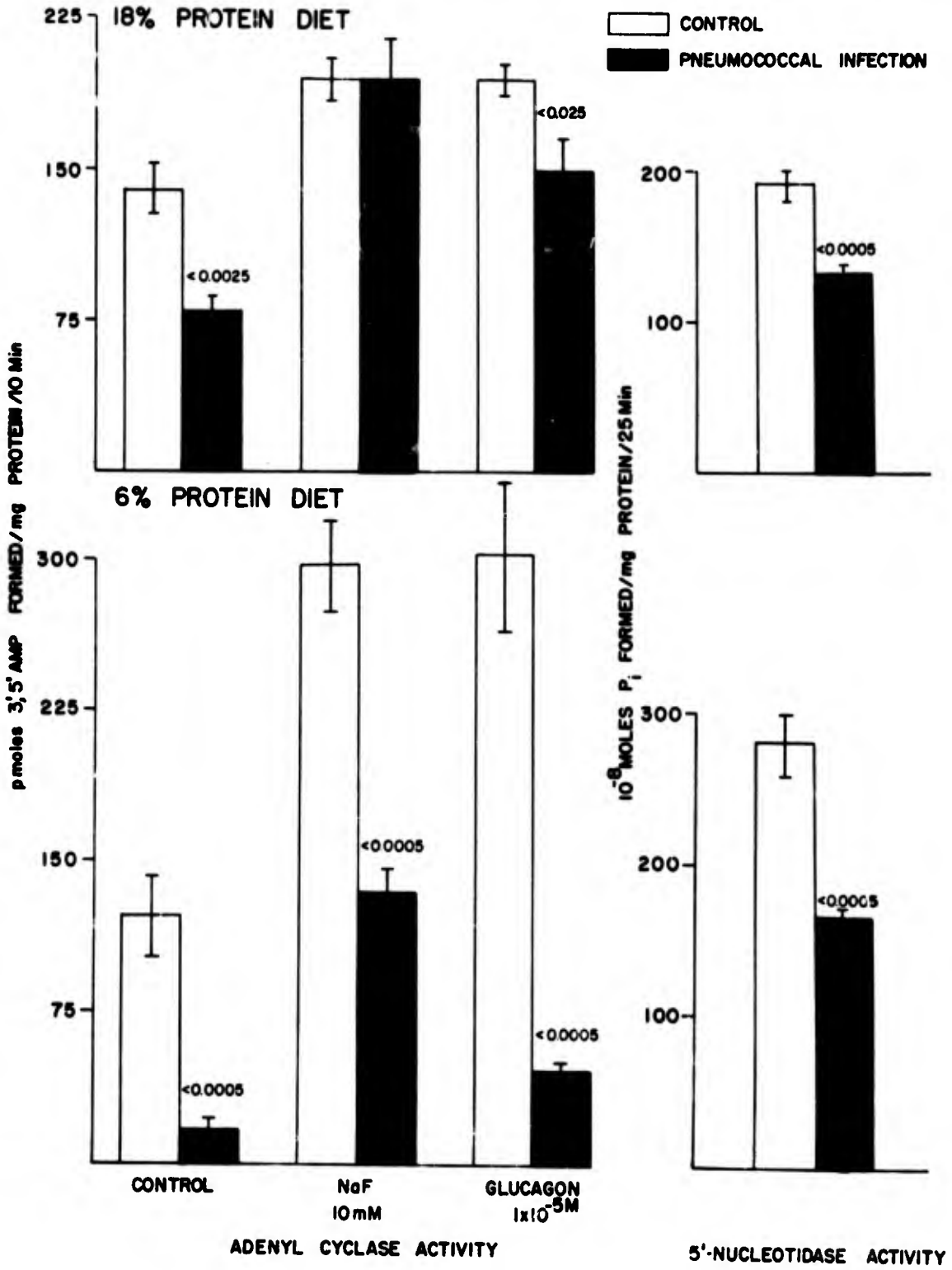

FIGURE 2. EFFECT OF PNEUMOCOCCAL INFECTION ON ADENYL CYCLASE AND 5'-NUCLEOTIDASE ACTIVITIES.

was damaged, the adenyl cyclase catalytic unit was capable of responding maximally to NaF. The activity of 5'-nucleotidase in the infected rats was 69% of control values indicating further that the plasma membrane was damaged. Therefore, although no tissue or membrane lamage could be measured by conventional methods such changes were shown to exist.

The basal, glucagon stimulated, and NaF stimulated adenyl cyclase activities in 6%-diet rats, shown at the bottom of Figure 2 were 13, 15, and 40%, respectively, of the control levels, while the 5'-nucleotidase activity in the infected rats was 60% of the control level. The extreme loss in adenyl cyclase activity in 6%-protein diet animals infected with D. pneumoniae compared to the more selective loss in the 18%-protein diet animals implies an increase in the severity of the infection in animals fed a low protein diet; this is further substantiated by the high level of serum enzymes and earlier death in the latter group. The 5'-nucleotidase activity changed in response to infection, but did not change in proportion to the severity of the infection. On the other hand, adenyl cyclase not only changed activity in response to infection, but also changed in proportion to the severity of the infection.

In conclusion, the changes observed in in vitro hepatic adenyl cyclase activity following injection with virulent diplococci suggest that the adenyl cyclase/cAMP system may be very useful in detecting selective membrane changes in the host during infection which have up to now been impossible to measure.

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EFFECTS OF MITOGENS AND HORMONES ON THE ADENYL CYCLASE-cAMP SYSTEM OF MOUSE AND HUMAN LYMPHOID CELLS

Frederick R. DeRubertis, Major, MC*

Nonspecific mitogenic agents such as phytohemagglutinin (PHA) and specific antigens can activate lymphoid cells in vitro, leading to cell division with blastic transformation and release of a number of soluble factors involved in the immune response.[1] Evaluation of the in vitro responsiveness of lymphocytes to mitogens and antigens often provides useful information about the in vivo status of host immune processes. Although the mechanisms by which mitogenic stimuli initiate lymphocyte transformation are uncertain, the events triggering this process may occur at the cell surface. Insoluble phytomitogens[2] and antigens,[3] conjugated to sepharose, have been reported to activate lymphocytes, presumably through an interaction limited to cell plasma membrane. Smith and co-workers[4] demonstrated a rapid increase in the plasma membrane adenyl cyclase activity and in the intracellular cAMP levels of human peripheral lymphocytes incubated with PHA. These observations and others have suggested that the action of mitogens, like those of many hormones,[5] might be mediated through this intracellular second messenger system.

In order to assess this possibility, we examined the in vitro effects of the mitogens staphylococcal enterotoxin B (SEB), PHA, and endotoxin (LPS) as well as the effects of several hormones on the adenyl cyclase - cAMP system of mouse spleen and thymus cells and on human peripheral lymphocytes. Cellular cAMP levels were determined by the protein binding assay of Gilman[6] and adenyl cyclase activity by the method of White and Zenser.[7] The incorporation of ^{3}H-thymidine into DNA was used as an in vitro index of lymphoid cell activation.

Figure 1 shows changes in mouse thymocyte cAMP levels in response to several hormones known to influence the adenyl cyclase - cAMP system. cAMP levels were significantly increased in response to PGE_1, isuprel and epinephrine but not thyrotropin (TSH) or corticotropin (ACTH). Prostaglandin was the most potent stimulus of cAMP generation tested in both thymus and spleen cells.

Figure 2 shows changes in cAMP levels in thymocytes in response to several prostaglandins, tested over a wide dose range. As depicted, this response showed selectivity insofar as the cells were less responsive to PGA_1 than to PGE_1 or PGE_2 and they were unresponsive to $PGF_{1\alpha}$. The incubation time of these experiments was 10 min but an 8-fold increase in cAMP levels occurred when cells were exposed to PGE_1 for only 4 min. In all subsequent experiments in which the effect of mitogens were

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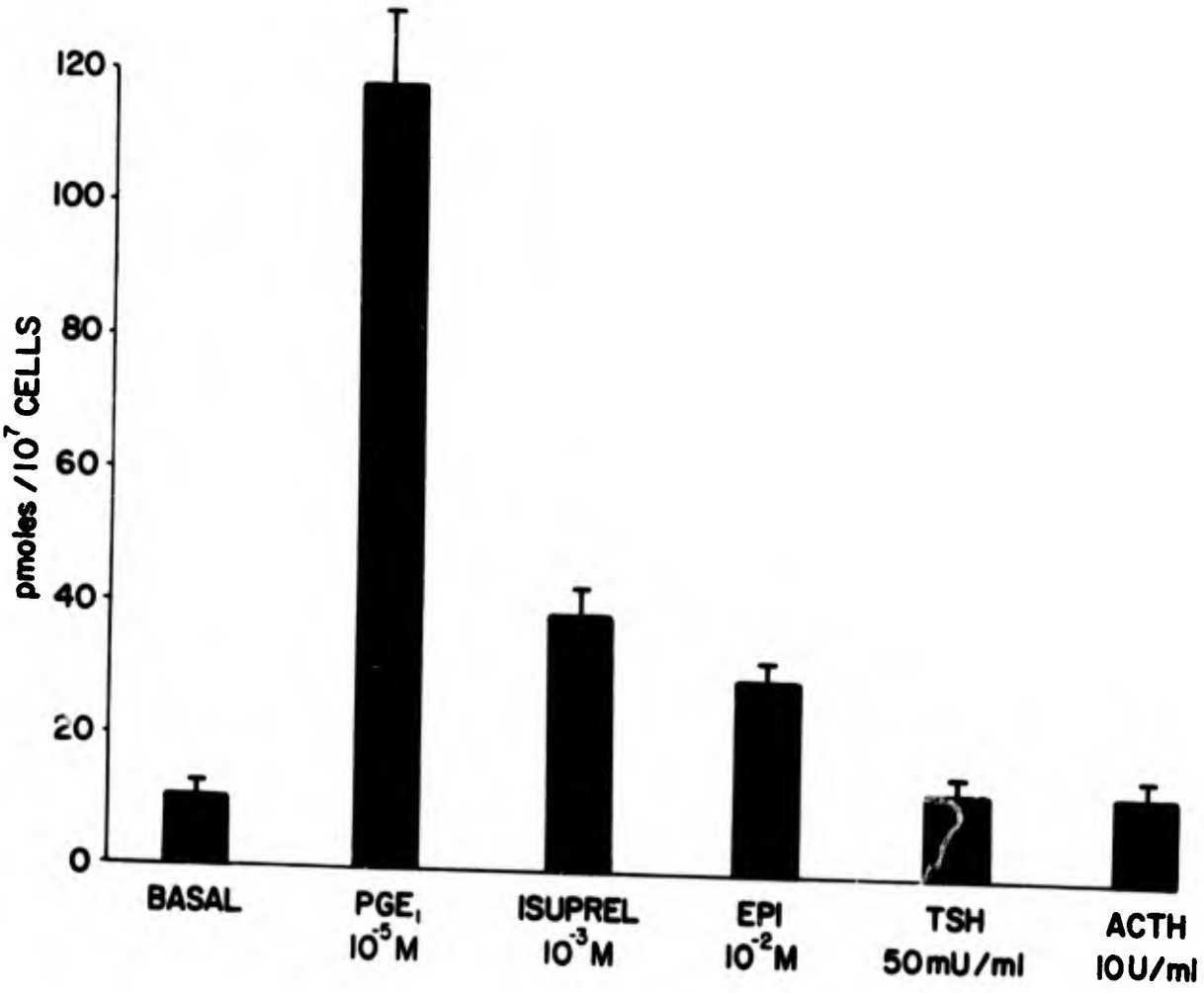

FIGURE 1. EFFECT OF HORMONES ON MOUSE THYMOCYTE cAMP.

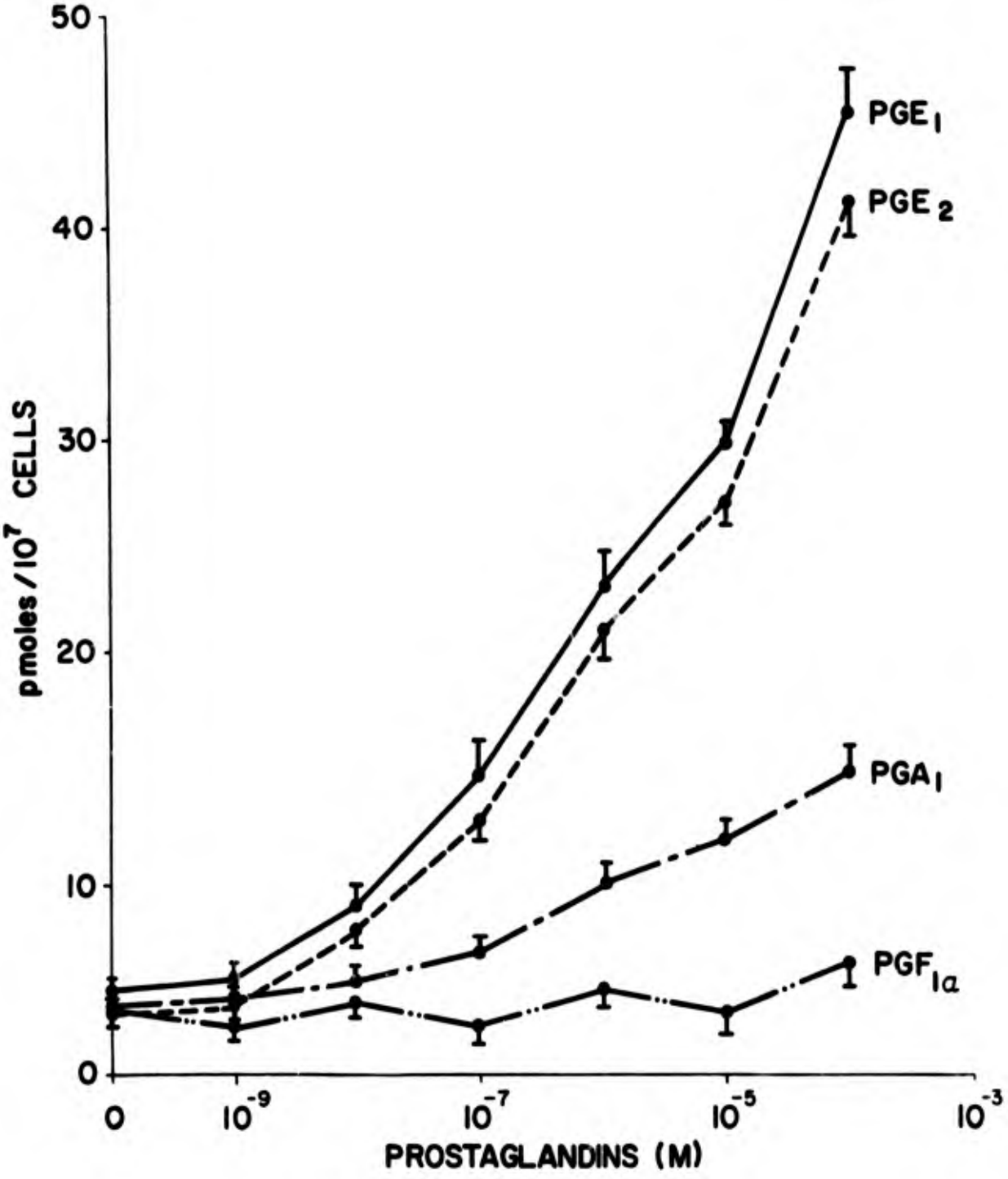

FIGURE 2. EFFECT OF PROSTAGLANDINS ON THYMOCYTE cAMP.

assessed, PGE_1 was run concomitantly as a positive control to verify the presence of a responsive adenyl cyclase - cAMP system in the cell preparation employed.

Figure 3 depicts changes in cAMP levels in human peripheral lymphocytes in response to PHA, SEB, and PGE_1. Cells were incubated with or without theophylline, an inhibitor of cyclic phosphodiesterase. Theophylline prevents cAMP degradation and should make small increases in cAMP more readily detectable. In human lymphocytes, clear increases in cAMP levels were noted in response to both PHA and PGE_1, but not in response to SEB. The incubation time of these experiments was 10 min. However, no increase in cAMP levels was detected in human lymphocytes incubated with SEB for up to 2 hr.

In contrast to the rapid and marked increases in cellular cAMP levels seen in response to PGE_1 in mouse lymphoid cells and in response to both PGE_1 and PHA in human peripheral lymphocytes, SEB, PHA, and LPS, tested over a wide dose range, all failed to increase cAMP levels in mouse spleen and thymus cells (Figure 4). The results of 10 min incubations are shown in Figure 4 but similar results were obtained in mouse lymphoid cells incubated with these mitogens for time intervals from 4 min up to 3 hr. Very high doses of PHA tended to depress cAMP levels but also inhibited DNA synthesis.

Figure 5 shows the effects of several agents on the adenyl cyclase activity of partially purified mouse spleen and thymus plasma membrane preparations. Significant increases in enzyme activity were seen in response to NaF and PGE_1 but not $PGF_1\alpha$. As depicted no increase in adenyl cyclase activity was observed in response to the mitogens PHA, SEB, or LPS. High doses of PHA were inhibitory in spleen cell plasma membranes.

Figure 6 demonstrates the stimulatory effects of PHA, LPS and SEB on mouse spleen cell DNA synthesis. In these studies ^3H-thymidine was present for the last 20 hr of a 48-hr incubation. An inhibitory effect of high doses of PHA was noted. In similar experiments, there has been no increase in DNA synthesis in mouse lymphoid cells in response to either PGE_1 or exogenously added cAMP.

Figure 7 shows the effects on DNA synthesis of brief vs. 48-hr exposure of cells to increasing doses of SEB. At 10 min, 1 hr and 2 hr, the cells were washed twice and resuspended in an SEB-free culture media for the remainder of the 48-hr incubation period. As shown washing the cells at these time intervals did not prevent subsequent cell activation. Studies with ^{125}I-labeled SEB indicated that in excess of 96% of a 25 μg dose of SEB was removed by this washing procedure. The calculated residual concentration of SEB was not sufficient to activate cells on full-time exposure. These studies suggest that a 10-min exposure of lymphoid cells to SEB is adequate to trigger activation. As indicated previously, no increase in cellular cAMP levels was seen during this time period in response to SEB in mouse lymphoid cells.

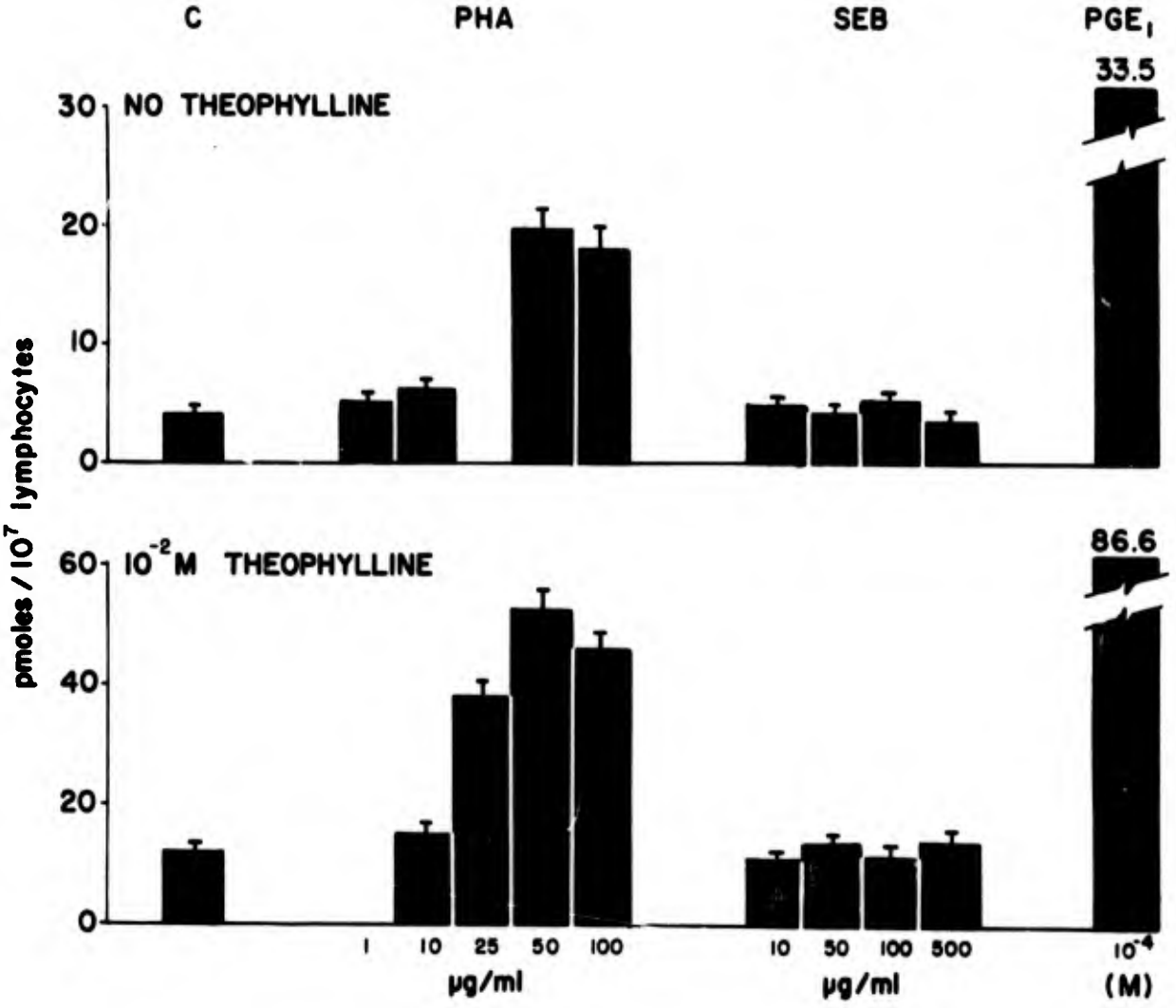

FIGURE 3. EFFECT OF PHA, SEB AND PGE_1 ON cAMP CONTENT OF HUMAN LYMPHOCYTES.

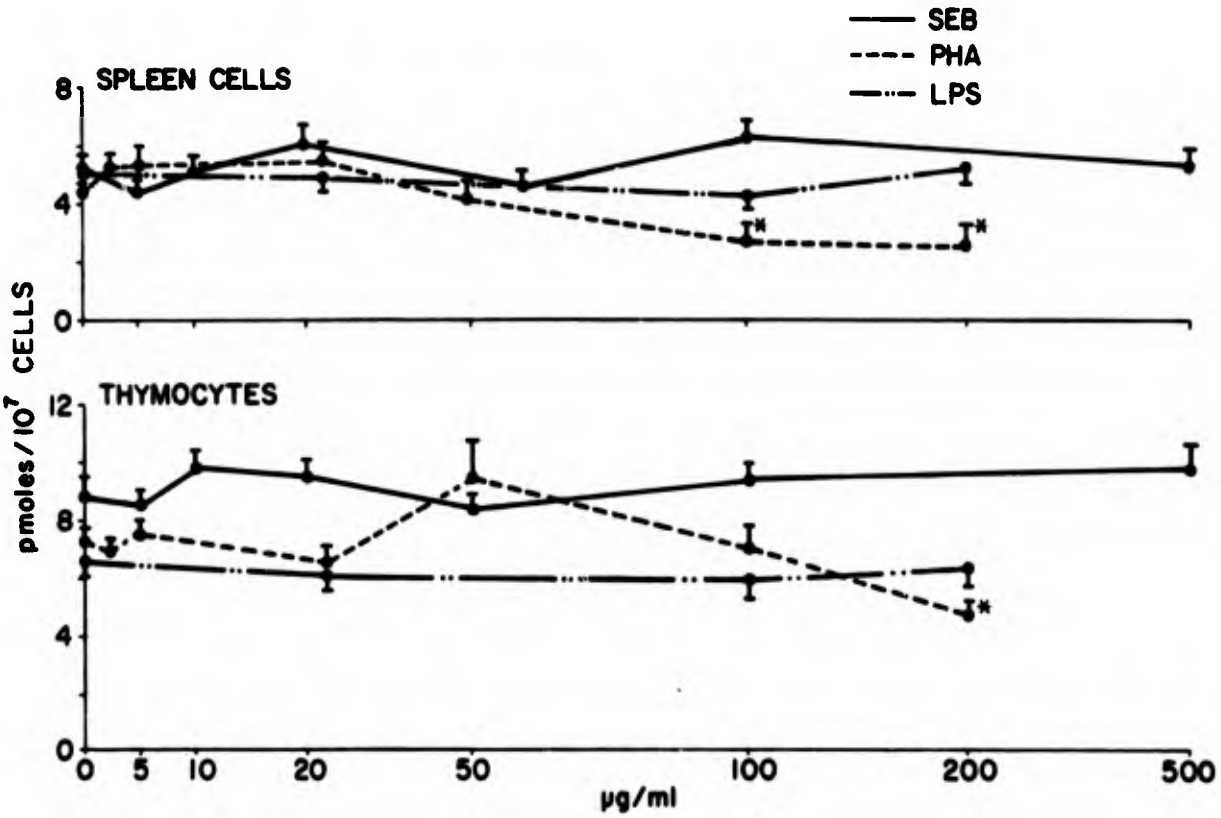

FIGURE 4. EFFECT OF MITOGENS, SEB, PHA AND LPS, ON cAMP CONTENT OF MOUSE LYMPHOID CELLS.

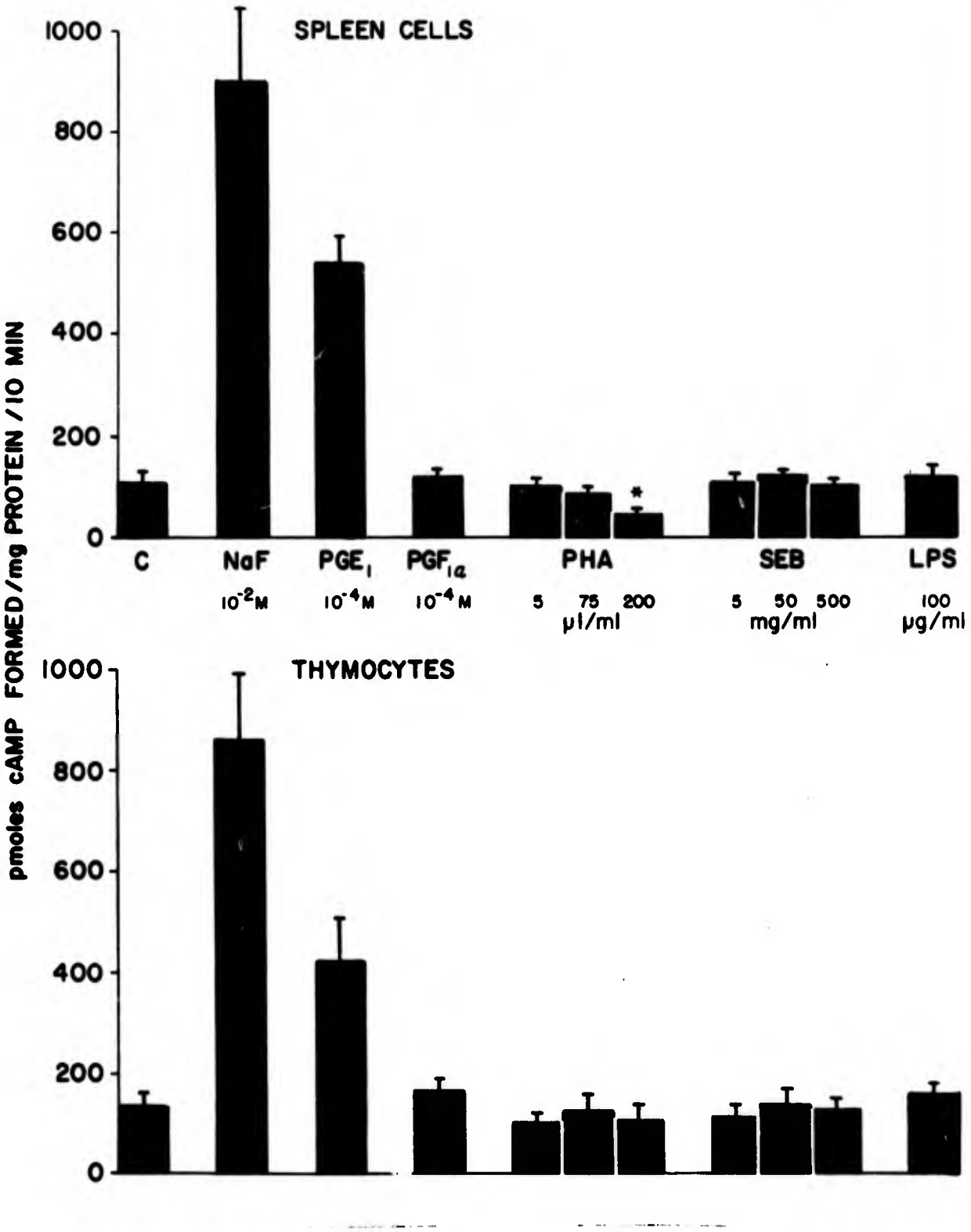

FIGURE 5. ADENYL CYCLASE ACTIVITY IN MOUSE LYMPHOID CELLS.

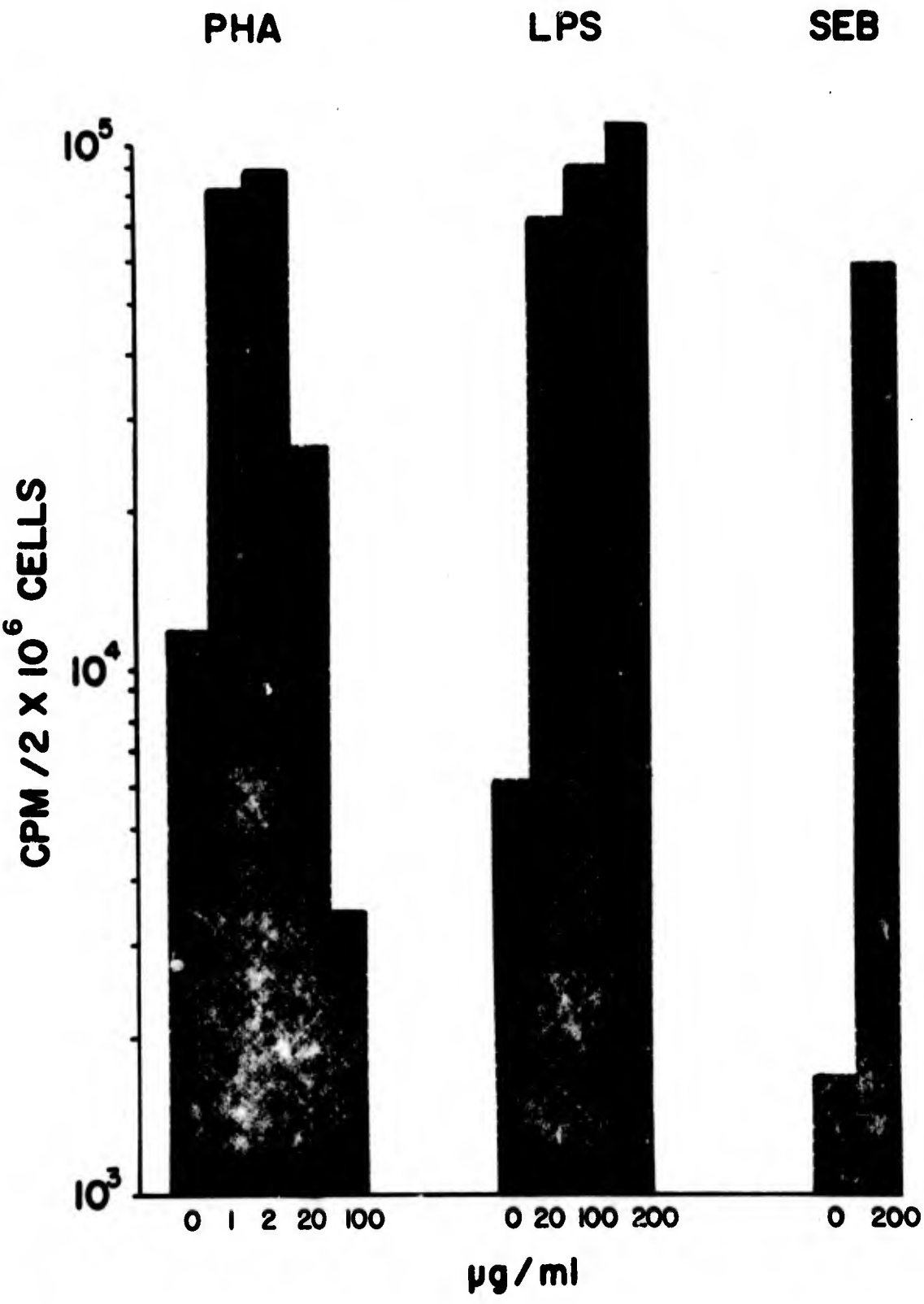

FIGURE 6. ^{3}H-THYMIDINE INCORPORATION INTO MOUSE SPLEEN CELL DNA (48 HR INCUBATION).

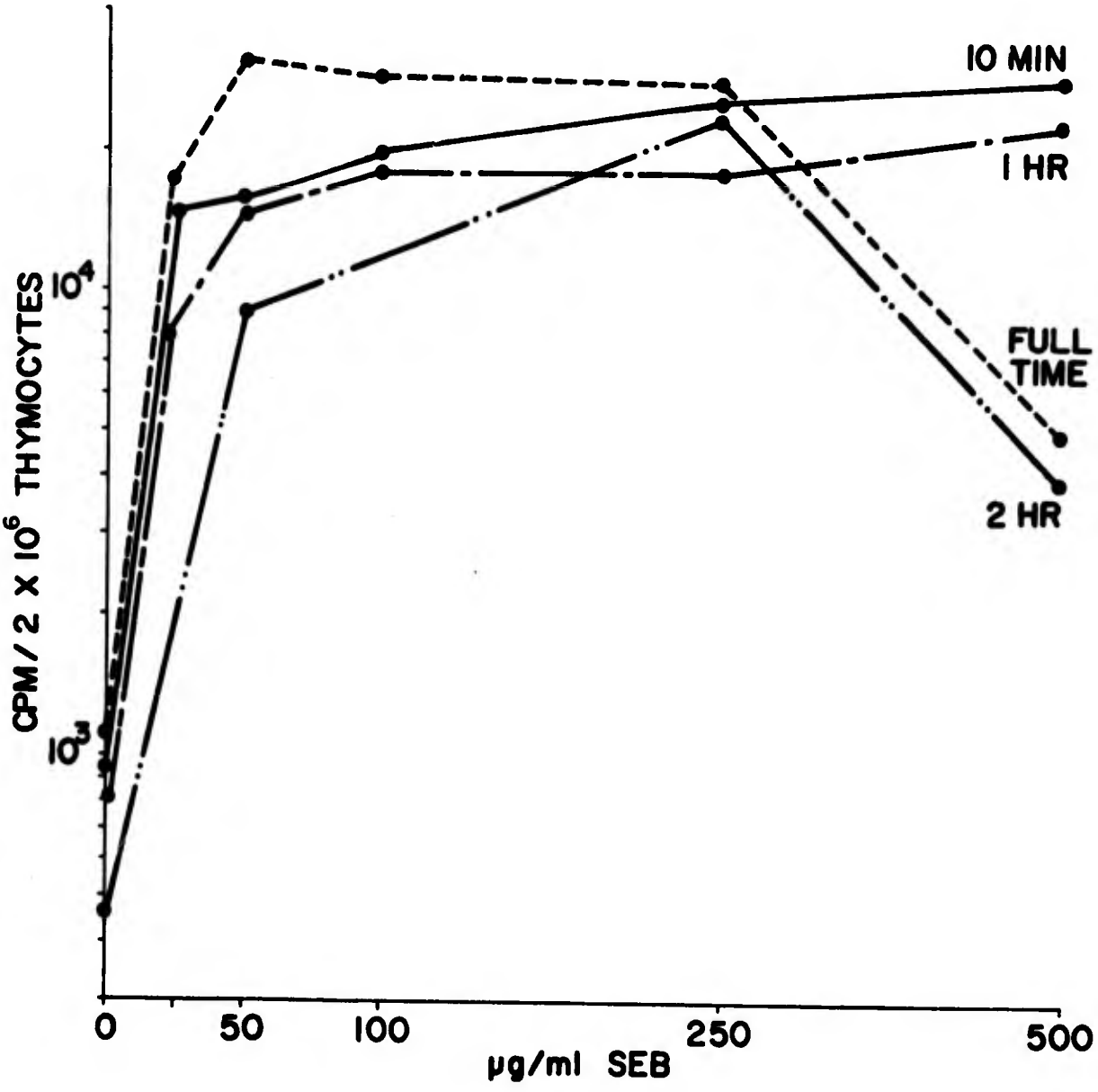

FIGURE 7. EFFECT OF CELL WASHING ON SEB STIMULATION OF ^{3}H-THYMIDINE INCORPORATION INTO MOUSE THYMOCYTE DNA (48 HR INCUBATION).

