BIOLOGICAL ACTIVITY OF THE LIPIDS FROM ESCHERICHIA COLI AND SHIGELLA DYSENTERIAE AND THEIR CHARACTERIZATION BY MEANS OF INFRARED SPECTROPHOTOMETRY

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by

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ABSTRACT

The lipids from two enteric gram negative organisms, Escherichia coli, considered to be non-pathogenic, and Shigella dysenteriae, an intestinal pathogen, were extracted and injected intraperitoneally into laboratory mice in an effort to learn if they possessed stimulatory activity for the reticulo-endothelial system.

By use of radioactive colloidal chromium phosphate and colloidal carbon preparations as indicators of the rate of phagocytosis of particulate matter from active circulation it was found that the lipids from S. dysenteriae possessed marked power to stimulate phagocytic activity to hyperfunction, while those of E. coli and its sub-fractions did similarly to a lesser degree.

Partial characterization of the sub-fractions from E. coli indicated that certain of them exhibited characteristics of phospholipids, since the most active fractions possessed an absorption band in the infrared region not shared by less active ones. The same band was observed in a known phosphatide, and it was tentatively identified as that produced by the phosphorous-containing group found in phosphatide structures.

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INTRODUCTION AND HISTORICAL REVIEW

The primary topic of this paper is a description of the physiological effects of cell-free bacterial lipid extracts and their partial characterization by physical means. The author explains the action of the total extractable, protein-free lipid obtained from Escherichia coli and Shigella dysenteriae, and the lipid sub-fractions obtained from E. coli. These were found to stimulate the rate of phagocytosis of a negatively charged colloidal particle from the circulating blood of laboratory mice. Infrared spectrophotometric analysis has been carried out upon each of these substances in order to demonstrate the uniqueness of each.

The data are presented in this paper to illustrate that the response of the reticulo-endothelial system as indicated by an elevation of phagocytic velocity of tracer particles within circulating blood is significantly stimulated by administration of lipids from the non-pathogenic Escherichia coli and by the total lipid from the enteric pathogen, Shigella dysenteriae.

The antigenicity of lipids has been almost universally negated among bacteriologists and immunologists. The stimulation of antibody production is commonly accepted as the criterion of antigenicity; however, particular

attention is given to other factors which may be similarly applicable as criteria also. One criterion which includes antibody production is the ability of the foreign substance to evoke a directly measurable or observable reaction within a host organism. The antigen-antibody reaction is one such phenomenon which is both observable and measurable in the laboratory, while an elevated opsonic index and an increased rate of phagocytosis are two others closely related which also conform to the criterion. While all of these functions belong to the reticulo-endothelial system or are very closely related to its activity, the stimulation of phagocytic velocity or elevated opsonic index are not commonly regarded singly as criteria of antigenicity.

Phagocytic velocity changes cannot be relied upon solely as a means of evaluating reticulo-endothelial function; however, a measure of phagocytic rate changes gives a very good indication of body defensive activity. Although other activities exist which are less well understood, the rate of disappearance of negatively charged colloid particles from active circulation and capillary beds may be considered prima facie evidence of changes taking place under certain stimuli. This paper makes no claim in favor of the antigenicity of lipids. However, factors which stimulate phagocytic velocity are of considerable interest because they are capable of elevating one activity phase of a very vital system.

Consideration should be made of the fact that the physiological activity of bacterial lipids as described in this work fits at least one of the criteria accepted by some persons as indicative of antigenicity. Since the mechanism for the observed activity described herein is not presently known, consideration of bacterial lipids and lipid fractions as haptens cannot be validly claimed either.

The ability of certain cells within human and animal bodies to engulf particulate matter and other cells has been observed and recorded for many years. Mallory (100) in 1898 observed that human endothelial cells of lymph nodes exhibited phagocytic powers and underwent rapid proliferation to form observable lesions found in autopsies of cadavers recently dead of typhoid fever. Cells of the splenic pulp, blood vessels, and intestinal lining behaved in a similar fashion during progress of the disease. He described the essential lesion in typhoid as a diffuse proliferation of endothelial cells giving rise to larger epithelioid cells having marked phagocytic properties. The organism responsible for the disease was cultured from all organs checked for such lesions: liver, spleen, blood vessels, and lymph nodes.

In an investigation of pneumococcus antiserum Bull (23) described a clumping of injected cells within the blood vessels and rapid removal of these clumps by cells of the spleen, liver, lungs, and possible other organs. An examination of stained tissue sections revealed the en-

gulfed bacteria. He described phagocytic cells as the chief defensive agents against infection (24). Excessive doses of the serum caused large clumps to form which, when taken up phagocytically, impeded circulation in lung capillaries. In another investigation Bull (25) found that typhoid bacilli were agglutinated and removed by polymorphonuclear leucocytes in the spleen, liver, and lungs and were digested and destroyed by the phagocytes. Blood drawn from the heart following intravenous injections into the rabbits produced cultures of successively fewer colonies until within a period of 15-20 minutes after initial injection the blood was very frequently sterile. No more than one colony per ml was ever produced by blood culture at this period.

Hopkins, (79) studying the effects of injections of hemolytic streptococci into rabbits, found that sublethal doses disappeared completely from the bloodstream within a few hours, while in lethal injections ninety percent of the organisms disappeared within a few minutes, and blood culture minima were obtained in two hours. However, in the latter case the engulfed organisms began to re-appear in the circulation from 4 to 6 hours following injection. He attributed the removal of bacteria to fixed endothelial cells. Some were reported removed by leucocytes, but sessile macrophages possessed the greatest removal activity, largely in the lung, liver, and spleen. Some

phagocytic activity was reported in the bone marrow, lymph nodes, and kidneys to a lesser degree. The phagocytosed bacteria were reported killed in 5 to 8 hours, and fatal septicemiae were indicated as probably not attributable to saturation of the macrophages, but rather to a physical lavage of the infected tissues.

Aschoff (6) in 1924 was the first to group all the activities of phagocytic cells previously described together for consideration as a single system. He called it the reticulo-endothelial metabolic apparatus, or just the reticulo-endothelial system, in order to include all cells acting as phagocytes irrespective of their location within the body. The system included reticulum cells of the spleen in the pulp and malphigian bodies, and those of the lymphatic glands and lymphoid tissue. It also included the endothelial cells which line the lymph sinuses of lymph glands, sinusoidal blood capillaries of the liver, bone marrow, suprarenal glands and pituitary gland. The Kupfer cells of the liver were described as being the most active of these. The system also included the amoeboid wandering macrophages and the mononuclear cells found in the splenic pulp and occasionally in the blood of intestinal organs.

Since the introduction of the concept of the reticulo-endothelial system several decades ago a nearly continuous effort has been put forth by medical and

biological investigators to study and measure its functions. By measurements of the activities of the system, its normalcy and integral function may be ascertained. Measurements of the functions of the system have presented special difficulties because the component parts are spread diffusely throughout the body and little can be learned through organ extirpation. Removal of a lung or liver usually resulted in death or gross abnormality of a laboratory animal; removal of the spleen alone gave little information about the functions of other parts of the system. As a result, most approaches to measurement of reticuloendothelial activity have involved use of the propensity of the system's cells to selectively engulf particulate matter of appropriate size and charge. Techniques of study have included the intravenous introduction of vital dyes, living organisms, inert colloids, carbon black and India ink, and in the more modern and sophisticated research efforts radioactive tracers have been used.

Drinker (42) and also Lund, et al. (98) utilized particles of manganese dioxide for intravenous administration to laboratory animals in order to determine its fate within the system. Manganese dioxide was insoluble in tissue fluids. It was prepared for injection in a suspension of 0.4 percent acacia solution. Animals were given 1.4 mg per kg of body weight. All particles greater than one micron in size were removed by selective centrifugation

of the preparation. The rate of removal from the blood was determined by performing gravimetric analyses on successively drawn 10 ml samples. All injected material disappeared from the blood within 18 minutes. Of the total particulate matter injected 90 percent went to the lung, liver, and spleen; 10 percent went to various of other body organs. The manganese dioxide was recoverable from these sites quantitatively within an hour following injection. Drinker concluded that the phagocytic power of the vascular endothelium for particulate matter was responsible for the results.

Lund, et al. worked with numerous other species of laboratory animals and found that for all the manganese dioxide went mainly to the liver. They also pointed out that the phagocytic effect was the same if an inert material such as manganese dioxide were used or if a protein substance were substituted.

Cappell (27,28) did extensive work using vital dyes, and he included studies of phagocytic uptake of these materials within animal systems. He used dyes such as trypan blue, lithium carmine, congo red, and a host of others. No attempt was made to determine the quantitative rates of dye uptake, but he did point out the tissues of Aschoff's system which took up dyes at a marked rate. He also indicated that phagocytosed dyes remained for long periods of time within reticulo-endothelial cells if the material were non-toxic, non-diffusable, and could not be

solubilized by the cell fluids.

Administration of carbon black or India ink dilutions via the intravenous route has been another method of measuring the phagocytic velocity rate of endothelial cells. Halpern and his co-workers have been the chief advocates of this method, and their work has spanned more than two decades. The method consisted of injecting a known amount of carbon per unit of body weight and determining quantitatively the amount removed from circulation as a function of time (19, 69, 70). Halpern, et al. (71) found that India ink which contained shellac slowed the rate of phagocytosis, and that an excess of carbon, i.e., greater than 24 mg per 100 gm of body weight in rats, caused a liberation of thromoboplastin. This tended to exhaust the reserves of blood anticoagulants and to cause the carbon to precipitate. Administration of thrombin or thromboplastin intravenously caused the rate of carbon disappearance to increase and modified the organ distribution of phagocytosed particles. Biozzi et al. (18) reported that the quantity of carbon injected and the degree of saturation of RE cells influenced the rate of phagocytosis of subsequent doses. The uptake under these conditions was found to be inversely related to the amount of carbon. The granulopoetic activity was also found to be directly related to the cube of the relative weights of the liver and spleen. An equation was established which related to

speed of uptake to the amount injected:

$-\frac{dC}{dT} = 0.06(1-\frac{P}{C_o})$ in which C = the concentration in the blood at any one time, C_o = the initial concentration of carbon in the blood, P = the amount phagocytosed by the RES, and T = the time from initial injection. They also established the concept of a granulopectic index:

$C = C_o 10^{-KT}$ in which K is the granulopectic or phagocytic index. Optimum carbon concentration for maximum uptake rate was found to be 8 mg per 100 gm of body weight. Benacerraf et al. (16) found that saturation of the system with excess carbon could effectively block all response. The animal could recover from a blocking dose but the presence of shellac in India ink preparations retarded the recovery making it four times the normal rate.

Jones et al. (81) were the first to use radioactive isotope tracer methods for the measurement of reticulo-endothelial function. A radioactive colloidal chromium phosphate was prepared from di-sodium phosphate containing P^{32}. The particle size of the colloid was less than one micron, and 10 microcuries of activity were injected into mice in a glucose suspension. Localization of the isotope was 90 percent in the liver, and most of the rest was found in the spleen, with the combined radioactivity of these two organs being 100 times that of the remaining tissue. Preparation of a non-radioactive colloid by the

same method resulted in material which stayed in the liver and spleen cells for over a year.

Gabrielli (53) used Jones' radioactive colloid to measure the rate at which disappearance from the blood-stream took place. He used biological half-life as a criterion of phagocytic velocity. This was defined as the amount of time in which half the activity of the colloid preparation had disappeared from circulation. The maximum T 2 value for rats was found to be 1.23 minutes, with the mean being 0.83 minutes. He established an equation similar to that of Halpern's co-workers: $A_x = A_0(e^{-\lambda T})$ in which A_x equals activity in the blood at any time T, A_0 equals the radioactivity in all the blood, and λ equals the velocity of phagocytosis. Gabrielli also revealed that particle size influences the rate of phagocytosis and the site of localization. When the particle size was 15-20 microns the lungs were found to absorb most of the 'colloid', while the liver removed most particles with a size of around one micron. Phagocytosis of the larger particles was slower than for the smaller ones (56). Gabrielli made the assertion that the distribution of radioactive chromium phosphate within the animal system is so constant that it seems the best possible agent to use for study of reticulo-endothelial rates and kinetics.

The mechanisms involved in phagocytosis and some of the substances and conditions which accelerate or retard

its progress have been investigated by numerous workers.

Ponder (111) found that phagocytosis is favored by a decrease in the surface energy of the cell or particle and an increase in the surface energy of the particle about to become phagocytosed. If the expression -- $\frac{(SE\ particle) - (SE\ cell\ particle)}{(SE\ cell)}$ has a value greater than 1.0, then phagocytic ingestion is favored. This was the basis for a paper by Berry (17) in which surface active agents were found to favor the in vitro ingestion of bacteria by human neutrophiles. Surface active agents decrease the surface energy of the cell and phagocytosis is thus enhanced. Berry and associates contended that bacteria are more readily ingested in the presence of serum because of an increase in their surface energy and a decrease in that of the neutrophile. A net decrease in free energy was the result described when bacteria were phagocytosed.

Gasselin (58) using radioactive colloidal gold described the removal of Au^{198} by rabbit peritoneal cavity macrophages in vitro as taking place in two stages. The gold colloid particles were reversibly adsorbed onto the macrophage cell surface. Ingestion of the surface bound particle into the cell became irreversible. The rate at which these two processes occurred was proportional to the amount of colloid adsorbed to the cell surface which could be in turn related to the concentration in the extra-

cellular fluid by the adsorption isotherm. The only limit which was noted was that imposed by the theoretical adsorption capacity of the external surface of the macrophage. In Gasselin's experiments less than one percent of the total surface was ever covered at any one time. He described no limit to the capacity of the macrophages to continue the reaction. At 37 C and pH 7.4, two to 20 percent of the adsorbed gold particles were ingested within one minute.

Waddell, et al. (134) noted the localization of fats dyed with Sudan IV and Sudan Black B in the spleens and livers of laboratory animals (rats). However, when the RE activity was blocked by excessive administration of carbon black, trypan blue, or lithium carmine, the fats did not localize as before. However, they continued to disappear from the bloodstream at the same rate as in untreated animals. Examination revealed concentrations of the colored fats in the hepatic parenchyma and splenic pulp.