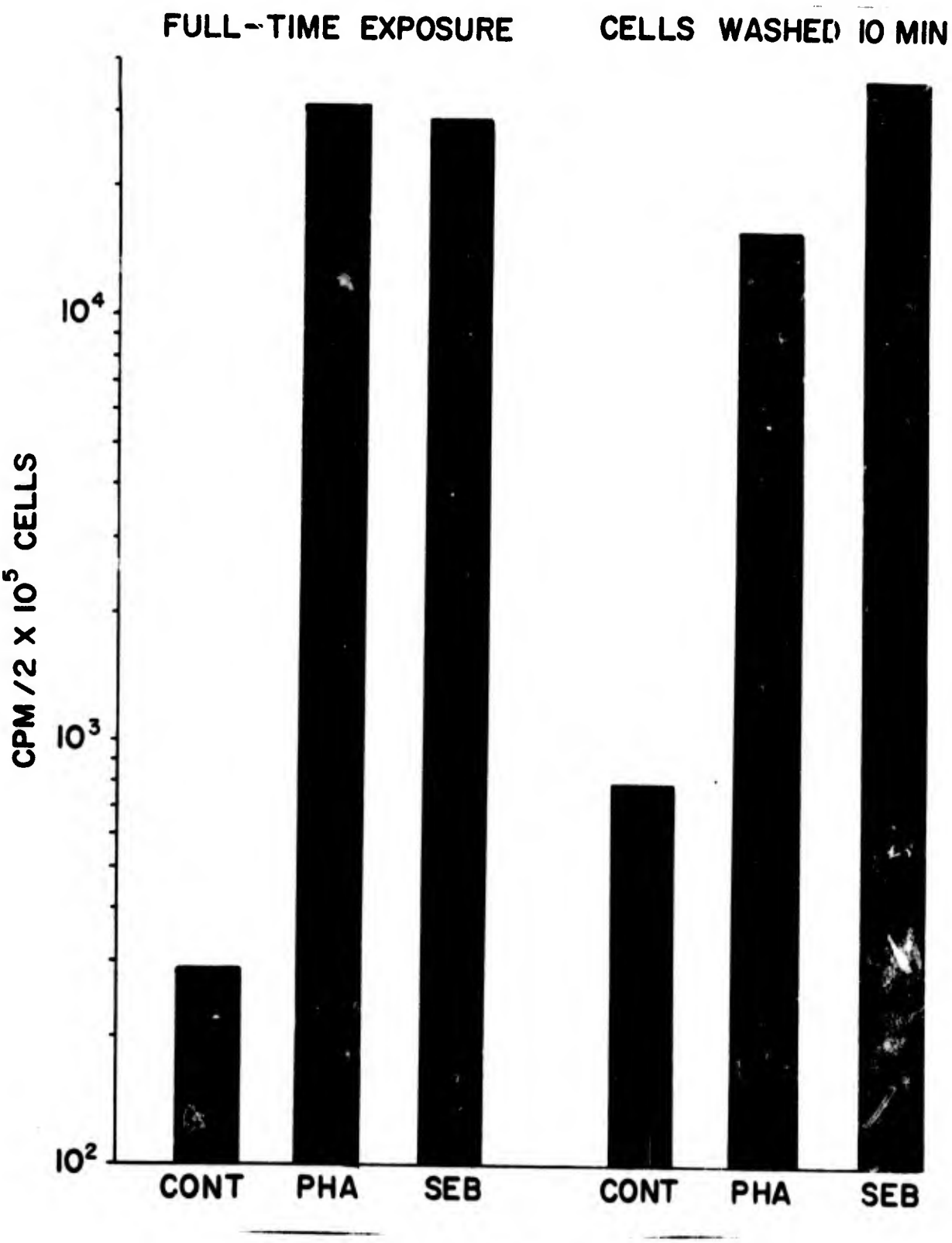

FIGURE 8. ^{3}H-THYMIDINE INCORPORATION INTO HUMAN LYMPHOCYTE DNA (5-DAY INCUBATION).

In similar experiments, both SEB and PHA enhanced DNA synthesis in human lymphocytes which were either washed at 10 min or exposed to the mitogenic agents full time (Figure 8). As previously indicated, PHA, but not SEB, increased cAMP levels in human lymphocytes within 10 min.

The significance of the PHA mediated increases in human lymphocyte cAMP levels is at present uncertain, but could possibly be related to nonmitogenic components of this heterogeneous extract[8] rather than implying a cAMP-dependent mechanism of cell activation. Unlike PHA, the highly purified preparation of SEB employed failed to increase cAMP levels in any of the cell types tested but was mitogenic in all.

In summary, these data suggest that an early or primary stimulation of the adenyl cyclase - cAMP system of lymphoid cells is not a general or essential feature of lymphoid cell activation by mitogens. It should be emphasized that these studies in no way exclude other important and complex roles for cyclic nucleotides in the regulation of lymphocyte function, such as the inhibitory effects of cAMP or the cytolytic activity of lymphoid cells.[9] Our data do suggest however, that the initiation of lymphocyte transformation is not a simple function of direct activation of the second messenger system of lymphoid cells by mitogenic stimuli.

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CURRENT CONCEPTS OF THE REGULATION OF EARLY METABOLIC CHANGES

Robert W. Wannemacher, Jr., Ph.D.*

It has been well established that infections or inflammatory responses are characterized by overall catabolic activity in the host.[1] However, previous investigators have emphasized the fact that many of these changes represent redistribution of the various metabolites within the host and alterations in many of the anabolic processes within various cells.[2,3] Three host tissues have received the greatest amount of study: (1) liver, because of the anabolic role it plays in the syrthesis of various acute-phase globulins; (2) skeletal muscle, because it is a reservoir of amino acids, proteins, and glycogen; and (3) blood, because of serum which reflects many of the movements of the metabolites, and white blood cells (neutrophils and lymphocytes), which play a significant role in host defense against infectious organisms. Most of these changes occur before clinical illness and are nonspecific, in that they do not involve humoral immunity.

The data presented in Figure 1 illustrate some of the sequential changes noted in the liver and serum of rats inoculated subcutaneously with virulent Diplococcus pneumoniae. By 4 hr postinoculation serum Zn as well as Fe (not shown) are significantly decreased. These trace metals continued to decrease in a linear fashion so that by the time of illness, at approximately 15 hr, the values are reduced to 50% of control concentrations. These decreases in serum trace metals represents a flux into liver tissue and not a loss by increased excretion.

Cycloleucine is a model amino acid which can give some insight into the rates of movement of other metabolized amino acids into various cells of the host. It is transported across the so-called "L" site; it is not metabolized or readily excreted by rats. When rats are equilibrated with this radioactive amino acid, ^{14}C-cycloleucine, and subsequently inoculated with D. pneumoniae, it concentration is significantly increased in the liver. Another model amino acid, amino isobutyrate (AIB), is also significantly accumulated in the liver of infected rats. Since these amino acids are transported at the major site on cell membrane it can be concluded that an infectious process stimulates increased transport of all amino acids into liver cells and accounts for the infection-related decrease in serum amino acids that has been observed in man and experimental animals. The cycloleucine continues to accumulate in the liver so that by the end of approximately 30 hr there are 3 times as much cycloleucine in the liver of the infected animals as in pair-fed controls.

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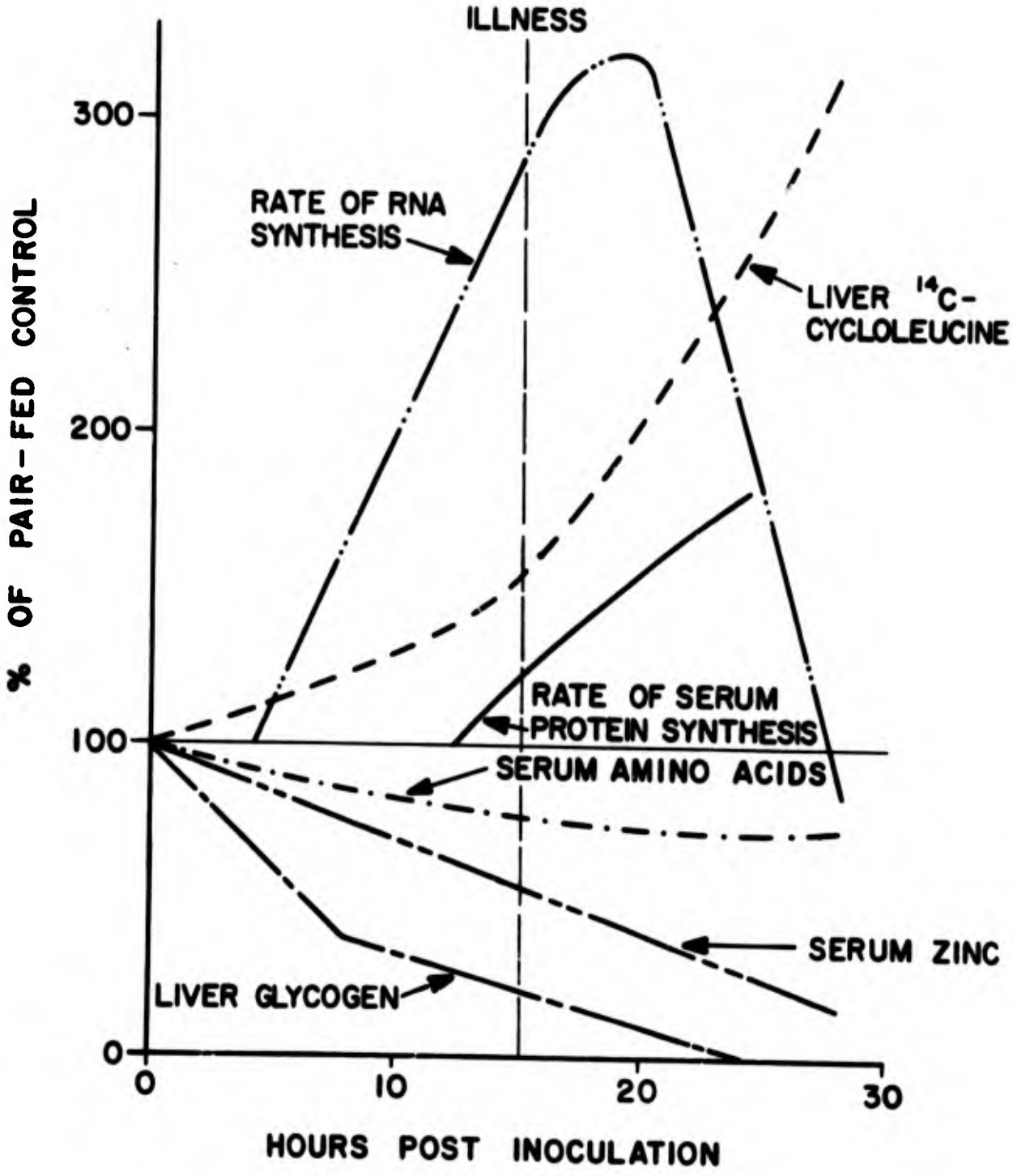

FIGURE 1. SEQUENTIAL CHANGES IN LIVER AND SERUM OF RATS INFECTED WITH *PNEUMONIAE*.

Serum amino acids are decreased very early in the infection. In muscle, the concentration of cycloleucine is progressively decreased. These changes are similar when expressed as concentration per milliliter of intracellular water or per gram of tissue. These data have been interpreted as representing a flux of amino acids from skeletal muscle to liver tissue. Following this flux there is a rapid increase in RNA synthesis in the liver, with most of the increase being associated with the so-called "bound ribosomal fraction." The increased rate of RNA synthesis is initially noted by 6 hr postinoculation and reaches a maximum nearly 3 times as high by 20 hr. At 12 hr the rate of serum protein synthesis is increased, representing an increase predominately in "acute phase globulins" or glycoglobulins, and a decrease in the rate of albumin synthesis.[4] In muscle protein synthesis is decreased very early after inoculation with D. pneumoniae.[2] Thus, an unaltered fractional catabolic rate of muscle protein plus a decrease in anabolic rate will result in a supply of amino acids which are readily moved to liver tissues for the rapid synthesis of certain serum proteins. Some of these amino acids will be utilized for gluconeogenesis or catabolized, resulting in increased secretion of urinary nitrogenous products, which has been associated with the catabolic activity of infectious disease. The increased need for fuels for energy is illustrated by the rapid loss of liver glycogen in infected animals as compared to pair-fed control.

As has been described many of the earlier biochemical changes can be produced by injecting serum from infected men or experimental animals into recipient rats.[5-7] These observations suggest that a humoral factor is present in the serum which mediates many of these biochemical changes. Also leukocytic endogenous mediator (LEM), a factor which is released from stimulated peritoneal leukocytes, elicits many of the changes shown in Figure 1.[6,7] Sequential changes in rats given a single 1-ml intraperitoneal injection of LEM are shown in Figure 2. Within 1 hr hepatic cycloleucine is significantly increased while serum Zn is decreased; the maximum accumulation of cycloleucine and depression of serum Zn occur in 4-6 hr. Within 4 hr the rate of synthesis of hepatic RNA, mostly bound ribosomes, is markedly increased and by 10 hr reaches a rate nearly 3 times that seen in saline-injected controls. By 12 hr the rate of serum protein, mainly "acute phase globulins," is significantly increased. This pattern is very similar to that observed in animals infected with D. pneumoniae but most of the changes are 2-3 hr earlier.

Dr. Cockerell has reported similar changes in liver serum and muscle of animals given subcutaneous injection of sterile turpentine (page 51, this report). Again, the timing is similar to that observed in rats infected with pneumococci. Our current theory as to the mechanism involved in the mediation of these changes is based on the concept that both the infectious organism and the turpentine stimulate phagocytosis by neutrophils in the circulation with the resulting release of LEM. It in turn mediates many of the biochemical changes noted in the infected and turpentine-injected animals.

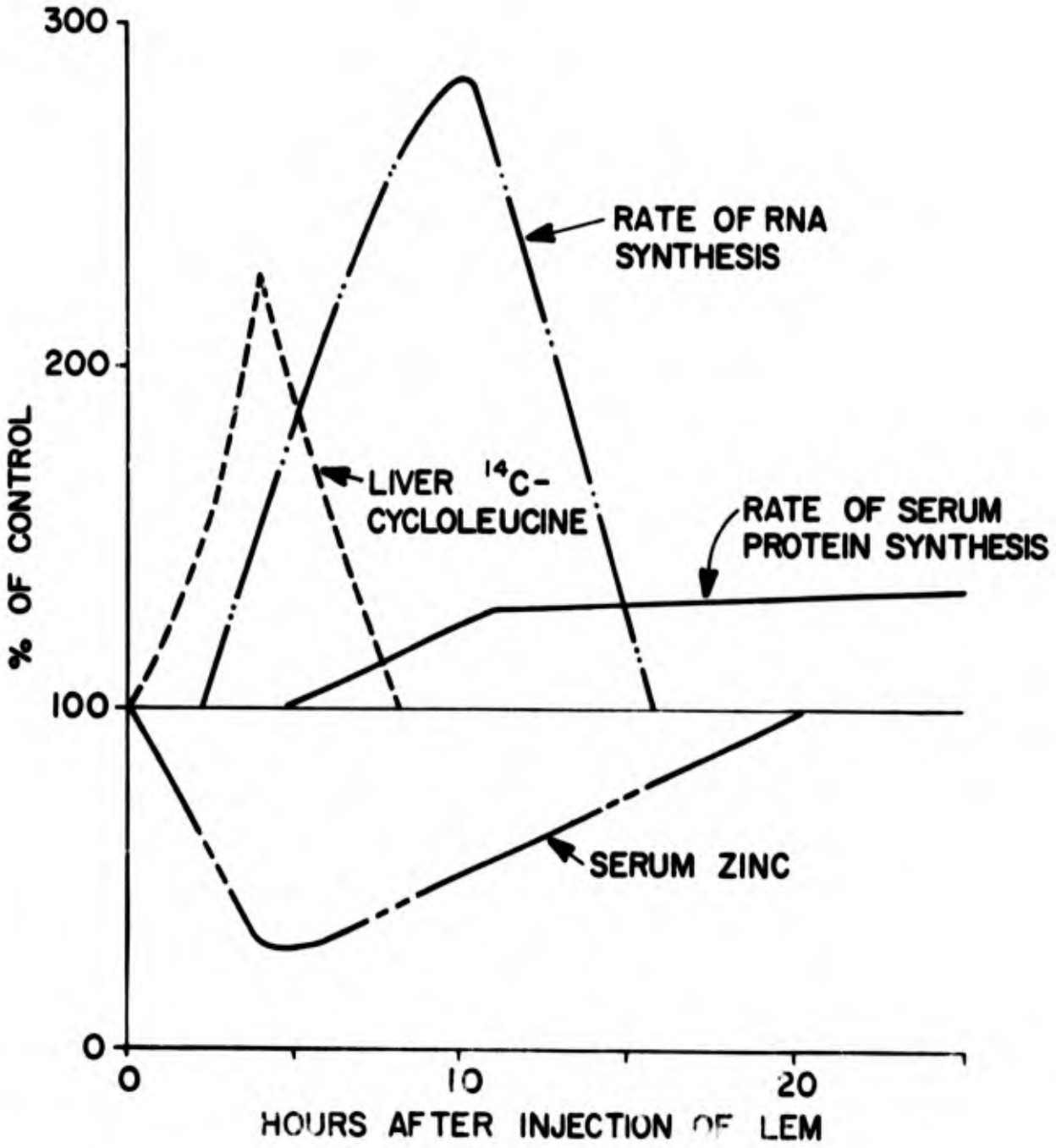

FIGURE 2. SEQUENTIAL CHANGES IN LIVER AND SERUM OF RATS INOCULATED WITH LEM.

In an attempt to describe the mechanisms and probable sites of metabolic effects on host cell functions, a schematic diagram of a model cell is shown in Figure 3. Many of the hormones have their action via the so-called "second messenger system." This involves stimulation of adenyl cyclase, a membrane bound enzyme, to stimulate more intracellular cyclic AMP. In turn, cAMP has been shown to regulate the metabolic activities of various cells, e.g. glycogen synthesis and breakdown and release of glucose. During infection glycogen is broken down and glucose storage is inhibited. This has lead to the concept that infection somehow affects the balance between insulin and glucagon, the 2 pancreatic hormones which regulate glucose release and storage, thus suggesting an increased need for metabolic fuels, such as glucose, as a source of energy in the infected host.

Previous data have indicated that fatty acids are mobilized and rapidly catabolized in infected hosts.[8] The utilization of fatty acids as a source of energy in infected animals is under investigation in Physical Sciences Division. In addition, the effects of infection on oxidative phosphorylation, a mitochondrial activity, are also being investigated. However, there is evidence from our laboratory and in the literature that certain bacterial products such as endotoxins can markedly affect this energy producing process.[9,10] At the lower left of the figure are listed 3 hormonal regulators, glucocorticoids, growth hormone and LEM?, which can affect the protein synthetic capacities of the cell. These hormones can stimulate amino acid transport, utilization of existing polysomes and the synthesis of new ribosomal and messenger RNA. Increased secretion of all 3 hormones have been demonstrated in the infected hosts.[7,11,12] LEM which is shown to be released early during the infectious process has a marked effect on the transport of amino acids, synthesis of bound ribosomal RNA (associated with synthesis of serum proteins) and subsequent synthesis and release of "acute phase glycoglobulins." These globulins are synthesized on the so-called "bound ribosomes" and are released into the endoplasmic reticulum, where the glycosugars are added. These glycoproteins are transported to the golgi apparatus and subsequently released into circulation. Ceruloplasmin, the copper-containing protein of the serum, is a good example of a protein in this category. Similarly, triglycerides and cholesterol are added to these proteins in the golgi apparatus thus accounting for the movement of fatty acids between various tissue compartments of the host.

Lysosomal and peroxysomal enzymes are involved in the catabolic activities of the cell and represent some of the intensive research activity on infection-related effects on these cytoplasmic particles now being carried forth in Bacteriology Division. Prostaglandins are a fatty acid hormone, synthesized and released from the membrane of various cells of the body. This hormone stimulates the so-called "second messenger system" of various immunocompetent cells, but apparently is unrelated to their mitogenic activity. However, there is a possibility that prostaglandins could be involved in the mediation of the inflammatory response and modulation of the release of cytotoxic products associated with cellular immunity.

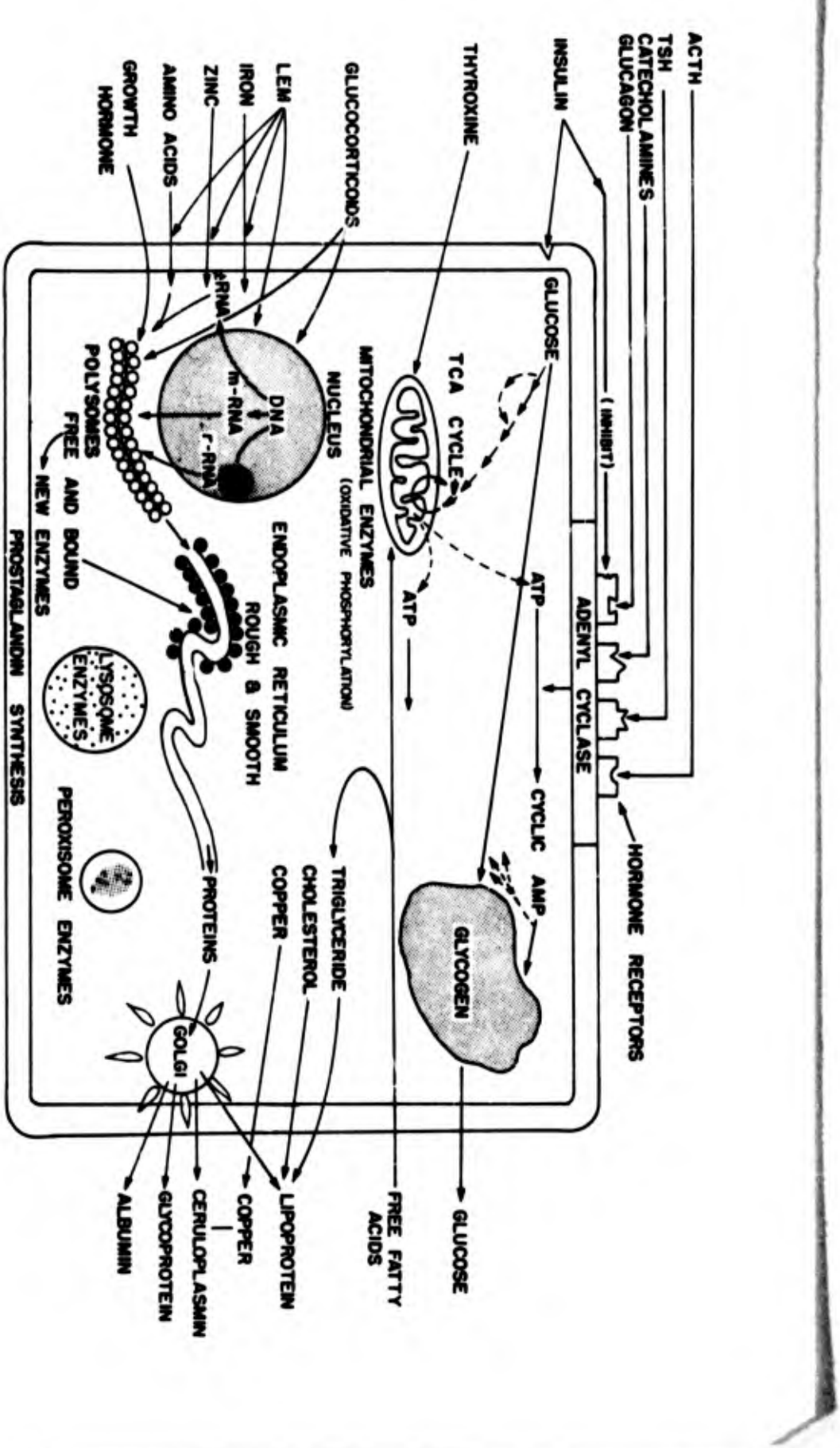

FIGURE 3. SCHEMATIC DIAGRAM OF A MODEL CELL.

The mechanism by which various hormones or metabolites can affect the pattern of protein synthesis within the cell can best be described schematically (Figure 4). Looking at DNA chromatin, it is realized that within the nucleus of every cell within the body, there is the genetic information for the synthesis of every protein also present within the body. Thus, liver contains not only those genomes which are involved in the synthesis of various hepatic enzymes and serum proteins, but also the proteins for the lens of the eye. Obviously, liver does not synthesize the proteins for the lens; therefore we can consider that this genome is highly repressed as dramatically illustrated on the extreme right. Other genomes such as serum albumin are relatively derepressed, in that the codes of messenger RNA are continually being made for these proteins in the liver (extreme left). For other proteins there can be varying degrees of repression or derepression. Various proteins and RNA classes have been associated with the repression of genone sites. Corticoids have been shown to derepress those genomes associated with the synthesis of certain hepatic enzymes such as tyrosine transaminase and tryptophan oxygenase.[13] The degree of amino acid acetylation of transfer RNA, zinc, and LEM have been suggested as being capable of repressing or derepressing certain specific genomes involved in the synthesis of specific proteins within a cell. We believe that LEM either directly or indirectly can affect those genomes responsible for the synthesis of messenger RNA for the various "acute phase globulins" by causing a derepression of these messengers as well as affecting the synthesis of ribosomal RNA and proteins associated with the so-called bound ribosomal fraction. We are currently investigating the effects of infection and LEM on chromatin template activity as well as RNA polymerase activity in the liver of the infected host. We hope to gain further insight into the mechanisms by which various infection-related stimuli are able to alter host metabolism.

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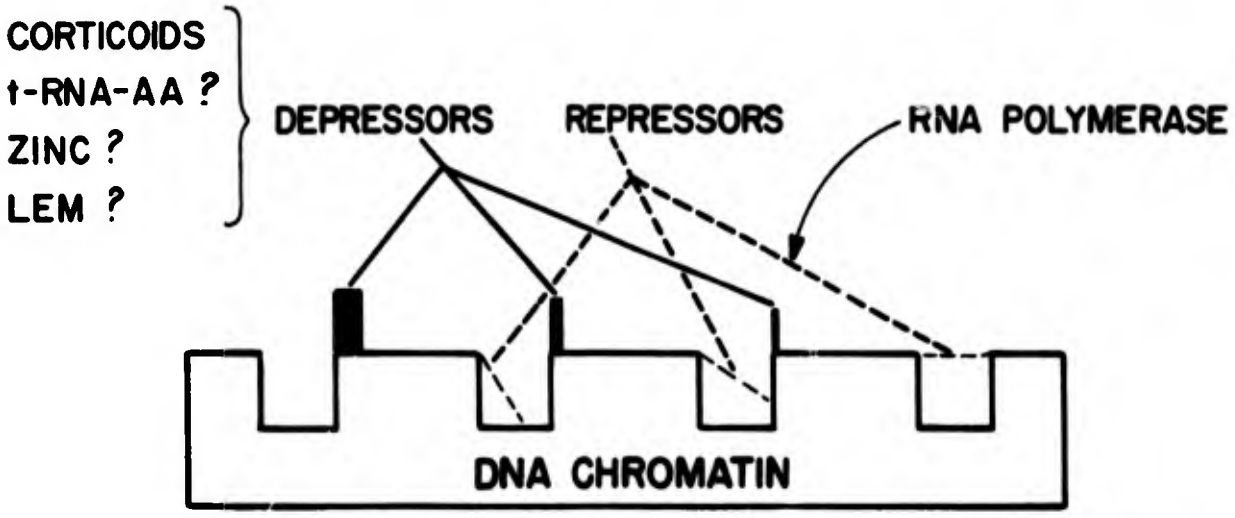

FIGURE 4. SCHEMATIC DIAGRAM OF DNA CHROMATIN REPRESSION AND DEREPRESSION.

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THE INTERACTION OF FORMALDEHYDE WITH AQUEOUS STAPHYLOCOCCAL ENTEROTOXIN B

John R. Warren, Major, MC,* Leonard Spero, Ph.D., and Joseph F. Metzger, Colonel, MC

Diminution in toxicity of bacterial exoproteins unaccompanied by a corresponding loss of specific antigenicity upon treatment with aqueous formaldehyde is well established. Yet little is known about specific biochemical mechanisms by which formaldehyde inactivates bacterial exotoxins.[1] During the past year a study was initiated on the effect of aqueous formaldehyde upon the conformation and biologic activity of staphylococcal enterotoxin B (SEB). Staphylococcal enterotoxin used in all experiments was obtained in highly purified form from appropriate bacterial culture supernatants by the carboxylic acid resin chromatographic procedure suggested by Schantz and his co-workers.[2] In our studies 2 mg/ml of SEB were added to 0.15 M phosphate buffer, pH 5.0 or 7.5, or 0.05 M carbonate buffer, pH 9.5. Formaldehyde was dialyzed into toxin-containing buffer to a final concentration of 1 gm %, giving a 4300 molar excess of formaldehyde to protein. During formalization of toxin for 30 days at 37 C, a change in charge of the toxin was followed by cellulose acetate electrophoresis and alteration in the shape and size by Sephadex G-100 exclusion chromatography. At the end of the 30-day reaction period loosely bound formaldehyde was removed from enterotoxin by exhaustive dialysis against large volumes of phosphate buffer.

Figure 1 shows the cathodic mobility of SEB on cellulose acetate strips, the protein migrating about 8 mm from the origin (O) after 15 min. After only 4 hr of exposure to 1% formaldehyde at pH 9.5, SEB migrated toward the anode (Figure 1b). Identical behavior of the 30-day product after dialysis against dilute buffer was observed. Furthermore, heating the 30-day formalized derivative at 100 C for 60 min had little effect upon its electrophoretic behavior (Figure 1c). Similar changes were found for enterotoxin exposed formaldehyde at pH 7.5. By 4 hr the native cathodic mobility was reversed to anodic (Figure 1d). After 16 hr or 30 days of treatment the formalized derivative migrated about 15 mm from the origin (Figure 1e). Again the 30-day product retained its large negative charge after exhaustive dialysis or heating at 100 C (Figure 1f). Reaction of formaldehyde with toxin was relatively slow at pH 5. After 4 hr in pH 5 buffered formaldehyde, toxin still migrated toward the cathode (Figure 1g) equilibrium not being obtained until after 4 days of reaction (Figure 1h). Formaldehyde bound to the 30-day toxin derivative was stable to prolonged dialysis but decomposed after heating at 100 C for an hour (Figure 1i).

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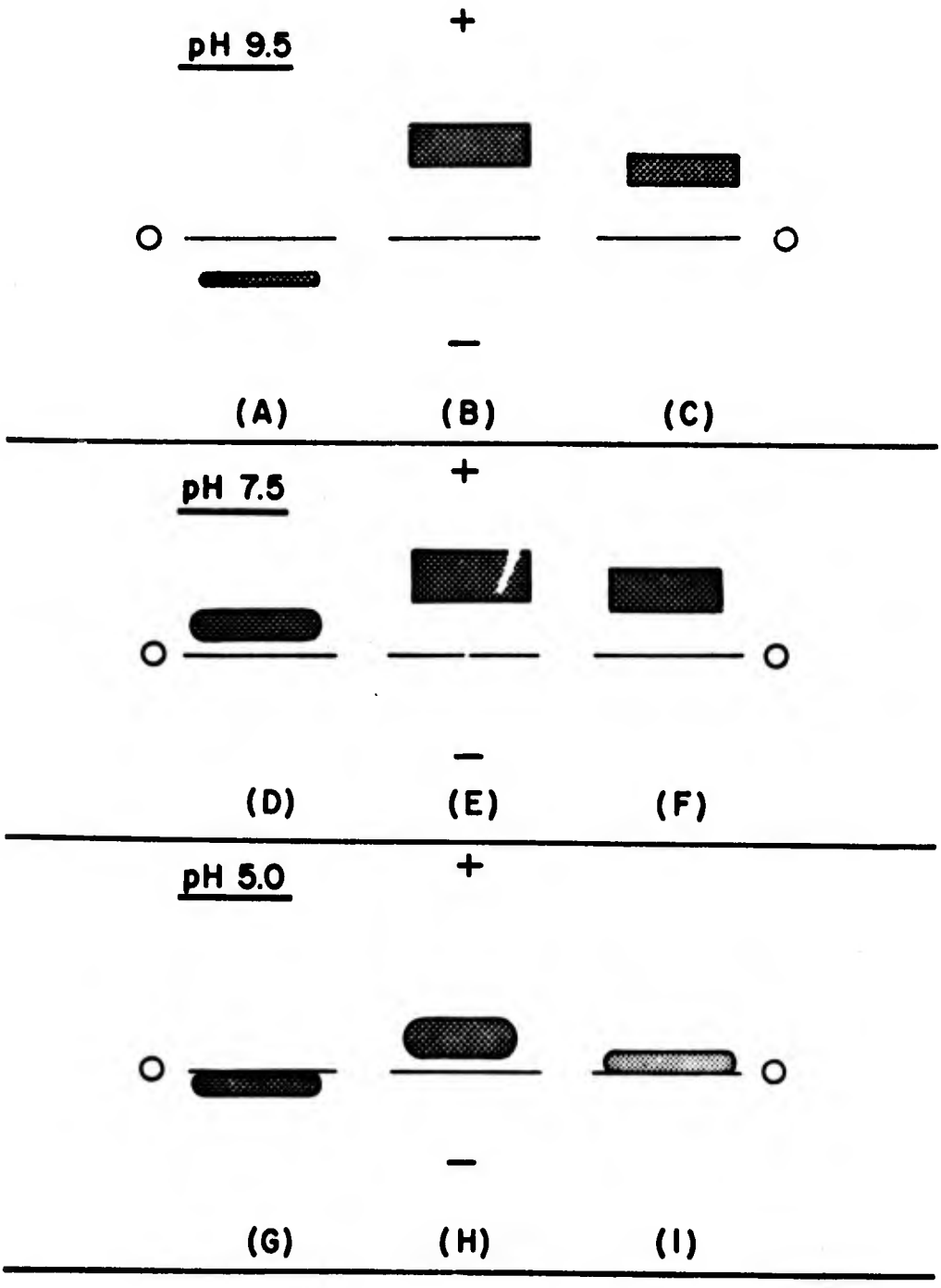

FIGURE 1. CELLULOSE ACETATE ELECTROPHORESIS, pH 8.6 BORATE BUFFER
a. 15 MIN, b. 4 HR, c. 30 DAYS (INACTIVATED);
d. 4 HR, e. 16 HR, f. 30 DAYS (INACTIVATED);
g. 4 HR, h. 4 DAYS, i. 30 DAYS (INACTIVATED).