According to Heller (73) the reticulo-endothelial system has several vital roles including production of antibodies, phagocytosis of negatively charged particles, and intermediary metabolism of fats. Because of the vital nature of this system a substance or condition which can elevate the system to a state of hyperfunction is to be considered of the utmost importance. The number of such

substances or conditions is, up to this period, limited. Therefore, any new stimulatory materials have a unique importance.

Klotz (88) stated that fat administered intravenously to rabbits was filtered out by lung capillary beds. Fats given intravenously tended to form emboli which stimulated proliferation of endothelial cells having phagocytic activity upon them. The vascular endothelial phagocytic activity of the spleen, adrenal glands, and arteries was stimulated by administration of cholesterin (cholesterol). During phagocytosis of cholesterin fat there was an associated proliferative response which tended to distort the vessel lumen.

The cytotoxic sera produced by Bogomoletz (21, 101, 126) have a pronounced elevating effect on the reticulo-endothelial system in very minute quantities. Heller's work cited previously indicated that choline is capable of pronounced stimulation of phagocytic response, and Gabrielli (54) described the activity of histamine as being similarly stimulatory. The response of the system can also be depressed by various means. Whole body irradiation (57) and administration of cortisone (76) are capable of such effects.

Ideas of looking toward cell-free products of bacteria are not new with respect to physiological activity. Coley (35) successfully treated cases of malignancy with

preparations of the toxins from Streptococcus erysipelatis. Included among the successfully treated cases were several of reticulum cell sarcoma of bone. Not until late in the course of Coley's work was it realized that one of the effects of injections of such toxins was an elevation of the reticulo-endothelial system and phagocytic activity. The literature containing reports of Coley's work is too extensive to more than make brief mention here, but it served to point out that bacterial toxins and perhaps other bacterial products were important in this respect.[1]

Girard and Murray (61, 62) have revealed that the total lipids extracted from a pathogenic Listeria monocytogenes can elicit certain of the responses characteristically observed in infections caused by this organism. Their MPA (monocyte producing agent) was a chloroform extract of protein-free total cell lipids. When injected with sodium lauryl sulfate as an emulsifying agent, the MPA

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[1] When reviewing the works of Bogomoletz and Coley one must be aware that their publications in this country have aroused considerable controversy among medical and biological researchers. Many investigators feel that merit exists in each, and that they may provide groundwork for future approaches to malignancy therapy. Others disagree and regard these as perhaps representing a bizarre type of research having little validity or reproducibility. In citing these works as part of this review the author does not wish to subscribe to either school of thought. The purpose in their citation is to indicate that they represent two individual lines of approach to the study of methods of reticulo-endothelial stimulation

caused monocytosis which persisted in rabbits for 20 days or longer. Animals immunized against typhoid and staphylococcus toxins gave higher titers during monocytosis than did controls. This was the type of situation observed by these workers during actual infections by the organism. Girard and Murray reported the MPA to be non-toxic and non-antigenic.

Stanley (122) preceded Girard and Murray with an MPA substance, but he reported that the MPA was probably bound to a polysaccharide within the cell. When the polysaccharide was precipitated independently of the MPA it was found not to have the same monocyte stimulating capacity as the lipids. Stanley later reported that lipids from Aspergillus fumigatii and Listeria monocytogenes when mixed with lecithin greatly augmented the antibody production to Salmonella typhimurium when all substances were injected simultaneously (123).

The pigment complex, violacein, obtained by ethanol extraction of Chromobacterium violaceum, has been used also to stimulate the rate of phagocytic velocity in laboratory mice. (Unpublished data from the New England Institute for Medical Research). This substance has been shown to possess infra-red absorption bands which are identical to certain of those found in bacterial lipid preparations.

The lipids of certain bacteria have been extracted, isolated, and characterized by several workers, but none

seem to have used them directly as agents to experimentally stimulate reticulo-endothelial activity.

Nicolle and Alilaire (103) grew enteric bacteria in large quantities and studied their lipids. When grown on potato agar at 37 C for 24 hours they reported the colon bacillus as having 9 to 16 percent total extractable lipid. They seem to have been the first to report phospholipids from this group of organisms. Dawson (38) grew E. coli on a peptone-meat extract medium and reported a lipid content of 4 to 5 percent on the basis of cell dry weight. Addition of one percent glycerol to the medium resulted in an increase to 8 percent lipid.

Eckstein and Soule (46) reported the lipids of E. coli to be characterized by low iodine numbers and by having fatty acids more saturated than oleic acid. When organisms were grown on a medium containing a small quantity of alanine they were found to have a greatly elevated rate of phospholipid synthesis. The phosphatide was reduced to a very low amount when cysteine was substituted for alanine. The iodine number for total lipids was 25.6, while that for fatty acid fraction was 37.4. The saponification number was 214. Negative reactions were obtained for the Liebermann-Burchard and Salkowski tests for sterols.

Anderson and his fellow workers (1, 2, 3, 4, 106, 133) did extensive work on members of the Mycobacteria. They reported 36.7 percent phosphatide from the avian

tubercle bacillus which contained mainly stearic and palmitic acids. Data for other members of the group were essentially the same with minor characteristic variations. However, the leprosy bacillus was found to contain only 2.25 percent phospholipids.

Williams et al.(136) cultivated 9 strains of enteric organisms including Shigella, Escherichia, and Aerobacter on a peptone-beef extract medium. Shigella paradysenteriae was found to have a total lipid content of 5.5 percent of its dry weight, with 73 percent of that being phospholipid. E. coli was found to have 8 percent total lipid and 60 percent of the amount was phospholipid. Iodine numbers were found to vary between 42 and 82, tests for sterols were negative, and P:N ratios were approximately 1:1 for the phospholipid fractions.

Folch (50) described a method for the separation of various phosphatide fractions from brain tissue. He recognized cephalin as a complex of several fractions, and he used acetone and ethanol for their separation by differential precipitation. Levene and Rolf (93) also described a method for the separation and purification of lecithin using the insolubility of its complex with cadmium chloride as a basis for separation.

Dawson, (39) and Lea, et al. (91) described techniques for chromatographic separations of phospholipids. Lea and his associates used silicated papers as an aid to

better separations, and they have reported a method of fractionation through a column using a fraction collector which would tend to eliminate error and loss through separation by routine chemical means.

The characterization and identification of complex molecules from bacteria and even of whole cells have been successfully undertaken by several investigators. Levine et al. (96) applied the infra-red spectrophotometric method for the identification of glycogen within whole bacterial cells, while Shirk and Greathouse (119) did essentially the same thing for a determination of the presence of bacterial cellulose. Stevenson and Bolduan (125) were among those using the infra-red spectrum for the identification of whole bacteria. Complex polymers such as glycogen, cellulose, protein, and complexes found in cells present some degree of difficulty in infra-red determinations not experienced with simpler substances, yet the results obtained by these workers seem to have justified their use of the technique.

METHODS AND PROCEDURE

Cultivation of Organisms; Extraction and Fractionation

E. coli received the greatest emphasis in this investigation. The strain used was one isolated from raw sewage which gave typical coliform reactions: acid and gas production from lactose broth, and a characteristic (+) (+) (-) (-) reaction to the IMViC test. The *S. dysenteriae* used was Dubos' strain 2308.

The lipid content of 300 gm of dried Shigella cells was extracted using a mixture of two parts chloroform to one part methanol. The extraction was conducted passively, i.e., by shaking cells in the presence of the solvent without mechanical rupture for about 120 hours.

E. coli was grown on a chemically defined medium containing the following per liter:

NaCl	5.0	gm
K_2HPO_4	1.0	gm
$(NH_4)_2HPO_4$	1.0	gm
$MgSO_4$	1.0	gm
glucose	2.0	gm
glycerol	2.0	ml

These constituents were incorporated into agar or utilized as liquid medium as demand required. Petri dishes, 150 mm in diameter, were used in early phases of the work for cultivation of organisms. Inoculations were made by swabbing from broth starter cultures, and growth was removed

by washing into the chloroform-methanol solvent. More rapid production of cells was achieved later when a super centrifuge became available. It was then possible to cultivate cells in large containers of aerated liquid and to recover them by centrifugation.

Use of an anhydrous, water-immiscible solvent such as chloroform or ethyl ether alone for extraction purposes was unsatisfactory. The presence of a solvent miscible with water was necessary in the system to break weakly bonded complexes in order to release lipid material.

Several methods of treatment of E. coli cells were followed. Extractions were conducted on moist and dry cells, with and without mechanical rupture. Experience indicated that violent methods such as grinding produced large quantities of objectionable denatured protein which had to be eliminated. Extraction of moist cells in the presence of a water-miscible solvent and chloroform (miscible) seemed to give more rapid removal of lipids.

Following passive extraction of cells with chloroform-methanol solution, water was carefully layered over the extracted liquid. Shaking and agitation had to be avoided because of the presence of surface active substances in the extract which tended to form stable emulsions with water. A large, spherical type separatory funnel which gave maximum interface area was used for this purpose. Successive separations against water in the cold (4 to 7 C)

were conducted until the extract no longer gave positive reactions for protein or carbohydrate. Most of the methanol passed into the water phase on the first separation.

The chloroform phase, containing the lipid extract, was concentrated by vacuum distillation at temperatures not exceeding 40 C. Total crude lipid left after removal of solvent was dehydrated in vacuo over sulfuric acid.

Extracts from both organisms were brown oils at this stage. Both extracts were tested for stimulatory effects in animals. The Shigella extract was not fractionated further.

The crude extract from E. coli was dissolved in analytical grade re-distilled chloroform. Approximately 10 ml per gm of crude material were used. To this was added a double volume of acetone. Upon standing, small amounts of a yellowish-white precipitate formed. Two distinct liquid layers were also evident. The precipitate was separated by centrifugation at 7500 RPM for 20 minutes at 4 C. The supernatant was decanted and retained. The precipitate was dissolved in 5 ml of re-distilled chloroform and transferred to a watchglass for evaporation under nitrogen. It was taken up in 10 ml of anhydrous ethyl ether and then transferred to a tared glass vial. The ether was removed under a reduced atmosphere of nitrogen, and the sample was labelled Fraction I.

The supernatant liquid containing lipid and solvent

was concentrated by vacuum distillation to a viscous liquid which was dissolved in 25 ml of re-distilled chloroform and transferred to a small separatory funnel. To the funnel was added 25 ml of absolute ethanol, and it was allowed to stand for 24 hours at 4 to 7 C. Two layers were visible at the end of this period. The lower layer was carefully drained from the funnel. Its volume was 18 ml, and upon standing several hours at 4 C it separated into two phases in the approximate ratio of 1.5:1. Centrifugation at 4000 RPM at a temperature of -25 C for 40 minutes separated the lower layer into a solid sediment. The supernatant layer was labelled Fraction II, and the material solid at -25 C was labelled Fraction III.

The portion remaining in the separatory funnel was allowed to stand in order to determine if more layering would take place. None did in 24 hours. More absolute ethanol was added, and the mixture was again allowed to stand 24 hours at 4 C. A semi-solid material formed on the surface of the liquid mixture. The liquid was drained away, and the floating layer was recovered in ethyl ether. It was labelled Fraction IV. The liquid phase from the funnel was concentrated by vacuum distillation to approximately half volume. Addition of absolute ethanol to this resulted in a layer forming which had a higher viscosity. This separated out as a solid when centrifuged at -25 C. The supernatant was decanted and retained. The fraction

solid at -25 C only partially dissolved in ethyl ether. The portion completely dissolving was labelled Fraction V, and the white portion having no ether solubility but dissolving in chloroform was labelled Fraction VI.

An excess of acetone was added to the supernatant from the previous procedure, and the tube was stored under desiccation at -20 C. A small amount of white precipitate was formed on standing 36 hours in the cold. The liquid was decanted while still cold, and the solid phase was dissolved in ethyl ether and transferred to a tared vial. The latter was labelled Fraction VIII. The supernatant was concentrated by vacuum distillation to a viscous oil which was taken up in ether, transferred to a tared vial and labelled Fraction IX.

Fraction II separated into two distinct layers upon standing at room temperature for 8 days. The upper layer was much darker than the lower. Centrifugation at -25 C caused the lower layer to solidify. The supernatant was retained as Fraction II, while the phase which solidified at reduced temperature was dissolved in ether, transferred to a tared vial, and labelled Fraction VII.

Vials containing the fractions were stored in a desiccator jar at a reduced atmosphere of nitrogen pending animal injection and analytical procedures.

Two extractions were carried out with E. coli cells, while only one was performed upon S. dysenteriae cells.

Measurement of Phagocytic Velocities

The stimulatory activity of the lipid fractions from *E. coli* and the lipid extract from *S. dysenteriae* upon phagocytic velocity as an indication of reticulo-endothelial function was measured by two procedures:

In the first procedure male white mice, obtained from Armer Farms, Croton Falls, New York, 90 to 100 days of age, averaging 30 gm weight, received via intraperitoneal injection 0.2 ml of the undiluted fraction whose activity was to be measured. Intravenous injections of lipids caused fat emboli and frequently death of the animal. All lipid fractions were oils at body temperature with one exception. A semi-solid fraction was dissolved in four parts of sesame oil for injection. A sesame oil control was necessary for animals receiving this preparation. Commercial cephalin and the *E. coli* extract were emulsified in sodium lauryl sulfate solution also for injection. These were prepared by suspending 0.2 ml of the sample in 10 ml of a 0.1 percent aqueous solution of sodium lauryl sulfate.

After a period of 72 hours following injections of lipids, each animal was given an injection of 10 microcuries activity of radioactive colloidal chromium phosphate according to the method of Jones (100). Phosphorous 32 in the form of di-sodium phosphate was added to an equal amount of sodium bicarbonate solution, and an excess of chromic

nitrate was added. The precipitate which formed was insoluble in cold water; but it was soluble in hot water, acids, and bases. It was washed and centrifuged twice in cold water and dried at 110 C. After drying it was heated at 600 C for 12 hours in an electric furnace. The amorphous product which resulted was insoluble in aqua regia, strong acids, and alkali. The precipitate was transferred to a heavy-walled glass-stoppered flask half full of glass beads and rotated for 12 hours with one to two ml of isotonic glucose per mg of material. This procedure gave a particle size of approximately one micron, and the predominating charge was negative. The radioactivity of an accurately measured aliquant was determined by evaporation under an infra-red heat lamp and counting under a Geiger tube. Dilutions into glucose were made according to radioactivity level. This preparation was insoluble in tissue fluids.

Injections were made via the caudal vein, and the experimenter cut the tip of the tail exactly 30 seconds following injection of the last bit of colloid. Five lambda (5×10^{-6} liter) blood samples were drawn by means of micro-pipettes each 30 seconds for a period of three minutes. These blood samples were quickly blown onto filter paper planchets and their radioactivity was determined under the Geiger tube of an LKB (Sweden) automatic Robot Scaler. Control animals received only the radioactive colloid except in the case of the sample containing sesame oil.

A plot of radioactive count was made on a log scale vs. a linear scale of elapsed time. The slope of the resulting lines was used to determine $T_{\frac{1}{2}}$, the biological half life value. This was the time in which half the activity had disappeared from the blood.

In the second procedure the sequence of events was essentially the same except that a preparation of colloidal carbon was injected instead of the radioactive colloid. The amount of carbon suspension which each animal received was determined on the basis of body weight. No animal was given more than 8 mg of carbon per 100 gm of body weight. A 30 gm animal would be given no more than 2.66 mg by such a procedure. Samples were drawn by means of micro-pipettes from the tip of the cut tail every 20 minutes. The sample representing zero time was taken 30 seconds after completion of injection. This was repeated until 6 samples had been drawn. The volume of each was 20 lambdas, and the first sample was diluted and lysed in 0.1 percent sodium carbonate solution such that its optical density read on a Coleman Jr. spectrophotometer at 600 mmu was not greater than 1.0. Lysed control blood was used as a standard for setting the instrument. Other samples drawn successively were given parallel dilution, and the optical density was plotted against time in minutes.

In each of the procedures a minimum of 6 animals

was used for each of the fractions tested. Six controls were used for establishment of normal T 2 value. In many cases more animals were used because of the difficulties experienced in making caudal vein injections without damage to the vessel wall. Animals were kept in restraint by means of a specially constructed tubular cage which permitted only the tail to protrude over an illuminated slit for injection.

The carbon used was obtained from National Midland Company of Natonah, New York, and it was prepared and stabilized in gelatin solution according to the methods of Benacerraf, et al. (16). The particle size was claimed by the manufacturer to average 0.5 micron.

Characterization of Lipids

The infra-red spectra included in this paper were made on a Perkin-Elmer model 21 double beam recording infra-red spectrophotometer at the Perkin-Elmer plant in Norwalk, Connecticut. Spectra were run for each fraction at cell spacings of 0.1 mm and 25 microns respectively. The thinner films gave greater detail than was observed in spectra obtained at the larger spacing. This was especially true in the case of several of the fractions which were much more opaque than others.

Only the spectra of the isolated fractions were obtained. This was by advice of the person scanning the spectra. The opinion was offered that, because of the nature of the mixture present in the whole cell lipid extracts, any spectrum which might be obtained would not necessarily convey any useful information. A rapid scan of the sample of total lipid from E. coli and that from S. dysenteriae revealed very little difference between them, so it was decided to abandon the attempt to determine characteristic spectra.

Fractions II, III, IV, and VII were subjected to chromatographic separation by ascending technique using silica treated Whatman #1 paper. Spots containing 10 lambdas of the fractions were placed two inches above the solvent level, and after 30 minutes equilibration in a one

liter glass-stoppered mixing cylinder irrigation was begun. Chloroform-methanol 8:2 with two percent aqueous sodium acetate-acetic acid buffer at pH 3.5 added was used as a solvent. Ethylene glycol monomethyl ether containing 10 percent of the buffer was also used. The buffer was added to stop objectionable trailing evident in previous chromatograms. Commercial cephalin was chromatographed in each of the solvents.

Spots were stained for location using Sudan IV, Sudan Black B, Oil Red O, and Rhodamine G with ultraviolet viewing. Sudan Black B proved most useful. Using various lipid samples, it was possible to detect $1:10^5$ lipid dilution on paper by means of Sudan Black B. Tests for choline were performed according to Chargaff, et al. (32). Papers were heated at 95 C for 30 to 60 minutes, dipped into two percent phosphomolybdic acid solution, immersed in n-butanol for 5 minutes, washed in tap water, and dipped into fresh 0.4 percent stannous chloride in 3N HCl. Drying by heating produced blue spots indicating choline. Free amino groups were detected by spraying papers with 0.1 percent ninhydrin in n-butanol and heating at 95 C for 5 to 15 minutes.

RESULTS

Disappearance rate of the injected material from peripheral circulation is used as a measure of phagocytic velocity. In plots of sample optical density vs. time the result is a straight line obeying the Beer-Lambert law. A straight line also results from a plot of radioactive count vs. time. The disappearance rate of radioactivity from the bloodstream produces an exponential function. In both methods of representing data the lines have the characteristic function $Y = MX + B$ which possesses validity for any linear graphic plot. Rather than use line slope as a measure of increase or decrease of phagocytic uptake, consideration of the biological half-life or T 2 value was regarded as being more convenient. This value represents the time at which 50 percent of the material injected initially has disappeared from active circulation. Specifically, it is the amount of time with respect to the first sample drawn that the concentration or activity of the particles has decreased to 50 percent of the initial level. An elevated phagocytic rate was evidenced by a plot line of greater slope; a decreased T 2 value gave the same evidence. In references to these results it is perhaps more meaningful to use T 2 values or biological half-lives as criteria in experiments of this type rather than line slope.

The values for T 2 demonstrated graphically or presented in tabular form have been determined against optical density calibration curves in the case of the carbon suspension or against corrected count rate for the P^{32} activity. In the latter case, the count rate as indicated on the Y axis was obtained from the number of disintegrations picked up by the Geiger tube, corrected by a factor determined for the geometry of the particular counting castle. The purpose of such a correction was to give so near as possible the actual radioactivity of a five lambda sample of blood.

Values of mean T 2 induced by the various lipid fractions are tabulated in decreasing order of phagocytic stimulation, and the shortest mean T 2 values are also represented graphically. Ranking of these values determined by either the carbon technique or the radioactive P^{32} tracer technique is parallel.

The S. dysenteriae lipid extract gives the shortest mean T 2 value. Fractions II and IV are more active than the total extract from E. coli, while both the total extract emulsified in sodium lauryl sulfate are more active than the rest of the fractions. Emulsification with the surface active agent seems to have reduced the activity of the extract from E. coli.

Examination of the infra-red spectra reveals a small and somewhat weak absorption band ranging from

2700 cm^{-1} to 2850 cm^{-1} evident in Fractions II, III, IV, and VII. This band is also present on the spectrum of commercial cephalin at 2720 cm^{-1}. (cm^{-1} is $\frac{1}{\text{Wavelength in microns}}$.) The less active fractions do not show the same absorption characteristics at this wave number.

The data in Table II reveal that the active fractions from E. coli have more bands in common with the spectrum of a known cephalin than any of the other fractions. Seven bands are shared with the cephalin spectrum: 4230 cm^{-1}, 2900 cm^{-1}, 2700 cm^{-1}, 1730 cm^{-1}, 1460 cm^{-1}, 1074 cm^{-1}, and 720 cm^{-1}. An exception is noted in that Fraction II does not exhibit an absorption band at 720 cm^{-1}.

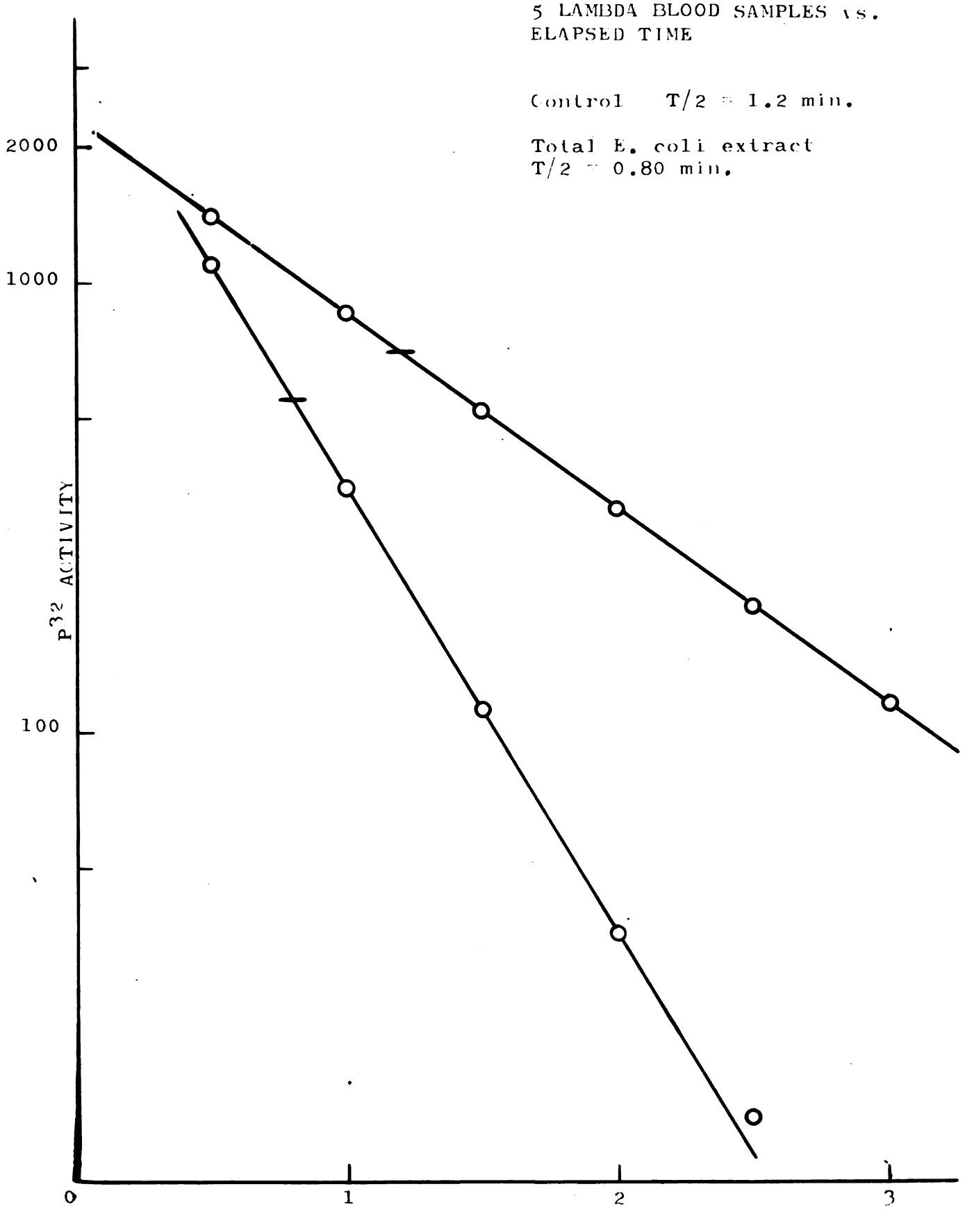
Fraction II had an Rf value of 0.91 in chloroform-methanol, and 0.86 in buffered cellosolve. It reacted positively to a phosphomolybdic acid-stannous chloride test. A spot which migrated poorly and diffusely indicated the presence of impurities. Fraction IV was found to give an Rf value of 0.80 by the chloroform-methanol mixture, and 0.76 by the irrigation in buffered cellosolve. The major portion reacted positively to both ninhydrin reagent and the phosphomolybdic acid-stannous chloride test. Impurities were evident. Fractions III and VII both gave data similar to the above. Commercial cephalin showed an Rf value of 0.79 in chloroform-methanol, but it migrated very poorly and gave inconclusive results in the buffered cellosolve mixture. It reacted positively to ninhydrin reagent.

FIGURE 1

PLOT OF P^{32} ACTIVITY IN 5 LAMBDA BLOOD SAMPLES vs. ELAPSED TIME

Control T/2 = 1.2 min.

Total E. coli extract T/2 = 0.80 min.



2700 cm^{-1} to 2850 cm^{-1} evident in Fractions II, III, IV, and VII. This band is also present on the spectrum of commercial cephalin at 2720 cm^{-1}. (cm^{-1} is $\frac{1}{\text{Wavelength in microns}}$.) The less active fractions do not show the same absorption characteristics at this wave number.

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FIGURE I

PLOT OF P^{32} ACTIVITY IN 5 LAMBDA BLOOD SAMPLES vs. ELAPSED TIME

Control T/2 = 1.2 min.

Total E. coli extract T/2 = 0.80 min.