Only a faint smudge was seen on the anodic side of the origin following heat treatment. In summary, formaldehyde is introduced into SEB at all three pH values in a form very stable toward continued dialysis against formaldehyde-free buffers. Since hydroxymethyl groups added to single amino acid residues readily dissociate into free formaldehyde, this indicated that formaldehyde formed stable covalent bridges between pairs of amino acids in the toxin molecule.[1] Also, reaction of formaldehyde with enterotoxin slowed in rate with decreasing pH and bonds introduced into toxin by formaldehyde at pH 5 were heat-labile.

In deciding whether formaldehyde forms intramolecular covalent bridges within or intermolecular covalent bridges between enterotoxin molecules, consideration of the G-100 gel filtration data is necessary. About 80% of the protein eluted in the void volume (at about 40 ml of eluant) of 1.5 x 83 cm columns of Sephadex G-100 after less than one day's incubation in formaldehyde buffered at pH 7.5. Since native SEB showed a single, symmetrical peak with an elution volume of around 85 ml upon gel filtration on the same G-100 columns, rapid and extensive polymerization of toxin by formaldehyde at neutral pH is apparent. Similar although slower polymerization of toxin in acid formalin occurred. Thus, after one day's exposure no void volume material was detectable. However, after 2 days, protein began to appear in the void volume fractions; by the 4th day most of the pH 5 formalized toxin was completely excluded by the G-100 gel. In contrast, enterotoxin formalized at pH 9.5 did not polymerize extensively. Even after 30 days only a trace amount of protein was found in the void volume eluant. Most of the pH 5 material showed an elution volume of about 65 ml. The Sephadex G-100 column was calibrated with 8 protein markers and the smooth curve constructed from the data relating elution volume to void volume ratios to molecular weights was used to estimate the apparent molecular weights of the different gel filtration fractions of the three formalized toxins. Most of the toxin treated with formaldehyde at pH 5 or 7.5 was polymerized into derivatives with molecular weights exceeding 150,000 (Table I). Smaller

TABLE I. MOLECULAR WEIGHTS FROM EXCLUSION CHROMATOGRAPHY

pH	Ve/Vo ± 0.1	MW ± 20%
5.0	1.0	> 150,000
	1.3	75,000
	1.7	35,000
7.5	1.0	> 150,000
	1.3	75,000
	1.6	40,000
9.5	1.2	100,000
	1.5	50,000

amounts of formaldehyde-denatured monomer and dimer were also present. At pH 9.5, formalized toxin formed primarily a formaldehyde-denatured monomer with only a small amount of dimer. Upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the 30-day pH 5, 7.5, or 9.5 formalized toxin, a series of polymeric species distributed from the origin to the monomeric form was observed with the pH 5 or 7.5 derivative. Only a very broad monomeric band and some dimer were present in the pH 9.5 product. The width of the pH monomeric band relative to the narrow bands formed by protein markers indicated extensive intramolecular cross-linking at pH 9.5 with formation of heterogeneous monomeric conformers. In summary, at the alkaline pH 9.5, formaldenyde introduced intramolecular methylene bridges into the SEB molecule with formation of a large number of monomeric species. At neutral or acid pH values, however the methylene bridges formed by formaldehyde were directed between enterotoxin molecules with polymerization of the protein into high molecular weight species.

Only a few brief points about specific mechanisms for the observed pH dependence of the formalization of enterotoxin can be made here. Since formaldehyde can react only with unprotonated amino groups[3] and since only 1 of the 36 lysyl epsilon-amino groups of enterotoxin is unprotonated at pH 7.5, increased reactivity of lysines in the toxin molecule toward formaldehyde cannot be offered as an explanation for polymerization at neutral pH. Fraenkel-Conrat and Olcott[4] in their extensive investigations of protein-formaldehyde interaction demonstrated covalent linking of aromatics to serum albumin by formaldehyde at neutral pH in a form stable to steam distillation. Thus, extensive polymerization of toxin by formaldehyde at pH 7.5 is compatible with participation of its tyrosines in the formation of heat-stable cross-links between protein molecules (Figure 2). A large number of cross-links could be formed by such a mechanism since 21 of the 239 amino acid residues in SEB are tyrosines.[5] Also, Fraenkel-Conrat et al.[6] demonstrated that amide-formaldehyde bonds introduced into proteins at acid pH values were very slow in forming and easily destroyed by steam distillation. Thus, the slow polymerization of toxin at pH 5 and the heat lability of the 30-day derivative strongly suggest that cross-links introduced between proteins at this pH involve asparagine and glutamine residues (Figure 2). Again, extensive polymerization by such cross-linking is certainly consistent with the 30 or so residues of asparagine and glutamine found in each SEB molecule.[5] The ability of formaldehyde to react with different amino acid residues in proteins at different pH values and the consequences of this altered reactivity for the structure and biologic activity of final reaction products is of far-reaching theoretical and practical importance and is under further investigation.

The antigenic activity of the 3 formalized derivatives has been compared both *in vitro* and *in vivo*. Quantitative precipitin tests with rabbit anti-enterotoxin B showed a sharp zone of equivalence at 30 μg of added native enterotoxin (Figure 3). The pH 5 30-day formalized toxin gave a much

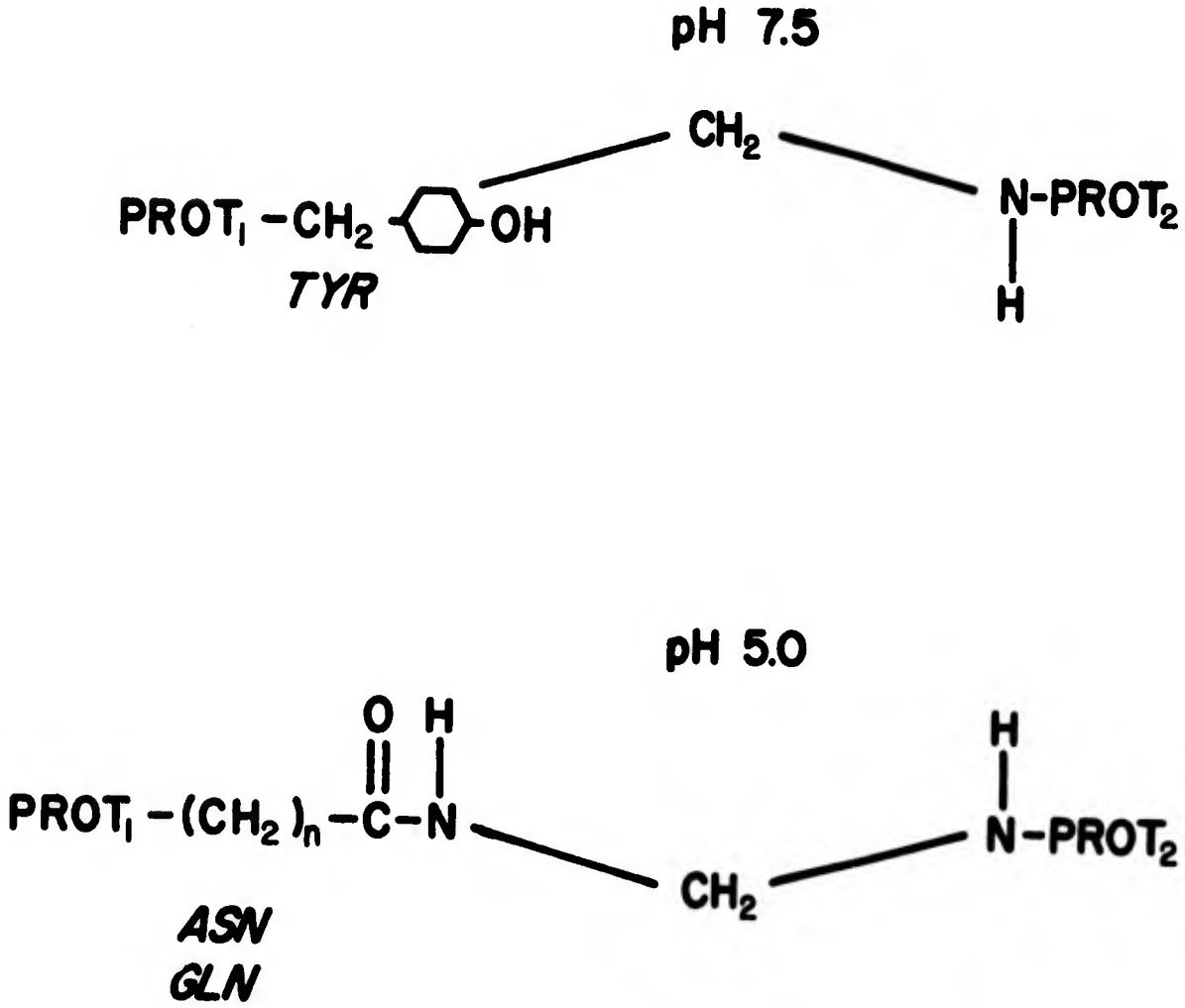

FIGURE 2. METHYLENE BRIDGE FORMATION INVOLVING SIDE-CHAINS OF TYROSINE AT pH 7.5 ON ASPARAGINE AND GLUTAMINE AT pH 5.0.

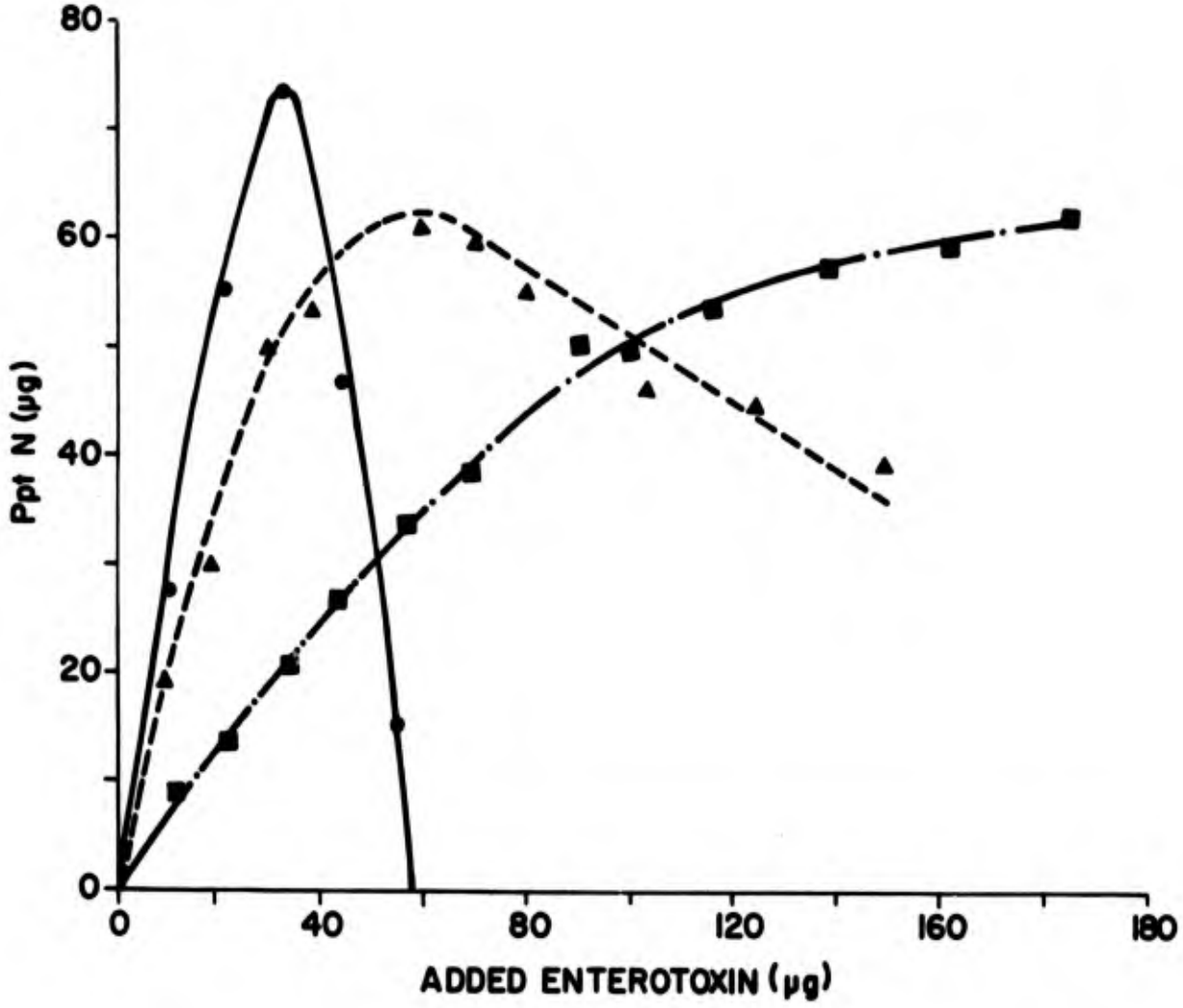

FIGURE 3. REACTIONS OF NATIVE, pH 5.0 (---) AND pH 7.5 (—·—·) FORMALINIZED TOXIN WITH RABBIT ANTI-SEB PRECIPITINS.

broader zone of equivalence at around 70 μg of added protein. No clear zone of equivalence was noted with pH 7.5 30-day formalized toxin. However, if it is assumed that all added protein between 120 and 180 μg is precipitated by specific antibody, then for purposes of comparison 35 μg of antibody N are present in the precipitate with pH 7.5 formalized toxin, 54 μg with pH 5 formalized toxin, and 70 μg with native toxin. Thus, at equivalence pH 5 formalized toxin combines with 75% as much specific antibody as native enterotoxin, pH 7.5 formalized toxin 50% as much. No precipitate was formed by adding up to 100 μg of the pH-9.5, formalized toxin.

Five milligrams of pH 5, 7.5, or 9.5 inactivated toxin were injected subcutaneously into 4 rhesus monkeys with no subsequent signs of enterotoxemia, that is emesis, diarrhea, or death. Within 1 month of injection a brisk, anti-enterotoxin hemagglutinin response was apparent in the 4 animals injected with pH 7.5 toxin, reciprocal titers ranging from 1:160-1:1280 (Figure 4). With one exception, the hemagglutinin response to pH 5 toxin was somewhat smaller and slower. No significant hemagglutinin response was observed in monkeys exposed to pH 9.5 formalized toxin. In conclusion, only polymeric toxin products derived from formaldehyde treatment at acid or neutral pH demonstrated detectable *in vitro* and *in vivo* antigenic activity. Therefore, while both intra- and intermolecular bridges appear to distort or "mask" toxic sites on the exoprotein molecule, integrity of the antigenic sites is maintained only when cross-linking occurs predominantly between enterotoxin molecules.

ACKNOWLEDGMENT

The authors wish to thank both Dr. Virginia McGann for performing the monkey sera hemagglutination determinations and SP4 Donald Dohlman for expert technical assistance in the laboratory.

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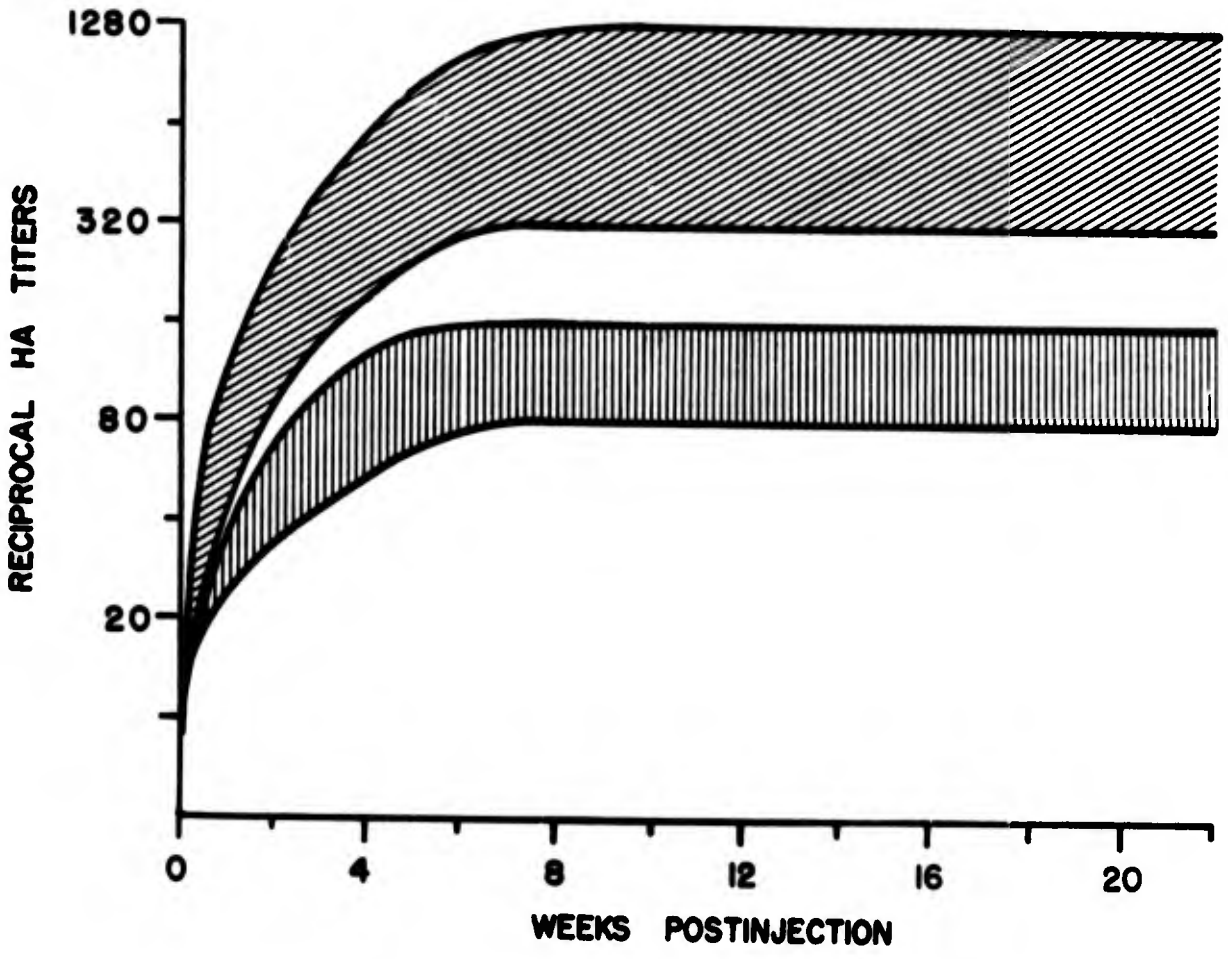

FIGURE 4. HEMAGGLUTININ RESPONSES OF RHESUS MONKEYS AFTER PARENTERAL ADMINISTRATION OF SEB FORMALINIZED AT pH 9.5 (DIAGONAL) AND 7.5 (VERTICAL).

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LIMITED DIGESTION OF STAPHYLOCOCCAL ENTEROTOXIN B BY TRYPSIN

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Joseph F. Metzger, Colonel, MC

The limited digestion of proteins by enzymes has found wide use by protein chemists in the study of the relation of structure and function. The work of Richards[1] on the cleavage of ribonuclease by subtilisin is perhaps the most outstanding example. A single peptide bond is split yielding 2 fragments which are individually inactive, but fully active when recombined.

Limited digestion occurs naturally in the activation of many enzymes and hormones, the conversion of trypsinogen to trypsin,[2] and of proinsulin to insulin.[3] A striking example has recently been observed in the field of microbial toxins. Diphtheria toxin is synthesized as a single polypeptide chain[4] whose toxicity is attributed to its catalysis of a reaction which inactivates an enzyme required in protein synthesis.[5,6] The intact toxin is enzymically inactive; brief exposure to low concentrations of trypsin converts it to 2 fragments held together by a single disulfide bond.[7] This has been called "nicked" toxin. In the presence of a thiol-reducing agent the enzymic activity is generated in one of the 2 fragments. "Nicking" occurs to a small but varying extent in all preparations of diphtheria toxin even before treatment with trypsin. It presumably results from enzymatic action during fermentation or subsequent purification. Figure 1 (based on a figure from a paper by Gill and Dinius[4]) illustrates these relationships. The solid line is the peptide chain with the amino terminus at the left and the carboxyl terminus on the right. There are 2 disulfide bridges and the trypsin sensitive site marked X is within one of these. Enzymatic hydrolysis at X yields nicked toxin and after reduction 2 separable fragments A and B. y is a less sensitive site of hydrolysis. Fragment A is enzymically active; fragment B although inactive is required for attachment to the host cell.

We have been investigating the digestion of highly purified staphylococcal enterotoxin B (SEB) by trypsin. The results offer great promise as a means for the identification of toxic and antigenic sites. Native enterotoxin has been reported to be resistant to trypsin; however, we have observed that it does undergo a rapid but limited digestion.

Figure 2 shows the results of a typical pH stat experiment. SEB was at a concentration of 5 mg/ml and the weight ratio of substrate to enzyme was 50:1. The ordinate represents the volume of alkali required to maintain a constant pH, here 9.0. The dashed line extrapolated to 0 time

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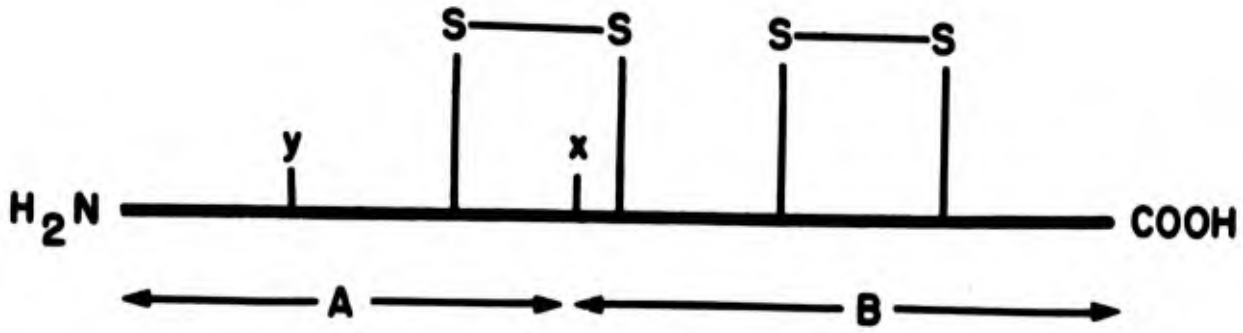

FIGURE 1. SCHEMATIC REPRESENTATION OF THE STRUCTURE OF DIPHTHERIA TOXIN.

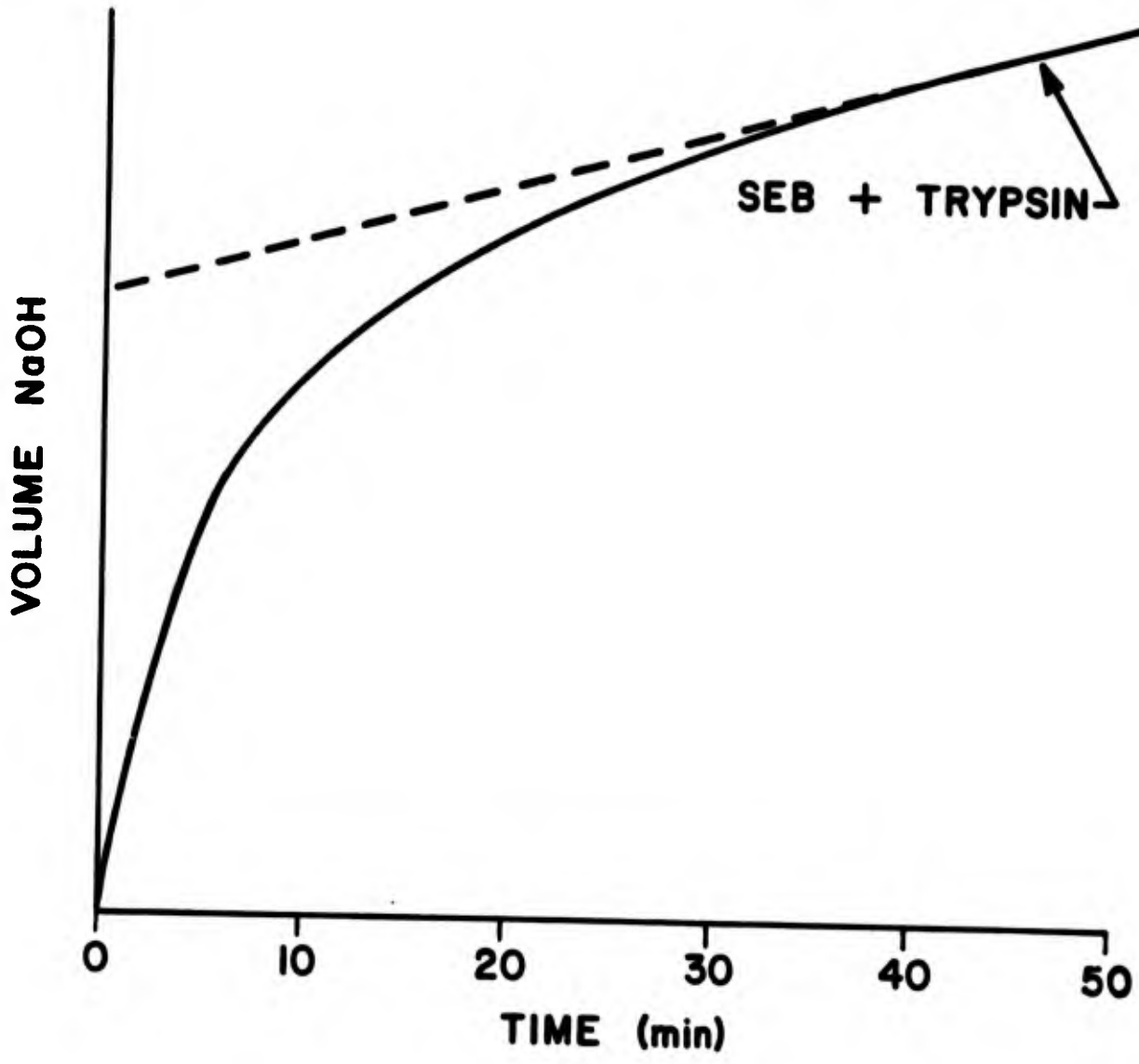

FIGURE 2. pH STAT CURVE OF THE DIGESTION OF SEB BY TRYPSIN.

permits a calculation of the number of bonds split in the initial rapid reaction. The figure obtained was 1.87 per molecule. At pH 8.5 and 8.0 values of 1.83 and 1.56, respectively, were obtained. This decrease suggests that the use of a single pKa for the calculation is inadequate and that there is at least partial cleavage of a bond whose amino group is unusually basic.

One would expect the molecule to be broken into at least 2 fragments. However, when this product, called SEB-T, was examined in the ultracentrifuge and by Sephadex gel filtration, only one component was found with the same molecular size as untreated SEB.

In order to verify that there actually was a specific reaction taking place and not the relatively complete breakdown of some denatured enterotoxin we looked for a terminal residue in SEB-T. Using the Sanger fluorodinitrobenzene technique[8] significant amounts of DNP-threonine in addition to the original N-terminus of DNP-glutamic acid were identified.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was used for clarification.[9] The first column in Figure 3 shows diagrammatically the kind of pattern obtained with 4 standards: bovine serum albumin with a molecular weight of 68,000, ovalbumin, 43,000, chymotrypsinogen, 26,000, and myoglobin, 17,000. Sodium dodecyl sulfate is used as a denaturant to convert all the proteins to random coils and to impart to them a high negative charge. In addition, the proteins are normally incubated in the presence of β-mercaptoethanol to reduce all disulfide bridges. A plot of the migration distance vs. the log MW yields a straight line and permits the determination of the MW of unknown proteins or peptides. The second column shows the position in this system of untreated SEB with a molecular weight of 28,500, without mercaptoethanol. The application of a large sample permits the demonstration of a low level of 2 smaller polypeptides. Thus SEB is nicked during bacterial fermentation to a small extent, just as diphtheria toxin is. This phenomenon has been observed in all preparations of SEB examined.

The next-to-last pattern shows SEB-T in the absence of reducing agent. The single line at the same position of SEB confirms the findings in the ultracentrifuge. When mercaptoethanol is added to SEB-T a completely different pattern is obtained, as seen on the extreme right. The line at the original SEB position has virtually disappeared and 2 new polypeptides are formed with molecular weights of about 16,400 and 13,000. Their sum, 29,400, is 3% higher than the value estimated for the molecular weight of SEB from its amino acid sequence. This deviation is well within the error of the method and indicates that the fragments are formed by a single cleavage and together comprise the entire molecule.

The effect of trypsin then must be on that portion of the peptide chain within the disulfide bridge between positions 92 and 112. Examination of the sequence of SEB[10] (Figure 4) confirms this. Trypsin is specific for

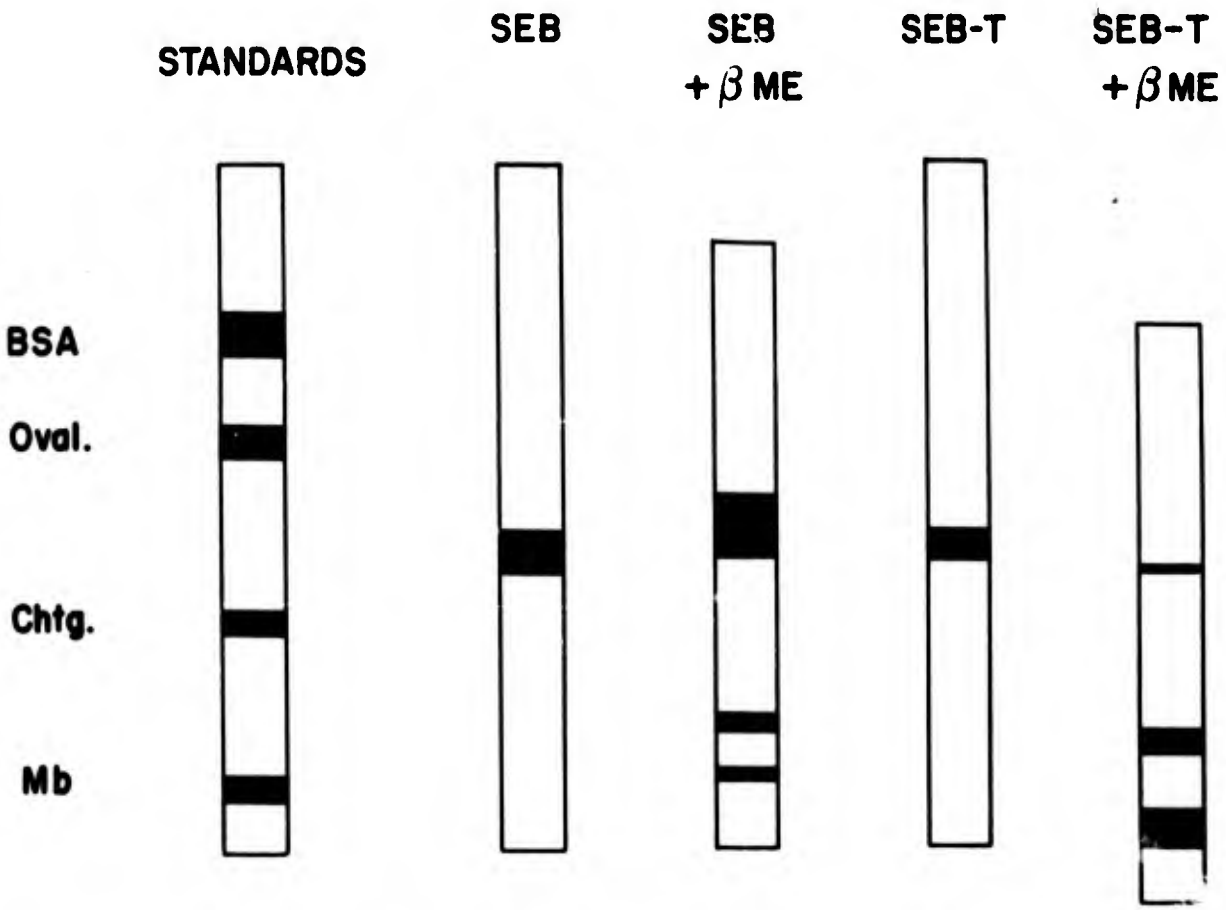

FIGURE 3. DIAGRAM OF POLYACRYLAMIDE GEL ELECTROPHORESIS OF SEB AND SEB-T.

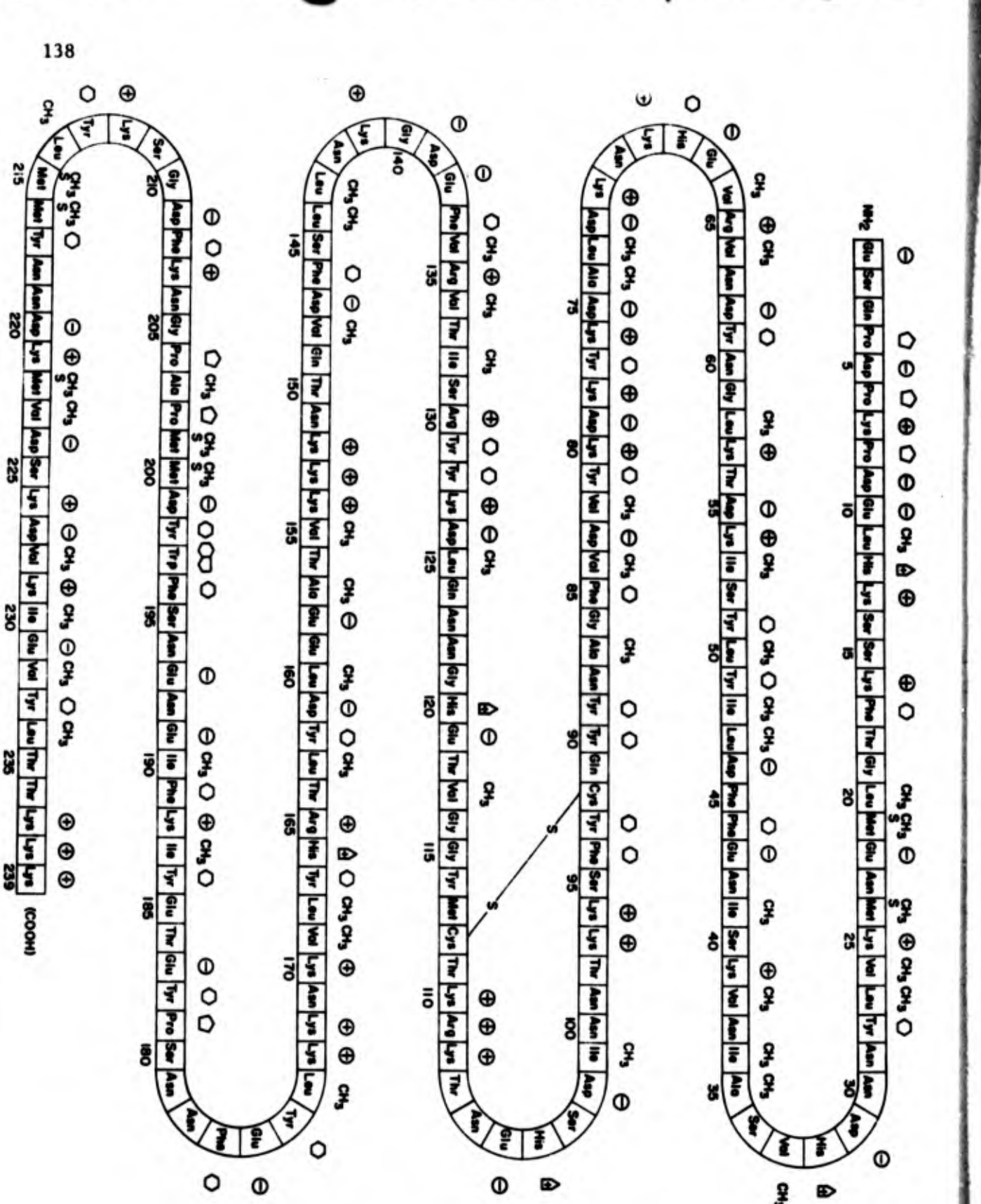

FIGURE 4. SCHEMATIC REPRESENTATION OF THE AMINO ACID SEQUENCE OF SEB (AFTER HUANG AND BERGDOLL[10]).

arginine and lysine peptide bonds. There are no Arg-Thr bonds and only 2 Lys-Thr sequences in the entire molecule and they occur at positions 97 and 110. Both, as predicted are within the disulfide loop. This linear representation of the sequence makes it easy to see why after trypsin action the molecule still appears to have the same size until the disulfide is reduced and the 2 halves can separate.

Hydrolysis at position 97 would yield, upon reduction, fragments of 17,050 and 11,450 molecular weight while hydrolysis at position 110 would yield fragments of 15,500 and 13,000. The molecular weights determined by gel electrophoresis, 16,400 and 13,000, are closer to the latter and suggest that the split occurs at position 110.

The optical density of the stained zones of the disc electrophoretic patterns are readily obtained by a linear scan and we have found that the area under the scan is linear with respect to the concentration of applied protein. This has enabled us to determine the amount of SEB remaining at any time during trypsin treatment. The kinetics of the limited lysis are shown in Figure 5 for 3 substrate:enzyme ratios. The digestions were carried out at 30 C at pH 9.0 in 0.05 M Tris buffer in the presence of 0.01 M calcium. The reaction was stopped by the addition of each aliquot to an excess of soy bean trypsin inhibitor. It is apparent that the fast reaction is virtually complete at 30 min with a 50:1 ratio. If the reaction is continued for longer periods a secondary cleavage is observed with formation of a molecule of about 22,000 MW. All the molecules of this type are nicked since they disappear upon reduction. This reaction is relatively slow, < 10% complete after 2 hr.

SEB-T is remarkably similar to native enterotoxin in physical and chemical properties. We have been unable to detect either a liberated peptide or free amino acids after trypsin treatment. The additional amino terminal residue, threonine, is a constituent of the intact molecule. There are no differences in amino acid composition. The molecular weights and schlieren patterns in the ultracentrifuge are also indistinguishable. We have measured 2 sensitive parameters of conformational change, intrinsic viscosity and ease of reduction of the disulfide bridge. No significant changes were observed by either method between the native protein and the trypsin-treated derivatives.

The biological activity of SEB-T was of great interest and Figure 6 shows no difference in the quantitative precipitin curves of SEB and SEB-T against rabbit anti-SEB serum. Ouchterlony immunodiffusion also gives a reaction of identity.

If the disulfide bridge of SEB-T is reduced and the material is reacted with antibody, there is essentially no change (Figure 7). Control data indicate that neither prior reduction with 0.5 M mercaptoethanol nor carrying out the reaction with antibody in the presence of 0.01 M mercaptoethanol altered the normal precipitin curve, suggesting that one or both of the 2 trypsin fragments had high serological activity.

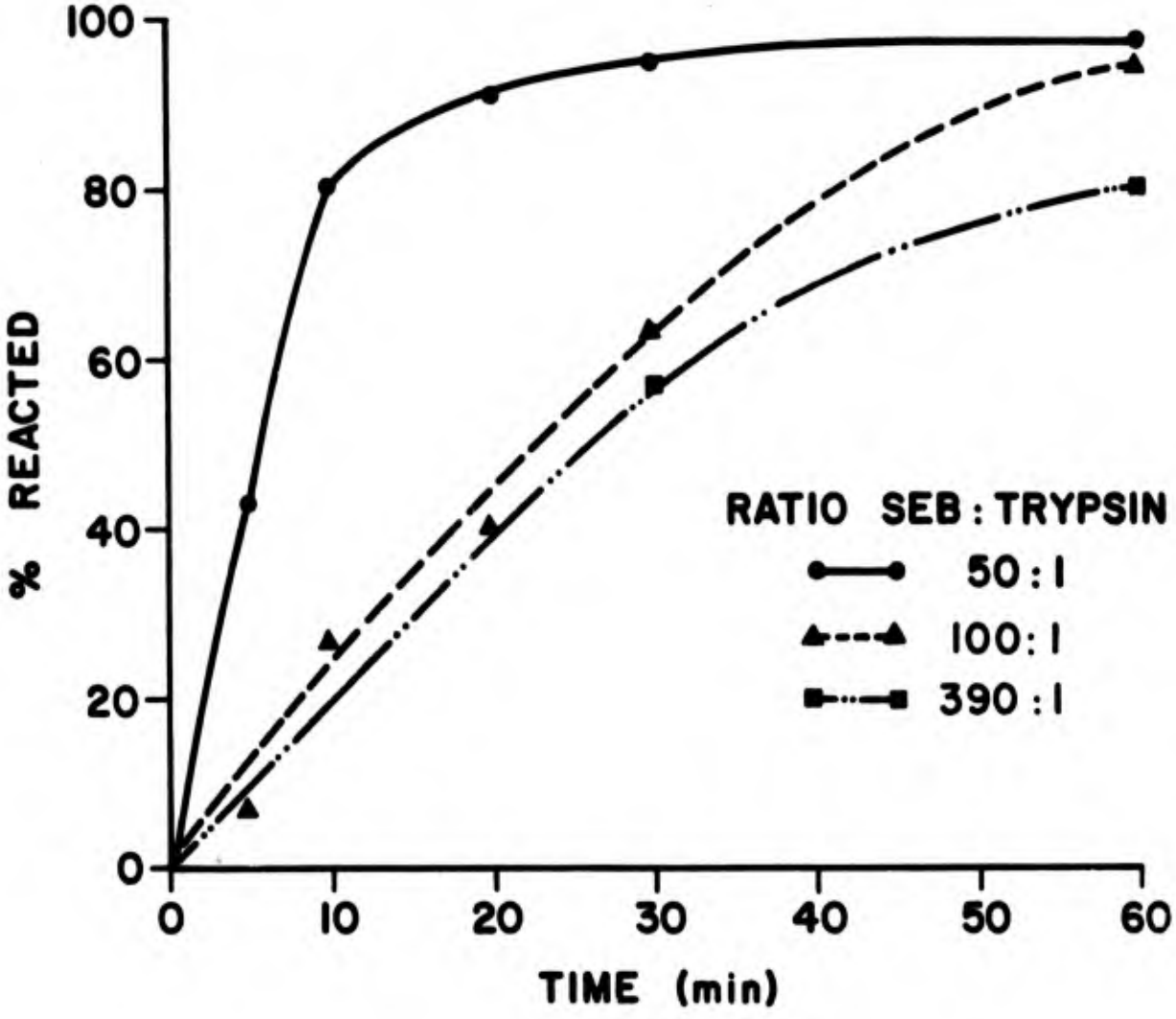

FIGURE 5. KINETICS OF DIGESTION OF SEB BY TRYPSIN AT VARYING RATIOS OF SUBSTRATE TO ENZYME.

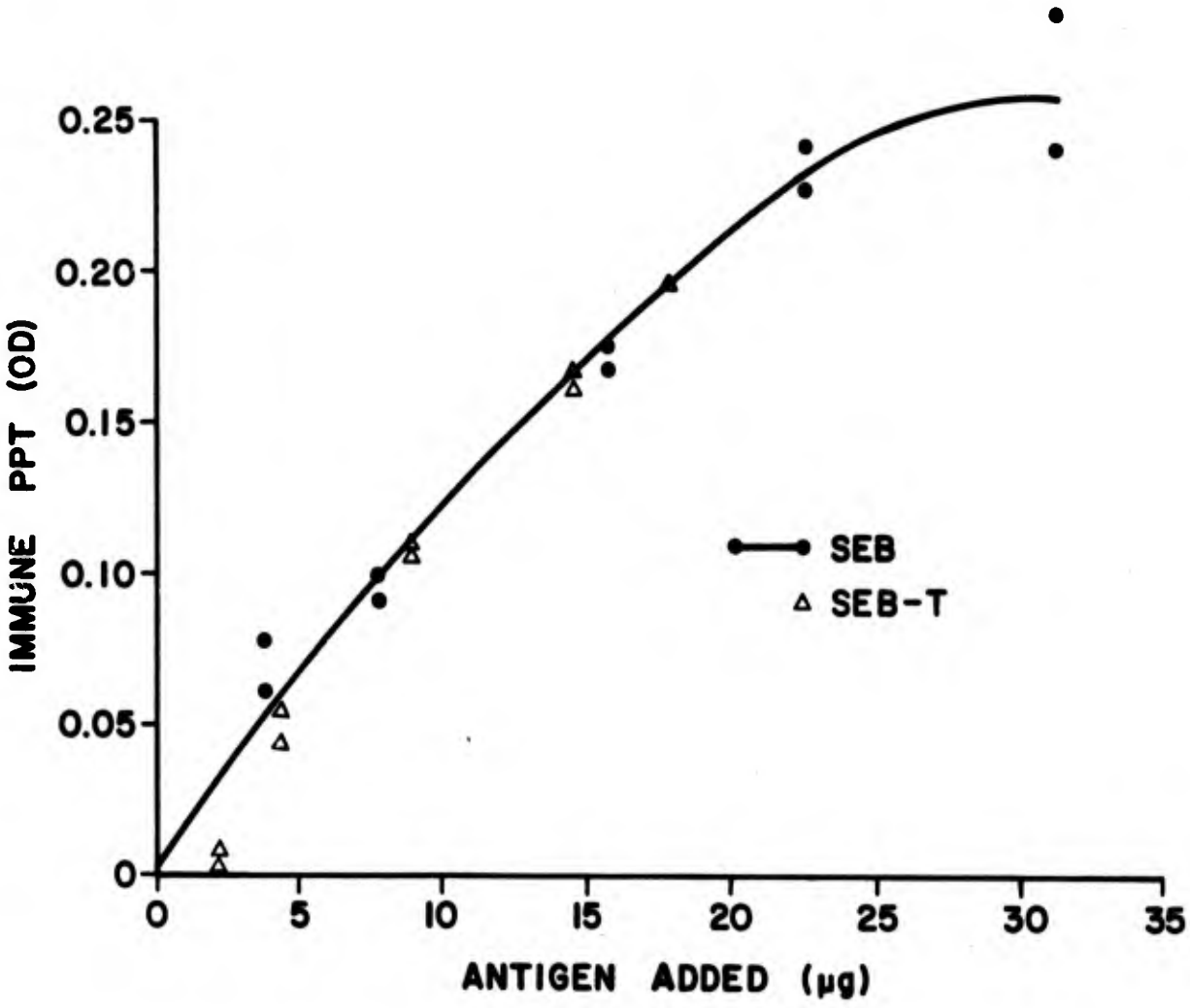

FIGURE 6. QUANTITATIVE PRECIPITIN CURVE OF SEB AND SEB-T AGAINST RABBIT ANTI-SEB SERUM.

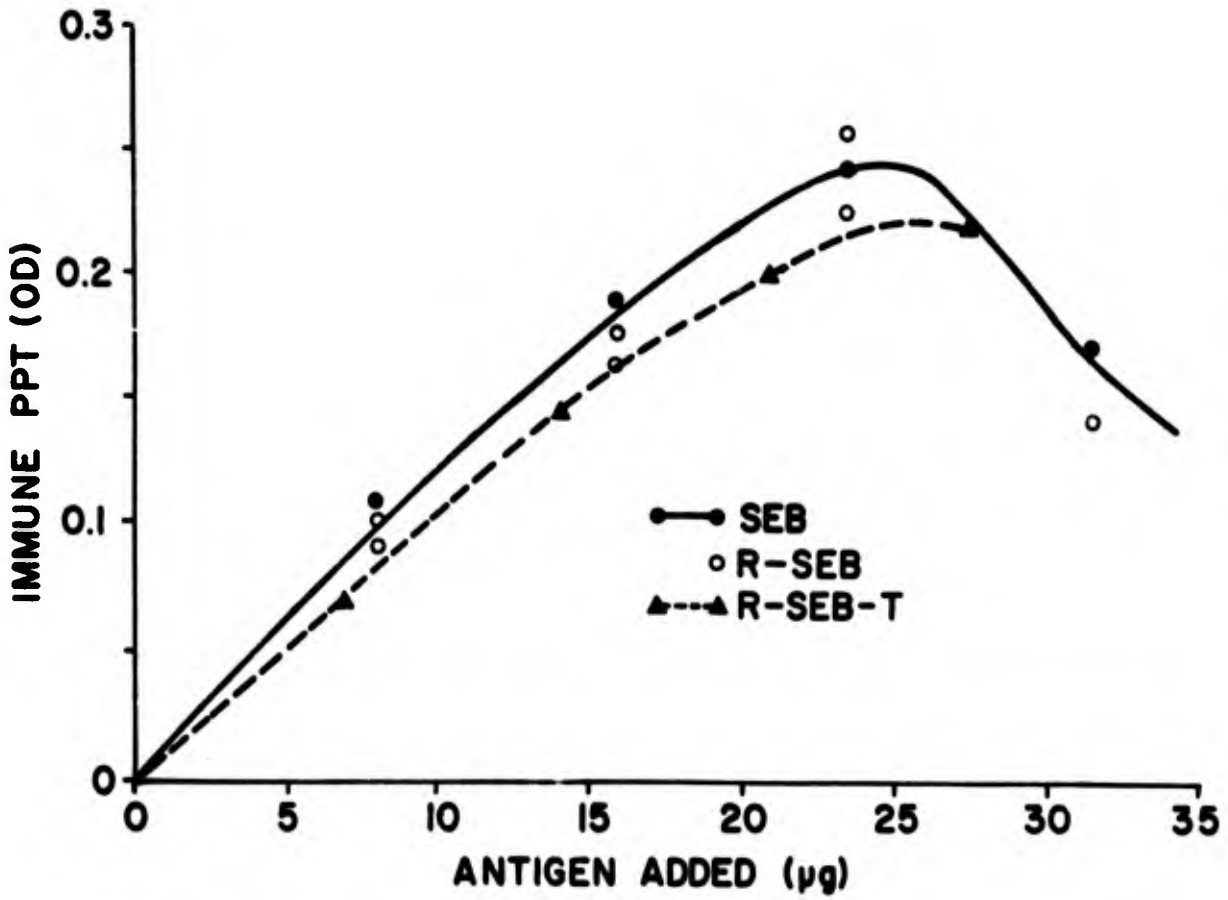

FIGURE 7. QUANTITATIVE PRECIPITIN CURVES FOR SEB, REDUCED SEB, AND REDUCED SEB-T.

The emetic activity of SEB-T was measured in rhesus monkeys (Table I). The intravenous median illness dose (ID_{50}) of SEB for monkeys is 0.1 μg/kg.[11] The preparation of SEB employed in this work is apparently fully active, as is the SEB-T derived from it. Latent periods were similar for both materials.

TABLE I. RESPONSE OF RHESUS MONKEYS TO SEB AND TRYPSIN-DIGESTED SEB

DOSAGE μg/kg	NO. ILL/TOTAL	
	SEB	SEB-T
1.0	ND	3/3
0.3	4/4	2/3
0.1	ND	2/3

Because of the high serological activity of reduced trypsin-treated SEB, it was important to verify that the molecule actually did separate into 2 fragments. Accordingly gel filtration was carried out on Sephadex G-50. Results are shown in Figure 8. It is apparent that SEB and SEB-T emerge at identical volumes after reduction with β-mercaptoethanol. The second peak is the reducing agent. Under these conditions the fragments of SEB-T are held together by noncovalent forces, illustrated conceptually in Figure 9. It is easy to see how the many points of contact could keep the 2 fragments together even though the bond at 110 is split and the disulfide is reduced. Actually these convolutions are not entirely fanciful for the model is basically that of chymotrypsinogen.[12]

The structural analogy with diphtheria toxin is so exact and so striking that we considered the possibility that this might represent a general method of toxin activation. Accordingly 2 other highly purified staphylococcal enterotoxins, A and C_1, were investigated. SEC_1 behaves very much like SEB. It is nicked to a small extent during the process of fermentation and reacts rapidly with trypsin in the disulfide bridge region to form 2 peptides. SEA, however, is not nicked and appears to be completely resistant to trypsin. All the enterotoxins have the same physiological manifestations and presumably, mode of action. Based on the differences between SEA and SEC_1 enzymatic activation probably plays no role in the physiological action of the staphylococcal enterotoxins. In a strict sense, only a trypsin-like activation is precluded.

The secondary cleavage observed with SEB is also seen in SEC_1. The bond is much more labile however (Figure 10). Essentially complete disappearance of 28,000 molecular weight material is seen as digestion

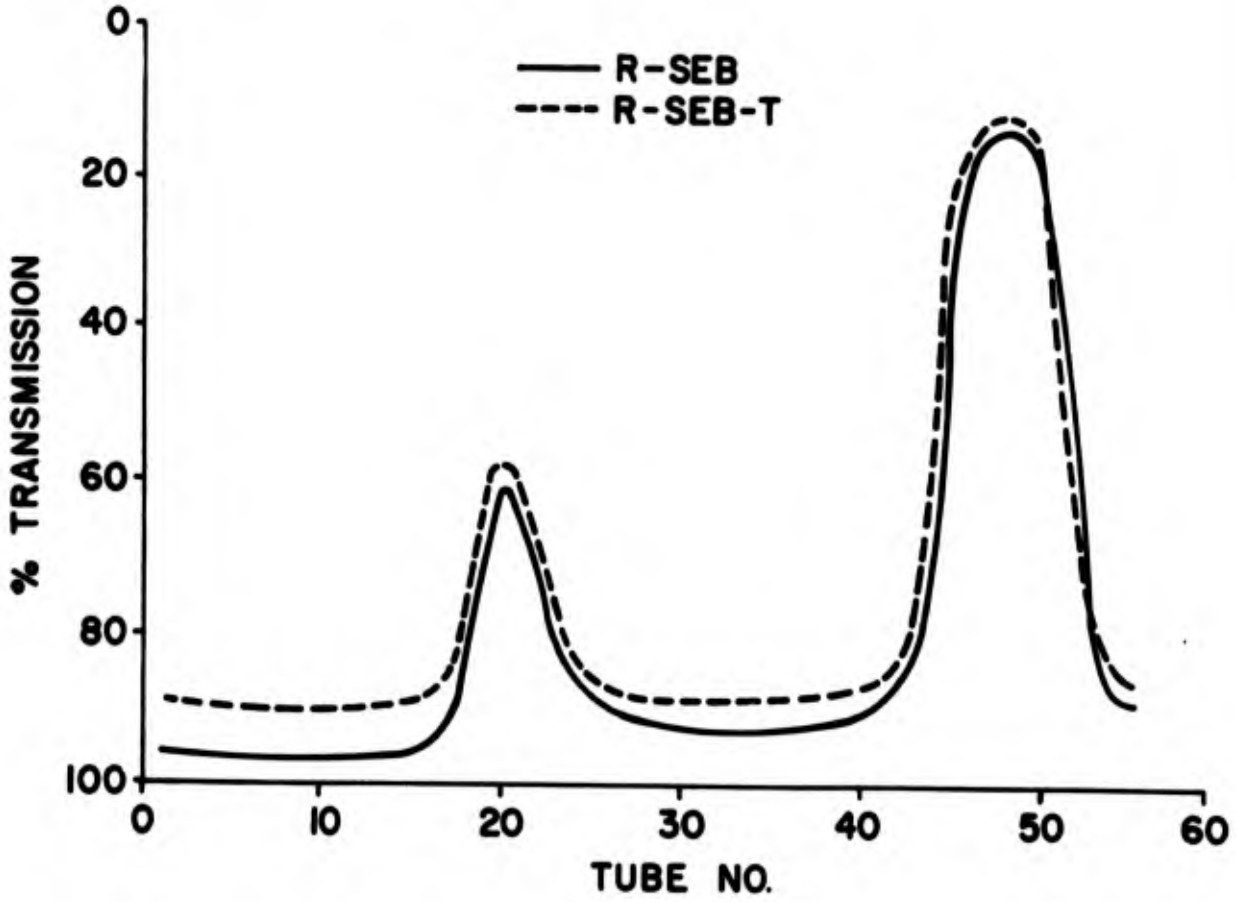

FIGURE 8. GEL FILTRATION OF SEB AND SEB-T.

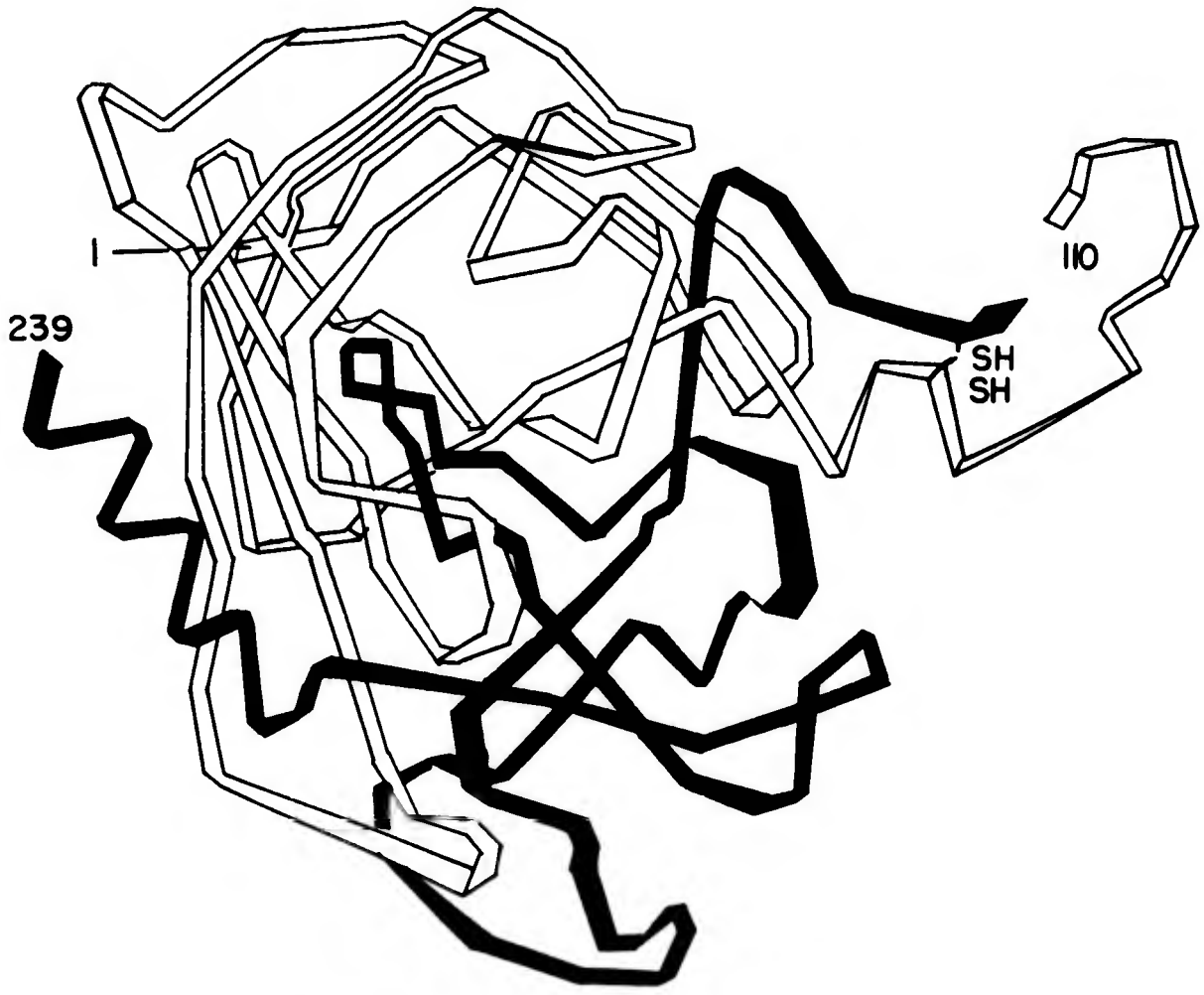

FIGURE 9. CONCEPTUAL MODEL OF REDUCED SEB-T.

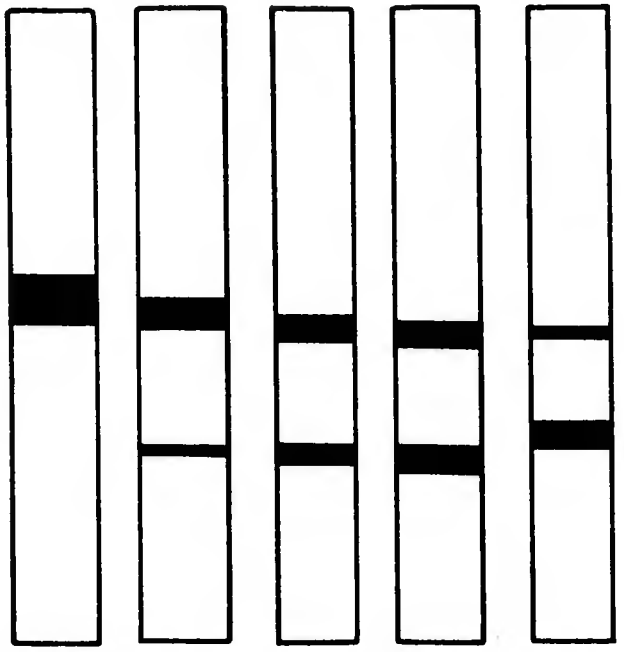

FIGURE 10. DIAGRAM OF POLYACRYLAMIDE GEL ELECTROPHORESIS OF SEC, AT VARIOUS TIMES AFTER TREATMENT WITH TRYPSIN.

time is lengthened. Since the loss of 6,000 daltons is equivalent to about 50 amino acid residues it should be interesting to measure the serological and emetic activities of this material.

Reduced and carboxamidomethylated SEB-T has been prepared.[13] It has full emetic and serological activity; it is susceptible to treatment by trypsin in the same manner as SEB.

In summary, we have found, in contrast to reports in the literature that SEB is resistant to trypsin, that it undergoes rapid cleavage of a single bond in the disulfide region of the molecule and a second slower cleavage outside of this region. The primary product retains full serological and emetic activity. Reduction of the disulfide bridge yields 2 peptides with molecular weights of about 16,400 and 13,000. These however, are held together by noncovalent forces under normal solution conditions.

There is a remarkable structural analogy with diphtheria toxin. It appears unlikely, however, that there is comparable physiological activation, since SEA does not undergo trypsin cleavage.

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LYSOSOMAL DEGRADATION OF STAPHYLOCOCCAL ENTEROTOXIN B

Peter G. Canonico, Ph.D.*

Limited data were presented concerning the distribution of fluoresceinated staphylococcal enterotoxin B (SEB) in kidney cells and its degradation by lysosomal proteases were reported previously.[1] We have reexamined and extended those initial studies employing enzymatically iodinated toxin.

^{125}I labeled SEB was prepared by a modification of the enzymatic method of Marchalonis[2] using lactoperoxidase (LPO) isolated from bovine milk. In this reaction milligram quantities of SEB were reacted in the presence of ^{125}I, H_2O_2 and microgram quantities of LPO for $\leq$ 10 min at room temperature. An iodination efficiency > 98% was consistently obtained. The iodinated toxin reacted with specific antisera and migrated electrophoretically in a manner similar to native toxin.

Shown in Figure 1 is the distribution of radioactivity and β-glucuronidase in subcellular fractions of rabbit kidney homogenates 30 min after intravenous (IV) injection of labeled SEB. Homogenates were centrifuged at 95,000 x g for 180 min. Radioactivity concentrated within the small granule fractions, the mitochondrial (M) and the light mitochondrial (L), and was similar to the distribution of the lysosomal enzyme, β-glucuronidase. Little radioactivity was detected in the nuclear (N) and soluble (S) fractions. Protein extracted with saline from the M and L fractions and qualitatively analyzed for reaction with goat anti-SEB serum by Ouchterlony double diffusion technique yielded a radioactive precipitin line which identified with native toxin. This observation indicated that the radioactive label remained attached to SEB and reflected the real distribution of toxin in subcellular fractions.

Following isopycnic centrifugation of the postnuclear fraction, the equilibrium density distribution of radioactivity was distinctly different from the mitochondrial marker, cytochrome oxidase, but paralleled the distribution of β-glucuronidase and acid RNase, 2 lysosomal enzymes which also reflect the heterogenic physical and enzymatic nature of kidney lysosomes (Figure 2). While other workers have demonstrated by fluorescent labeling methods that SEB is cleared from the circulation and accumulated within the proximal tubular cells of the kidney, the present data demonstrate the sequestration of toxin within lysosomes of kidney cells. The handling of SEB by the kidney therefore seems analogous to the uptake and sequestration of other relatively low molecular weight proteins such as albumin, horseradish peroxidase and lysozyme.

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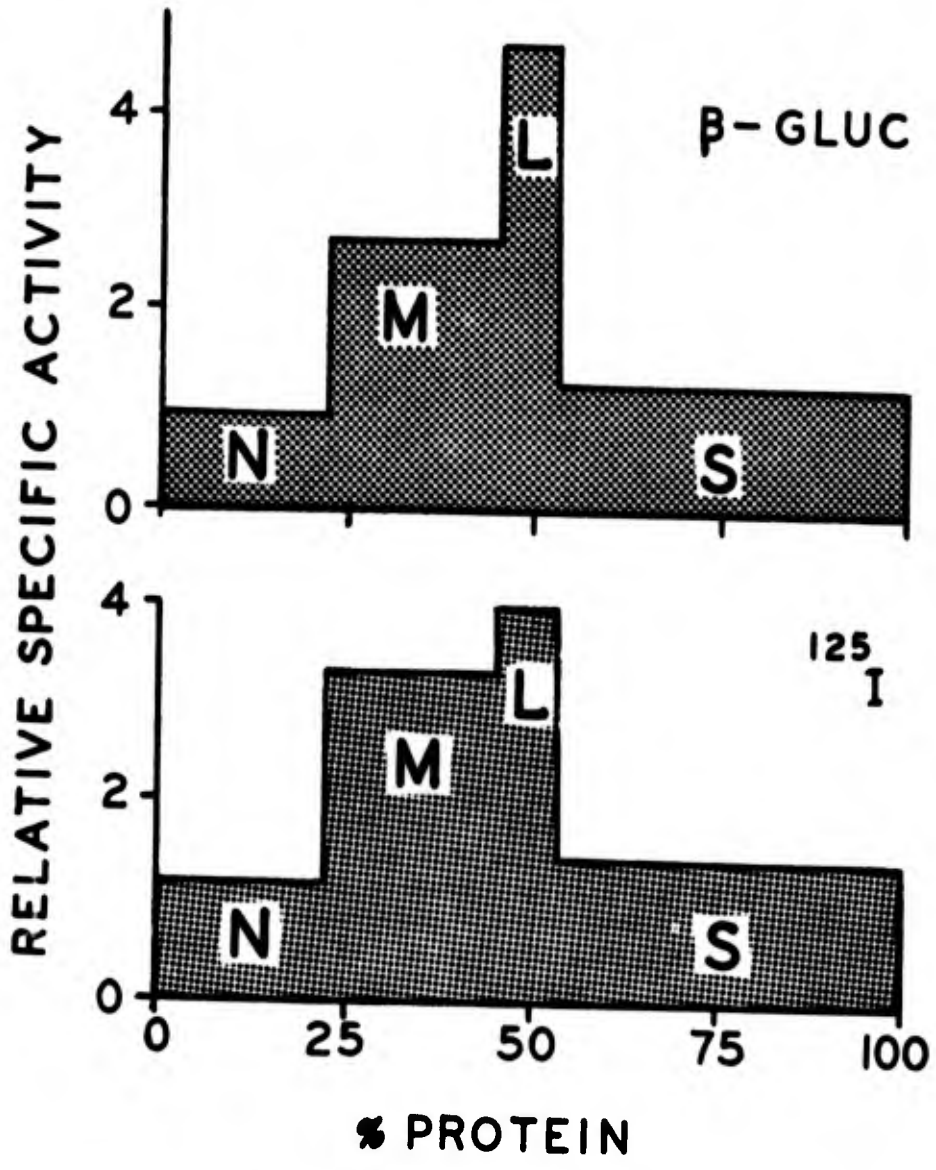

FIGURE 1. SUBCELLULAR DISTRIBUTION OF β-GLUCURONIDASE AND ^{125}I-SEB.

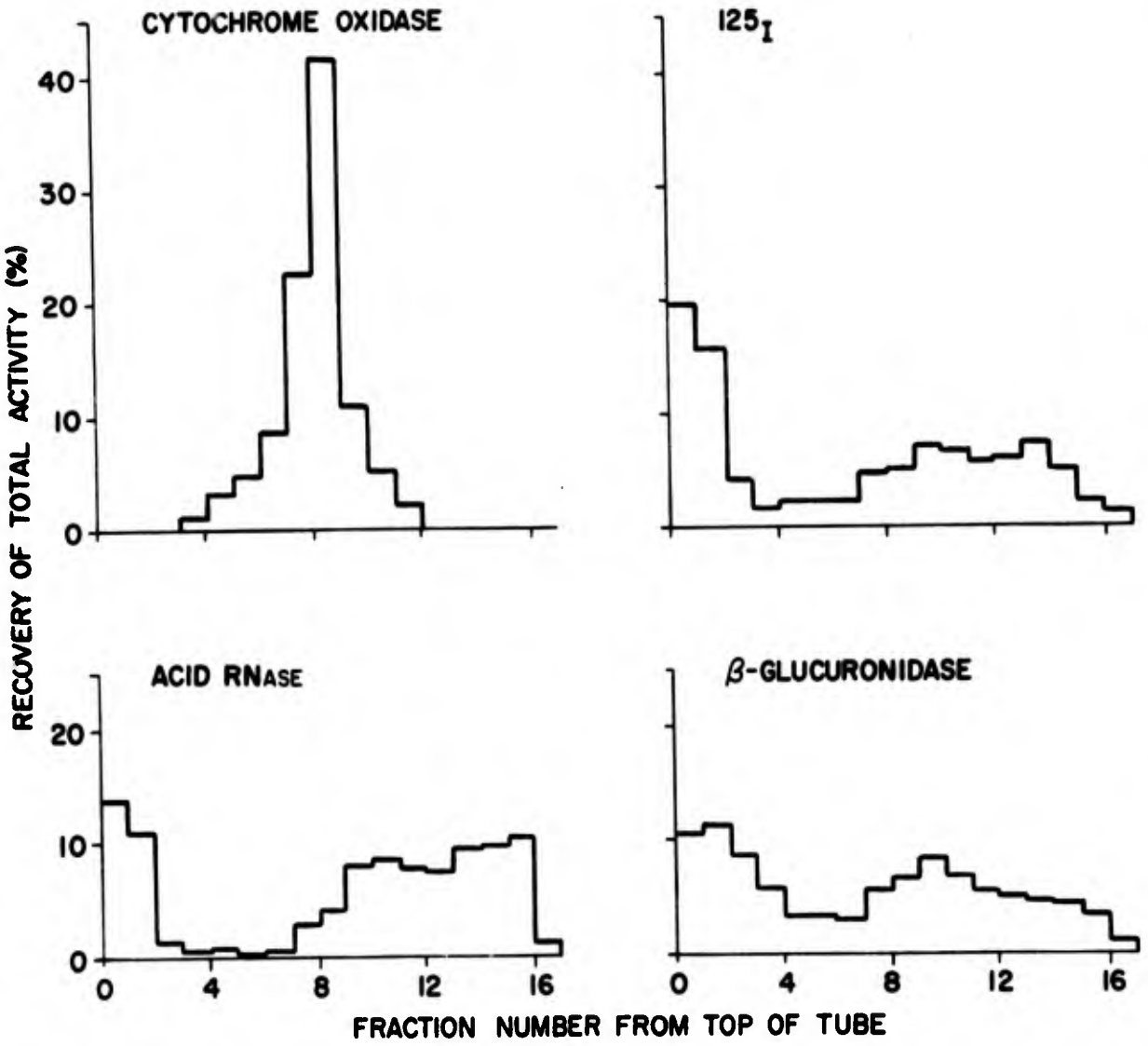

FIGURE 2. EQUILIBRIUM DENSITY DISTRIBUTION OF PARTICLE-BOUND ENZYMATIC ACTIVITY AND RADIOACTIVITY AFTER FRACTIONATION OF POSTNUCLEAR RABBIT KIDNEY HOMOGENATE ON SUCROSE GRADIENT.