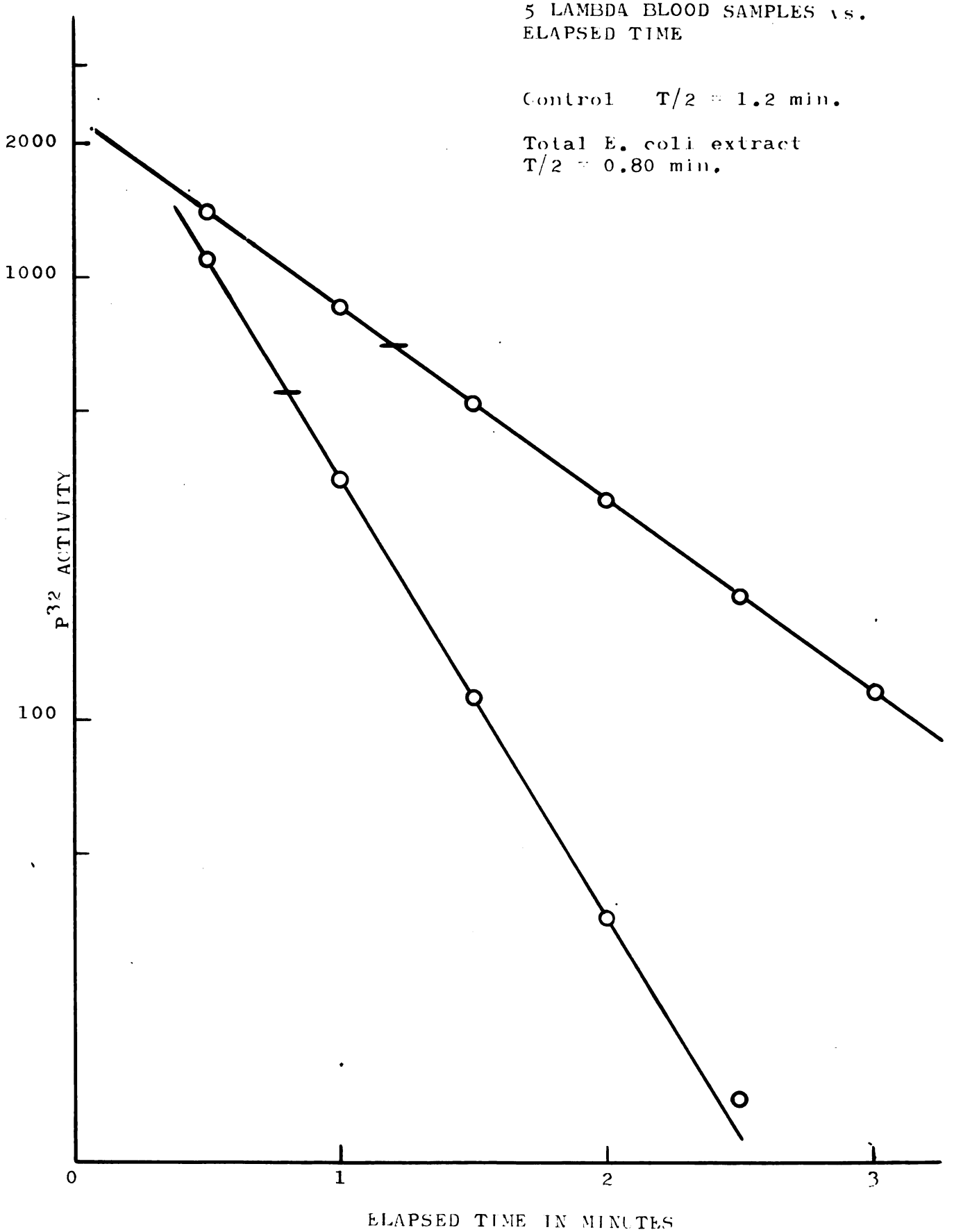


FIGURE 2

PLOT OF P^{32} ACTIVITY IN CPM OF 5 LAMBDA BLOOD SAMPLES VS. ELAPSED TIME

Control T/2 = 1.2 min.

Total Shigella lipid
T/2 - 0.65 min.

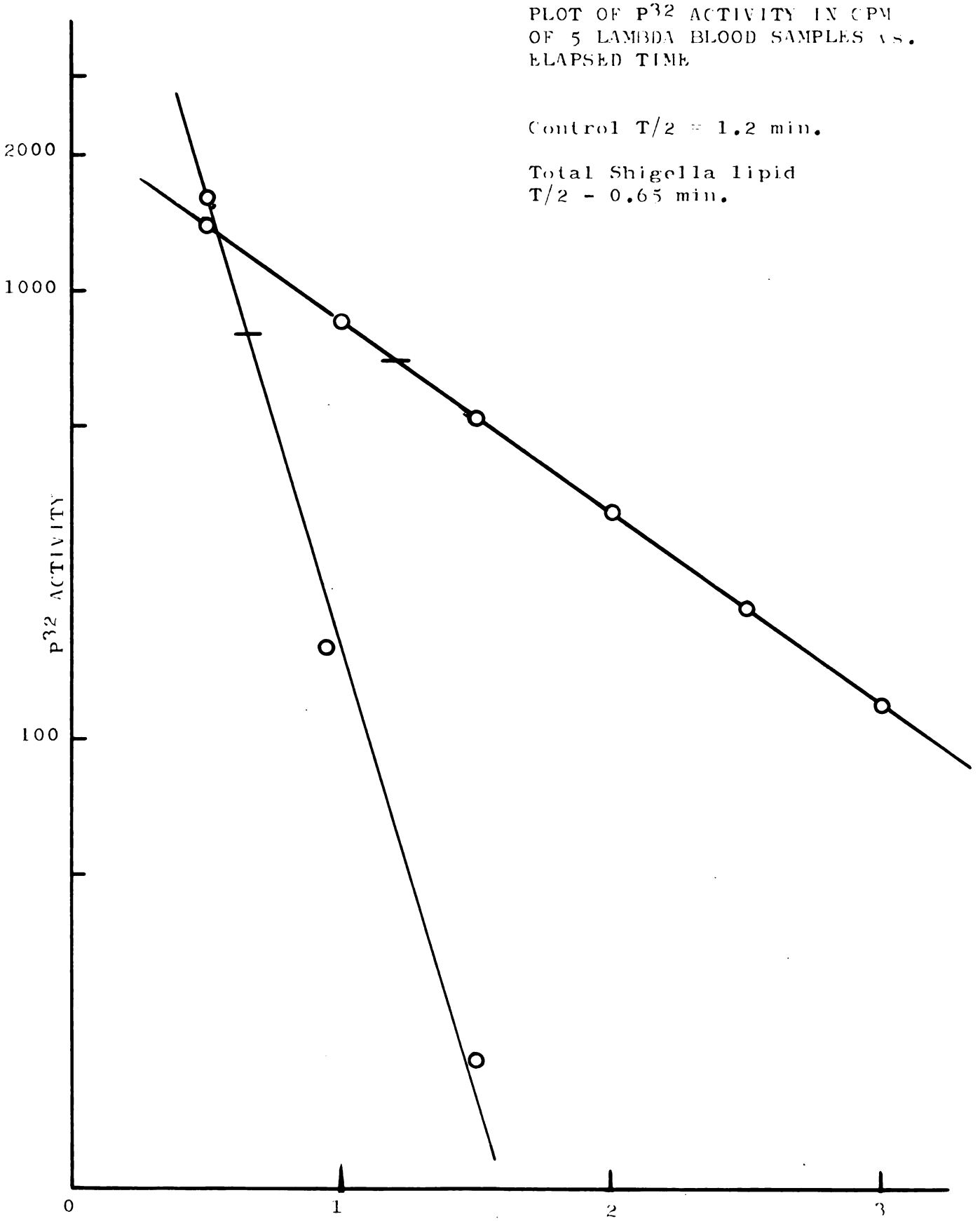


FIGURE 5

PLOT OF OPTICAL DENSITY OF CARBON FROM 20 LAMBDA BLOOD SAMPLES vs. ELAPSED TIME

Control T/2 = 51.9 min.

Shigella lipid T/2 = 22.9 min.

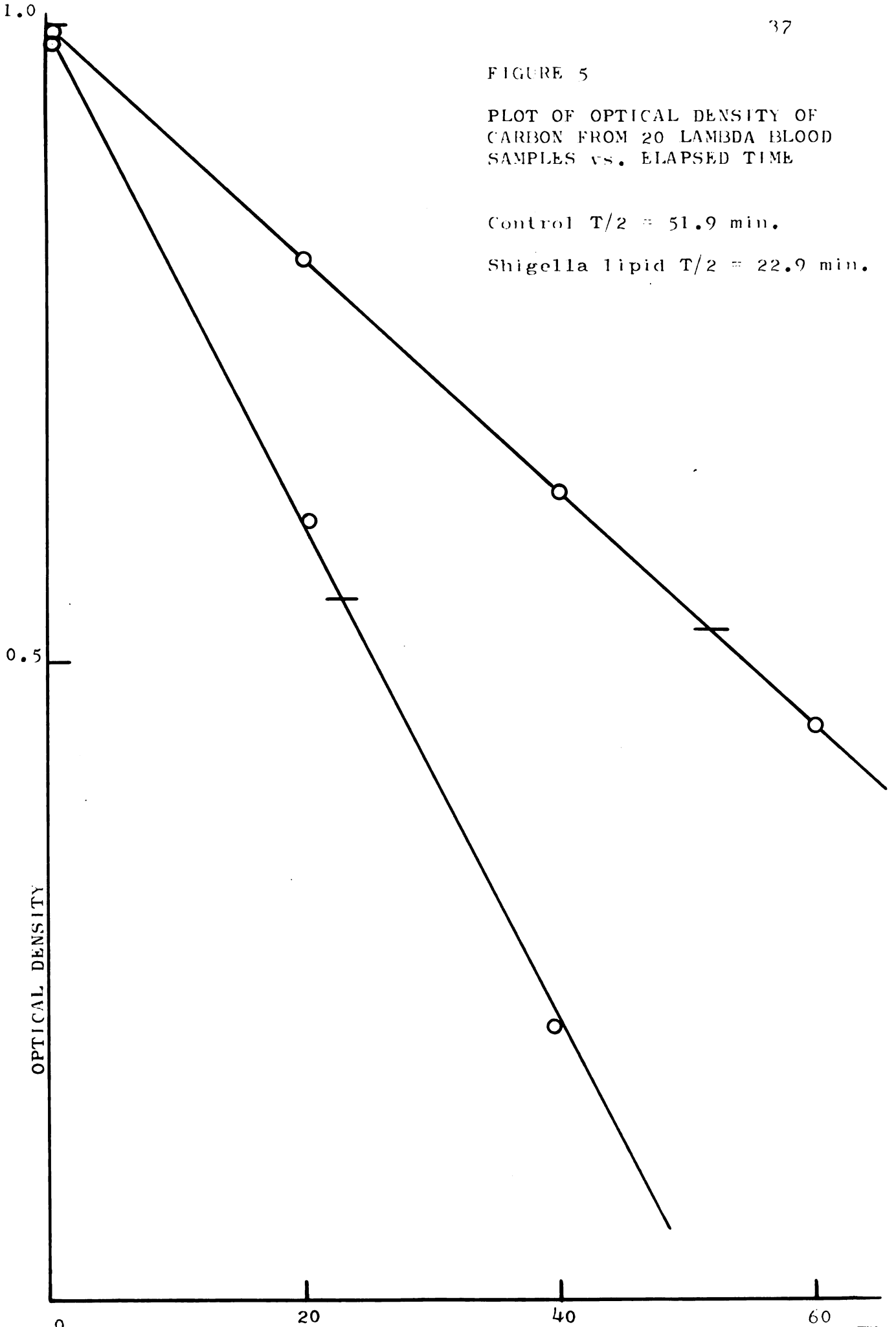


FIGURE 4

PLOT OF P^{32} ACTIVITY IN CPM OF 5 LAMBDA BLOOD SAMPLES VS. ELAPSED TIME

Control T/2 = 1.2 min.

Fraction II T/2 = 0.78 min.

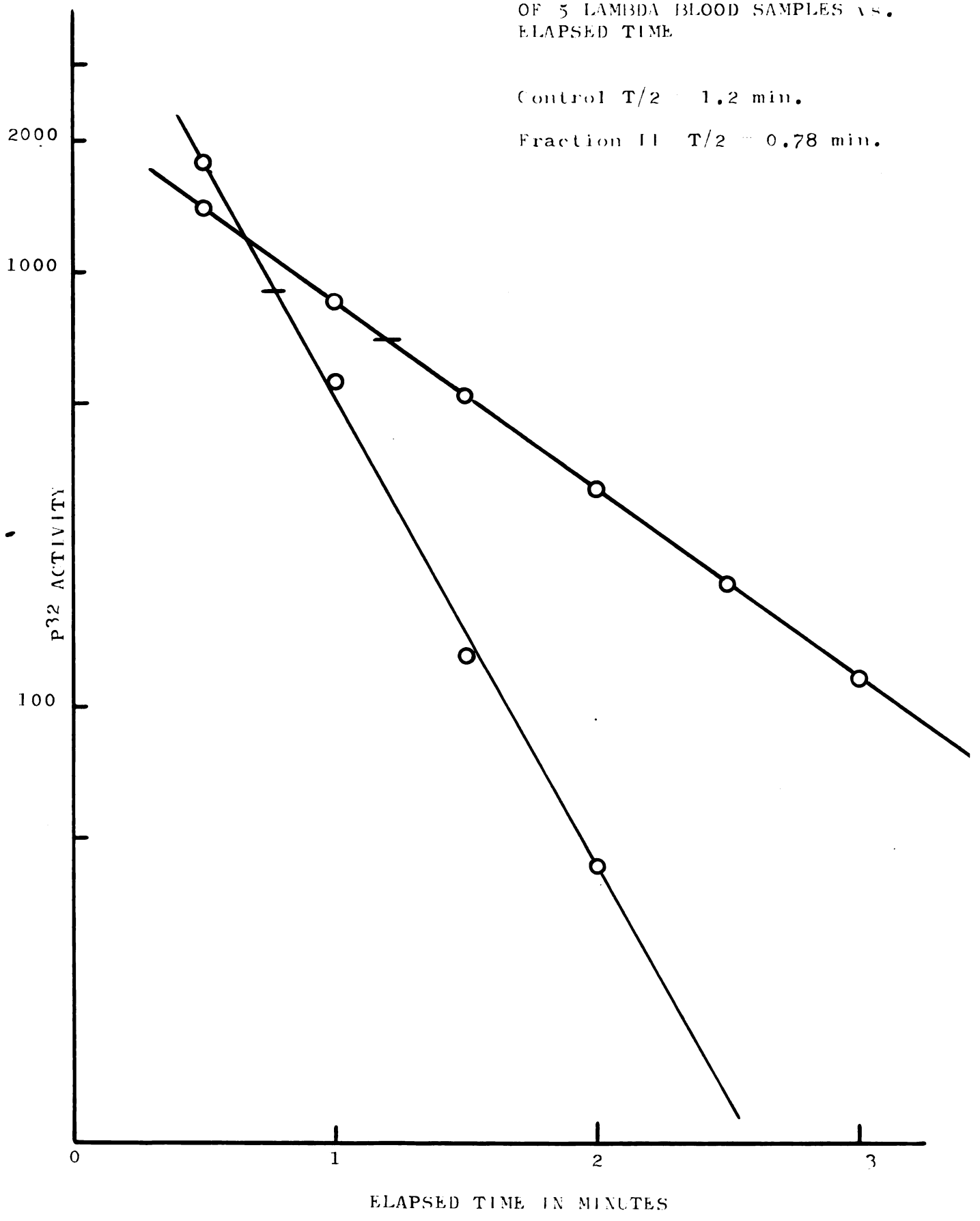


FIGURE 7

PLOT OF OPTICAL DENSITY OF CARBON FROM 20 LAMBDA BLOOD SAMPLES vs. ELAPSED TIME

Control T/2 = 51.9 min.