To investigate the ability of lysosomal proteases to degrade SEB, in vitro radiolabeled SEB was incubated with lysosomal enzymes. Purified lysosomes were prepared from rabbit liver, after an in vivo injection of the non-ionic detergent, Triton WR-1339. These triton filled lysosomes are often called tritosomes. Their enlarged size and bouyant properties facilitate their isolation and purification. The SEB digestion mixture, varying in volume from 50 µl - 2 ml, depending on the purpose of the experiment, contained toxin at a final concentration of 200 µg/ml and sufficient tritosome enzyme protein to give 1 unit of cathepsin D activity/ml of incubation mixture. Incubations were carried out for 12-18 hr at 37 C with continuous shaking. Reactions were stopped by addition of 6 volumes cold 10% trichloracetic acid (TCA), clarified by centrifugation; percentage of TCA-soluble radioactivity was then assayed. In some experiments, the release of TCA-soluble ninhydrine-positive material was also determined.

Extensive hydrolysis of ^{125}I-toxin occurred in the pH range 2.25 and 3.25. The pH profile for the digestion of unlabeled toxin, determined by the release of ninhydrin positive material, was similar to that obtained for the enzymatically labeled derivative (Figure 3a). The pH optima for both curves occurred at pH 2.75. This is in contrast with the pH optima of 3.2 found by other workers for the hydrolysis of fluoresceinated toxin. As previously reported little digestion of toxin occurred above pH 3.5.

Hydrolysis of toxin (Figure 3b) was more extensive in citrate buffer than HCL-glycine or acetate buffer. Digestion was not altered by 1 mM iodoacetamide suggesting that cathepsin D is the principle protease responsible for the in vitro catabolism of toxin (Figure 3c). In the presence of cysteine an increase in proteolytic activity was observed, accompanied by a broadening of the pH activity profile. This observation suggests that a SH-dependent protease probably cathepsin C also participates in digestion of toxin.

A loss of the emetic properties of SEB was observed when toxin digested at pH 2.5 was fed to a monkey. This was accompanied by a complete loss in the immunoelectrophoretic properties of the toxin. These data suggest that fragmentation of the molecule into biologically active fragments does not occur under our experimental conditions. When digestions were performed at pH 3.5 or 4.5 the toxin retained its immunoelectrophoretic and emetic activities.

It is appropriate at this time to mention that proteins in their native configurations are poor substrates for lysosomal endopeptidases. SEB is resistant to acid denaturation and retains a native configuration at $\geq$ pH 3. At lower pH the native configuration is altered; this allows the toxin to become susceptible to hydrolytic attack. This view is consistent with the hypothesis that denaturation of proteins within the internal acid environment of lysosomes may be a prerequisite to lysosomal hydrolysis.

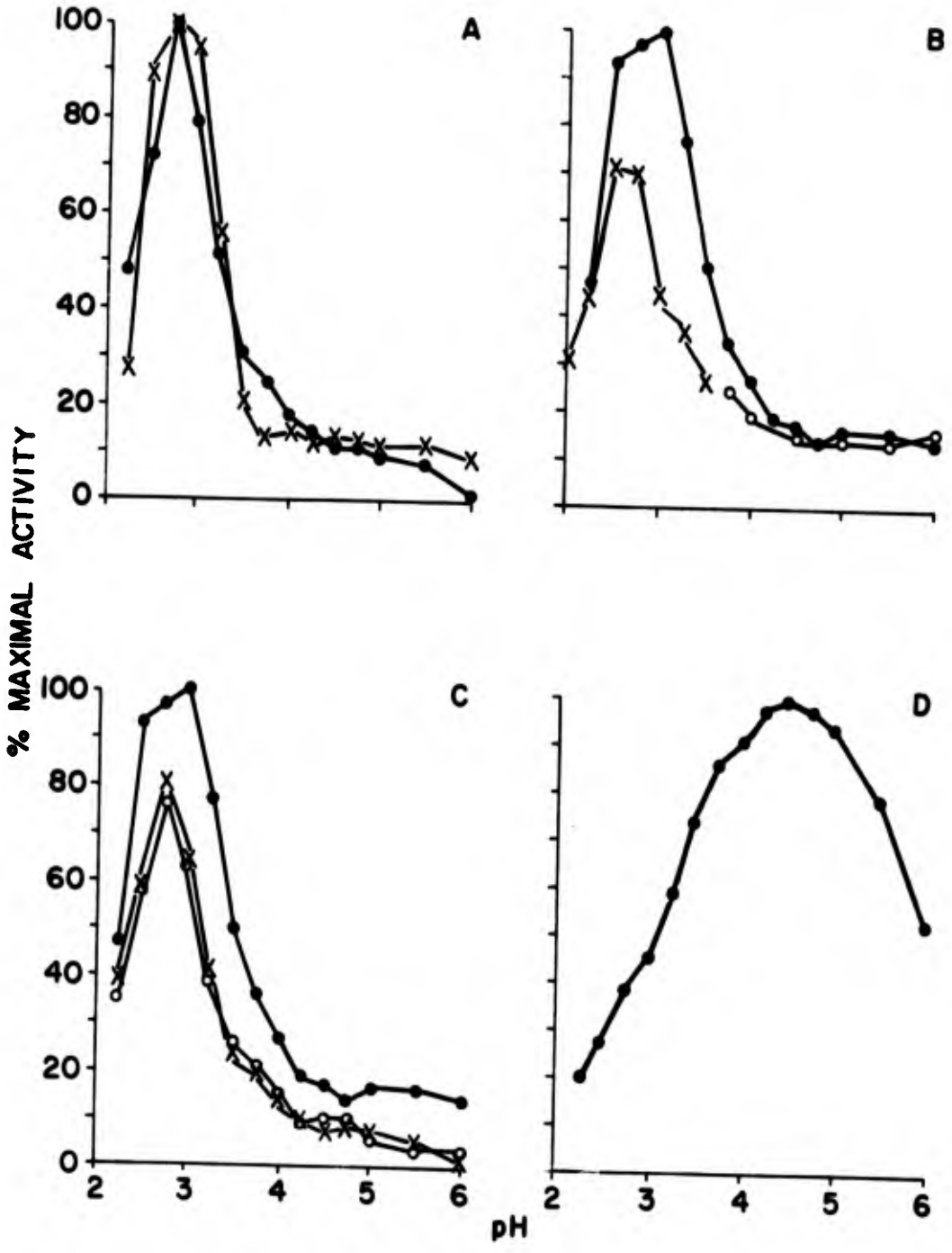

FIGURE 3. pH ACTIVITY PROFILES FOR THE DIGESTION OF ^{125}I-SEB BY RABBIT LIVER TRITOSOME ENZYMES.
A. ●-0.1M CITRATE BUFFER. X- NINHYDRINE POSITIVE.
B. 5mM CYSTEINE +: ●-0.1M CITRATE BUFFER; X-0.1M GLYCINE-HCl BUFFER; O- 0.1M ACETATE BUFFER.
C. X-0.1M CITRATE BUFFER; ●-BUFFER + 5mM CYSTEINE; O-BUFFER + 1mM IODOACETAMIDE.
D. HEAT DENATURED.

Proteins which are more susceptible to acid denaturation such as albumin or hemoglobin are easily hydrolyzed by lysosomal cathepsins in the pH range around 4.5. Under these conditions, however, SEB retains its native configuration and is resistant to extensive digestion.

Data are presented in Table I which compare the ability of proteases from the granule fraction of various tissue homogenates to digest enzymatically labeled SEB with respect to the purified tritosome preparation. At the pH indicated liver granule fraction enzymes hydrolyzed SEB similarly to tritosome proteases. A similar picture was obtained for the digestion of toxin by granule fraction enzymes of kidney and polymorphonuclear cells.

It would be appropriate, based on the data presented to conclude that SEB cleared from the circulation is sequestered within lysosomes of proximal tubular cells, detoxified and digested by the action of lysosomal proteases. However, it remains an open question whether a pH sufficiently low to alter the relatively stable molecular structure of SEB is attained within lysosomes in vivo. For example, data in the recent literature suggests that the lowest pH attained within secondary lysosomes of PMN cells is between 4.0 and 4.4. In this environment SEB is stable and resists proteolytic attack. The possibility cannot be excluded that other intralysosomal factors may alter the conformation of SEB into a molecular form which would be susceptible to hydrolysis at a physiologically compatible pH. In support of the suggestion that the pH optima for the hydrolysis of toxin may be altered depending on the "natured state" of the molecule, Figure 3d shows the pH-dependent profile for hydrolysis of heat-denatured toxin. Under these conditions the toxin was effectively hydrolyzed in the pH range of 3.5-5.5, with a pH optima at pH 4.5.

Kidney and PMN lysosomal proteases were most effective in hydrolyzing heat denatured SEB at pH 4.5 (Table I). When SEB is oxidized with performic acid, it is extensively digested by all enzyme preparations throughout the total range of pH tested. Denaturation, however, does not necessarily lead to increased susceptibility to hydrolysis. For example, when SEB was subjected to lactoperoxidase, and H_2O_2 for 24 hr a denatured derivative was obtained which did not react with specific immunoglobulins or migrate electrophoretically. This denatured protein, however, was more resistant to hydrolysis than the native molecule. Similarly, chemical iodination of SEB using chloramine T, also yielded a derivative which, though electrophoretically and immunologically indistinguishable from native toxin, was less susceptible to lysosomal catabolism.

The point that these data illustrate is that the rate and extent a protein can be hydrolyzed by intracellular proteases is largely dependent on the natured state of the molecule. This is not often taken into consideration by investigators who study the in vivo distribution, localization, degradation and persistence of protein toxins and immunogens topically labeled by chemical procedures.

TABLE I. IN VITRO HYDROLYSIS OF ^{125}I-SEB BY LYSOSOMAL PROTEASES

SEB TREATMENT		% HYDROLYSIS[a] (SOURCE OF LYSOSOMAL ENZYMES)			
		Tritosome[b]	Kidney G.F.[c]	Liver G.F.	PMN G.F.
Enzymatically Iodinated					
pH	2.5[d]	55	40	59	49
	3.5	28	20	19	16
	4.5	13	14	13	6
	7.5[e]	--	--	--	4
Performic Acid Oxidation					
pH	2.5	92	86	84	
	3.5	80	96	89	
	4.5	87	93	90	
100 C for 60 min					
pH	2.5	10	27	13	46
	3.5	33	63	35	64
	4.5	44	80	44	76
	7.5	--	--	--	32
Hydrogen Peroxide					
pH	2.5	11	10		12
	3.5	11	10		14
	4.5	9	14		8
	7.5	--	--		10
Chloramine T					
pH	2.5	10	11		5
	3.5	9	10		4
	4.5	8	7		3
	7.5	--	--		3

a. Expressed as % of total ^{125}I recovered as TCA soluble after 18-hr hydrolysis.
b. Liver lysosomes isolated after Triton WR-1339 loading.
c. Large granule fraction isolated by differential centrifugation of homogenates.
d. 0.1 M citrate buffer with 5 mM cysteine.
e. 0.1 M phosphate buffer with 1.0 M KCl.

Finally, the observations may have some relevance to the production of toxoids and killed particulate vaccines. The chemical manipulations encountered in the preparation of such vaccines can alter the rate and extent that these immunogens are degraded by antigen processing cells. One might, therefore, be able to control the susceptibility of killed particulate vaccines to lysosomal hydrolysis in the hope of improving the efficacy of the products.

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HOST DEFENSES IN VENEZUELAN EQUINE ENCEPHALOMYELITIS INFECTION

Stanley G. Rabinowitz, Major, MC*

In recent years it has been demonstrated that an immune response is critical in recovery from a number of experimental, primary viral infections.[1-3] Possible mechanisms by which the immune response might contribute to recovery include elaboration of neutralizing antibody, interferon, or the recruitment of cell-mediated immune processes. Obviously, these mechanisms are not mutually exclusive.

At this meeting last year[4] we discussed the relative contribution of humoral antibody and cell-mediated immunity in protection against experimental infection of mice with Venezuelan equine encephalomyelitis (VEE) virus. Both humoral antibody and cellular immunity were shown to play important roles.

The present study extends our observations on the nature of cell-mediated immunity in VEE infection following the procedure shown in Figure 1. In most experiments, spleens from donor mice were harvested 7 days following immunization with attenuated, TC-83 strain, VEE virus. On occasion, mice were given a booster injection 35 days after primary immunization and spleen cells were harvested 8 days later. Washed spleen cell suspensions were divided into 2 portions, one for *in vivo* passive transfer experiments and the second for *in vitro* lymphocyte stimulation studies, using recently developed methods for culturing mouse lymphocytes. In the *in vivo* studies, cell harvest were transferred to an adoptive host at time of challenge with the Trinidad strain of VEE virus; survival was used as the measure of protection. In *in vitro* lymphocyte stimulation studies, incorporation of radioactively labeled thymidine by cell harvests was used as a measure of lymphocyte proliferation, or reactivity. Specificity of response was evaluated by exposure of cell cultures to homologous or heterologous antigens or to cell mitogens, i.e. PHA or LPS. The responsive lymphocyte population was characterized by pretreating cultures with various cytotoxic antisera to deplete selectively the spleen cell population of thymic-dependent or bone marrow derived lymphocytes. Antisera against θ antigen, a surface antigen present on thymic-dependent lymphocytes, against thymocyte antigens or against mouse γ-globulin were used in these experiments.

Figure 2 summarizes results from several experiments in which immune spleen cells or immune sera were transferred to nonimmune mice. Protection conferred by immune cells to nonimmune recipients was

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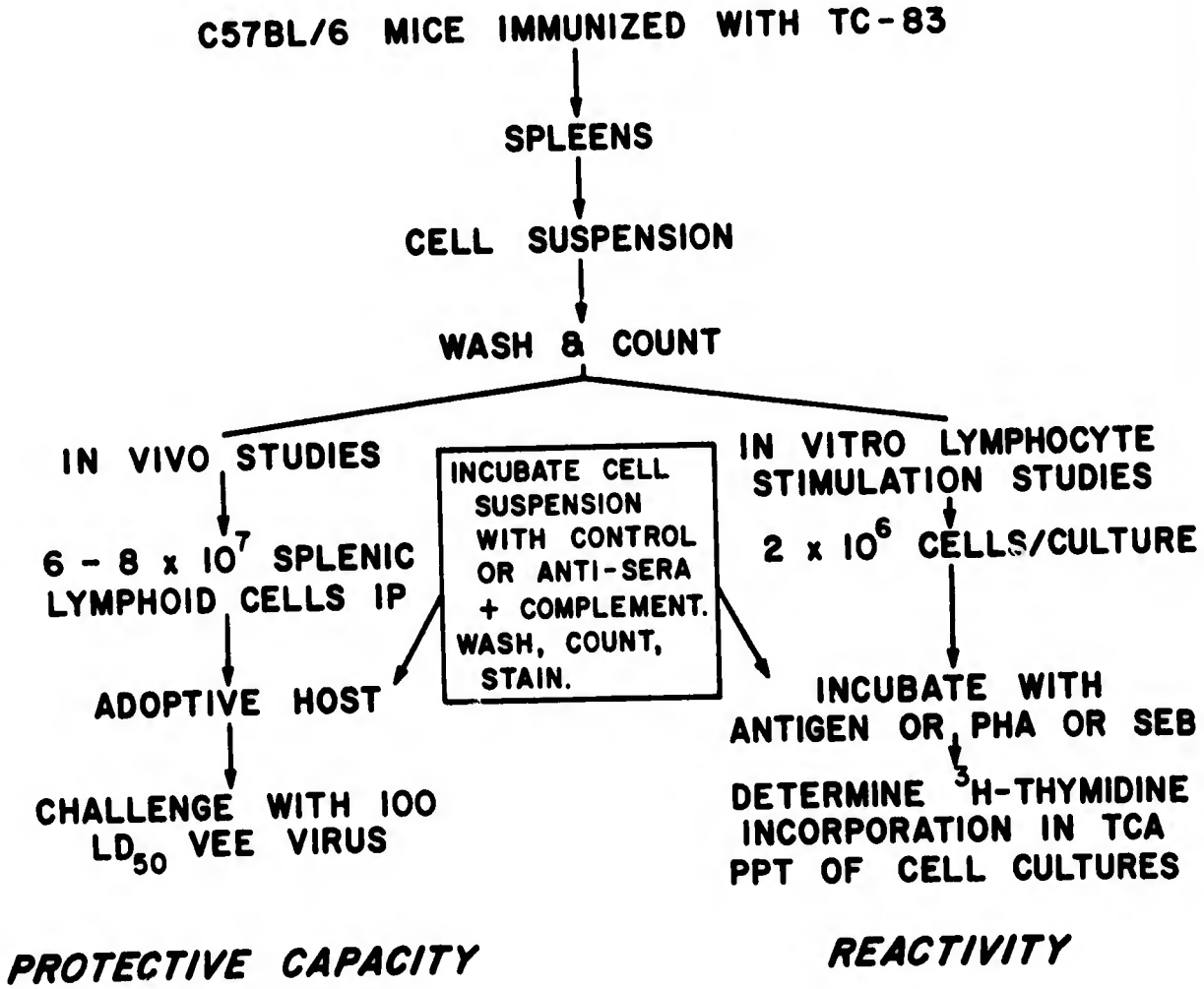

FIGURE 1. EXPERIMENTAL SCHEME.

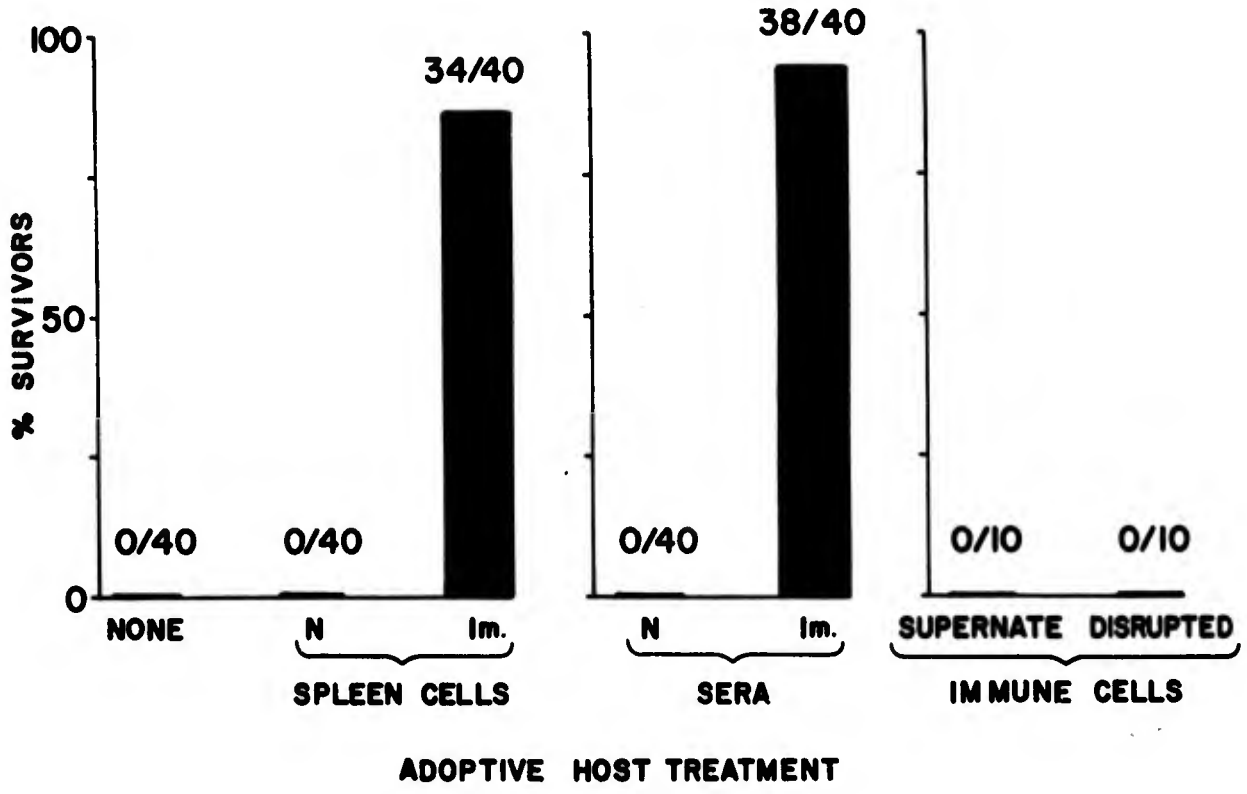

FIGURE 2. PROTECTIVE EFFECTS OF IMMUNE SERUM OR SPLEEN CELLS TRANSFERRED TO NORMAL MICE AT TIME OF VEE CHALLENGE.

essentially the same as that observed after passive transfer of antiserum. Recipients treated with cells from nonimmune mice or with the supernates or sonically-disrupted preparation of immune spleen cells succumbed to infection like challenged, untreated control mice, indicating that intact viable immune cells were necessary for protection. This observation led to further experiments to outline the mechanism of immune cell transfer of protection.

To determine the period of time during which donor cells were competent to confer adoptive immunity, spleen cells were harvested at intervals for 27 days following immunization. Recipients of each cell harvest were challenged in the usual manner. Figure 3 shows that time following donor immunization is a critical factor in determining both the capacity to confer adoptive immunity and to react in vitro to viral antigen. The circles depict survival for groups of adoptively immunized mice, the bars, the difference between proliferative responses of VEE-stimulated and control, nonantigen-treated lymphocytes as measured by in vitro incorporation of thymidine. Peak in vitro reactivity to antigen occurred 7-10 days after immunization, and thereafter decreased. Note also that the protective capacity of the immune spleen cells was also time-dependent and closely followed the degree of in vitro reactivity. Cells from nonimmune animals had no protective capability and were unresponsive to in vitro stimulation with antigen. Of great interest also is the fact that splenic harvests obtained after booster immunization were as reactive to antigen in vitro as those obtained after primary immunization, but were incapable of protecting an adoptive host against virulent VEE challenge.

The protective capability of immune cells was specific. Cells from a mouse immunized with attenuated VEE virus protected about 75% of the recipients against subcutaneous challenge with 100 median lethal doses of virulent VEE virus, but not against, Semliki Forest virus (SFV) at the same challenge dose.

Antigen recognition and spleen cell reactivity were also highly specific (Table I). Cells from nonimmune mice or from mice immunized with tissue culture supernates Western equine encephalitis (WEE) or SFV vaccine exhibited no activation following exposure to VEE antigen. Spleen cells from mice immunized with attenuated SFV responded to inactivated SFV antigen, but not to VEE antigen; likewise, WEE immune cells were activated only by homologous antigen.

Since immune spleen cells were capable of conferring adoptive immunity and of reacting in vitro specifically to VEE antigen, experiments were performed to determine whether the thymic-dependent lymphocyte, which correlates with cell-mediated immunity, was the cell responsible for the results observed. Other investigators have reported that certain mitogens, such as PHA, will selectively stimulate thymic-dependent, or T, lymphocytes, while others, such as cell wall lipopolysaccharide (LPS) from gram negative

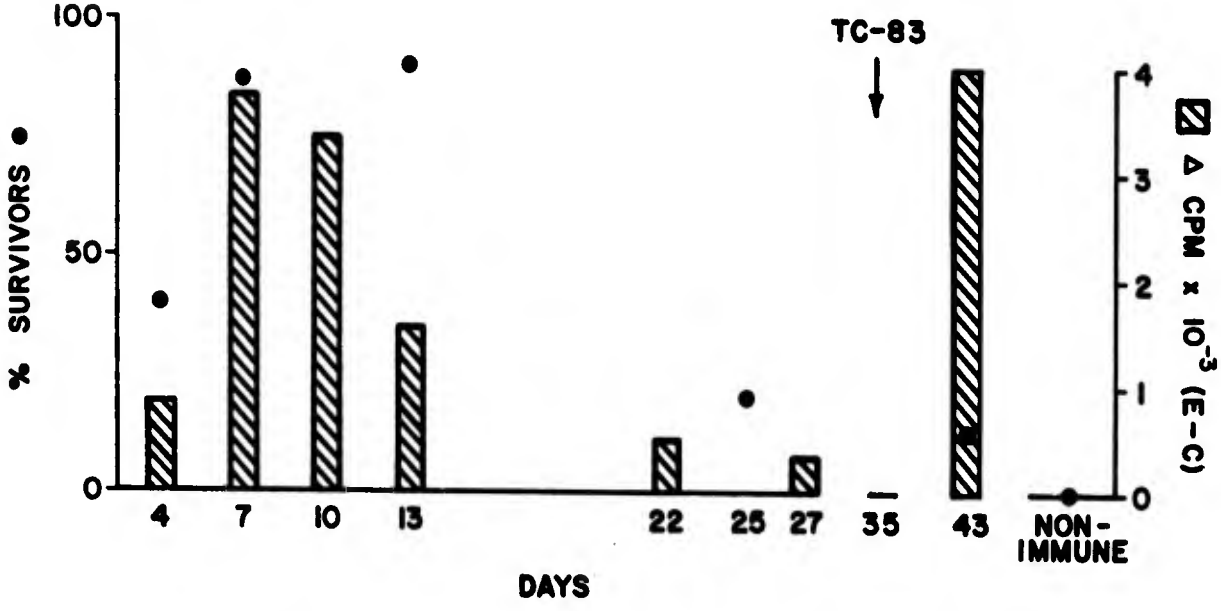

FIGURE 3. IMMUNE SPLEEN CELL ACTIVITY AT VARIOUS TIMES AFTER VEE IMMUNIZATION.

TABLE I. SPECIFICITY OF RECOGNITION OF VIRAL ANTIGEN BY IMMUNE SPLEEN CELLS ASSAYED BY IN VITRO LYMPHOCYTE STIMULATION

IMMUNIZATION OF CELL DONORS	ANTIGEN ADDED TO CULTURE	LYMPHOCYTE UPTAKE ^{3}H-THYMIDINE (Mean)		
		Control	Peak Antigen Response	Δ
NONIMMUNE MEDIA + SERUM	---	2723	2869	146
	VEE	3509	3911	402
VEE (Attenuated)	SFV	5899	5809	92
	VEE	2934	6469	3535
SFV (Attenuated)	VEE	3162	3180	18
	SFV	3775	6393	2168
WEE (Inactivated)	VEE	8369	8229	140
	WEE	1514	7392	5878

organisms, affect bone marrow-derived, or B, lymphocytes. By making use of this information, various cytotoxic antisera were employed in the presence of complement to deplete spleen cell populations of either T or B lymphocytes and then assaying the responsiveness of the treated cell populations to PHA or LPS. Prior to use, all antisera were absorbed with C57 fibroblasts and complement, with C57 spleen cells.

When mouse spleen cells were treated with either rabbit anti-mouse thymocyte serum or AKR anti-C_3H θ serum, there was markedly reduced PHA responsiveness but no effect on LPS responsiveness. Conversely, prior treatment of spleen cells with goat anti-mouse γ-globulin failed to effect PHA responsiveness but markedly reduced LPS responsiveness (Figure 4).

Since it was possible to utilize cytotoxic antisera to identify T and B lymphocyte populations in spleen cell cultures, we then examined the effect of these antisera on the capacity of treated immune cell populations to respond in vitro to VEE antigen or to confer adoptive immunity to recipients.

Figure 5 demonstrates the in vitro reactivity of spleen cells that were harvested 7 days after VEE immunization and incubated for 30 min at 37 C prior to addition of VEE antigen. It can be seen that treatment with rabbit-ATS or anti-θ serum eliminated the responsiveness of cell populations to

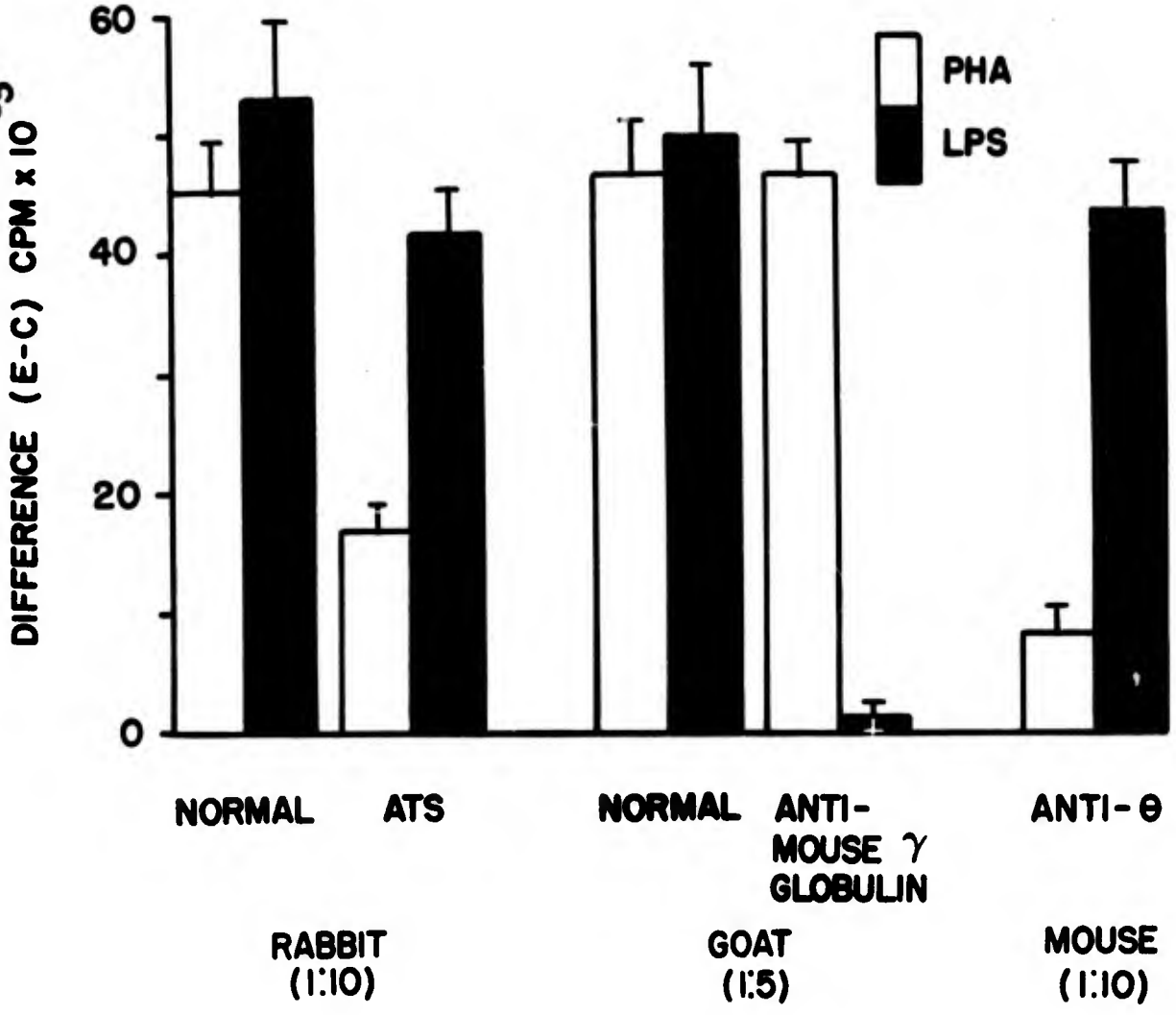

FIGURE 4. EFFECT OF CYTOTOXIC ANTISERA ON *IN VITRO* REACTIVITY OF IMMUNE SPLEEN CELLS TO PHA AND LPS.

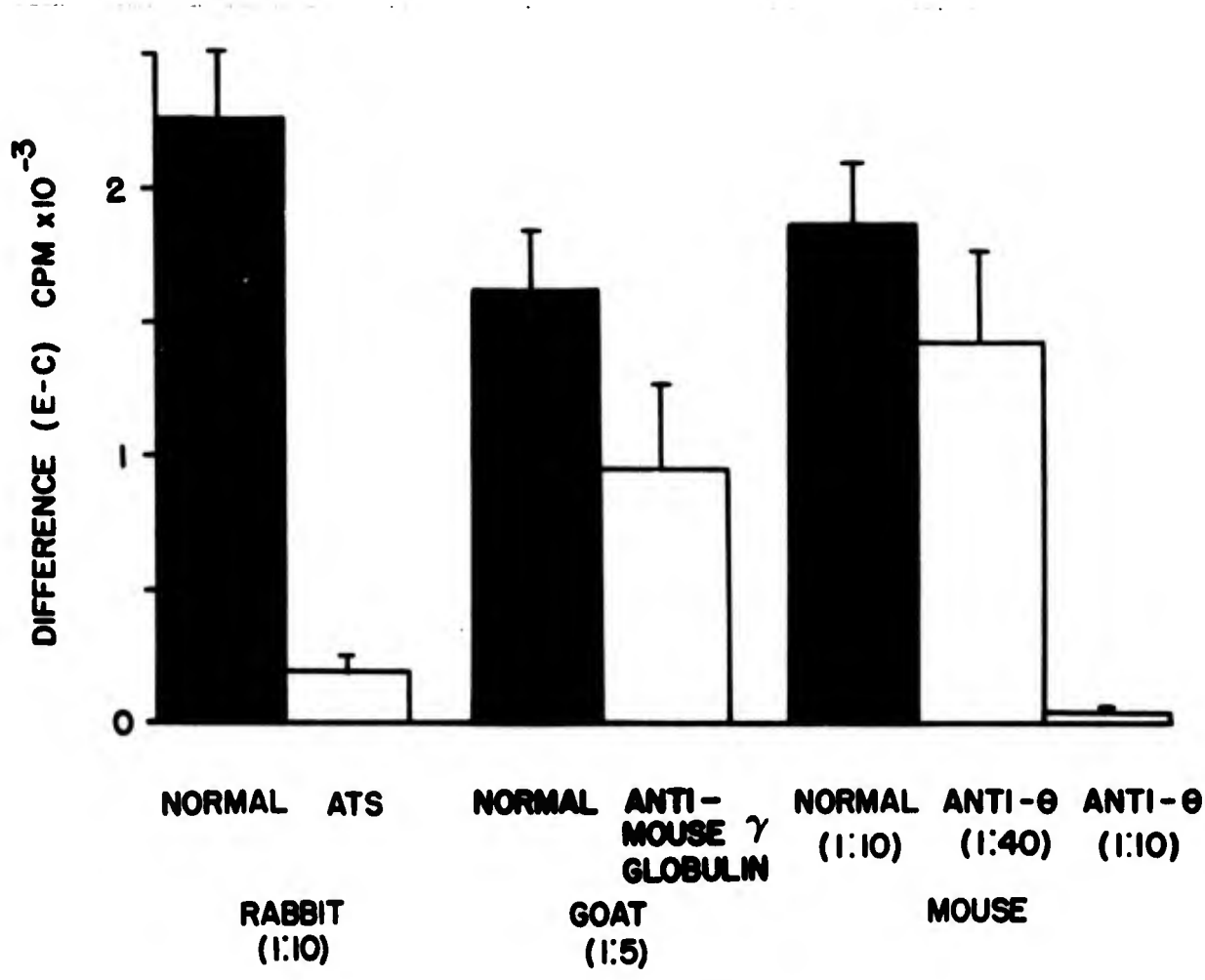

FIGURE 5. EFFECT OF CYTOTOXIC ANTISERA ON *IN VITRO* REACTIVITY OF IMMUNE SPLEEN CELLS AFTER PRIMARY IMMUNIZATION WITH ATTENUATED VEE VIRUS.

VEE antigen, whereas treatment with goat anti-mouse γ-globulin did not interfere with activation by VEE antigen. Likewse, in passive transfer experiments, similar immune cell harvests treated with anti-θ or anti-thymocyte sera were unable to confer adoptive immunity, whereas immune cells treated with anti-mouse γ-globulin protected adoptively immunized recipients.

Cell populations harvested after booster immunization with attenuated VEE virus vaccine were notably different. The effect of cytotoxic antisera on *in vitro* lymphocyte stimulation of an 8-day harvest of spleen cells obtained from donor mice that received a booster injection 35 days after primary VEE immunization in shown in Figure 6. Although responsiveness of immune cells was somewhat decreased by treatment with anti-thymocyte serum, reactivity was completely abolished by treatment with anti-mouse γ-globulin serum. Moreover, spleen cells harvested 5-28 days after booster immunization werc incapable of protecting adoptively immunized recipients against virulent VEE challenge.

These findings suggest that after primary immunization of mice with VEE vaccine both antigen recognition and proliferation and the capacity to confer adoptive immunity are associated with θ-containing, non-γ-globulin coated, thymic-dependent lymphocytes, but that after secondary immunization, these functions are under the control of non-thymic-dependent, γ-globulin-coated, β-lymphocytes.

The effect of specific antibody on spleen cell reactivity to VEE antigen is shown in Figure 7. When VEE antigen and VEE antiserum were added to cultures prior to the incubation period, antigen-induced stimulation of immune spleen cells was abolished, indicating that humoral antibody recognized, and competed for, viral antigenic determinants associated with lymphocyte stimulation. In the absence of specific antigen, VEE antibody had no effect on incorporation of ^{3}H-thymidine by immune cells or by PHA stimulated cultures of immune cells.

An antigen-sensitive T cell population was detectable for only a relatively short period of time in the spleen. Furthermore, there was apparently an inverse relationship between the temporal course for the disappearance of antigen-reactive cells and the appearance of increasing amounts of humoral antibody. Hence, it is possible that competition between humoral antibody and sensitive lymphocytes for available antigen suppresses antigen-driven T-cell division *in vivo* and as a consequence, T-cell reactivity to antigen *in vitro* disappears.

In summary, antigen-sensitive splenic cell populations that appeared after primary immunization consisted of T-lymphocytes, as was indicated by the ablative effect of ATS or anti-θ serum, but not of anti- mouse γ-globulin, on their *in vitro* proliferation. These cell populations were able to protect an adoptive host against VEE infection; their *in vitro*

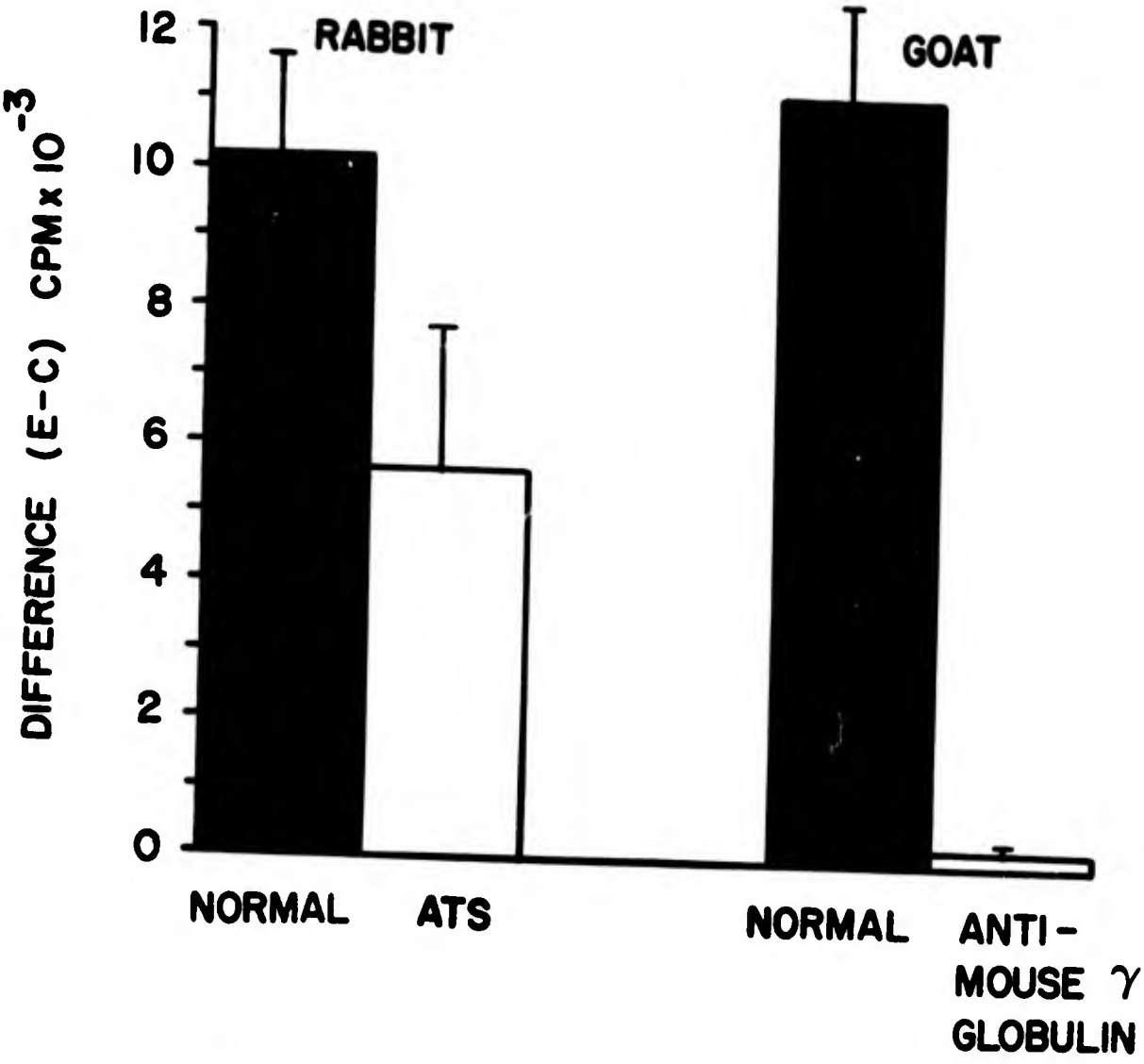

FIGURE 6. EFFECT OF CYTOTOXIC ANTISERA ON *IN VITRO* REACTIVITY OF IMMUNE SPLEEN CELLS AFTER BOOSTER IMMUNIZATION WITH ATTENUATED VEE VIRUS.

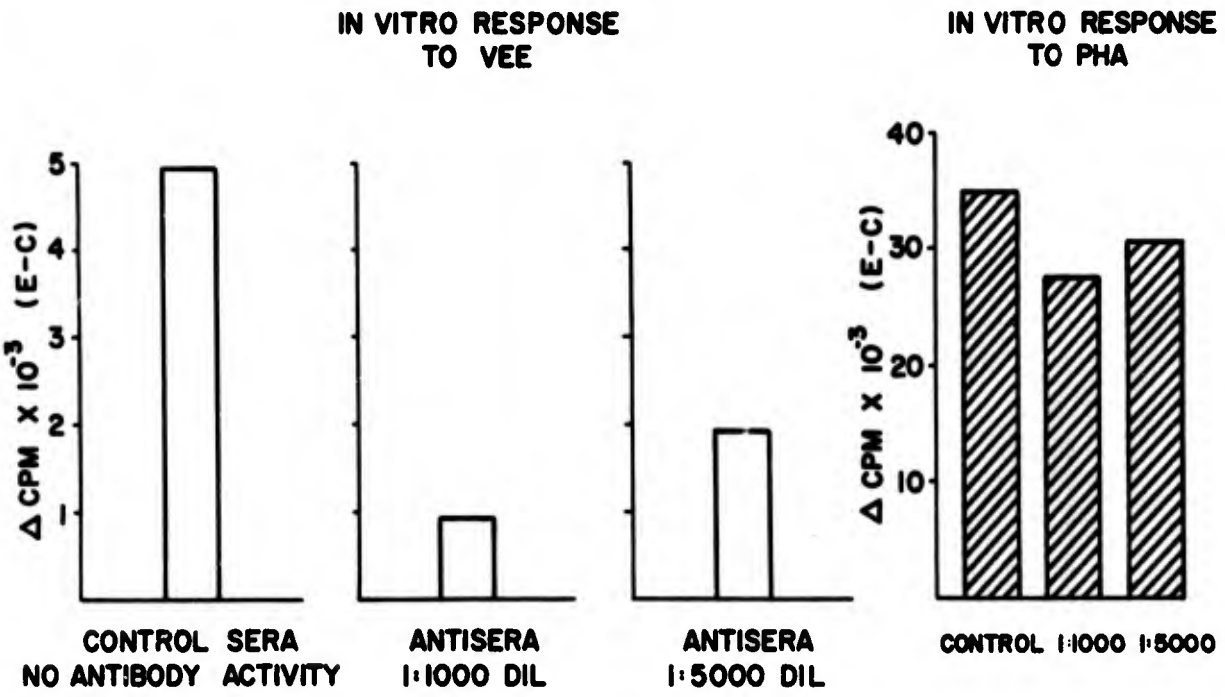

FIGURE 7. EFFECT OF *IN VITRO* ANTI-VEE ANTISERA ON THE *IN VITRO* RESPONSIVENESS OF IMMUNE SPLEEN CELLS.

reactivity to specific antigen was a valid measure of cellular immunity. This splenic T-cell response was shortlived; reimmunization with attenuated vaccine did not result in its restoration. Instead an antigen-sensitive B-cell population developed. This B-cell population was incapable of protecting an adoptive host against VEE challenge, and *in vitro* reactivity to specific antigen was not indicative of cellular immunity.

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ROCKY MOUNTAIN SPOTTED FEVER IN RHESUS MONKEYS

Gerald L. Ruch, Captain, VC, and James R. Moe, Captain, VC*

PART I. CLINICAL RESPONSE
(G. L. Ruch)

Last year, Dr. Kenyon[1] presented data on a Rocky Mountain spotted fever (RMSF) vaccine prepared in duck embryo cell (DEC) culture. Prior to testing this vaccine in monkeys, a suitable model for RMSF in Macaca mulatta monkeys was sought. A variety of inocula and routes of administration were examined.

This report describes the clinical response of monkeys following intravenous (IV) or intraperitoneal (IP) administration of approximately 10^7 chick embryo median lethal doses of the Shelia Smith[2] strain of Rickettsia rickettsii grown in duck embryo yolk sac (YS) or DEC.

Healthy rhesus monkeys were administered the DEC material 6 each via the IP and IV routes or YS material, 6 IP and 4 IV. Only one monkey given DEC material IP failed to have a clinical response. The severity and duration of illness did not differ significantly between those monkeys administered the YS or DEC material. However, a marked difference in response was observed between the IV and IP groups.

In general, the monkeys challenged IP could be divided into 2 groups: (1) death ensued within 7 days, and (2) prolonged illness with survival. The clinical disease in the latter group is more representative of the classic disease as seen in man and monkeys following aerosol exposure.[3]

In general, a febrile response developed 2-3 days postexposure, with a peak of 105-106 F which lasted 7-11 days. Monkeys became lethargic, anorexic and progessively weaker, with developing dehydration and anemia; episodes of shivering were observed and signs of muscular pain on handling were apparent. Scrotal areas of males became progressively more edematous and hemorrhagic. Occasional slight nose bleeds occurred. One monkey developed echymotic lesions on the thigh and back, eventually spreading to the extremities and face; echymoses of the ears were followed by progressive peripheral necrosis.

The disease in monkeys which died was more acute with terminally developing severe dehydration and coma. Although pulmonary lesions were prominent histologically (Part II of this section) respiratory distress was not a prominent finding. Beginning scrotal lesions were observed in 2 of 4 male monkeys.

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In all of the IV-challenged monkeys, the disease was peracute. Onset of fever occurred 4-30 hr and death generally ensued within 5 days. Coincident with onset of fever, monkeys developed pronounced depression, lethargy, anorexia and weakness, followed by coma and death. No cutaneous hemorrhagic lesions were observed.

As previously described, the major difference in clinical response of RMSF-infected monkeys were related to the route of exposure. This is in agreement with the data of Sawlaw and Carlisle on aerosol-infected monkeys[3] (Table I). While the infectivity rate did not change with the routes of exposure, lethality and incubation period varied markedly.

TABLE I. RESPONSE OF MONKEYS TO RMSF BY ROUTE OF EXPOSURE

ROUTE OF EXPOSURE	NUMBER	INCUBATION PERIOD hr	INFECTED No.	INFECTED %	NO. DIED	NO. WITH RASH
IP	12	48-72	11	92	6	1
IV	10	4-30	10	100	10	0
Aerosol[3]	60	120-168	56	93	42	38
SC	2	24	1	50	0	0

Clinical manifestation of the classical disseminated hemorrhagic rash, commonly associated with RMSF in man, was seen in only 1 monkey exposed by the IP route, but was common in aerosol-challenged monkeys.[3] Since clinical hemorrhagic lesions do not generally develop until 9-11 days after initial exposure, the acute onset of disease with death or recovery occurring < 10 days postexposure, may have precluded the development of disseminated rash in monkeys inoculated IV and IP.

In summary, clinical responses of monkeys inoculated by various routes with RMSF were examined. Although the studies were preliminary, they indicate the usefulness of the monkey. We now have a model in which we can control the time of onset, duration of disease, and mortality, enabling studies related to the early onset of this infection; e.g., the possible role of rickettsial toxin in the pathogenesis of spotted fever infections, other facets of the pathogenesis and pathophysiology of this disease, and testing of candidate vaccines in a subhuman primate.

PART II. HISTOPATHOLOGY
(J. B. Moe)

The histologic lesions of RMSF in man and experimentally-induced RMSF in the rhesus monkey have been described.[4] Sequential development of the lesions of RMSF and distribution of the causative agent, R. rickettsii, after aerosol exposure of rhesus monkeys has been reported.[5] The distribution of histologic lesions of 15 of the 16 monkeys infected IV and IP is presented.

Nine of the 10 IV-inoculated group and the 6 in the IP group were necropsied shortly after death. Tissues were fixed, sectioned and stained according to standard techniques.

Tissues from all organ systems were studied by light microscopy. Lesions observed were recorded according to organ systems affected; percentages by organ systems were calculated.

It may be seen in Table II that there were no siginificant differences in percentage of cases with parencymal change attributable to RMSF. Lesions occurred very frequently in the respiratory tract, skin, urogenital system, adrenal gland, lymphoid organs, gastrointestinal tract and liver.

TABLE II. SYSTEMIC PATHOLOGY OF RMSF IN MONKEYS

ORGAN/SYSTEM	% AFFECTED BY CHALLENGE ROUTE IV	IP	TYPE OF LESIONS DESCENDING ORDER BOTH GROUPS
Respiratory tract	100	100	T,A,E,S,V,N,K
Cutaneous (ear and scrotum)	88	100	V,T,H,N,E,C
Urogenital	89	83	V,N,T,H,E,K
Adrenal gland	89	83	A,C,H,N
Lymphoid (including spleen)	89	67	A,C,L,T,H,E,N,D
Gastrointestinal	89	67	T,H,V,C,E,A
Liver	44	67	N,T,F,V,A
Brain	56	40	H,A,C,M
Skeletal muscle	17	75	T
Heart	22	50	V,T
Pancreas	13	0	T

A - acute inflammation C - congestion D - depletion
E - edema F - fatty metamorhosis H - hemorrhage
K - chronic inflammation L - lymphoid hyperplasia M - malacia
N - necrosis S - subacute inflammation T - thrombosis
V - vasculitis

Lesions of the respiratory system were recorded in all of the monkeys and consisted of patchy, acute to subacute interstitial pneumonia (in 10) and pulmonary edema in 9 monkeys.

Vasculitis and thrombosis, with subsequent congestion, hemorrhage, edema and dermal necrosis were evident in skin from ear or scrotum of 14 of 15.

The urogenital system was affected in 13 of 15 monkeys. Most striking were vasculitis, hemorrhage, and ischemic necrosis in the testicles.

Lesions ranging from acute coagulative ischemic necrosis to infiltration of lymphocytes and plasma cells were observed in the adrenal glands of 13 monkeys.

Multifocal areas of ischemic coagulation necrosis occurred in the livers of 6 of 15 monkeys. Necrotic areas were well defined with clear lines of demarcation from normal hepatic parenchyma.

Principal parenchymal lesions in lymph nodes and spleen were lymphoid necrosis and depletion, occasionally with evidence of reticuloendothelial hyperplasia.

A malacic area was detected in the medulla oblongata of 1 monkey. Hemorrhage and congestion were also present in his brain and in those of 6 others.

Although purely vascular lesions occurred in the gastrointestinal tract, skeletal muscle, heart and pancreas of many of the animals, there was no parenchymal necrosis in these tissues. Vascular lesions in all organs included endothelial swelling and proliferation and thrombosis followed by inflammation and necrosis of the vessel walls.

In summary, there were no significant differences in lesions of RMSF between the 2 groups infected IV and IP. Lesions were highly reproducible within both groups. Multifocal ischemic coagulation necrosis in the liver occurred at an apparently higher incidence than reported by other investigators.[4,5] Otherwise, the lesions were comparable to those observed in monkeys exposed to R. rickettsii by aerosol,[5] in humans afflicted with RMSF,[4] and our observations in guinea pigs (with LTC Stookey). Findings indicate that the rhesus monkey is an excellent model for the study of RMSF.

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DOSE-RESPONSE RELATIONSHIP IN YELLOW FEVER INFECTED RHESUS MONKEYS

Philip C. Kosch, Captain, VC*

Although much has been written on yellow fever (YF),[1-6] there is little current work being reported. Use of a monkey model has been presented in isolated reports; Tigertt et al.[7] reported its possibilities for early diagnosis in the YF-infected rhesus monkey (Macaca mulatta), and Dennis et al.[8] described various hematologic findings in infected rhesus monkeys. YF infection in the rhesus monkey is a fulminating process characterized by hepatic failure, albuminuria, hemorrhagic manifestation, and, invariably, death.

Our Institute has recently undertaken a multidisciplinary approach to the investigation of this infection, establishing the YF-infected rhesus monkey as an acute viral infection model for studies of trace metals, free amino acids, renal and hepatic function, sites of tissue damage and effects on various organ systems. Studies involving thyroid dynamics and carbohydrate homeostasis are currently in progress. A prospective study in the rhesus monkey model characterizing the course of the disease relative to the relationship of inoculum to incubation period, early onset of clinical signs and length of illness is reported here.

A dose range of virus from 0.001-1000 mouse intracerebral median lethal doses ($MICLD_{50}$), was utilized for subcutaneous (SC) challenge of 35 monkeys. Only monkeys with insignificant or no serum neutralization indices were used. Body temperature was continuously monitored by an implanted thermocouple deep in the paraspinal muscle. The incubation period lasted from the time of SC inoculation to the initial upward deviation from normal daily temperature of each monkey. Length of illness was that period measured from fever onset to time of death, which was arbitrarily the time when core body temperature was $\leq$ 94 F. In general, the typical pattern of illness consisted of a variable incubation period followed by the onset of a rapid and marked rise in body temperature, peaking within approximately 2 days and terminating precipitously with hypothermia and death. Clinical signs and abnormal laboratory findings were detected predominately during the febrile stage of the disease, reflecting target organ damage and dysfunction. The titration and responses in the experimental monkeys are shown in Table I.

The incubation period of monkeys inoculated with 10 $MICLD_{50}$ was not markedly different from that of monkeys given 1000 $MICLD_{50}$. However, when monkeys were inoculated with $\leq$ 1 $MICLD_{50}$, there was marked lengthening. With 0.1 $MICLD_{50}$, the incubation period was lengthened still further.

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TABLE 1. YELLOW FEVER IN M. MULATTA

DOSE $MICLD_{50}$	NO. DEAD/TOTAL	INCUBATION PERIOD hr	LENGTH OF ILLNESS hr	TIME OF DEATH hr
1000.0	3/3	60 (60)	40 (34-42)	100 (94-104)
10.0	9/9	67 (54-76)	44 (38-50)	111 (94-124)
1.0	6/6	84 (72-100)	40 (30-45)	124 (110-138)
0.1	7/7	183 (124-245)	43 (33-66)	223 (159-294)
0.01	3/5	145 (72-264)	39 (33-46)	184 (109-310)
0.001	0/5			

Of the 5 monkeys inoculated with 0.01 $MICLD_{50}$ 3 developed signs of disease, with an average incubation period quite similar to that at the next higher dosage. All 5 monkeys inoculated with 0.001 $MICLD_{50}$ remained clinically well. Two surviving monkeys in the 0.01-group had no signs of disease, no abnormal clinical findings and no circulating virus or specific neutralizing antibody up to 44 days postinoculation. Length of clinical illness was very similar in regard to all parameters measured regardless of dosage.

Since only the incubation period was dose-dependent, other data were combined for the 28 sick monkeys. Data were then plotted from death time backwards.

The average temperature curve of the 28 monkeys (Figure 1) showed a rapid and marked elevation from normal, occurring at approximately 48 hr before death with a peak of about 105 F, 30 hr before death. A precipitous fall in body temperature occurred terminally resulting in coma and death. Viremia was measured in 11 of the 28 monkeys. Ten of these 11 were viremic < 24 hr before onset of fever.

Figure 2 presents selected laboratory findings. There was a progressive fall in total white blood cell (WBC) count beginning about 24 hr before the onset of fever. A terminal rebound was observed in 10 of 28 monkeys. The absolute lymphopenia and corresponding relative neutrophilia did not become evident until well into the febrile stage of the disease.

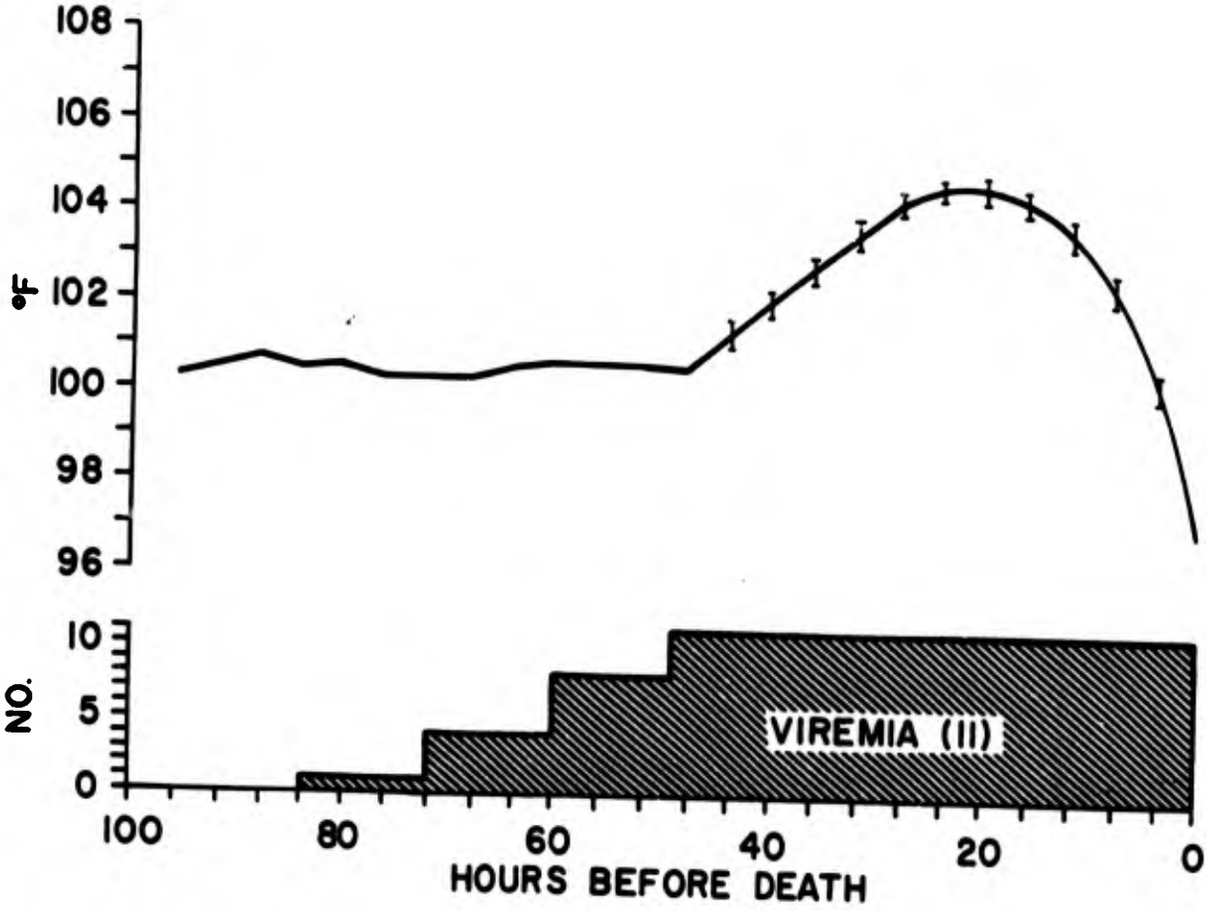

FIGURE 1. TEMPERATURE AND VIREMIA (II OF 28) YF-INFECTED MONKEYS.

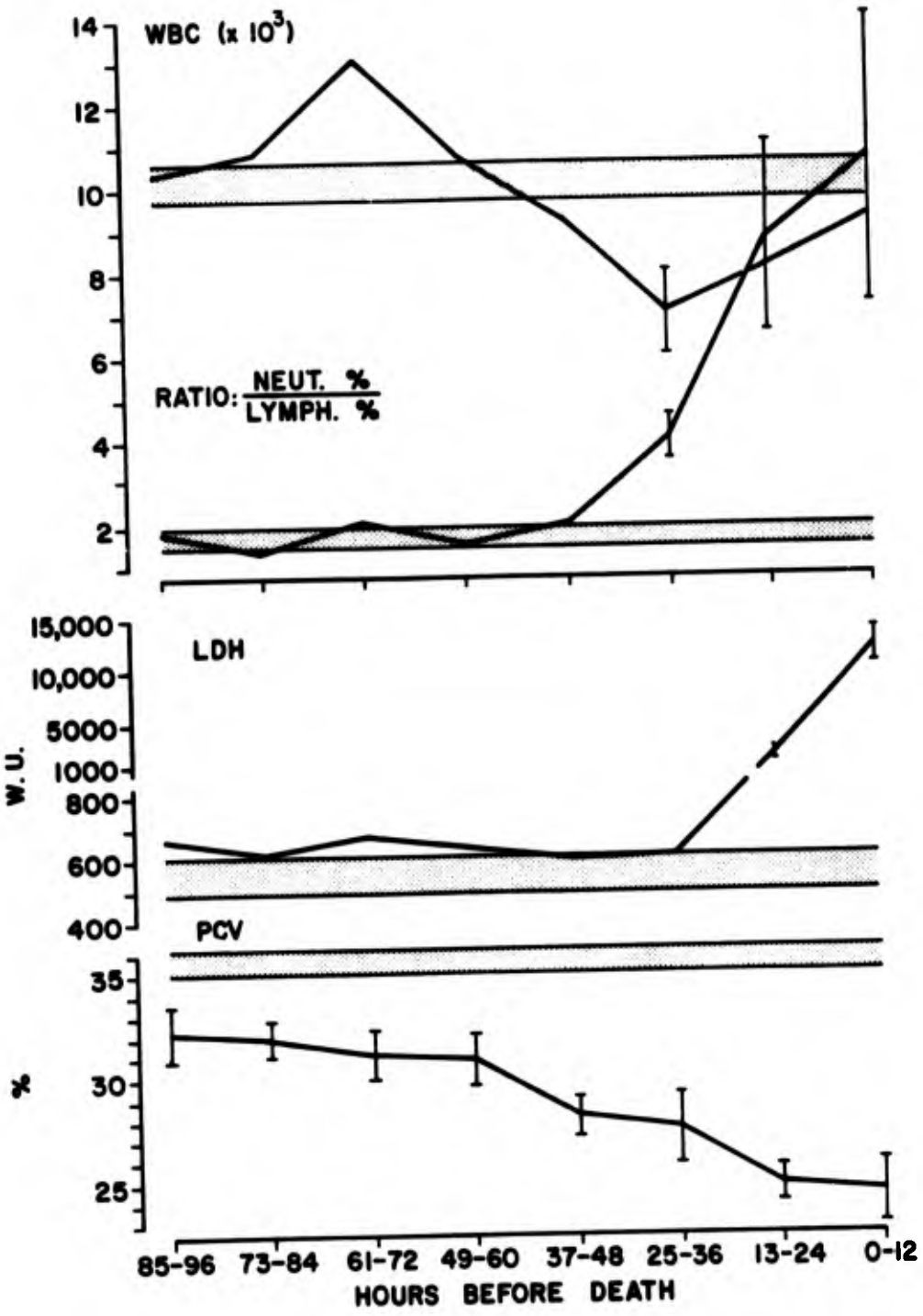

FIGURE 2. WBC, NEUTROPHILS, LYMPHOCYTES, RATIO, LDH AND PCV IN YF-INFECTED MONKEYS.

A gradual fall in packed cell volume (PCV) due to serial bleedings was observed in all animals. An additional marked decrease was observed during the febrile stage of the disease, probably associated with the bleeding diathesis seen in man[4] and primates.[8] The 7 monkeys not developing disease did not show this marked fall, but continued the gradual decline in hematocrit, eventually stabilizing within a week. Although occurring late in the clinical illness, 13-24 hr preceding death, the rise in lactic dehydrogenase (LDH) activity was dramatic; it probably represents tissue damage in target organs.

Diseased monkeys did not demonstrate any outward abnormal clinical signs before fever. Coincident with its onset monkeys exhibited only signs of mild depression and inappetence. Later in the course of illness, moribund monkeys became extremely debilitated, frequently had jaundice and hepatic insufficiency, followed by coma. Both gross and micropathologic changes were compatible with reported YF lesions.[9,10]

In summary, YF in monkeys is a fulminating disease regardless of dosage of virus administered. Although each infected monkey did not exhibit all clinical and laboratory abnormalities measured, a composite picture of the disease developed. Monkeys become viremic on or before the onset of the rapid and marked elevation in body temperature. There is a terminal rise in WBC, with predominance of neutrophils, confirming the findings of Tigertt et al.[7] that this occurs in approximately 36% of monkeys. In mild cases reported in man, the leukopenia disappears rapidly; in severe cases there may be a transition to leukocytosis, with neutrophilia.[4] Surviving monkeys are susceptible to rechallenge. The median infective dose of YF virus in the rhesus monkey is apparently one median lethal dose. One monkey subcutaneous LD_{50} is approximately equal to 0.01 $MICLD_{50}$.

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A PROPOSED MODEL FOR THE STUDY OF SEQUENTIAL RESPIRATORY INFECTIONS

Richard F. Berendt, Ph.D.*

The sequence of influenza followed by bacterial pneumonia is frequently observed among the population. Staphylococci are the most frequently isolated cause of fatality following influenza, but the pneumococci cause the greatest morbidity.[1-4] As a model for the study of sequential infection, therefore, aerosol exposure to influenza virus followed by Diplococcus pneumoniae in rhesus monkeys was chosen. This species was chosen as host mainly because it is the only common laboratory animal, other than the dog, with a respiratory tract of sufficient size to be considered similar to that of man with respect to particle size selectivity. This parameter is expected to be of critical importance in planned investigations because it affects the site of deposition and degree of retention of airborne microorganisms.

Inital investigations have involved several phases including: (1) choice of aerosol equipment and determination of the viable concentration and particle size distribution of short-aged aerosols of influenza virus and D. pneumoniae; (2) selection of criteria of infection, and determination of the response of monkeys to the virus and to the bacterium administered separately; and, finally, (3) determination of response of monkeys to the organisms administered sequentially.

The device used for aerosol exposures consisted of a Henderson tube[5] connected to a plastic helmet which was fitted with a diaphragm to provide a relatively snug fit around a monkey's neck (Figure 1). An all-glass impinger (AGI-30)[6] was connected to the helmet both to draw the aerosol over the monkey's head at 12.5 l/min, and to obtain a sample of the aerosol for estimation of viable concentration. During exposure, the monkeys were restrained in a sitting position in an aluminum chair. The relative humidity during the experiments ranged from 65-85%; the temperature from 70-80 F.