Fraction IV T/2 = 25.4 min.

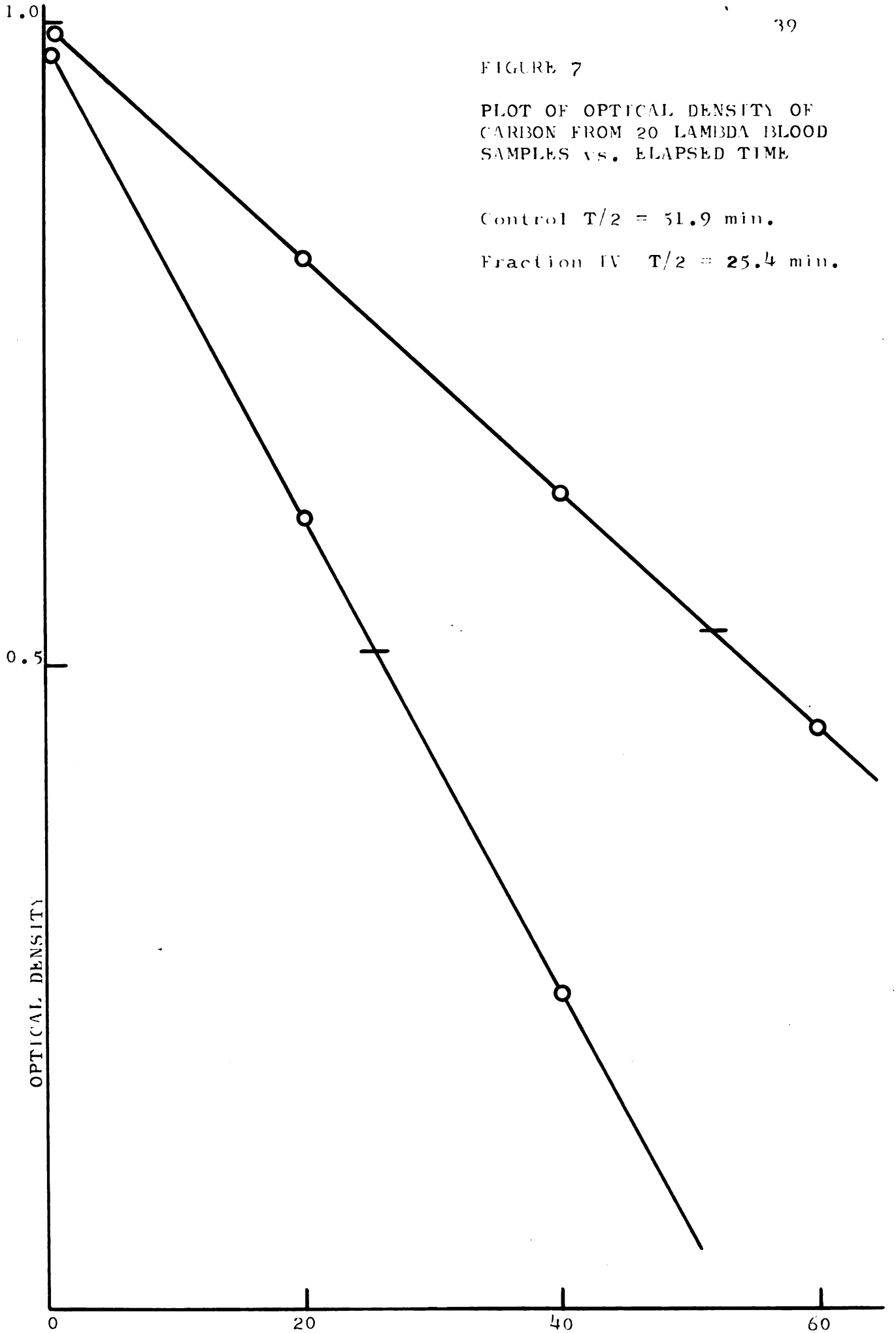


FIGURE 8

PLOT OF OPTICAL DENSITY OF CARBON FROM 20 LAMBDA BLOOD SAMPLES vs. ELAPSED TIME

Control T/2 = 51.9 min.

Fraction II T/2 = 28.2 min.

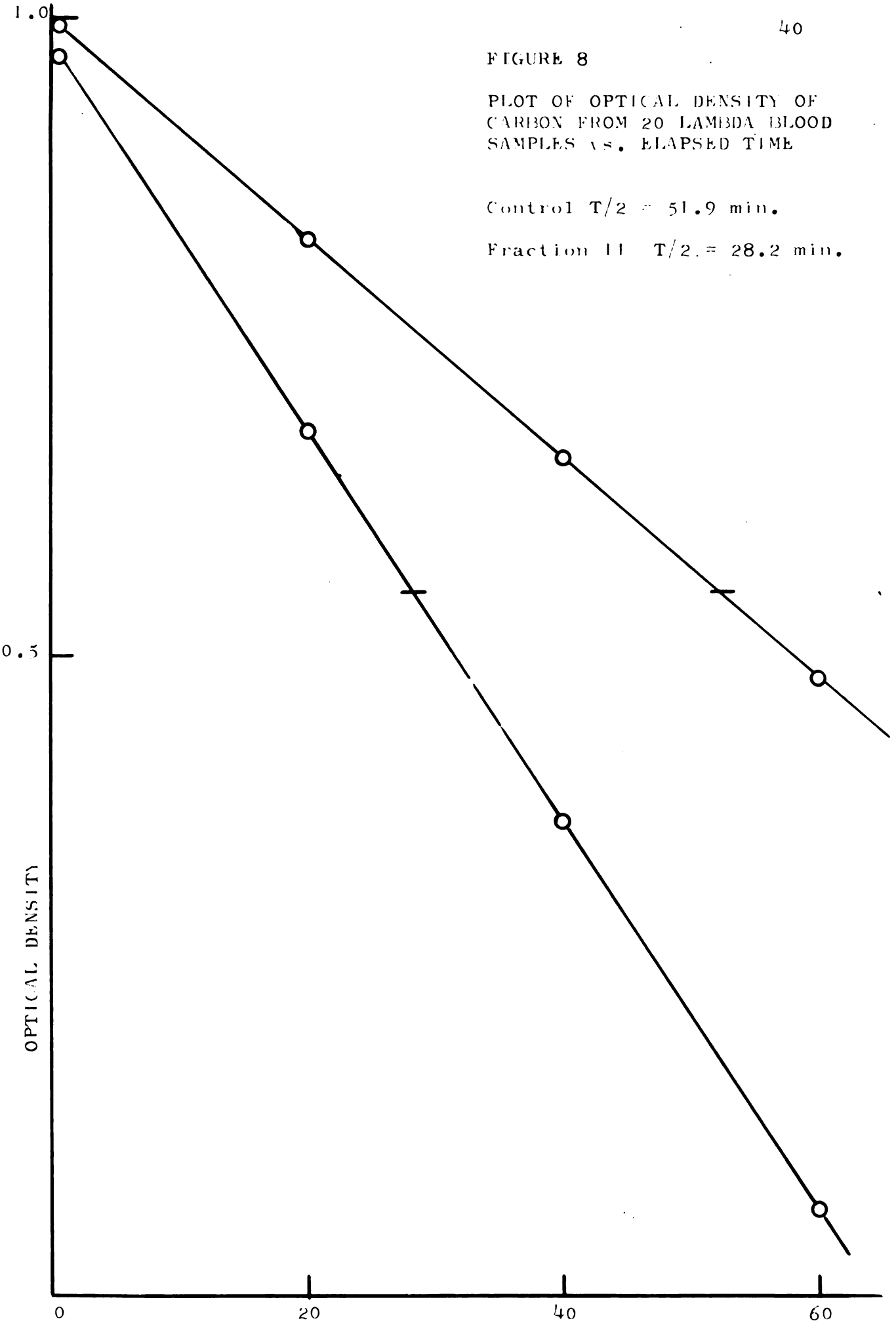


FIGURE 8

PLOT OF OPTICAL DENSITY OF CARBON FROM 20 LAMBDA BLOOD SAMPLES vs. ELAPSED TIME

Control T/2 = 51.9 min.

Fraction II T/2 = 28.2 min.

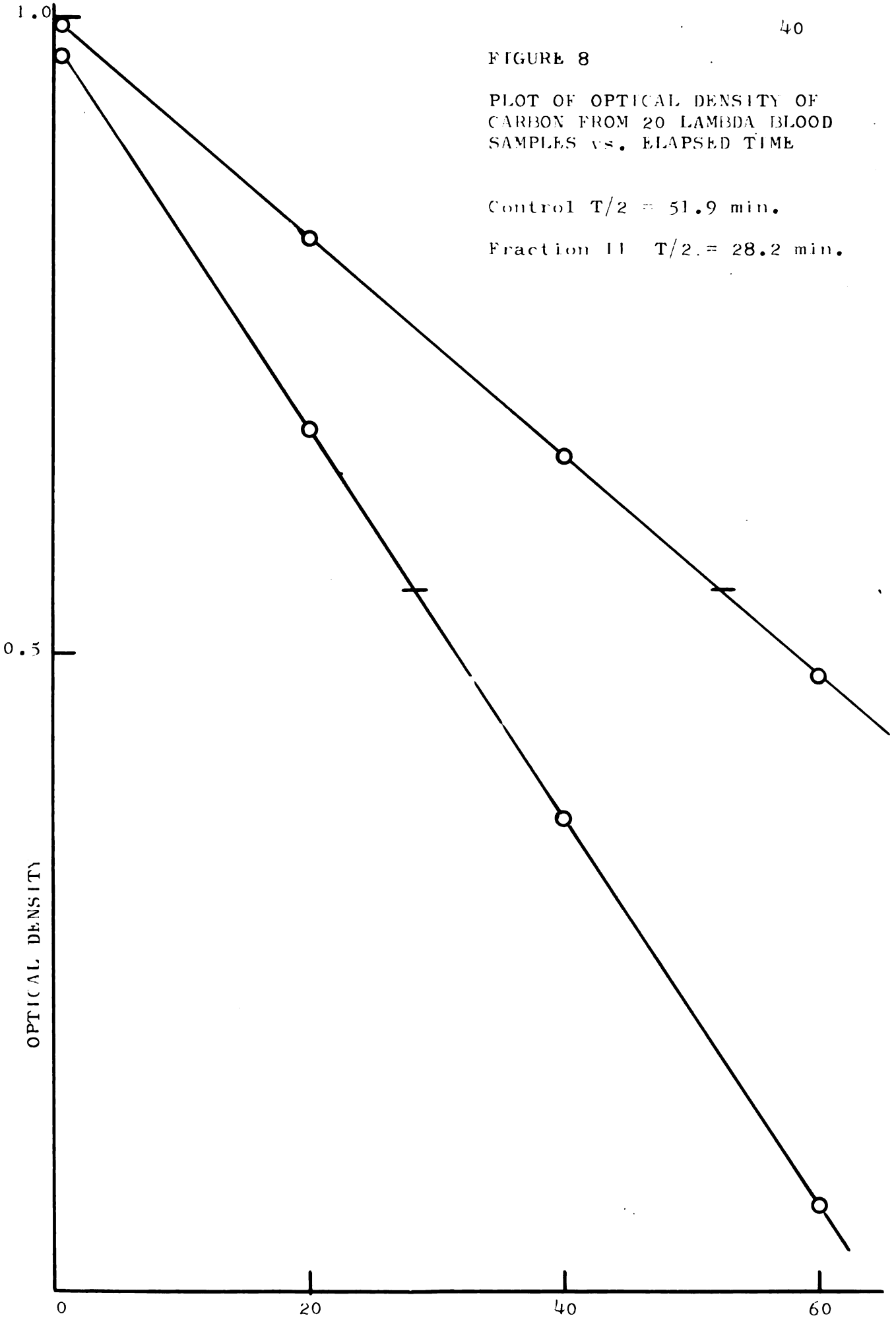


TABLE I.
MEAN T/2 VALUES FOR THE VARIOUS LIPID FRACTIONS

LIPID	^{32}P TECHNIQUE	CARBON TECHNIQUE
Control	1.2 min.	51.9 min.
Sesame oil control	1.1 min.	49.2 min.
Fraction VI	1.1 min.	49.0 min.
Fraction IX	0.98 min.	43.7 min.
Fraction VIII (In sesame oil)	0.95 min.	48.3 min.
Fraction I	0.96 min.	47.0 min.
Fraction V	0.92 min.	43.5 min.
Fraction III	0.95 min.	39.2 min.
Fraction VII	0.95 min.	33.6 min.
Cephalin in SLS	0.93 min.	32.8 min.
Total E. coli Extract in SLS	0.91 min.	31.6 min.
Total E. coli Extract	0.80 min.	30.4 min.
Fraction II	0.78 min.	28.2 min.
Fraction IV	0.72 min.	25.4 min.
Total Shigella Extract	0.65 min.	22.9 min.

TABLE II

ABSORPTION BANDS OF FRACTIONS COMMON TO A KNOWN CEPHALIN PHOSPHATIDE

Cephalin	4230 cm^{-1}	3330 cm^{-1}	2900 cm^{-1}	2700 cm^{-1}	1730 cm^{-1}
Fraction I	(±)	(-)	(±)	(-)	(-)
Fraction II*	(±)	(-)	(±)	(±)	(±)
Fraction III*	(±)	(-)	(±)	(±)	(±)
Fraction IV*	(±)	(-)	(±)	(±)	(±)
Fraction V	(±)	(±)	(±)	(-)	(±)
Fraction VI	(±)	(-)	(±)	(-)	(±)
Fraction VII*	(±)	(-)	(±)	(±)	(±)
Fraction VIII	(-)	(±)	(±)	(-)	(±)
Fraction IX	(±)	(±)	(±)	(-)	(±)

Cephalin	1640 cm^{-1}	1460 cm^{-1}	1074 cm^{-1}	720 cm^{-1}
Fraction I	(±)	(±)	(-)	(±)
Fraction II*	(-)	(±)	(±)	(-)
Fraction III*	(-)	(±)	(±)	(±)
Fraction IV*	(-)	(±)	(±)	(±)
Fraction V	(±)	(±)	(±)	(±)
Fraction VI	(±)	(±)	(-)	(±)
Fraction VII*	(-)	(±)	(±)	(±)
Fraction VIII	(±)	(±)	(-)	(-)
Fraction IX	(-)	(±)	(-)	(-)

*Denotes most active fractions

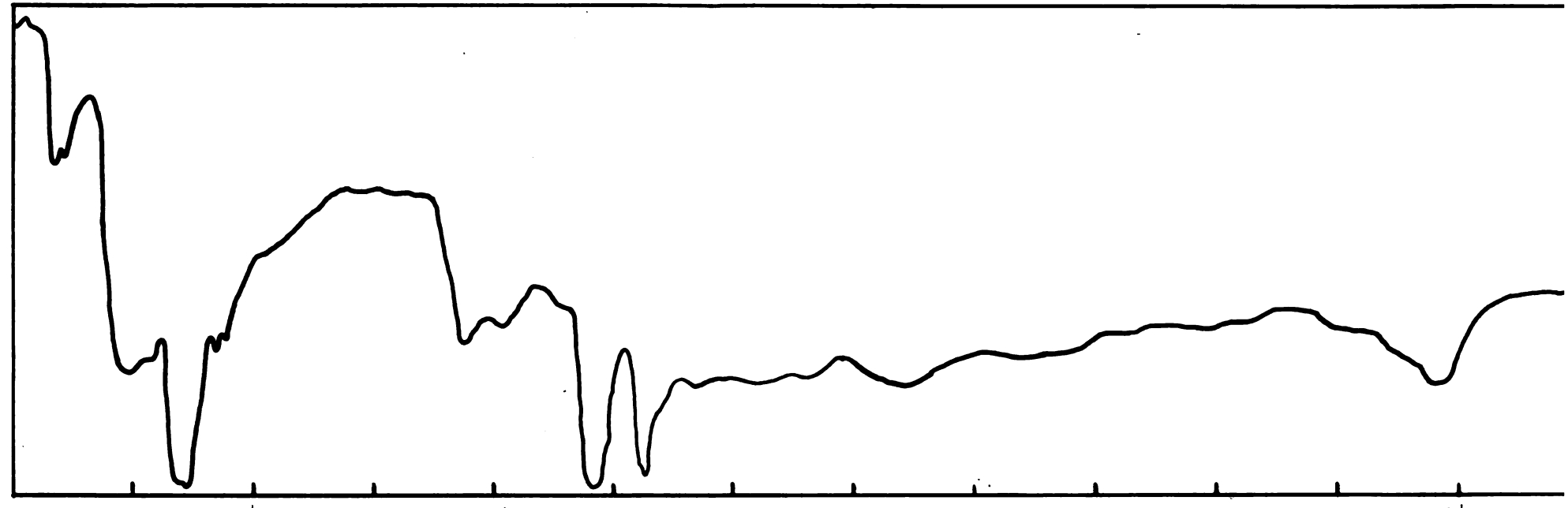
(+) Indicates band in common with cephalin

(-) Indicates band not shared with cephalin

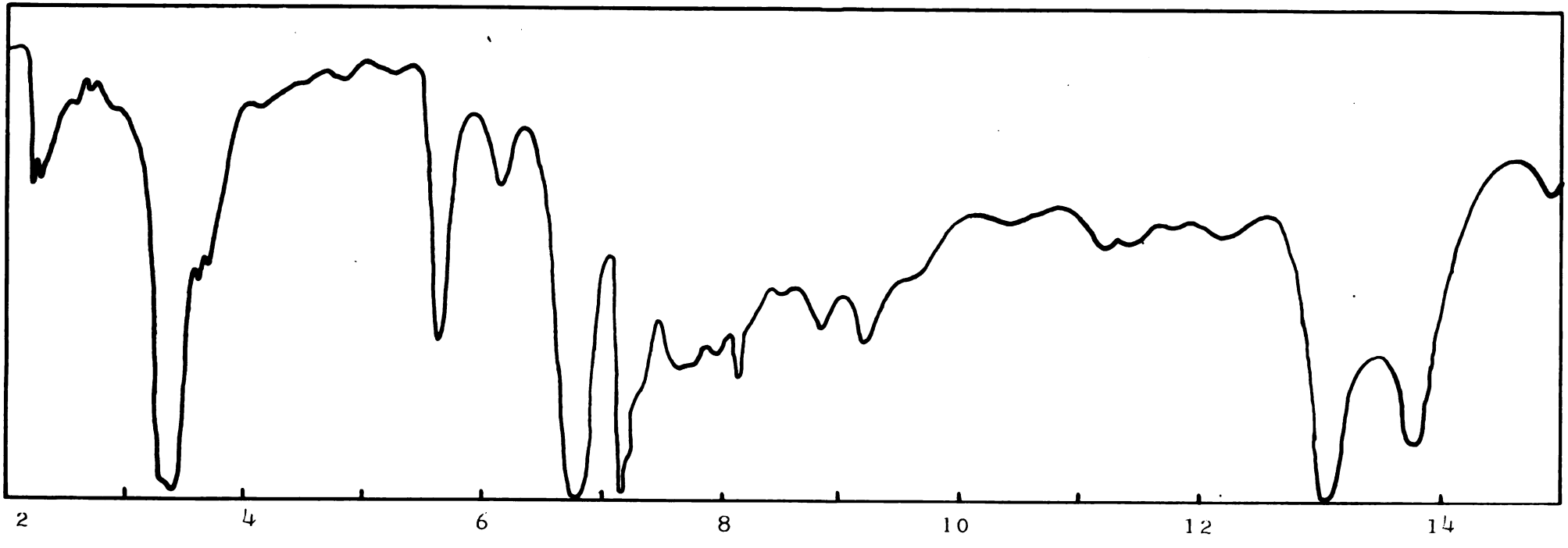
Wave number in $cm.^{-1}$ is the reciprocal of wavelength in microns.

Infrared Spectra

On the following pages are infrared spectra of the fractions listed in order of decreasing biological activity. These spectra were obtained on a Perkin-Elmer model 21 recording spectrophotmeter with cell spacings as indicated.