On the assumption that the influenza virus usually infects the respiratory tract at a point somewhat higher than does the pneumococcus and, consequently, would be infective in larger particles than would the bacterium, the size distribution of aerosol particles of both organisms was measured. In the case of the pneumococci the distribution of viable organisms with respect to particle size was determined. Measurements were carried out with single-stage impactors (SSI).[7]

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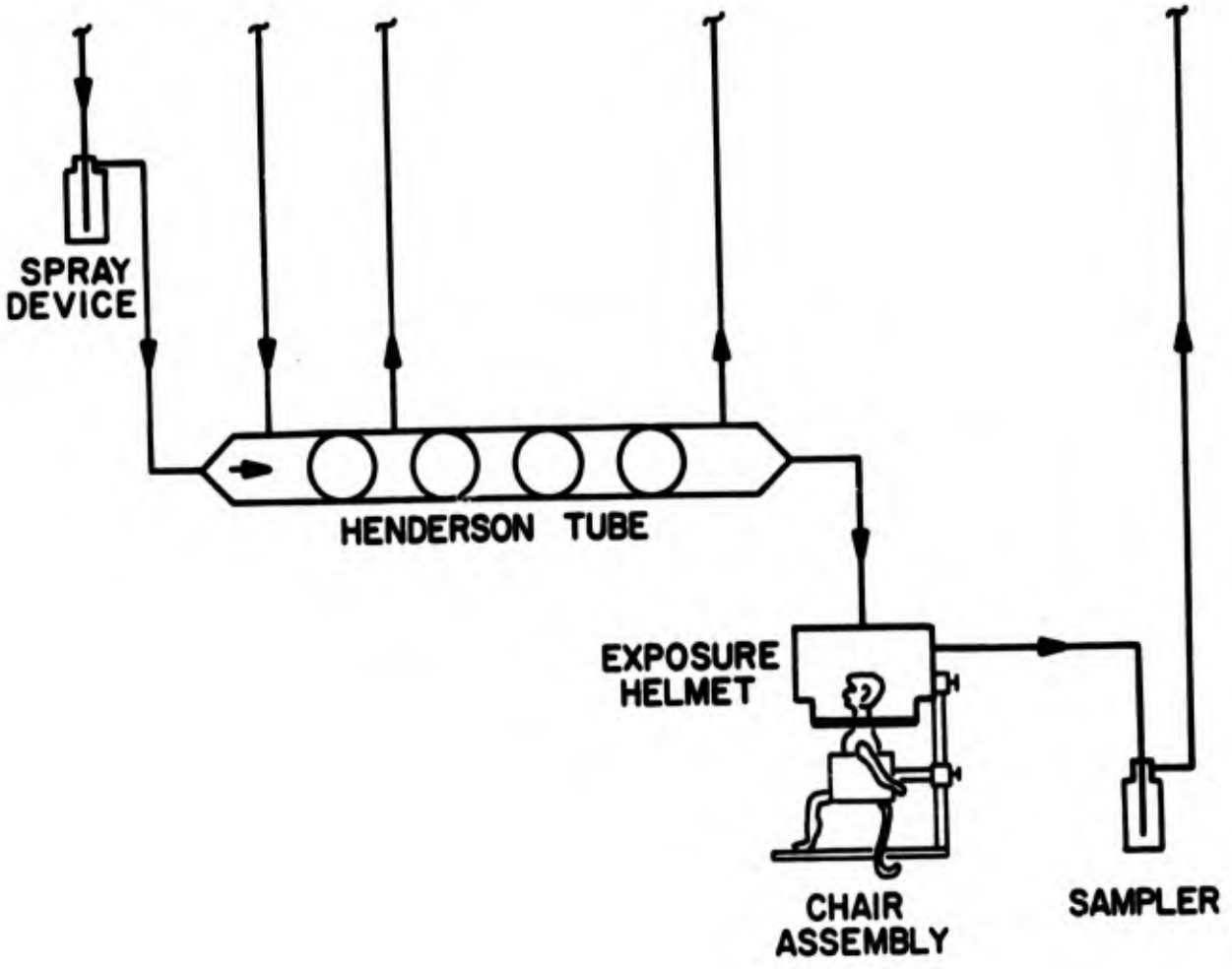

FIGURE 1. HENDERSON TUBE-HELMET EXPOSURE SYSTEM.

The results obtained from 4 replicate experiments in which influenza virus, simulated by normal allantoic fluid containing 1 mg/ml of sodium fluorescein, was disseminated from an atomizer into the aerosol exposure system are shown in Table I. The concentration of dye collected from the exposure helmet was determined with a fluorometer. Particle size was determined with a SSI. If the influenza virus was distributed throughout the aerosol in approximately the same manner as the dye, then half of the virus was located in particles < 2.0 μm, and approximately 90% in particles ≤ 5 μm.

TABLE I. PARTICLE SIZE OF ALLANTOIC FLUID (INFLUENZA VIRUS) AEROSOLS IN THE COLLISON ATOMIZER-HENDERSON TUBE-HELMET SYSTEM

SSI CUTOFF μm	% ≤ INDICATED SIZE	MASS MEDIAN DIAMETER μm	COEFFICIENT OF VARIATION %
1	14.8		
3	71.3		
5	89.5	2.13[a/]	10.3
7	114.1		
11	100.6		

a. Mean of 4 replicate trials.

In a subsequent experiment, the viable concentration of Type 1 D. pneumoniae in particles of various sizes collected from the exposure helmet was determined. The average of duplicate trials made with the Collison atomizer are shown in Table II. The pneumococcal aerosols were larger than the viral, one-half of the organisms were found in particles < 3.5 μm in contrast to the 2.1 μm value obtained for influenza virus; most importantly, no organisms were found in 1 μm particles. Considerable foaming was seen in the atomizer. In an effort to reduce the size of the D. pneumoniae aerosol particles, and thereby increase lung penetration, the techniques of growth and dissemination were carefully examined. The methods that were finally adopted were to grow the organisms in heart infusion broth containing added normal rabbit serum and sheep red blood cells, and to add 2.5% of antifoam just prior to aerosolization. Investigation did not reveal any adverse effect of antifoam on the viability of the pneumococci. The diameter of aerosol particles was also reduced by employing a Dautrebande nebulizer[8] that provided considerably more baffling of large particles than the atomizer used for most experiments. The average of duplicate particle size experiments using these methods are also given in Table II. Although foaming was still extensive and determinations varied considerably, about 18% of the bacteria were placed in 1 μm particles, about 80% were found in particles 5 μm; the median diameter decreased from 3.5-2.2 μm.

TABLE II. PARTICLE SIZE OF D. PNEUMONIAE AEROSOLS IN HENDERSON TUBE-HELMET SYSTEM

SSI CUTOFF μm	CUMULATIVE % ORGANISM DISTRIBUTION		MASS MEDIAN DIAMETER (μm)	
	Collison atomizer	Dautrebande nebulizer	Collison atomizer	Dautrebande nebulizer
1	0.0	17.8		
3	39.3	64.6		
5	61.6	79.7	3.5	2.2
7	86.4	84.4		
9		81.8		
11	93.2			

The third phase of investigation has involved the exposure of monkeys to aerosols of virus or bacteria. Six monkeys, weighing 2.5-3.5 kg were given an estimated inhaled dose of about 10^7 median egg infectious doses (EID_{50}) of the Aichi strain of type A2 influenza virus. Groups of 3 animals were examined on alternate days to reduce the total volume of blood withdrawn from any one monkey. The examinations performed included rectal temperature, nasal wash for virus, viremia, serum zinc, total and differential white blood cell counts, hematocrit, hemoglobin, and hemagglutination-inhibition (HI) tests for antibody in serum and nasal washes. On this, and all subsequent experiments, 2-4 preexposure samples were obtained, so that each monkey was its own control. These tests were also carried out on one nonexposed control monkey to determine the effect of withdrawing frequent blood samples. The significant findings are summarized in Table III. Five of 6 monkeys showed

TABLE III. FINDINGS IN MONKEYS EXPOSED TO AEROSOLS OF INFLUENZA VIRUS

FINDING	NO. RESPONDING/TOTAL	RESPONSE	
		Extent	Duration
Leukopenia	5/6	25-50% of baseline	48-72 hr
Virus in nasal secretions	6/6	Not quantitated	96-192 hr
Increased HI titer	5/5[a]	> 4-fold increase	Starting on 9th day

a. 1 monkey died day 9 with a 2-fold increase.

leukopenia beginning at 24 hr and lasting about 48 hr. Virus was recovered from the nasopharynx of one monkey 24 hr after exposure. All animals shed virus in nasal secretions within 72 hr of exposure for a period of 96 to 192 hours. All remained afebrile. HI titrations were performed with formalin-treated virus as antigen; sera were treated with trypsin and periodate and absorbed with guinea pig erythrocytes to remove nonspecific inhibitors. Five of 6 monkeys had baseline titers of 1:40, the other, 1:80. One monkey died on the 9th day of a cause not related to influenza; at that time, its titer had increased from 1:40 to 1:80. Of the 5 remaining monkeys, all showed an increase in titer beginning on day 9 or 10, reaching 1:640 on the 14th day in one case and measuring 1:160 for all five on the 21st day after exposure. The control animal had a baseline titer of 1:40 which did not change during the observation period. No antibody was detected in the nasal secretions of experimental animals, possibly because the samples were too diluted. These samples have been concentrated and will be titrated again. No significant changes were seen in serum Zn, hematocrit, or hemoglobin levels, and no viremia was detected. To summarize, clinical illness was not produced; the findings of virus multiplication evidenced by virus recovery from the nasopharynx after a lag period lasting as long as 48 hr in some cases, the increase in HI titer, and leukopenia, suggest that infection was established.

In an initial experiment with the pneumococcus, 6 monkeys were exposed to calculated inhaled doses of about 33,000 bacteria. Previous experiments indicated that the number of bacteria in $\leq$ 1-μm particles in these aerosols was about 6,000 cells. A single monkey again provided a control on the effect of frequent blood sampling.

A summary of data is given in Table IV. Two of 6 monkeys showed signs of illness manifested by anorexia and apathy on day 5 or 6. At the same

TABLE IV. FINDINGS IN 6 MONKEYS EXPOSED TO AEROSOLS OF D. PNEUMONIAE

FINDING	NO.	MONKEY DESIGNATION	RESPONSE	
			Extent	Duration hr
Anorexia, apathy	2	C,F	Not measured	24
Febrile response	4	C,D,E,F	> 103 F	24
Leukocytosis, neutrophilia	3	B,C,D	100% > baseline 50% > baseline	24
Serum Zn depression	2	C,D	$\leq$ 50 μg/100 ml	24-48

time, both of these monkeys and 2 others showed a "borderline" febrile reaction, ranging from 103 to 104 F which lasted about 24 hr. Three monkeys had leukocytosis with neutrophilia on the day associated with other signs, and 2 also showed depressed serum Zn values on the 4th - 6th days. No bacteremia was detected. Serological determinations have not yet been carried out on the sera from these animals. In summary, pneumococcal infection and mild clinical illness were produced in some monkeys. It seems likely that the number of bacteria inhaled will have to be increased to exacerbate symptoms and increase the number of monkeys responding.

In our first sequential infection experiment, 8 monkeys were exposed to aerosols of influenza virus followed 4 days later by aerosols of D. pneumoniae. Controls were provided by exposing 4 monkeys to influenza virus alone and 4 to pneumococci. Two additional monkeys were used as controls for the effect of blood sampling. The 4-day interval was chosen because the previously-described study indicated that all animals were likely to be infected with influenza virus by that time. Tests carried out included hematocrit, total and differential leukocyte counts, nasal wash for virus and bacterial isolation, bacteremia, serology for influenza and rectal temperature determinations. Unfortunately, the large requirement for blood precluded serum Zn determinations. A partial summary of the data is given graphically in Figure 2. The most significant finding was that of considerably greater recovery of pneumococci from the nasal secretions of the sequentially-infected monkeys than from the pneumococcal controls. Other findings were about the same as those observed with the controls.

The attainment of the major objective of this project, the establishment of a model for sequential infection, has not yet been achieved, but monkeys have been successfully infected with both microorganisms. The preexposure titers seen in influenza experiments make interpretation of serological data somewhat difficult, particularly with regard to specificity. Experimentation currently in progress indicates that the preexposure titers may be nonspecific. This finding, if confirmed, suggests that it would be advantageous to employ virus neutralization or other serological tests as well. An alternative approach, if the baseline titers are shown to be specific, involves the use of another serotype, e.g., influenza B, which is less likely to have infected monkeys previously.

Whether further effort must be expended to decrease the particle diameter of pneumococcal aerosols remains an open question. An extended investigation of the infectivity of D. pneumoniae as a function of particle size would undoubtedly help to establish a model. On the other hand, if the relatively large median diameters observed in previous experiments are due to the inherent characteristics of the capsular material of the bacteria or of the mucus secretions, which normally constitute the material in which the organisms are excreted, then a different approach may prove more informative. Namely, examine the means by which the pneumococcal infection may be established in the pharynx and subsequently aspirated to the deep respiratory

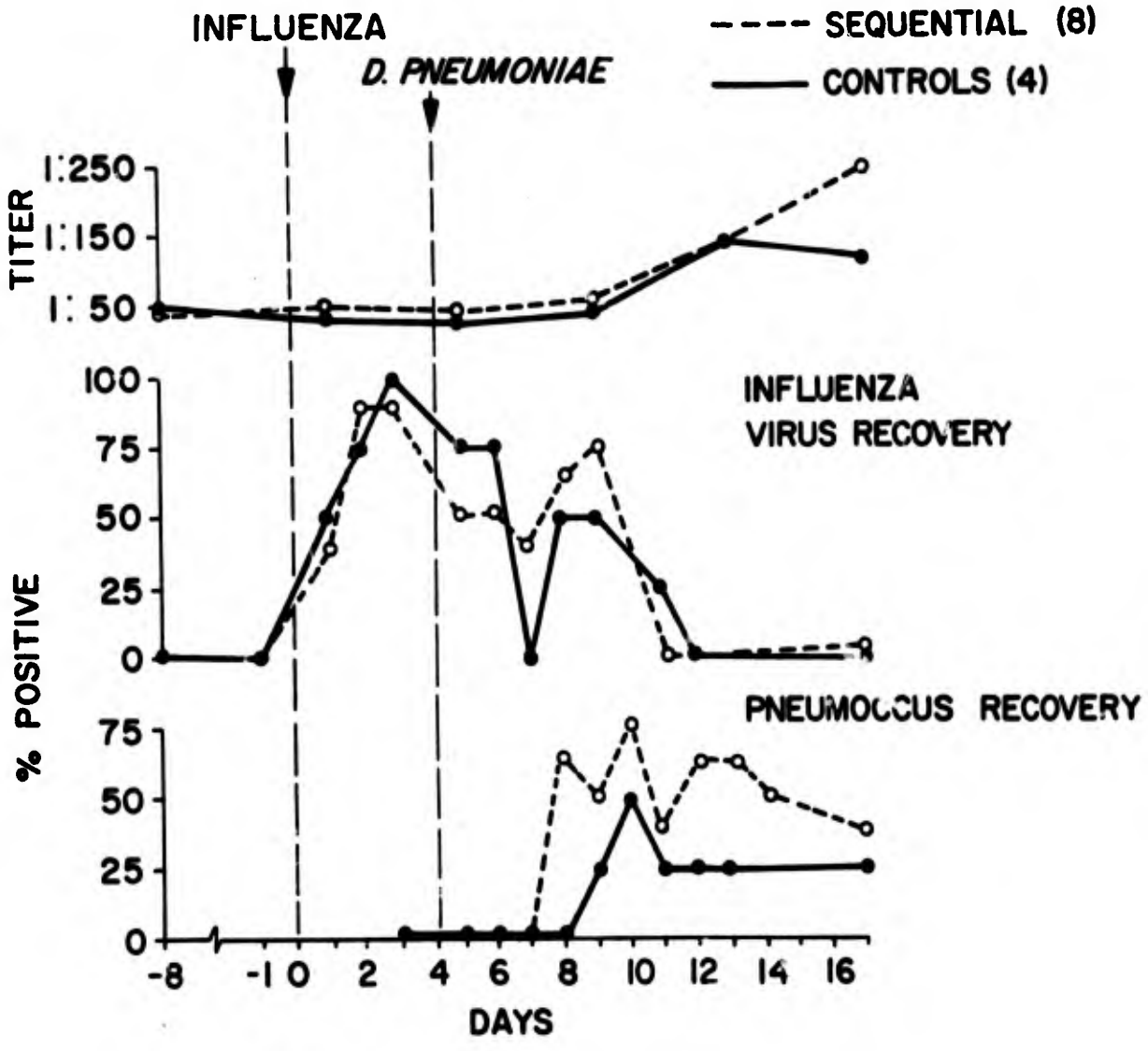

FIGURE 2. EFFECT OF SEQUENTIAL INFECTION ON INFLUENZA HI-TITER, AND RECOVERY OF INFLUENZA VIRUS AND *D. PNEUMONIAE*.

passages. In this regard, it is important to confirm the preliminary finding in the sequential infection experiments that coexisting influenza virus infection favors colonization of the pharynx by pneumococci.

To determine further the suitability of the model, a careful study of temporal relationships must be undertaken to determine the time intervals between exposures best suited to produce sequential infection. Histopathological examination of tissues obtained from serially-sacrificed monkeys may also aid in the determination of the most appropriate times for exposures.

In conclusion, current efforts to establish a model system have been briefly reviewed. It must be emphasized that the primary aim of the research is not to elucidate the mechanisms of these particular infections in a susceptible host, although that may be a highly useful result of the investigations. Rather, the main purpose of this research is to investigate the selected systems as a model for the generalized case of sequential respiratory infections, reasoning that the information obtained might be broadly applied to other respiratory infections in a range of susceptible hosts.

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SEQUENTIAL IMMUNIZATION AGAINST GROUP B ARBOVIRUSES

Winston H. Price,*

with Jordi Casals, Inderjit Thind, and Walter O'Leary

Since 1956 reports have been published from this laboratory on the development of a scheme utilizing selected attenuated living Group B viruses to protect spider monkeys against many Group B viruses.[1-10] Other investigators have also reported on the possibility of using certain Group B arboviruses to protect against related viruses of the same group.[11-21]

A sequential immunization procedure consisting of living 17D yellow fever (17D YF) virus followed by living attenuated strains of Langat E5 virus, dengue 2 virus, and Japanese encephalitis (JE) virus protected monkeys against 28 Group B arboviruses.[9] However, very broad hemagglutination-inhibition (HI) and neutralizing antibody responses resulted when only 17D YF, Langat E5, and dengue 2 virus (New Guinea C strain) vaccine strains were employed with JE omitted; indeed, its addition resulted in lower antibody levels against related Group B viruses.

The sequential use of 17D YF, Langat E5,[22] and attenuated dengue 2[23] afforded protection to spider monkeys against 4 dengue serotypes, Russian spring-summer encephalitis (RSSE), Kyasanur Forest disease (KFD), Omsk hemorrhagic fever (Omsk HF), JE, St. Louis encephalitis (SLE), Murray Valley encephalitis (MVE) virus, West Nile (WN) virus, and Kadam viruses. Neutralizing antibodies were produced against all 20 Group B arboviruses tested.

All of the virus strains employed and techniques have been described.[1-10] For examining brains and spinal cords standard techniques were used for celloidin infiltration of brain and paraffin infiltration of spinal cord; 30-μ serial sections were cut, but only about every hundredth section was stained with gallocyanin. Usually, 6 sections were examined from the brain stem, 5 from cerebellum and 12 from cortex. Serial 15-μ sections were collected from the C6, T6 and L3 levels of spinal cord; 60-90 sections were studied. All sections were graded according to the lesions observed. Animals were killed at 30 days. All histopathologic examinations were done with slides coded by numbers.

The spider monkey, Ateles geoffroyi, was used throughout. Before use the serum of each monkey was screened by HI test for Group B arboviruses, using a serum dilution of 1:10 against MVE, JE, dengues types 1-4, and RSSE viruses. Undiluted serum was also screened for neutralizing

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antibodies against the challenge virus using approximately 50 intraperitoneal (IP) or intracerebral (IC) doses. Any animal that showed detectable antibody titers in any of these tests was excluded from the study.

Unless otherwise stated the sequential immunization was carried out in the following manner. The spider monkey was inoculated intramuscularly (IM) at time (1) with approximately 100,000 suckling mouse median lethal doses ($SMICLD_{50}$) of 17D YF; time (2) ca. 3×10^3 $SMICLD_{50}$ of Langat E5, time (3) one month later, ca. 3×10^6 $SMICLD_{50}$ of dengue 2. Four months later they were challenged subcutaneously (SC) with the appropriate virulent virus.

Table I summarizes the HI antibody responses at each time after the sequential immunization procedure.

In experiments shown in Table II the 3-virus sequential immunization scheme produced the highest HI titers and in some instances broader responses than the other schemes shown. Four monkeys inoculated sequentially with normal chick embryo suspension, followed by normal chick embryo suspension, followed by uninoculated monkey kidney tissue culture fluid, to simulate the 3-virus immunization procedure, showed no HI titers to any of the 21 antigens 4 weeks after each inoculation.

Table I also shows the results of neutralization tests after time (3).

The 3-virus sequential immunization procedure protected spider monkeys against challenge with approximately 10,000 infectious doses of dengue serotypes 1-4. Previous results had showed that the attenuated dengue 2 virus alone protected against homologous virulent challenge.[23] The protection is based on lower viremias in vaccinated monkeys and the absence of a dengue antibody booster response.

The 3-virus sequential immunization procedure protected spider monkeys challenged with JE, SLE, MVE, WNV and RSSE viruses based on histologic examination of the central nervous system (CNS) (Table III).

Groups of sequentially vaccinated and control monkeys were challenged SC with 50,000 $MICLD_{50}$ of KFD, 25,000 $MICLD_{50}$ of Omsk HF, or 1,200,000 $MICLD_{50}$ of Kadam viruses. No histologic evidence of CNS lesions was seen in brain and spinal cord. Vaccinated monkeys were protected against challenge as indicated by marked depressing effect on viremia.

The results show that spider monkeys inoculated sequentially with living 17D YF, living attenuated Langat 5, and living attenuated dengue 2 viruses (New Guinea C isolate) were protected against dengue serotypes 1-4, JE, MVE, SLE, WN, KFD, RSSE, Omsk HF, and Kadam viruses as determined by viremia studies, histologic examination of the brain and spinal cord, or antibody booster responses. It should be noted that not only were the sequentially immunized monkeys protected against all 4 dengue serotypes as determined by

TABLE 1. RECIPROCAL HI AND NEUTRALIZING ANTIBODY RESPONSES OF 17 MONKEYS AFTER 3-VIRUS SEQUENTIAL IMMUNIZATION

ANTIGEN	CONTROL	1 MON AFTER						NEUTRALIZING ANTIBODIES
		17D YF		Langat E5		Dengue 2		
		No.	Titer	No.	Titer	No.	Titer	No. positive[d]
Asibi YF	0	16[a]	≤ 160	16[a]	≤ 320	17	≤ 1280+	17
17D YF	0	17	≤ 160	17	≤ 160	17	≤ 320	Not done
JE	0	1[b]	20	14	≤ 320	17	≤ 640	16
Dengue 1	0	2	10	5	≤ 40	16	≤ 160	16
2	0	1	10	5	≤ 80	17	≤ 320	16
3	0	1	10	6	≤ 80	16	≤ 320	16
4	0	1	20	13	≤ 160	17	≤ 1280+	16
Langat E5	0	1	10	15	≤ 160	16	≤ 640	16
RSSE	0	1	40	16	≤ 160	16	≤ 1280+	16
WN	0	1	20	4	≤ 160	16	≤ 640	16
Omsk HF	0	0		14	≤ 20	16	≤ 160	16
Powassan	0	1	20	16	≤ 80	16	≤ 640	16
KFD	0	1	10	16	≤ 80	16	≤ 320	16
SLE	0	9	≤ 160	15	≤ 1280+[c]	17	≤ 1280+	16
Zika	0	5	≤ 40	15	≤ 320	17	≤ 1280+	16
Wesselsbron	0	11	≤ 160	15	≤ 640	17	≤ 1280+	16
Kadam	0	1	20	7	≤ 80	16	≤ 320	15
3-Arch	0	1	≤ 20	4	≤ 160	16	≤ 640	16
U. S. bat salivary gland	0	1	20	10	≤ 160	16	≤ 640	16
Entebbe bat	0	16	≤ 320	16	≤ 1280+	17	≤ 1280+	16
MVE	0	2	10	14	≤ 20	17	≤ 1280+	16
WEE		0		0		0		0
TIME		(1)		(2)		(3)		(3)

a. Monkey No. 71-9 negative for HI for all but 17D YF at times (1) and (2). Scattered positives at time (3).
b. Monkey No. 71-14 positive for all but dengue 1 and Omsk HF at time (1) and at highest titers at all times.
c. 1:1280 highest dilution tested.
d. Positive = serum neutralized 100-500 $SMICLD_{50}$, IP except dengue, Kadam and 3-Arch.

TABLE II. COMPARISON OF HI ANTIBODY RESPONSES AFTER 4 IMMUNIZATION SCHEDULES

ANTIGEN	MEDIAN RECIPROCAL HI TITERS 1 MON AFTER			
	3-Virus	Langat E5 - Dengue 2	17D YF - Dengue 2	Dengue 2 - Dengue 2
Asibi YF	320	10	40	20
17D YF	80	10	40	0
JE	160	10	20	10
Dengue 1	80	20	20	20
2	80	40	40	80
3	80	10	20	10
4	160	20	40	20
Langat E5	160	20	20	0
RSSE	160	10		0
WN	160	160	160	40
Omsk HF	80	10	10	0
Powassan	80	10	20	10
KFD	80	10	10	0
SLE	640	40	160	40
Zika	320	40	80	40
Wesselsbron	1280+	160	320	160
Kadam	80	10	10	10
3-Arch	80	10	40	10
U. S. bat salivary gland	80	20	40	20
Entebbe bat	1280+	80	320	80
MVE	640	40	80	40
NO. OF MONKEYS	17	7	7	7

TABLE III. EFFECT OF 3-VIRUS IMMUNIZATION PROCEDURE ON CNS LESIONS

CHALLENGE VIRUS	NO. WITH HISTOLOGIC CHANGES		
	Immunization Procedure[a]	Brain	Spinal cord
JE 1.2×10^5 $MICLD_{50}$	3-virus	0/5	0/5
	Control	4/5	3/5
SLE 7.5×10^4 $MICLD_{50}$	3-virus	0/5	0/5
	Control	3/5	Not done
MVE 8×10^4 $MICLD_{50}$	3-virus	0/5	0/5
	Control	3/5	3/5
WN 1×10^5 $MICLD_{50}$	3-virus	0/5	0/5
	Control	3/5	Not done
RSSE 3×10^4 $MICLD_{50}$	3-virus	0/5	0/5
	Control	4/5	4/5

a. Controls inoculated on same schedule but with normal chick embryo suspension, 2 times, followed by monkey kidney tissue culture fluid.

viremia studies, but these monkeys showed no dengue antibody booster HI response indicating that they were solidly immune against the dengue viruses. This is important in view of the theory put forward by Halstead[24] that hemorrhagic shock caused by the dengue viruses is due to infection with 2 different serotypes. Based on our experiments, it appears that even if the above theory is correct, 2 dengue infections with different serotypes do not cause the shock syndrome. However, whether man reacts similarly to spider monkeys must await further experimentation.

It is also of interest that the 3-virus sequential immunization protects spider monkeys against the newly discovered Group B arbovirus, Kadam virus, in spite of the fact that Kadam virus is not closely related to any of the viruses used in the sequential immunization procedure.

One problem that may arise in the sequential immunization scheme in man is the cross-sensitization that may occur in an individual inoculated with the virus preparation. This may precipitate a reaction in a person sensitized to Group B antigens. In an attempt to study this phenomenon, monkey organs, tissues, and blood are being examined for signs of sensitization after each step in the 3-virus immunization procedure and after challenge with dengue serotypes and other Group B viruses.*

Preliminary experiments indicate that if the sequence is reversed so that dengue 2 virus is given first followed by Langat E5 virus followed by 17D YF, protection is not as broad. The importance of the sequence of infecting Group B viruses in producing a broad neutralizing antibody response was shown first by Smithburn[11] in 1954 and later in this laboratory.[6]

In the development of a sequential vaccination procedure aimed at protecting against a number of Group B arboviruses, we have considered the following points:

(1) Attenuated viruses are preferable to inactivated viral vaccines.

(2) The vaccine must be safe for inoculation into humans.

(3) The viruses chosen for use in the vaccination procedure must be those that give the broadest heterologous protection.

(4) The viruses must be inoculated in the correct sequence at the right time-intervals.

The fact that the sequence is important could be explained by the following hypothesis: the best heterologous responses are obtained when the first virus sensitizes the immune mechanism of the host in such a way that the multiplication of the 2nd virus is not inhibited. The heterologous antigenic components of virus no. 2 may then reach a high enough concentration to simulate the antibody-producing system of the host. If the first virus is too closely related serologically to the 2nd virus, it will inhibit the multiplication of the latter to such an extent that the host will react as if he had not received a secondary stimulus. If the first virus given is not closely enough related serologically to the 2nd, there will be no sensitization, and thus no broad heterologous responses.

* These experiments are being carried out in collaboration with the U. S. Army Medical Research Institute of Infectious Diseases.

The sequential vaccination procedure depends on rather subtle responses of the host to related antigens. A further understanding of the mechanisms involved in this procedure would be desirable both from the basic and practical aspects of immunization and antibody responses to these viruses. Of considerable importance in this connection is the level of neutralizing antibodies required to protect the host from the involvement of the CNS which occurs with some of the virulent Group B viruses. Morris and co-workers[25] showed that low levels of neutralizing antibodies, induced by previous infection with JE or RSSE viruses, protected monkeys against CNS involvement after homologous challenge. Using passive immunization techniques, low levels of neutralizing antibody induced by inoculation of monkeys with Langat TP-21 virus protect against CNS lesions due to challenge by RSSE virus.[6]

(5) The efficacy of the vaccination procedure must be judged upon protection against disease and not simply on antibody response. With Group B arboviruses, antigenic relationships show greater group reactivity when measured by the HI test than when measured by neutralization tests. In studying combinations of Group B viruses that might be suitable for a vaccine, however, the neutralizing antibody levels are what must be considered, since these antibodies are the principal ones concerned in resistance. Thus, various combinations of Group B viruses may produce high levels of HI antibodies to RSSE viruses in monkeys but little if any neutralizing antibody; when such monkeys are challenged with RSSE virus no protection is noted.[6] Since by various non-neural routes of inoculation in laboratory animals, viremia may be considered to be a natural precursor to involvement of the CNS, moderate or low levels of circulating neutralizing antibodies should protect against CNS involvement by some of the Group B viruses by controlling the viremia; this they do.[6,25-27]

(6) The duration of protection should be substantial.

(7) The attenuated viruses used in the vaccination procedures should be of such low infectivity as to render the subject incapable of infecting arthropods that might feed upon him at the time of vaccination. Both the attenuated Langat E5 virus[22] and dengue 2 virus[23] meet this criterion.

(8) The attenuated viruses should be distinguishable from wild strains by at least 3 genetic markers. The attenuated characteristics should be stable. Both attenuated Langat E5 virus[22] and dengue 2 virus[23] meet these criteria.

It is well known that clinical illnesses due to Group B viruses have occurred in individuals who had Group B antibodies prior to their infection. In view of the wealth of evidence that heterologous neutralizing antibody responses can protect a laboratory host, as judged by the lowering of viremia, or decreased neuropathogenicity, against Group B virus infections,

one might draw the conclusion that man differs completely from various laboratory hosts, including primates. At the present time there is no evidence which would allow one to draw such a conclusion. Of critical importance would be (a) whether the person had neutralizing antibodies to the infecting virus at the time of infection and the level of such antibodies, and (b) with what virus or viruses had he been previously infected and in what sequence. If we may extrapolate from the findings in primates, these factors would determine his neutralizing antibody levels and the ability to respond with an anamnestic neutralizing antibody response when infected with other heterologous Group B viruses. This latter fact would be of great importance for the efficacy of the vaccination procedure.[6] An anamnestic antibody response was observed in humans by Schlesinger and co-workers;[16] volunteers inoculated 7-7.5 years previously with 17D YF virus responsed with rapid production of neutralizing antibodies to YF, and dengue 1 and 2 viruses after inoculation with an attenuated type 2 dengue virus.

It appears that the 3-virus sequential immunization procedure reported in this paper is ready to be tested in man if the living attenuated dengue 2 virus is found to be safe for humans.

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CURRENT STUDIES ON IMMUNOPROPHYLAXIS

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Typhoid Fever

The Division of Infectious Diseases of the University of Maryland School of Medicine has been interested for a number of years in typhoid fever, from the standpoints of evaluation of various typhoid vaccine and treatment. The recent outbreak of antibiotic-resistant typhoid fever in Mexico prompted a study designed to (1) demonstrate that the strain causing the epidemic is in fact resistant to chloramphenicol and (2) select an effective antibiotic. Resistance to chloramphenicol for this strain had to be verified because some reports indicated that the chloramphenicol being used in Mexico was not up to U. S. standards. A simple protocol was designed to use a standardized known quality chloramphenicol in patients with mild to moderately severe disease who had not been ill for more than a week. Blood was drawn to determine drug levels in order to insure that there was appropriate absorption.

Results indicated that about 13% of the patients now being admitted to one of the pediatric hospitals in Mexico City are susceptible to treatment with chloramphenicol. The strain causing these infections is a phage type D and appears to be similar to the endemic strain that has been present for a number of years in Mexico. The other 8% were resistant to chloramphenicol treatment; *in vitro* sensitivity testing revealed > 1000 µg/ml resistance to the drug. Patients ill with this resistant strain have all of the complications that have been recorded in the past, ranging from ruptured gall bladders, perforated intestinal tract, meningitis, pneumonia, empyemas, myocarditis, hepatitis, to osteomyelitis. The strain appears to be susceptible to ampicillin which is presently the drug of choice. However, recently 3 strains were isolated which are completely resistant not only to chloramphenicol but also to ampicillin.

The basis of the resistance of this new epidemic strain appears to have arisen through the mechanism of episomal transfer from *Shigella* strains that have been present in southern Mexico for a number of years. They are the same *Shigella dysenteriae* strains first noted in Central America to have multiple antibiotic resistance and which then moved into Mexico. During our volunteer studies, aminoglycocides were shown to be effective *in vitro* but not *in vivo*. Rifampin has been tried in Mexico but was ineffective. The combination of Trimetnoprim and sulfonamide was also tried in Mexico with varying results.

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We are faced with a new epidemic strain of typhoid fever. This strain is not responding to currently available antibiotics except for ampicillin and may become resistant to it. The epidemic has shown some indication of waning in the last several months but cases have been imported into the United States. No attempt at control of carriers or widespread vaccination has been undertaken in Mexico. Our studies currently include *in vitro* analysis of combinations of antibiotics. There does appear to be synergism between the penicillin drugs and cephalosporin derivatives.

A tissue culture technique will be used so as to define better antibiotic activity in an intracellular environment. Hopefully some form of antibiotic therapy will be found from these techniques since it appears that the currently available epidemic in Mexico could become a worldwide problem.

Induced Cholera in Volunteers

Infectious diarrheal disease of man appears to be elicited by a limited number of pathogenic mechanisms: two of the most significant involve either actual penetration of the mucosal surface by invasive bacteria or alteration of the cell surfaces and fluid leakage as a consequence of exotoxin activity. Since both processes initially involve bacterial multiplication, antibacterial substances in the gut, either in the succus entericus or associated with the cell membrane, theoretically could inhibit 2 major bacterium-host interactions. An excellent *in vivo* example of the bactericidal activity has been found in volunteers previously ill with cholera diarrhea who were shown to have a significant vibriocidal activity at time of rechallenge. No evidence of bacterial multiplication could be detected; all stool cultures were negative for *Vibrio cholerae*. These men were solidly immune. The mechanisms involved in this protective barrier are unknown. Achievement of this level of intestinal immunity through vaccine administration by any route is a worthy goal. Oral vaccine administration may mimic the method of naturally acquired resistance, that is, by repeated ingestion of small numbers of pathogens. Oral vaccine trials have been initiated with the hope of defining how effective this route might be in stimulating local immunity and protecting against cholera. Previous encouraging experience with *Shigella* and typhoid vaccines have demonstrated that organisms that penetrate gut mucosa may be inhibited by the resistance induced by these vaccines. This experience has stimulated attempts to use cholera antigens as oral vaccines. Preliminary results are presented.