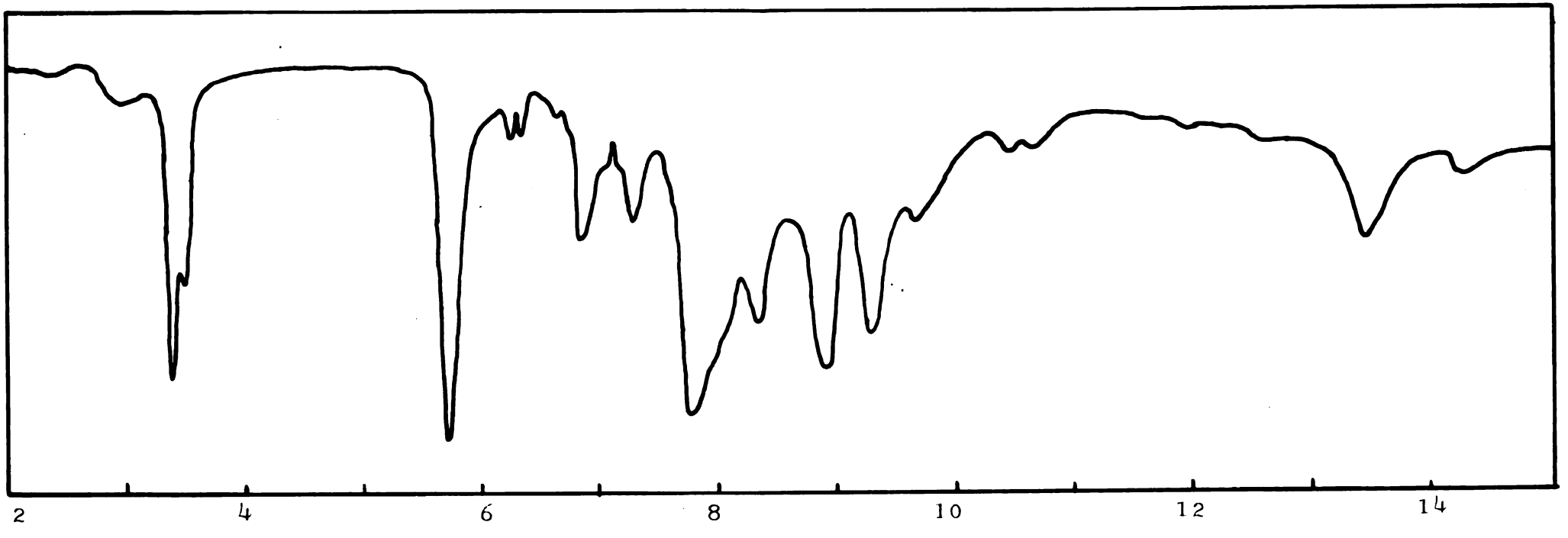


Cephalin as Evaporated Film



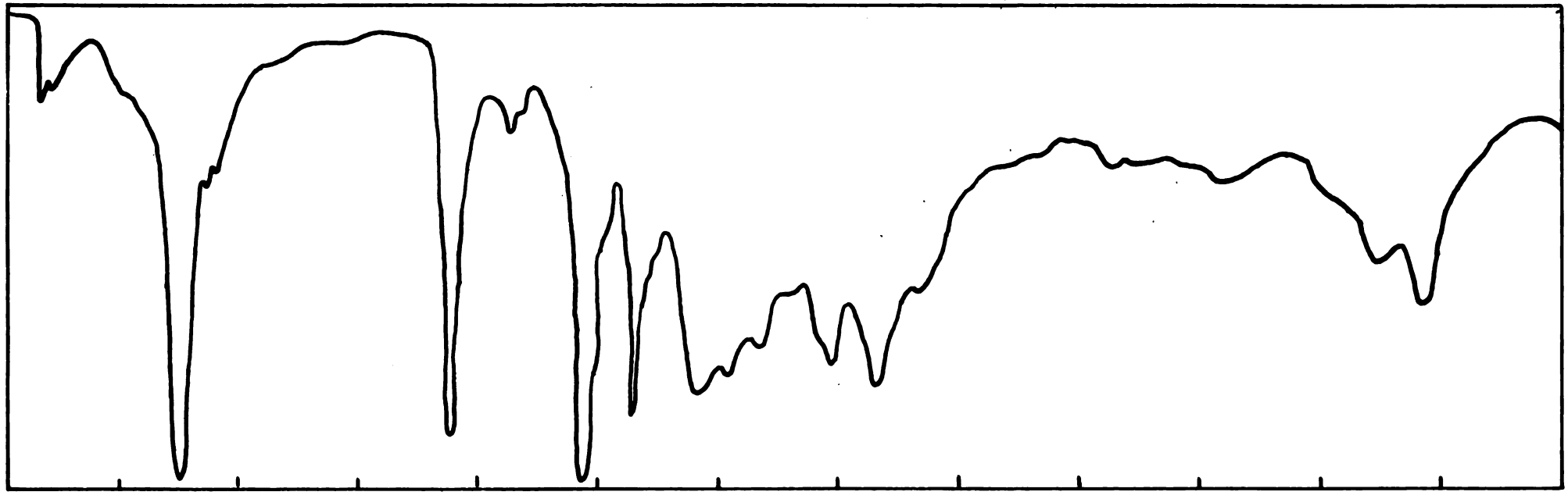
Wavelength in Microns

Fraction IV
25 Micron Spacing

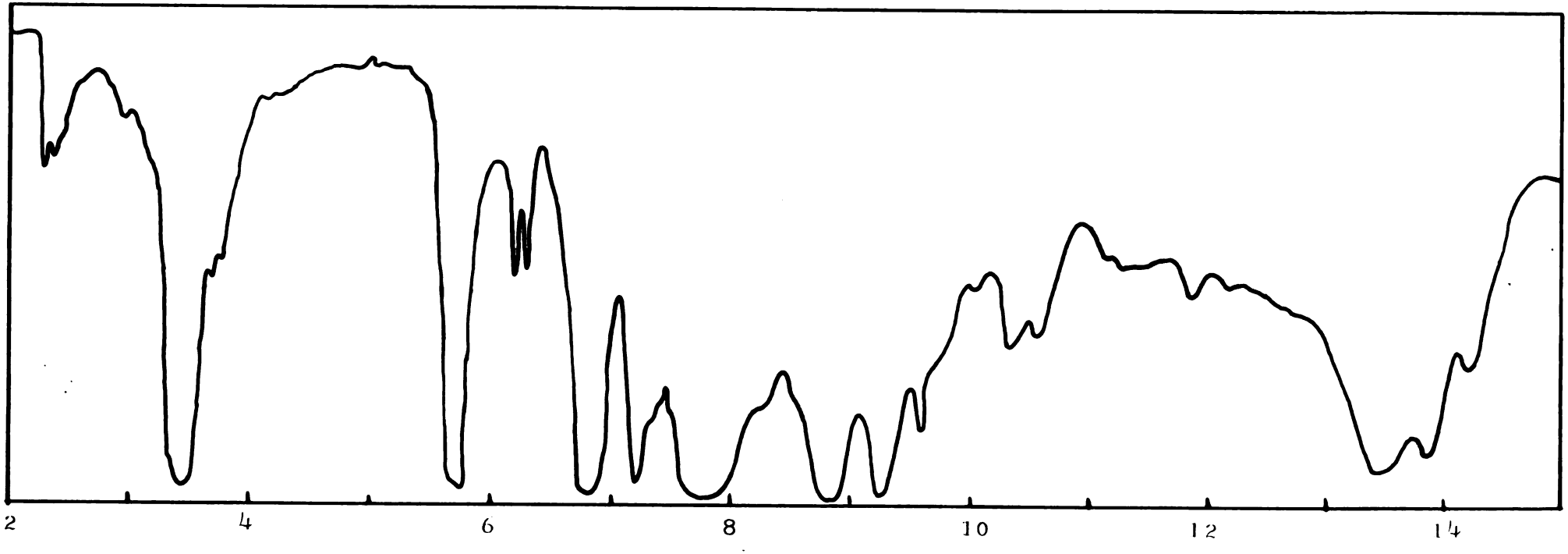


Wavelength in Microns

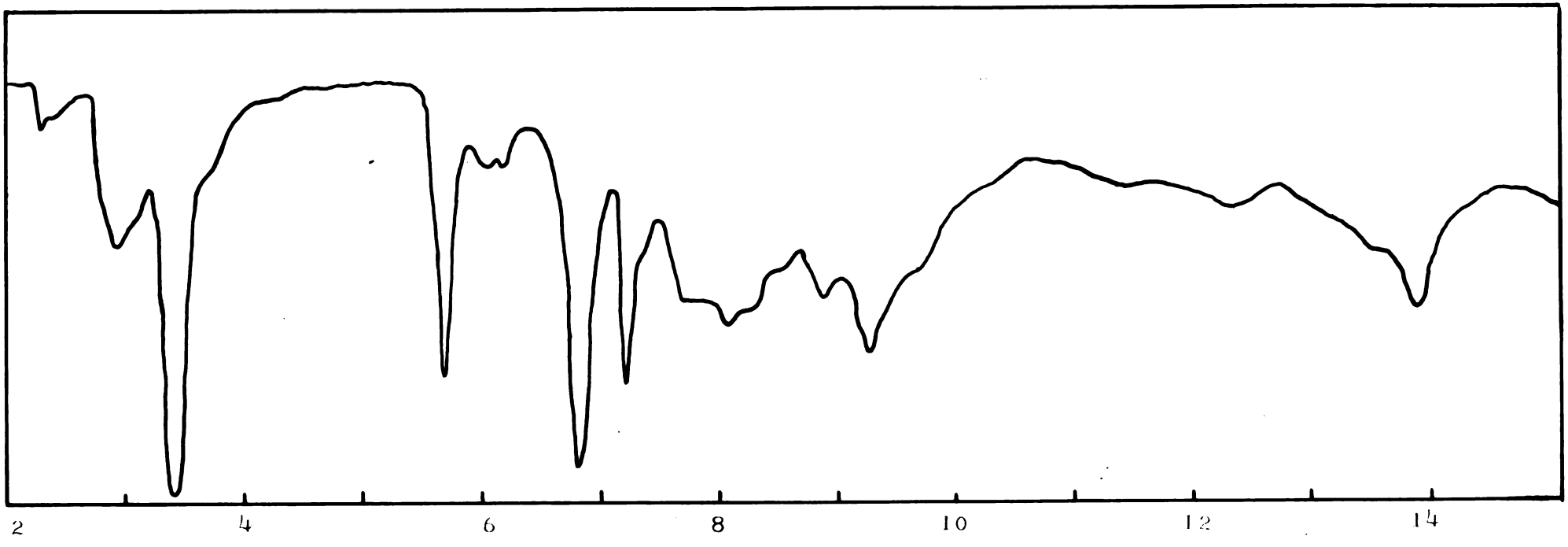
Fraction II
25 Micron Spacing



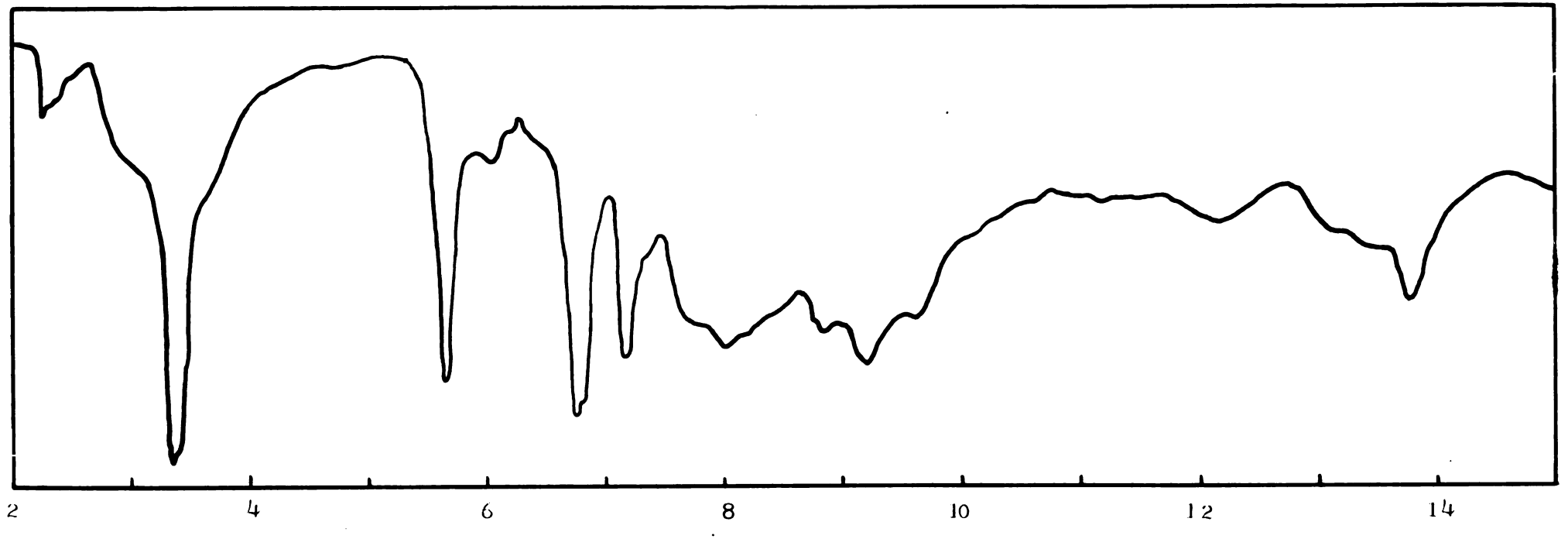
Fraction VII
25 Micron Spacing



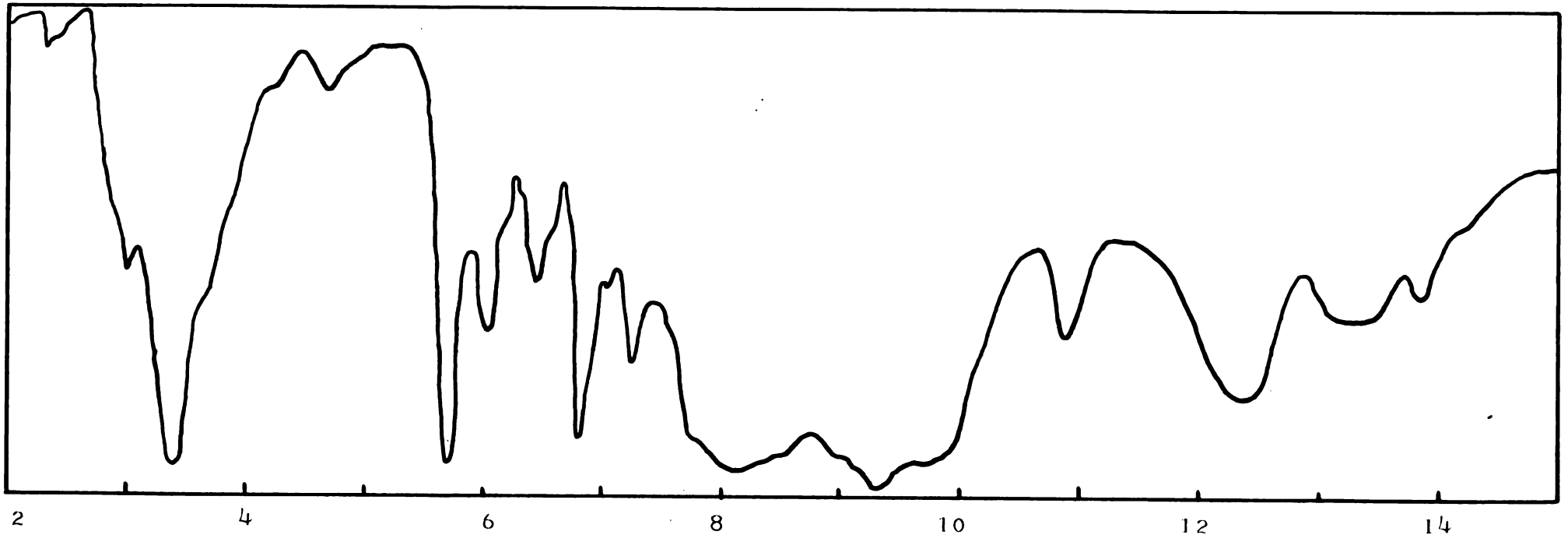
Fraction III
25 Micron Spacing



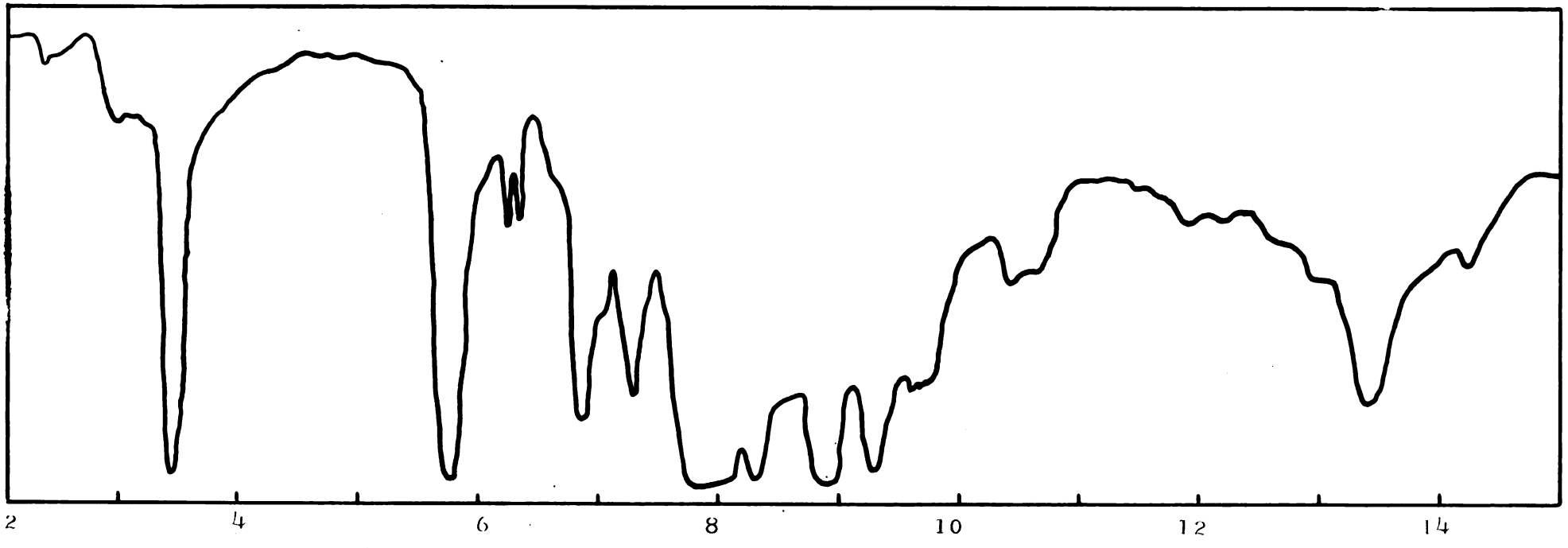
Fraction V
25 Micron Spacing



Fraction 1
25 Micron Spacing

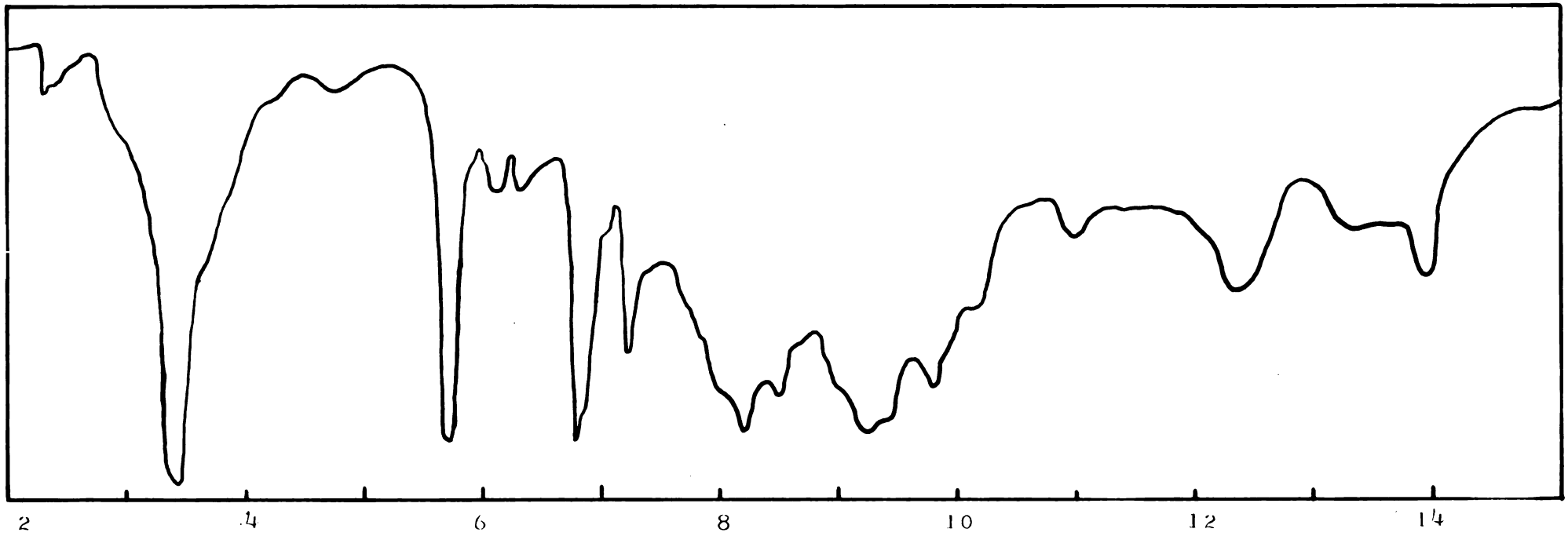


Fraction VIII
25 Micron Spacing



Wavelength in Microns

Fraction IX
25 Micron Spacing



Fraction VI
25 Micron Spacing

DISCUSSION

The results obtained reveal that the tendency toward elevation of phagocytic velocity by each of the fractions ran parallel whether the determination was made by means of the carbon technique or by using colloidal radioactive chromium phosphate as an indicator. The stimulation brought about by Fractions IV and II was greater than that of the total lipid from E. coli, while other fractions, although fairly stimulatory, were less so than the total extract. The reason for this is not known. The stimulatory power of the lipid extract from S. dysenteriae was noticeably greater than that from the other enteric organism. One could perhaps infer some relationship between the degree of stimulation of phagocytic activity and the pathogenicity. E. coli is regarded as non-pathogenic to mildly pathogenic, with some few strains being rather virulent, especially in infants. The Shiga bacillus is usually regarded with a great deal of respect so far as its capacity to cause intestinal disorders is concerned. No evidence has been presented to support such an inference, but it is an interesting point of consideration which more complete experimentation in a similar vein with other enteric pathogens and non-pathogens may either tend to confirm or negate. If such a relationship were to hold we might have

some idea as to what the mechanisms for phagocytic procession are.

The data gained by comparison of infra-red spectra indicate that the most active fractions from E. coli have a band at 2700 cm^{-1} which is not present in other fractions. All other fractions were less active and did not possess this band. This particular band, though weak, may hold some factor responsible for the difference in activity. It may be presumed with some validity that this is the absorption due to the phosphate group within the phosphatide structure. Bellamy (14) reports absorption of the phosphate group in cephalin at 2700 cm^{-1}. The method of separation of the fractions does not permit one to make any statement on the homogeneity of the preparations other than they are components partially separated from the total lipid extract. It was the opinion of the Perkin-Elmer spectroscopists that all of these fractions were mixtures of at least two or more components. Due to the presence of mixtures and the masking and shifting of absorption in the infra-red region there may exist a reason for the weak bands observed at 2700 cm^{-1}.

The infra-red spectrophotometer is based in its function upon the response of a molecule to interaction with radiant energy in the particular portion of the spectrum between 2 microns and 15 to 18 microns. The different molecular vibrations and rotational changes of the molecule taking place absorb energy and give identity to individual

molecules. In order to accurately make qualitative identification of molecular structure the compound under scrutiny must be a pure one. This was not the case with the lipid fractions. Phospholipids, especially cephalins, tend to complex among themselves and with other molecules, and they present unique difficulties of separation. The use of the infra-red spectrophotometer in this work was not for qualitative identification of these materials. Rather, its use was intended to serve much the same function as that of the investigators who used it for characterization of whole bacterial cells and complex substances within the cell such as cellulose and glycogen. The purpose in this work was to gain an idea of the characteristics which tended to make one fraction distinct from another, and if possible, to show absorption band differences and relationships among them.

Individual groups or radicles, such as the phosphate or phosphoryl of phosphatides will tend to exert their absorptive influences in the region of the spectrum below 7 microns (1430 cm^{-1}), while the vibrations of whole molecules tend to exert their characteristic influences in the longer wavelengths. Complex and large molecules tend to be very sluggish and good response to the infra-red beam is not achieved in the longer wavelengths. This is especially true if contaminating substances are present. For this reason, particular attention has been given to the bands in the

shorter regions of the spectrum, although the bands beyond 7 microns have been tabulated for their similarities of characteristics.

The reticulo-endothelial system functions as a main bulwark for defense of the animal body, and one of its primary functions is that of phagocytosis of foreign matter within the vascular system. Just exactly why the system responds by supplying increased numbers of wandering macrophages to localized lesions and general systemic infections alike is not known. One explanation found largely in textbooks, which offers partial clarification is that of chemotaxis. However, discrepancies exist which are not made entirely clear by the theory of chemotaxis. It does not explain the simultaneous propensity of sessile macrophages for removal of material from the vascular stream; neither does it give any indication why the phagocytes tend to engulf inert, insoluble substances, such as radiocolloids and carbon particles known to have no chemical by-products or secretions comparable to bacteria and polymorphonuclear cells. Because the increased activity of phagocytosis is extremely important any substance not harmful or non-toxic to the host which can stimulate phagocytic activity has tremendous importance. An understanding of the stimulation and depression of phagocytic response is fundamental in the host-pathogen relationship in infection.

The monocyte is one of the reticulo-endothelial

cells, of particular importance because it has the function of phagocytosis. Tompkins (128, 129) has reported that phospholipids stimulate an increase in numbers of monocytes in circulation and that monocytes are phagocytic toward such lipid moieties and metabolize them. If the fractions which have been shown to possess the highest activity as described herein can be conclusively identified as parts of the cell phospholipid complex, a partial explanation of the mechanism of phagocytic stimulation may be at hand. Such a partial solution seems to fit in well with the theory of chemotaxis.

Lipids have been considered as haptens by immunologists. According to the concept held, they are thought to combine with substances already formed within the system to produce antibodies. The data presented in this paper support the contention that lipids have the activity necessary to fulfill at least one of the criteria prescribed for an antigen: they stimulate phagocytic velocity to hyperfunction. Said stimulation is without the presence of a living bacterial cell.

If the theory of chemotaxis were to be accepted as applicable to the results described here, one might tend to gather that the responsible factor(s) were coming from the cell of the pathogen rather than being liberated by the tissue at the site of an infection or lesion. Except for the fact that the lipids used in this series of experiments

were administered intraperitoneally, the theories involved in chemotaxis might well apply. Early attempts at injection of the lipid intravenously met with little success, so no data have been gathered for comparative stimulatory rates of the fractions introduced by the two routes. Thus, if chemotaxis is dependent upon a secretion of metabolic by-product in the vascular stream for stimulatory effect upon phagocytosis and attraction of mobile macrophages, little consideration can be given it for applicability here.

Any agent which can stimulate reticulo-endothelial function to a marked degree has potential as a therapeutic agent with a number of possible applications. Many of the persons devoted to research on the reticulo-endothelial system recognize that the functions of the system are essentially the same whether it be a case of neoplastic disease or a microbial infection. The system is thought to have a limiting activity upon neoplastic cells, and in experimental tumor-bearing animals a proliferation of histiocytes, and Kupfer cells of the liver, and splenic hypertrophy have been described. Such observed histologic phenomena may perhaps be interpreted as the manifestation of a physiological defense mechanism on the part of the host. One of the possible applications of a reticulo-endothelial stimulating agent, such as the bacterial lipid, could be its use as a chemotherapeutic agent in the treatment of neoplastic disease. Further development of such a

possibility would require a great deal of refined experimentation, but the basic principle outlined in this work might serve as a beginning point.

The author has a great deal of interest in investigations of cell-free extracts. Progress has been made in past years in the areas of general, morphological, and taxonomic microbiology, and newer problems are being visualized every day. Many of these, including certain ones of pathogenesis and immunity, lie within the scope of fundamental cell chemistry and physiology. By cell chemistry is meant the investigations of reactions and phenomena which are related to products obtained from the living cell. The isolation and study of enzyme activities and kinetics fall in this category; the knowledge of cell enterotoxins and certain metabolic identities have been arrived at by investigation of these products apart from the cell. The research upon cell lipids in other than analytical fashion represents another form of effort delving into the complexities of the cell. The disciplines of biological and physical chemistry are very closely related to microbiology at this level, and microbiologists could do very little without knowledge of them.

Investigation of cell products represents a newer approach to study of microbiological problems. Findings from cell extract research indicate that certain cell products can be used to artificially induce phenomena which

have been normally associated with the presence of a micro-organism. The MPA substance of Girard and Murray, and Stanley is an example. The monocytosis produced by administration of the total cell lipid contents is the same as that produced by an infection of Listeria monocytogenes. The cell lipid work began as a source of this paper is perhaps another such example. Use of bacterial cell products as described here may also have introduced a new concept of therapy where the tissues are stimulated to greater anti-bacterial activity as compared with the use of an antibiotic agent to combat the organism.

While such study of cell-free products may represent a departure from classical microbiological procedures, its methods may possess shortcomings which could serve to offset some of its positive attributes. Any treatment of cells, regardless of tissue identity, which is disintegrative in its action creates gross abnormalities from which only data of questionable reliability may be obtained. The investigator may not be certain that he is recovering the desired fraction intact in a technique which utilizes blendors, homogenizers, or colloid mills. Doctor Erwin Chargaff of Columbia University enlarged this point at length while conducting a series of lectures in fundamental cell chemistry. He expressed an opinion that any resemblance to the original state after such drastic means of fraction separation was entirely a matter of chance, and that the milder the treat-

ment the more likely the investigator was to obtain a recovered product near its native state. The author has tried to make this clear in the section describing methods of extraction as a reason for carrying out passive extractions in preference to more violent treatment.

Future work upon bacterial lipids would necessitate much more refined and extensive techniques than employed in this investigation. For example, a major problem in biochemical work with lipids is their oxygen lability. Handling of all stages of the extraction and separation procedures in an inert atmosphere would greatly enhance accuracy, validity, and reproducibility. It was not possible to protect all stages of the work from oxidative changes either at the New England Institute or at the University because of limitations of materials and space.

Fractionation of crude lipid by column chromatography using a fraction collector would seem to be superior to the method of precipitation by chemical agents. The loss incurred in handling and repeated transfers would thus be minimized, and the accuracy of analytical determinations would thus be enhanced. This should be particularly emphasized in future work, because it will be extremely important to identify the fractions more precisely than was done here. By use of the column and fraction collecting technique one can obtain curves by plotting sample volume, recovery time, or sample number against the percentage of a given component

subject to analysis. These curves show maxima, and by observing such maxima one can determine which of the fractions collected, or what volume of eluate contains the greatest percentage; thus, one could determine where the greatest concentration of phosphorous was, similarly for nitrogen, etc.

A major handicap throughout the entire investigation was that of insufficient lipid with which to work. Large quantities of cells were required to provide sufficient extracts, and the time required for accumulation of such large quantities was the predominant prohibitive factor. Growth of cells in small quantities presented problems of storage until sufficient amounts were accumulated. Lyophilization in tissue containers seemed the best method, and it seems doubtful if future procedures would offer a great deal of improvement. Separation of large quantities of cells without a super-centrifuge is almost impossible.

The decision to use 0.2 ml as injection dose of lipid was an arbitrary one which resulted in the expenditure of over a milliliter of a lipid fraction with only 6 or 7 animals. The size of a minimal stimulatory dose should be determined as a part of future work. There is a distinct possibility that less lipid than was used in this series of experiments would demonstrate the same degree of activity and yet enable experimentation to be carried on with greater conservation of the active substance.

No explanation or complete hypothesis as to the mechanism of lipid stimulation has been offered here. The use of labelled fractions in future work might be of benefit in an investigation of the mechanism. Cultivation of bacteria on media containing radioisotopes with the aim of producing labelled fractions which could be followed in the animal system would be invaluable as a technique to permit determination of the fate or site of localization of the lipid fractions. Nitrogen 15 or phosphorous 32 would likely be the isotopes of choice. Carbon 14 would perhaps provide information of the localization of fractions other than phospholipids, but it would also present certain problems of counting technique not inherent with the use of the other isotopes.

In expanded future work investigation of possibility of relationship between pathogenicity of the bacterium and stimulatory activity of its lipid fractions upon phagocytic velocity should be considered imperative. As stated earlier, the data presented here seem to indicate a relation dependent upon pathogenicity of the organism, but the evidence is insufficient to draw any valid conclusion. Preparation of the total extractable lipid and its fractions from several organisms representing pathogens and non-pathogens would be necessary.

SUMMARY

The lipids from two enteric gram negative organisms, Escherichia coli, considered to be non-pathogenic, and Shigella dysenteriae, an intestinal pathogen, were extracted and injected intraperitoneally into laboratory mice in an effort to learn if they possessed stimulatory activity for the reticulo-endothelial system.

By use of radioactive colloidal chromium phosphate and colloidal carbon suspensions as indicators of the rate of phagocytosis of particulate matter from active circulation it was found that the lipids from S. dysenteriae possessed marked power to stimulate phagocytic activity to hyperfunction, while those of E. coli and the sub-fractions did similarly to a lesser degree.

Partial characterization of the sub-fractions from E. coli indicated that they exhibited many of the attributes of phospholipids, since the most active fractions possessed an absorption band in the infra-red region not shared by less active ones. The same band was observed in a known phosphatide, and it was tentatively identified as that produced by the phosphorous containing group found in phosphatide structures.

CONCLUSION

The total extractable lipids from Escherichia coli and Shigella dysenteriae and four of nine sub-fractions from E. coli found to possess physical characteristics of phosphatides are decidedly stimulatory to the phagocytic function of the reticulo-endothelial system of laboratory mice as measured by the uptake of radioactive chromium phosphate and colloidal carbon from the bloodstream.

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