Since cholera exotoxin has such a profound effect on the intestinal mucosa it is conceivable that antitoxin activity may be involved in acquired immunity following the disease. Drs. J. Craig and N. Pierce have suggested that antitoxin activity can be found in succus entericus. *In vitro* studies have suggested that cholera antitoxin can effectively neutralize the exotoxin of *Escherichia coli*. Could such similar *in vivo* activity be involved in the acquired immunity of adults living in endemic cholera - *E. coli* diarrheal countries? The volunteer model of induced disease allows for evaluation of cross resistance between the 2 diseases.

All volunteers were healthy, informed inmates of the Maryland House of Correction who freely agreed to participate. Each was able to withdraw at any time withour fear of recrimination. Several oral vaccine preparations were employed. Twenty-two men received killed Inaba whole cell vaccine orally (Merck, Sharp and Dohme Lot #4445G) by the following schedule: 2 ml for 5 days, a 2-day rest period, a second 5-day schedule of 2 ml each day, a 30-day rest period and a final 5 days of vaccine, 2 ml daily. This schedule presented a total of 30 ml of killed vaccine per man. Each ml of vaccine contained 8 x 10^9 freeze-dried Inaba organisms. No toxin could be demonstrated in this monovalent preparation. Vaccine was given concurrently with 2 gm of $NaHCO_3$.

Two attenuated strains of V. cholerae have been administered as potential vaccines. The first is C14-S5 strain, a streptomycin-resistant mutant of Ogawa type 162/P, isolated by Doctor Bhaskaran. It was administered to a small group of volunteers to test for virulence and ability to elicit antibodies. Strain EW-6 (El Tor) from Doctor Mukerjee in Calcutta was given to 5 volunteers in a dose of 10^9 organisms to evaluate reactions and production of antibodies.

Rechallenge of volunteers who recovered from cholera diarrhea was carried out using 2 enterotoxigenic strains of E. coli. Five volunteers, recovered from grade 2 or 3 cholera (i.e., with diarrhea), were given 10^8 organisms strain B_2C along with 5 controls. An additional 9 volunteers, recovered from cholera, and 3 controls were given 10^8 cells of E. coli strain H-10407. All inocula were administered in approximately 45 ml of milk.

Eleven volunteers given killed oral vaccine and 11 controls were challenged with Inaba 569B vibrios 5-10 weeks after the last dose of vaccine. Each ingested approximately 10^8 cells immediately following the drinking of a bicarbonate solution. All volunteers were hospitalized on the special research ward of the University of Maryland at the Maryland House of Correction. Fluid intake and output were carefully monitored. Each stool specimen was collected, measured and cultured. Intravenous (IV) fluid was administered when it became clinically apparent that oral replacement was not sufficient to maintain fluid balance. Tetracycline therapy was routinely begun when IV fluids were started. All other volunteers receiving Inaba 569B organisms were given a 5-day course of tetracycline (250 mg, 4 times a day) beginning 7-10 days after challenge.

Nineteen volunteers were given the V. cholerae strain C14-S5 in graded doses with bicarbonate solution. None of these 19 showed any clinical evidence of disease and none had diarrhea. No organisms were isolated from any stool specimens. Serological conversions were noted in serum specimens from 2 volunteers (one receiving 10^8 and one, 10^8 organisms): in one, from < 1:20-1:320 for both Ogawa and Inaba antigens and from < 1:20-1:160 for antitoxin. The other volunteer had only a rise in serum antitoxin titer from < 1:20-1:80. None of these volunteers have been challenged with virulent organisms.

Five volunteers were given 10^9 EW-6 organisms without incident. Stool cultures were consistently negative for vibrios; no serological response was noted. None have been challenged.

Twenty-two volunteers were begun on the oral killed vaccine. The vaccine was tolerated without difficulty. Evidence of a systemic serological response was meager but short-lived homologous vibriocidal antibody responses could be demonstrated (Table I). No antitoxin activity was apparent.

TABLE I. VIBRIOCIDAL AND ANTITOXIN TITERS FOLLOWING KILLED ORAL INABA CHOLERA VACCINE ADMINISTRATION

WEEKS	RECIPROCAL GEOMETRIC MEAN TITERS		
	Ogawa	Inaba	Antitoxin titers
0	21	31	< 20
2	31	70	< 20
4	24	47	< 20
6	21	34	< 20
8	32	67	< 20

Eleven of these volunteers were challenged 5 to 10 weeks after the last dose of vaccine with 10^6 Inaba strain 569B organisms with bicarbonate solution as were 11 controls. This inoculum had caused severe diarrhea (grade 3 cholera) in about 25% of nonimmune volunteers. Results of the challenge are outlined in Table II. It is apparent that no grade 3 disease occurred in any of the oral-vaccine group. Diarrhea, grades 2 and 3, was recorded in 27% of the volunteers who received oral vaccine and 73% of the controls, but there is no statistical difference ($p < 0.02$).

For comparison, the results of challenge of volunteers given the same monovalent Inaba vaccine by the parenteral route are listed in Table II. These men had received a total of 1.5 ml of 2 doses 30 days apart. Again no grade 3 disease was noted in any of the 13 volunteers. Superficially, the comparison between oral and parenteral vaccine administration reveals little difference in terms of efficacy in preventing diarrhea. Obviously, the numbers are small in each group but the results are encouraging. Adding the results of the 2 vaccine groups together and comparing them with the total number of men acting as controls, a significant difference can be calculated ($p < 0.01$). Thus, the administration of monovalent Inaba vaccine by mouth or subcutaneously appears to induce some protection against induced cholera. The lack of significant systemic antibodies following oral vaccine implies that perhaps local defenses of the gut were involved. These results further indict humoral antibodies as being related to individual protection against cholera.

TABLE II. EFFICACY OF ORAL OR SUBCUTANEOUS KILLED MONOVALENT INABA VACCINE IN PREVENTING INDUCED CHOLERA

VACCINE STRAINS	NO. OF VOLUNTEERS	NO. WITH GRADE OF CHOLERA				% WITH DIARRHEA (Grade 2 & 3)[a/]
		1	2	3	4	
Oral Vaccine	11	4	4	3	0	27
Control	11	2	2	5	2	64
Parenteral Vaccine	13	8	3	2	0	15
Control	5	0	1	2	2	80

a. χ^2 with Yates correction, $p < 0.02$ for oral vaccine vs. controls, $p < 0.01$ for all vaccinated vs. controls.

The serological responses following challenge of the oral vaccine group revealed that a few men without diarrhea had significant increases in vibriocidal titers (Table III). Mean antitoxin titers were consistently < 1:20. The baseline geometric mean titers between the 2 groups are, at a glance, strikingly different. However, in the groups that did not develop diarrhea, 2 men had similar titers as the controls, i.e. < 1:20 and 1:20. Not readily apparent on this table but obvious when comparing Tables I and III is evidence of a booster response in vibriocidal titers occurring probably as a consequence of the last 5 days of

TABLE III. SEROLOGICAL RESPONSES OF VOLUNTEERS GIVEN ORAL VACCINE FOLLOWING VIRULENT INABA 569B CHALLENGE

WEEKS	RECIPROCAL GEOMETRIC MEAN TITERS	
	Homologous vibriocidal	
	Diarrhea	No diarrhea
0	< 20	146
2	800	813
4	160	358
6		160
8		113

vaccine administration. In Table I the last Inaba vibriocidal titer is at 8 weeks (1:67) or one week after the last series of vaccine doses. In Table III the geometric mean titer is recorded at 1:146 for those without diarrhea or 1:80 for the group of 11. Thus there was a further increase in geometric titers between the last serum specimen postvaccine and the time of challenge $\geq$ 2 weeks later.

Why no antitoxin titers developed in this group following challenge especially the 3 who developed diarrhea may be a reflection primarily of minor illness and secondly, insensitivity of the test. Ordinarily, about 50% of grade 2 and 3 disease states result in detectable antitoxin titers.

The second part of this presentation deals with the attempt to demonstrate *in vivo* cross neutralization of *E. coli* and cholera exotoxins.

Preliminary studies, demonstrated no evidence of immunity to cholera or to *E. coli* diarrhea in a small number of volunteers who had recovered from the heterologous disease prior to subsequent rechallenge. Five volunteers previously exposed to *E. coli* (2 with no diarrhea) were exposed to cholera; all 5 developed grade 2 disease (Table IV). It seems unlikely that the exposure to *E. coli* enterotoxin provided any protection against cholera. Five volunteers having had grade 2 or 3 cholera were rechallenged with 10^8 B_2C strain of *E. coli*. Two of 5 developed diarrhea whereas 4 of 5 controls became ill. An additional 9 men who had recovered from cholera and 3 controls were challenged with 10^8 *E. coli* strain H 10407, originally isolated in Dacca. Three of the 9 recovered cholera patients developed *E. coli* disease, 1 severe, reminiscent of grade 2-3 cholera except with fever and vomiting. Two of the 3 controls developed disease, 1 severe and 1 mild. All 12 of these men had the etiological *E. coli* strain consistently isolated from the stools. It appears that no clear-cut demonstration of acquired capability of intestinal cross neutralization of the 2 toxins was shown.

TABLE IV. EFFICACY OF A HETEROLOGOUS DISEASE TO INDUCE CROSS-REACTING IMMUNITY

INITIAL DISEASE	NO. OF VOLUNTEERS	HETEROLOGOUS CHALLENGE	NO. WITH SECOND DISEASE	NO. CONTROLS INFECTED/TOTAL
E. coli	5	Cholera	5	
Cholera	5	10^8 B_2C, *E. coli*	2	4/5
Cholera	9	10^8 H 10407, *E. coli*	3	2/3

Investigation of oral vaccines in man to prevent typhoid fever, shigellosis and cholera have been carried out. Each study has suggested the feasibility of this approach but none has demonstrated clear-cut induction of complete immunity to subsequent challenge. Implicit in improving these results is to obtain more information pertaining to the best form of oral antigen, its proper dose, optimal dosage schedule and parameters indicative of an immune state. Thus far our studies have suggested that some protection can be achieved with an inactivated oral vaccine. Fujita and Finkelstein[1] have shown that oral toxoids can prevent cholera disease in mice. A similar approach should be tested in man. Apart from killed antigens given in repetitive large doses, it seems reasonable to try attenuated replicating strains that may induce long lasting resistance and obviate the need for repeated dosing. Preliminary results obtained with 2 such strains have been presented. These strains plus others require further evaluation. The ease and apparent safety of oral antigens tested to date plus the encouraging signs of efficacy obtained with typhoid and Shigella oral vaccines indicate that additional studies are warranted in cholera.

The investigations into the possible in vivo neutralization of E. coli exotoxin by cholera antitoxin induced by prior disease failed to demonstrate this or the reverse relationship. Conceivably, there may be little similarity between the 2 species of exotoxin involved and hence make it less likely for the totally unrelated antitoxin to be effective. Perhaps when further in vitro studies delineate the strains which induce cross neutralization, additional studies can be performed. The concept is intriguing and if proven would give some insight into how nonspecific defense mechanisms of the gut can develop.

In summary, large doses of monovalent Inaba killed vaccine given by mouth produced some evidence of protection against induced cholera. No evidence of cross protection in man by antitoxins to the enterotoxins of E. coli or cholera could be demonstrated. Additional investigation of oral cholera vaccines appears indicated using killed as well as living attenuated strains.

ACKNOWLEDGMENT

These studies could not have been performed without the willing, whole hearted, and courageous cooperation of the inmates of the Maryland House of Correction, Jessup, Md. Their continued support and desire to volunteer for additional studies attests to the acceptance these studies have in the prison. The officials of the institution have been generous in their administrative assistance to back our investigations. A special thanks is due to the hard working male nurses who have monitored these studies so very carefully allowing for the collection of significant data.

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EFFECT OF EXCHANGE TRANSFUSION ON ENDOTOXIN LETHALITY IN RABBITS

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When gram-negative bacterial endotoxin is administered intravenously (IV) to healthy man or animal, it is cleared from the circulation in exponential fashion. The initial clearance phase is rapid, with a slower phase generally becoming evident within 30 min.[1-3] The major contribution of the reticuloendothelial system (RES) to this endotoxin clearance has been documented previously.[1,2] When tolerance to endotoxin is induced by prior endotoxin injections, the rate as well as total RES uptake of circulating toxin becomes markedly enhanced.[2-5] Based upon such findings, together with the apparent abolition of tolerance following RES "blockade", Beeson proposed that enhanced RES removal of circulating endotoxin mediates tolerance; this mechanism presumably would act by protecting other more susceptible tissues from toxin injury.[5] This attractive hypothesis, however, has been challenged by a number of recent studies.[2,6-10] The significance of this challenge is of more than academic interest, since it is now feasible to enhance the removal of circulating endotoxin by physical means, e.g. exchange transfusion. Thus, following the introduction of a lethal dose of endotoxin into the bloodstream, mechanical assistance of the nontolerant RES with toxin removal by early and rapid exchange transfusion will simulate the enhanced clearance of the tolerant RES. Significant protection should now result if enhanced blood clearance *per se* is indeed the basis of tolerance. The present studies were designed to test this possibility.

A Boivin preparation of *Escherichia coli* endotoxin, 0127B8 (Difco) was labeled with $NaCr^{51}O_4$ by the method of Braude *et al.*[11] Control solutions of labeled chromate were treated identically except for omission of endotoxin to determine the quantity of $NaCr^{51}O_4$ that remained nondialyzable as a result of aggregation under the conditions of labeling. It was found that a maximum of only 1% of the labeled *E. coli* endotoxin preparation could be contaminated with unbound chromate ion.

Employing aseptic precautions, one femoral artery of 2.0-2.5 kg, healthy, albino New Zealand rabbits was cannulated with sterile polyethylene, PE 90 tubing previously rinsed repeatedly with pyrogen-free sterile saline. Unlabeled *E. coli* endotoxin expected to produce 80% deaths (LD_{80}), 2.5 mg, was administered via ear vein. Twenty minutes later, an exchange transfusion was carried out as follows: 10 ml blood was rapidly withdrawn via the cannula and 10 ml of freshly drawn, heparinized blood from healthy rabbit donors was immediately returned through the same cannula by turning a 3-way stopcock. The donor blood was pretreated at room temperature (70-72°F) by filtering through sterile, pyrogen-free polyethylene screens

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A version of this presentation will be published.

of the type used in human transfusion sets to remove microthrombi. Additional 10-ml aliquots of blood were exchanged repeatedly until the recipient had received the equivalent of 14% of body weight of donor blood (280-350 ml). This accomplished the exchange of approximately 80% of the recipients initial blood volume as determined by plasma protein labeling studies with Evans blue dye. The entire exchange transfusion was always completed within 20 min. Randomly selected control animals were concomitantly given 2.5 mg of the E. coli endotoxin by ear vein, and 20 min later 10-ml aliquots of blood rapidly withdrawn via a femoral artery cannula. In these controls, however, the blood was not discarded but was returned to the same animal after addition of sterile, pyrogen-free heparin equivalent to that utilized in the actual exchange transfusion, i.e approximately 4000 USP units. Following either the exchange or sham-exchange procedure, the femoral artery cannula was removed, the artery ligated, and the wound closed with sterile sutures. All animals were observed for 96 hr.

Additional control studies were carried out to determine whether exchange transfusion per se enhanced susceptibility to endotoxin lethality. For this purpose, femoral artery cannulation was performed in a group of 10 rabbits. Five animals were not exchanged but were given 4000 USP units heparin IV, the other 5 animals were exchange transfused. The cannulas were removed, the femoral artery ligated, and the femoral wound sutured with sterile precautions. All animals were then given an LD_{80} dose of E. coli endotoxin (0.5 mg) via ear vein, and monitored for 96 hr.

Blood clearance studies of the E. coli endotoxin were performed in 3 groups of animals - normal, normal exchange-transfused, and tolerant. The tolerant rabbits were studied on day 8 following 7 daily IV injections of 100 μg unlabeled E. coli endotoxin. In preliminary studies, 10 animals thus pretreated were found highly tolerant, i.e. exhibited no mortality after ear vein administration of 2,500 mg unlabeled E. coli endotoxin. The femoral artery of each test animal was cannulated; 2.5 mg of labeled E. coli endotoxin were injected via ear vein. At carefully timed intervals, 1-ml blood samples were removed from the femoral artery cannula and discarded (washout), and a second 1 ml sample removed and placed in plastic tubes of uniform size. Radioactivity was determined by counting in an automatic gamma well counter for sufficient time to permit reproducibility to within 5%. The amount of circulating endotoxin was expressed as the percentage of administered dose of radioactivity calculated to be present at each time interval in the total blood volume of each animal.

Ear vein administration of labeled E. coli endotoxin into healthy, non-tolerant rabbits resulted in typical blood clearance patterns, i.e. an initial rapid phase followed within 30 min by the slower phase. This latter phase was characterized by prolonged circulation of appreciable quantities of the initially injected dose of toxin - between 20-30%, Figure 1 (upper curve). In contrast, when the tagged toxin was injected into endotoxin-tolerant animals, the expected marked enhancement in blood clearance was

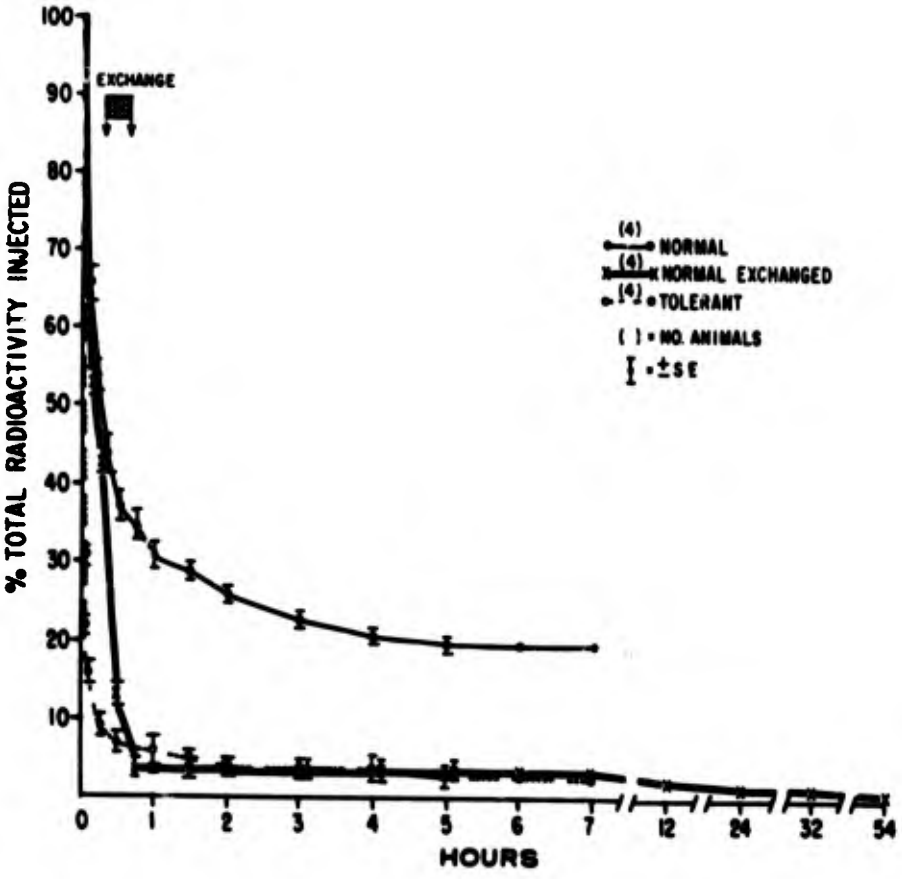

FIGURE 1. BLOOD CLEARANCE OF 2500 μg ^{51}CR-TAGGED *E. COLI* ENDOTOXIN IN NORMAL, NORMAL EXCHANGE-TRANSFUSED AND TOLERANT RABBITS.

seen (lower curve). When exchange transfusion was carried out in nontolerant animals, the circulating endotoxin was rapidly and permanently reduced to levels closely approximating those in tolerant animals (center curve).

Exchange transfusion did not enhance susceptibility to endotoxin (Figure 2). Thus, a 20% mortality occurred when 0.5 mg of the E. coli endotoxin was administered after exchange transfusion. When 2.5 mg of endotoxin was administered 20 min before exchange transfusion, 96 hr mortality was only slightly reduced.

By means of exchange-transfusion begun 20 min after sudden IV injection of an LD_{80} dose of E. coli endotoxin it was possible to minimize the high levels of endotoxin that otherwise continue to circulate for hours. The 20-min interval was carefully selected so as to couple maximum RES uptake of endotoxin with maximum clearance of circulating toxin reproducing as closely as possible the enhanced clearance of circulating endotoxin, characteristic of the tolerant state. It is emphasized that the enhanced rate of clearance of endotoxemia achieved by exchange transfusion did not precisely simulate that seen in the tolerant animal, since exchange was not begun until 20 min after toxin injection. Nevertheless, total endotoxin clearance, as well as the time at which the circulating levels of endotoxin attained their minimal levels after exchange transfusion, was virtually identical to that seen in the tolerant animal. Despite this ability of exchange transfusion to elicit drastic and sustained reduction of endotoxemia, only slight, statistically insignificant reduction in the subsequent 96-hr mortality ensued. This contrasted with the no mortality in tolerant animals. This apparent lack of protection by exchange transfusion could not be related to enhancement of endotoxin susceptibility secondary to the exchange procedure per se. These findings lend direct support to the accumulating evidence that enhancement of endotoxin clearance by the RES is not per se the primary mechanism responsible for the endotoxin-tolerant state. Thus, earlier studies have shown continued activity of the tolerant mechanisms despite RES "blockade,"[6] an enhanced, rather than reduced, endotoxin susceptibility after stimulation of RES phagocytic activity with zymosan, BCG, triolein, or glucan[2,7-10] a lapse of tolerance following discontinuance of endotoxin injections despite persistence of accelerated blood clearance of toxin[12] absence of accelerated endotoxin clearance during the early phase of tolerance,[13] toxicity of rough endotoxins despite their rapid blood clearance,[14] a dissociation of tolerance from enhanced blood clearance of endotoxin by transfer of tolerant phase serum,[15] and resistance of the RES cells of tolerant animals to endotoxin both in vivo[16] and in vitro.[17] Considered collectively, these earlier findings indicate that mechanisms other than enhanced RES phagocytic activity are key determinants of the endotoxin-tolerant state. The present findings are entirely consistent with these observations, and support the alternative concept developed during studies on pyrogenic tolerance to endotoxin, i.e., that tolerance is based primarily upon refractoriness of the highly susceptible RES to endotoxin injury, and that accelerated clearance represents only an ancillary protective mechanism in that toxin is brought more rapidly into these refractory cells.[18]

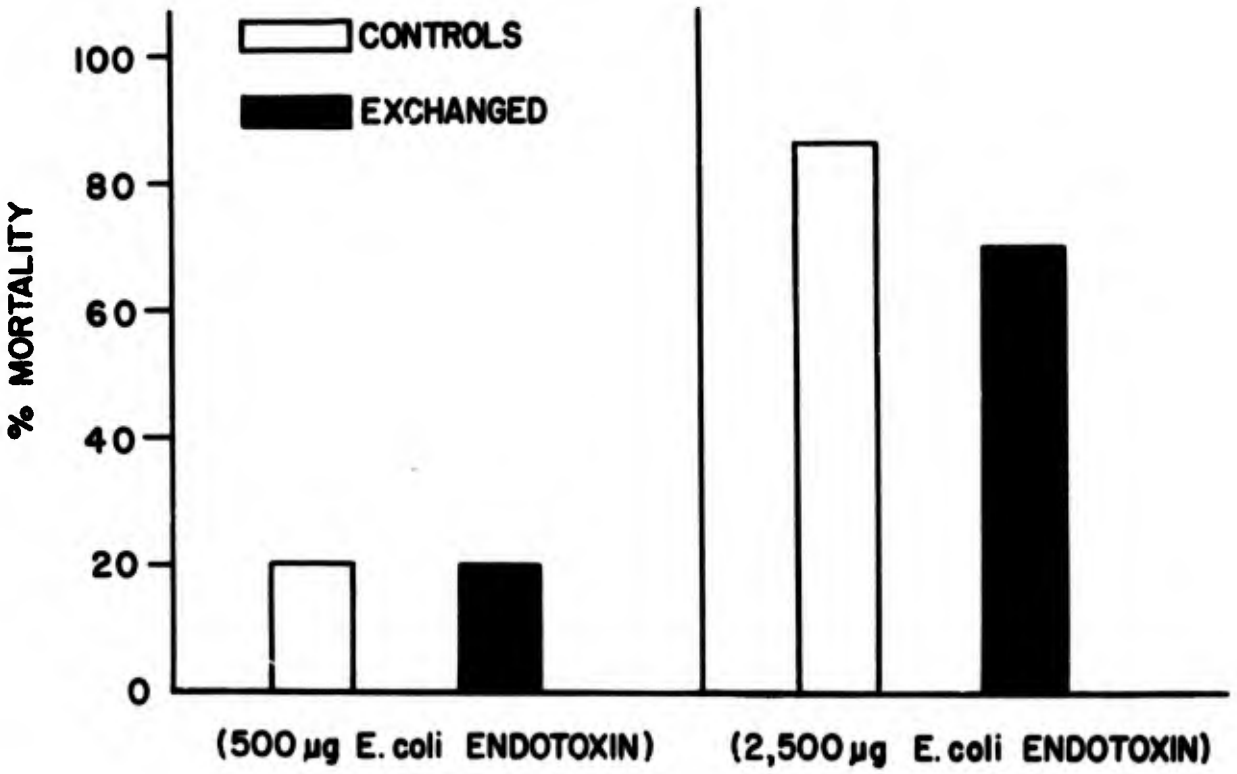

FIGURE 2. EFFECT OF EXCHANGE TRANSFUSION ON *E. COLI* ENDOTOXIN ON 96-HR MORTALITY.

In summary, healthy rabbits were injected IV with an LD_{80} dose of E. coli endotoxin. Twenty minutes later, an exchange transfusion was rapidly performed, accomplishing a drastic and sustained reduction in the level of endotoxemia approximating that in animals rendered highly tolerant by 7 prior sublethal injections of toxin. Despite such reduction in endotoxemia, the 96-hr mortality was only slightly reduced compared to sham-exchanged controls (70 vs. 83% respectively). Control studies indicated that the exchange transfusion per se did not enhance endotoxin mortality. The findings directly support the accumulating evidence that endotoxin tolerance is not based primarily upon enhanced RES clearance of circulating endotoxin.

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ORAL ENTERIC VACCINES

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with

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Epidemiologic studies have shown that naturally acquired shigellosis and typhoid fever are followed by a measurable degree of protective immunity against second exposures to disease. In the case of the former disease this immunity appears to be serotype-specific. The finding of protective immunity following exposure to enteric disease has given hope that vaccines might be successful in controlling the spread of infection. Killed typhoid vaccines have been ineffective in controlling the disease while killed Shigella preparations have been entirely ineffective in preventing disease in experimental animals.

An oral enteric vaccine should have certain advantages over parenteral preparations. The oral route would probably minimize local and systemic postvaccinal reactions found with parenteral vaccines. They would directly stimulate local intestinal immune mechanisms. Also the vaccine would be easier to manufacture and to administer in field situations.

Living attenuated strains of shigellae have been used as oral vaccines to protect animals against experimental challenge and man against naturally occurring dysentery. Formal and associates have worked with both spontaneously derived avirulent mutants and Shigella-Escherichia coli hybrid strains in monkeys. In Yugoslavia, Mel and co-workers used streptomycin-dependent (SmD) strains of various Shigella serotypes to confer resistance against the naturally occuring disease in both adults and children.

Over the past 6 years studies utilizing attenuated and virulent Shigella strains have been conducted at the Maryland House of Correction. These studies have allowed formation of important prerequisites necessary to immunize the intestinal tract against an enteric pathogen. Using these studies as a basis, evaluation of "killed" or "live" oral antityphoid antigens has been conducted.

Table I outlines important biologic properties of various enteric bacterial pathogens. As suggested by Sprinz, bacterial pathogens can be divided into 3 groups on the basis of their characteristic interaction with the intestinal mucosa. Group 1 pathogens typified by Vibrio cholerae produce their disease by proliferation in the small bowel and release of an absorbed enterotoxin. These bacterial toxins do not invade the intestinal mucosa. The second group of pathogens typified by virulent Shigella

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TABLE I. PATHOGENESIS OF BACTERIAL DISEASES

PATHOGEN	BACTERIA INTESTINAL MUCOSA INTERACTION	PATHOGENIC MECHANISM	INTESTINAL LOCALIZATION
V. cholerae E. coli	No invasion	Enterotoxin production	Small bowel
Shigella E. coli	Penetration, intra-epithelial multiplication	Mucosal inflammation with destruction	Early small bowel Later colon
Salmonella	Complete penetration epithelial cell	Inflammation of the lamina propria	Small bowel and colon

strains, characteristically invade the intestinal mucosa and lead to extensive ulceration and destruction of the mucosa. Bloody mucoid stools are observed which indicate an extensive destructive process in the colon. The third group of pathogens exemplified by Salmonella strains produce their illness by invasion of the intestinal epithelial lining without extensive destruction followed by multiplication in the lamina propria. The type of inflammatory response elicited by Salmonella appears to determine the disease expression. Typhoid strains elicit predominantly a monocytic response; organisms are carried into the blood stream where typical enteric fever is seen. On the other hand, nontyphoid salmonellae lead predominantly to a polymorphonuclear leukocytic reaction; the organisms are phagocytized, contained in the lamina propria and the clinical expression is that of gastroenteritis. The knowledge of the pathogenesis of these enteric pathogens has led to the development of a toxoid preparation for Group 1 pathogens and live attenuated vaccines to used orally for Groups 2 and 3 pathogens.

Three types of Shigella vaccines were evaluated in our prison volunteer model. The first is a SmD strain which is apparently avirulent, due to its inability to grown in the absence of the antibiotic streptomycin. This vaccine strain is identical to the one tested extensively in Yugoslavia by Mel. The second is that produced by Dr. Formal at Walter Reed Army Institute of Research; an avirulent Shigella mutant designated MH is hybridized with an E. coli strain rendering it avirulent by 2 separate mechanisms. The segment of the Shigella chromosome which is replaced by the E. coli chromosomal material is the region felt to be responsible for virulence. Not only are the organisms incapable of invading epithelial cells by its primary mutation but if reversion to epithelial penetration should occur, the organism has a

further check on its virulence by lacking the inherent portion of the Shigella chromosome responsible for virulence. The third vaccine is one obtained when an avirulent E. coli has received chromosomal substance from a Shigella strain which renders it capable of producing group- and type-specific Shigella antigens. While the organism resembles E. coli in all aspects it will agglutinate with type- and group-specific Shigella antiserum. Table II shows the reactions in man following immunization with multiple doses of the first two vaccines. Minimal diarrhea occurred on rare occasions; in no case was it long lasting or bothersome to the volunteer. Of particular significance was the finding that pretreatment with $NaHCO_3$ increased the recovery rate of vaccine organisms from the stool of approximately 1/3 of volunteers receiving either vaccine to more than 80% receiving the MH vaccine and 100%, the SmD vaccine. This pretreatment is essential to assure that viable organisms reach the small bowel. Table III shows the level of immunity obtained in volunteers immunized with oral Shigella vaccine when they swallow virulent homologous organisms. This measurable degree of immunity when compared to unexposed controls is less than that seen against naturally occurring disease as demonstrated by Mel. A similar degree of immunity has been seen in volunteers following administration of 2 doses of the third type of vaccine (E. coli with Shigella antigens). The finding that 2 doses may confer a significant degree of immunity to volunteers has given us hope for continuing studies utilizing this new vaccine.

The extent of immunity provided by previous typhoid fever and 3 vaccines to virulent oral challenge is shown in Table IV. When compared to controls the attack rates are comparable for groups 1 and 3; however, for groups 2 and 4 there are significant differences by χ^2 analysis ($p < 0.01$). That is, both killed parenteral and live oral vaccines are effective in reducing incidence of disease. When these same attack rates are compared to each other, there is no difference by χ^2 analysis. When stool isolations for the 4 groups are compared to their controls, there is a difference only for the live oral vaccine group, $p < 0.01$.

When groups are compared to each other, some significant differences are found (Table IV). Killed parenteral vaccine is significantly better than killed oral vaccines (2 vs. 3) with respect to attack rate, but not stool isolations. Live oral vaccine is significantly better (4 vs. 3) than killed oral $p < 0.05$ only in the second disease category. Among the control comparisons only groups 1 and 2 had significantly reduced numbers of positive stools when compared to group 4. We feel that these studies demonstrate that there is some antibacterial immunity stimulated by an oral vaccine.

We have also studied succus entericus obtained from volunteers vaccinated with oral Shigella vaccine. We have found an antibacterial substance in small bowel fluids which is heat stable and dialyzable and is not present in baseline specimens.

TABLE II. EFFECT OF 4 DOSES (5×10^{10} cells/dose) OF MH AND SmD VACCINE COMPARED TO MILK-FED CONTROLS

PARAMETER	MH VACCINE		SmD VACCINE		MILK-FED CONTROLS
No. of volunteers	25	28	27	28	20
$NaHCO_3$ pretreatment	-	+	-	+	-
Diarrhea					
Mild					
1-3 watery stools	5 20%	10 36%	4 15%	4 14%	2 10%
Severe					
> 3 watery stools	0	0	0	0	0
No. positive stools	8 32%	23 82%	9 33%	28 100%	0 0%
No. with 4-fold increase HA titer	6 24%	2 8%	4 15%	8 27%	1 5%

TABLE III. PROTECTION AFFORDED BY SmD AND MH IN VOLUNTEERS CHALLENGED WITH 10^4 VIRULENT SHIGELLAE

VACCINE HISTORY	NO. OF VOLUNTEERS	DISEASE[a]		POSITIVE STOOLS	
		No.	%	No.	%
SmD	53	16	30	32	60
MH	68	30	44	36	53
None - Controls	88	52	59	66/87	76

a. Fever $\geq$ 100 F or diarrhea $\geq$ 4 watery stools/24 hr.

TABLE IV. IMMUNITY INDUCED BY ILLNESS AND PARENTERAL OR ORAL TYPHOID FEVER VACCINES WITH χ^2 ANALYSES

GROUP	2ND ILLNESS NO./TOTAL	%	CONTROLS NO./TOTAL	%	χ^2 P
ATTACK RATE					
1. Previous illness	5/22	23	11/34	43	ns
2. Parenteral vaccine	6/74	8	15/58	26	< 0.01**
3. Killed oral vaccine	8/21	38	13/24	54	< 0.30
4. Live oral vaccine	4/30	13	12/26	46	< 0.01**
POSITIVE STOOLS					
1.	7/20	35	13/30	43	ns
2.	25/74	34	27/58	47	< 0.10
3.	7/21	33	15/24	63	< 0.10
4.	10/30	33	19/25	76	< 0.01**

COMPARISON	χ^2 ANALYSIS: PROBABILITY -- P[a] ATTACK RATES		POSITIVE STOOLS	
	2nd Disease	Controls	2nd Disease	Controls
2 vs 3	< 0.001***	< 0.05*	ns	ns
4 vs 3	< 0.05*	ns	ns	ns
1 vs 4	ns	ns	ns	< 0.05*
2 vs 4	ns	ns	ns	< 0.05*

a. 1 vs 2 and 1 vs 3, no significant difference.

In summary, these studies have given support to the theory that typhoid fever and shigellosis may be controlled by an orally administered attenuated bacterial strain. We are currently evaluating various candidate strains for their ability to proliferate in the human bowel. The vaccine appears to work through stimulation of an antibacterial substance which prevents multiplication of ingested virulent strain. This substance is probably locally produced antibody.

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